PN-ABM-073

FINAL REPORT

Advanced Training for Tabbin Institute of Metallurgical Studies (TIMS), Cairo, Egypt

USAID CONTRACT #263-010-C-00-2038-00
PROJECT #263-0101

VOLUME I

Prepared by
A. Curley,
Team Leader
Office of Research and Sponsored Programs
Clark Atlanta University
J.P. Brawley Drive at Fair Street, SW
Atlanta, GA 30314

August 1992

TABLE OF CONTENTS

VOLUME I

PART I **Technical Training**

Background & Introduction

- General
- Scope and Training Design
- Training Objectives and Expected Outputs

Section I: **Analytical Instrumentation: Laboratory Configuration**

Section II: Waste Water Training Module

Section IIA: **Water Quality Training Topics**

Section III: Air Emissions Training Module

Section IIIA: **Air Quality Training Topics**

Section IV: Air and Wastewater Laboratory Management Module
Quality Assurance/Quality Control (QA/QC) Training Topics

Section IVA: **Air and Wastewater Laboratory Management**

Section V: Training Schedule

Section VA: **Lectures/Laboratory Sessions**

Section VB: **References**

Section VI: Workstation Rearrangement

PART II **Conclusions and Recommendations**

PART IIA TIMS Technical Staff Assessments

PART III **Summary**

PART IV Supplies, Reagents and Equipment

APPENDIX **Volumes 2 - 4**

Volume 2 Handouts for Atomic Absorption Spectroscopy

Volume 3 Handouts for Gas Chromatography

Volume 4 Handouts for Microbiology

PART I

TECHNICAL TRAINING

BACKGROUND AND INTRODUCTION

The General Organization for Industrialization (GOFI) was mandated by the Ministry of Industry and Mineral Resources to develop a program of industrial pollution control and abatement in the public industrial sector of Egypt's national economy in concert with the on-site U.S. Environmental Advisor. The program received funding and technical assistance from USAID and was an integral part of a larger project, the Industrial Production Project, to enhance industrial productive capacity thereby stimulating a market driven economy.

During a period of several years, GOFI has been making in-house studies of the needs of the industrial pollution control program and, following the execution of a contract with Weston International, Inc., (USAID Contract No. 263-0101-02 HCC) GOFI instructed Weston International under the supervision of the Environmental Advisor to prepare a concept design report on establishing a National Environmental Laboratory dedicated to industrial pollution control; e.g., monitoring and management.

To ensure that the laboratory concept would become a reality in the shortest possible time, GOFI conducted a search for a building which could be readily adapted to use as a laboratory. A building at El-Tabbin Institute which had formerly been intended for use as a metallurgical laboratory to serve the needs of the steel industry was found and GOFI made arrangements to reserve a portion of that building for the environmental laboratory. Plans of the allocated area were furnished by GOFI to Weston International for their use in designing the laboratory.

During the pre-contract period, GOFI and the U.S. Environmental Advisor developed outline concepts of the complement of laboratory equipment and of the types of environmental analyses and tests which the environmental laboratory should be capable of conducting for monitoring the vast number of polluting industries. GOFI included lists of equipment which were intended to form the framework upon which the full equipment complement would be based.

With comments and guidance from GOFI and the U.S. Environmental Advisor, Weston International, Inc. prepared the final designs for the environmental laboratory. Acting as a procurement agent for GOFI, Weston International, Inc. prepared the procurement package for the purchase of the laboratory equipment, supplies and O&M training and arranged for shipment to Egypt. Where necessary, certain suppliers of complex or delicate apparatus would be required to visit Cairo to ensure that these pieces of equipment are properly installed and fully operational; they would also give whatever training is necessary in operation and maintenance of the equipment.

This report describes in detail the training conducted and the allocation of a time frame to the various laboratory functions to be performed. TIMS must ensure the availability of adequate utilities, chemicals and reagents to meet the training needs in order to produce a comprehensive environmental laboratory training program. The following are the major areas into which the laboratory is subdivided.

- Instrumentation Laboratory
- Air Quality Laboratory

- Wet Chemistry Laboratory
- Bacteriology
- BOD Laboratory
- Hot Room
- Balance Room
- Glassware Preparation Room
- Data Management Program
- Quality Assurance/Quality Control Program

Scope and Training Design

The training was scoped to consist of meeting the functional needs of the major subdivisions of the Laboratory. Therefore, the training was subdivided into training modules to provide state-of-the-art training on instrumentation and representative acceptable analysis and tests for pollutants emanating in industrial waste water effluents, air emissions and the work environment to alleviate public and worker exposure situations. The training modules and schedules are presented in the following sections as described later.

On-Site Training: Training Objectives and Expected Outputs

a. The On-Site Training services supplied by the Contractor to Employer personnel (El-Tabbin Laboratory staff) in Egypt was applied and theoretical training.

b. Upon conclusion of training on Analytical Methodology and tests, data management and quality assurance/quality control training on analytical instrumentation and accessories for Air and Water testing and monitoring, the trainee should at a minimum be able to:

    1) Startup and shutdown the subject apparatus without damage to the equipment;

    2) Reconfigure the subject apparatus without damage to the equipment;

    3) Perform operator maintenance checks on both primary items and their accessories to include operator-level troubleshooting and operator-level fault correction.

    4) Calibrate the subject apparatus in each of its principle configurations;

    5) Know when to request spare parts and supplies;

    6) Identify and request spare parts and supplies;

    7) Identify operational and maintenance problems beyond operator-level capabilities;

8) Know what constitutes unauthorized manipulation of the subject apparatus from both a warranty and an operability aspect;

9) Know when and how to request expert maintenance assistance;

10) Recognize environmental conditions which threaten the mechanical and/or electrical integrity of both the primary item and its accessories; and

11) Correctly configure, startup, operate, and shutdown the subject apparatus for one standard analytical method other than calibration.

c. Training on the microcomputer is to be for both operation of delivered software packages and on Operation and Maintenance of the system. Upon completion of training on the mini-computer, the operator should be at a minimum be able to:

1) Startup and shutdown the system without damage to either the system or to any accessories such as floppy disks;

2) Recognize environmental conditions which threaten the mechanical and/or electrical integrity of both the primary item and its accessories to include data loss;

3) Bootstrap all delivered software; and

4) Operate all delivered software.

d. Know how to conduct waste water characterization studies, collect and preserve appropriate samples and perform required analyses and tests.

e. Know how to conduct air emissions studies by characterizing both ambient air pollutants and point source emissions, how to use appropriate air monitoring and sampling instrumentation and perform the necessary pollutant analyses.

f. The estimated Level of Effort for the Training was man-hours and presented in Section V. The distribution of time between job titles or labor categories is described.

SECTION I

ANALYTICAL INSTRUMENTATION: LABORATORY CONFIGURATION

ANALYTICAL INSTRUMENTATION: LABORATORY CONFIGURATION

The original plan and layout of equipment for the El-Tabbin Laboratory is listed in Table 1 by room specifying the major equipment items in each room.

Training was developed by environmental media; e.g., water, air and workplace and performed in all aspects of use; e.g., operations and maintenance of the following equipment:

Table 1

List of Major Equipment Items by Laboratory Room

INSTRUMENT LAB

Spectrophotometer
Electrocolorimeter
Solid State Recorder
Specific ION Meter
Turbidimeter
Safety Station
Atomic Absorption Spectrophotometer
TOC Analyzer
Gas Chromatograph

AIR LAB

Twin Cylinder Compressor
Safety Station
Sulfur Analyzer
CO-Analyzer
NO-NO_2-NO_x Analyzer
Ozone analyzer
Gas Phase Titration Calibrators
Permeation Tube Calibrators
Strip Chart Recorders
Mercury Vapor Analyzer
Anemometer
Gas Storage Bags

WET CHEMISTRY LAB

Balance, Top Loading
pH Meter
Conductivity Bridge
Heating Mantles
Flameless Electric Burners
Magnetic Stirrers
Precision Universal Centrifuge

Refrigerator
Titration System
Single Stage Vacuum Pump
Safety Station
Evaporator
Oil/Fat Extractor
Soxhlet Extraction Assembly

BACTERIOLOGY LAB

Constant Temperature Bath
Incubator
Refrigerator
Microscope
Dry Bath Incubator
Colony Counter
Autoclave
Membrane Filter

BOD LAB

Oxygen Meter

HOT ROOM

Muffle Furnace
Forced Convection Oven
Gravity Convection Oven
Stirring Hot Plate
Hot Plates
Heating Mantles
Water Bath
Cyanide Distillation Apparatus
Safety Station

BALANCE ROOM

Balance, Analytical
Balance, Top Loading
Balance, Platform

GLASSWARE PREPARATION ROOM

Dishwasher
Glassware Dryer
Automatic Stills
Pure Water System
Demineralizer Cartridge

DATA MANAGEMENT ROOM

Mini-Computer
Freezer*
Dry Ice Maker*
Dry Ice Chest*

* Freezer Storage and Ice Maker stored in this room for lack of space.

STORAGE ROOMS

Wastewater Sampler
Portable pH Meters
Lithium Injectors
Sonic Flow Meter
Portable Lab Kit
Industrial Hygiene Test Kit
Portable Meteorology Station
Suspended Particulates Sampling System
Stack Sampling Trains

SECTION II

WASTE WATER TRAINING MODULE

CHARACTERIZATION OF WASTE WATER EFFLUENTS

Wastewater Characterization studies are conducted to determine (1) the physical, biological, and chemical characteristics and the concentrations of constituents in the wastewater, and (2) the best means of reducing the pollutant concentrations.

Procedures for wastewater sampling methods, sample analysis and expressions used to present the results will be presented.

1. Sampling

The sampling techniques used in a wastewater survey must assure that representative samples are obtained, because the data from the analysis of the samples will ultimately serve as a basis for designing treatment facilities. There are no universal procedures for sampling programs. These must be individually tailored to fit local situations. Special procedures are necessary to handle problems when sampling wastes vary considerably in composition. Thus suitable sampling locations must be selected and the frequency and type of sample to be collected must be determined.

2. Sampling Locations Procedures

3. Sampling Intervals Determination

4. Sampling Equipment Selection

5. Sample Preservation Procedures

6. Methods for Sample Analysis

The analyses used to characterize wastewater vary from precise quantitative chemical determinations to the more qualitative biological and physical determinations. Many of the parameters are interrelated. For example, temperature, a physical parameter, affects both the biological activity in the wastewater and the amounts of gases dissolved in the wastewater. The accepted reference that details the conduct of water and wastewater analyses. Simplified techniques of analysis for selected constituents may be found in the U.S. Environmental Protection Agency publication, *Methods of Wastewater Analysis.* Although Analysis of *Water and Sewage* is an older reference, it is useful. As a general reference, *Chemistry for Environmental Engineering* is recommended. *Aquatic Chemistry*, an advanced text. should be consulted for chemical-equilibrium problems, especially in natural waters.

Training was provided on the following analyses and tests for characterization of waste water effluents;

- acidity-alkalinity
- BOD

- COD
- cyanide
- color
- Cl_2
- Halides - Cl; F, I, Br.
- Total & fecal coliform
- Hardness
- Metals - Cd, Cr, Cu, Hg, Ni, Fe, Co, Mn, Mg, Na
- Ammonia
- TKN
- Nitrite
- Nitrate
- Phosphate
- Sulphate
- pH
- Turbidity
- Specific conductance
- Dissolved oxygen
- Phenols
- TOC
- Sulfides
- Oil & grease
- Sulfite
- Surfactants

SECTION IIA

WATER QUALITY TRAINING

WATER QUALITY TRAINING TOPICS

I. Standard Analytical Methods for Chemical Analysis of Water and Wastewater Effluents. 1 week

- Measurement

  Simple Instrumental:
  - pH
  - Conductivity
  - Turbidity
  - Color
  - Dissolved Oxygen (Probe)
  - Fluoride (Probe)

  Simple Volumetric:
  - Alkalinity (Potentiometric)
  - Acidity (Potentiometric)
  - Chloride
  - Hardness
  - Dissolved Oxygen (Winkler)

  Simple Gravimetric:
  - Solids, Suspended
  - Solids, Dissolved
  - Solids, Total
  - Solids, Volatile

  Simple Colorimetric:
  - Nitrite N (Manual)
  - Nitrate N (Manual)
  - Sulfate (Turbidimetric)
  - Silica
  - Arsenic

  Complex, Volumetric, or Colorimetric:
  - Biochemical Oxygen Demand (BOD)
  - Chemical Oxygen Demand (COD)
  - Total Kjeldahl Nitrogen (TKN)
  - Ammonia
  - Phosphorus, Total
  - Phenol (Distillation Included)
  - Oil and Grease
  - Fluoride (Distillation Included)
  - Cyanide

  Special Instrumental:
  - TOC
  - Metals (by AA), No Preliminary Treatment
  - Metals (by AA), With Preliminary Treatment
  - Organics (by GC), Pesticides, Without Cleanup
  - Organics (by GC), Pesticides, With Cleanup

II. Water and Wastewater Sampling 1 week

- Program Objectives
- Collection of representative samples
- Site Selection
- Basic types of Sampling
- Automatic Samplers
- Flow Measurement
- Special Sampling Procedures
- Sample Preservation and Handling
- Chain-of-Custody Procedures
- QA in Field Sampling
- Duplicate, Split and Spiked Samples
- Sample Preservation Blanks
- Precision, Accuracy and Control Charts
- Calibration of Field Equipment
- Sample Preparation for Analysis with or without preliminary treatment or cleanup

III. Instrumentation — 1 week

- Operation and Maintenance
- Analytical Balances
- pH/Selective-Ion Meters
- Conductivity Meters
- Turbidimeters (Nephelometers)
- Spectormeters; Atomic Absorption Spectrophotometers
- Organic Carbon Analyzes
- Gas Chromatography
- Centrifuges
- Tritration Systems
- Colorimeters

IV. Data Management and Analysis — 1 week

- Laboratory Record Keeping
- Operation and Maintenance of Automated Data Management Systems
- The Analytical Value
- Basic Statistics Glossary
- Report Forms

SECTION III

AIR EMISSIONS TRAINING

AIR EMISSIONS - STATIONARY SOURCES AND AMBIENT AND POLLUTANTS

This section of training provided guidelines for quality assurance in performance of air emissions testing of stationary sources by USEPA prescribed procedures. The guidelines may be applied to all categories of sources commonly monitored.

The purpose of emissions testing (also called "source sampling" or "stack sampling") is to extract from the stack or duct a sample that is representative of emissions from that source during a time period in which the process is under a desired operating condition. The sampling methods prescribed by Federal agencies are for specific substances and types of sources and are designed to provide representative and reliable data. Since the Federal New Source Performance Standards are promulgated from data obtained by these methods, adherence to these standard procedures for sampling and analysis is essential.

The following training guidelines for assurance of high quality emissions test data will be presented in four major phases:

- Planning the test program,
- Performing the test
- Chain-of-custody procedures
- Data acquisition and management

Specific method descriptions will be given for specific sample/pollutant analysis.

Ambient air emissions were included in meteorological phase of course.

SECTION IIIA

AIR QUALITY TRAINING

AIR QUALITY TRAINING TOPICS

I. Emission Testing and Air Sampling — 2 weeks

- Stationary Source Emissions Testing
  - Stack Gas Velocity and Volumetric Flow Rate
  - Apparatus and Supplies
  - Calibration of apparatus
  - Presampling operations
  - On-site measurements
  - Post-sampling operations
  - Data, calculations and analysis
  - Maintenance
  - Auditing
  - Reference Method

- Air Pollutant Analyses
  - Determination of CO_2, O_2, Excess air and dry molecular weight
  - Determination of Particulates
  - Determination of SO_2 Emissions
  - Determination of Nitrogen Oxide Emissions

- Miscellaneous Laboratory Analyses

- Field Tests

- Special Tests

II. Ambient and Toxic Air Meteorological Monitoring — 2 weeks

- Continuous/Criteria Ambient Air Monitoring

- Site Surveys and Selection of Monitoring Sites

- Use and calibration of Air Monitoring Instrumentation
  - SO_2 Analyzer
  - Sulfur Analyzer
  - CO-Analyzer
  - NO-NO_2-NO_x Analyzer
  - Hydrocarbon Analyzer
  - Mercury Vapor Analyzer
  - Anemometers
  - Gas Phase Titration Calibrators
  - Permention Tube Calibrators
  - Portable Meteorology Station

- Suspended Particulates Sampling System
- Stack Sampling Trains

- Operation of Data Acquisition/Data Support Systems
  - Laboratory Records Keeping
  - Data Analysis

- Toxic Air Monitoring

SECTION IV

AIR AND WASTEWATER LABORATORY MANAGEMENT

AIR AND WASTEWATER LABORATORY MANAGEMENT

Quality Assurance/Quality Control (QA/QC) Training Module

The environmental monitoring laboratory provides qualitative and quantitative data for use in decision-making. To be valuable, the data must accurately describe the characteristics and concentrations of constituents in the samples submitted to the laboratory. In many cases, because they lead to faulty interpretations, approximate or incorrect results are worse than no result at all.

The TIMS Environmental Laboratory was designed for both air emissions and water/wastewater analyses. Hence, it is basically an analytical laboratory.

Ambient water quality standards for pH, dissolved oxygen, heavy metals, and pesticides are set to establish satisfactory conditions for drinking water, fishing, irrigation, power generation, or other water uses. The laboratory data define whether conditions are being met and whether the water can be used for its intended purposed. In wastewater analyses, the laboratory data identify the characteristics of the treatment plant influent and the final load imposed upon receiving water resources, as well as the effectiveness of steps in the treatment process. Decisions on process changes, plant modifications, or the construction of new facilities may be based upon the results of water laboratory analyses. The financial implications of such decisions suggest that extreme care be taken in analysis.

Effective research in water pollution control also depends upon a valid laboratory data base, which in turn may contribute to sound evaluations of both the progress of the research itself and the viability of available water pollution-control alternatives.

The analytical data from air, water and wastewater laboratories may also be used to determine the extent of compliance of a polluting industry with discharge or surface and ground water standards or air standards. If the laboratory results indicate a violation of a standard, remedial action is required by the responsible parties. Both legal and social pressures can be brought to bear to protect the environment. The analyst should realize not only that he has considerable responsibility for providing reliable laboratory descriptions of the samples at issue, but also that his professional competence, the validity of the procedures used, and the resulting values reported may be challenged (perhaps in court). For the analyst to meet such challenges, he should support the laboratory data with an adequate documentation program that provides valid records of the control measures applied to all factors bearing on the final results of investigations.

Because of the importance of laboratory analysis in determining practical courses of action that may be followed, quality assurance programs to insure the reliability of the air water and wastewater data are essential. Although all analysts practice quality control (QC) in amounts depending upon their training, professional pride, and the importance of their particular projects, under actual working conditions

sufficiently detailed QC may be neglected. An established, routine, quality assurance program applied to each analytical test can relieve analysts of the necessity of originating individual QC efforts.

Quality assurance programs have two primary functions in the laboratory. First, the programs should continually monitor the reliability (accuracy and precision) of the results reported; i.e., they should continually provide answers to the question "How good (accurate and precise) are the results obtained?" This function is the *determination* of quality. The second function is the *control* of quality (to meet the program requirements for reliability). As an example of the distinction between the two functions, the processing of spiked samples may be a determination of measurement quality, but the use of analytical grade reagents is a control measure.

Each analytical method has a rigid protocol. Similarly, QC associated with a test must include definite required steps for monitoring the test and insuring that its results are correct. The steps in QC vary with the type of analysis. For example, in a titration, standardization of the titrant on a frequent basis is an element of QC functions. All of the experimental variables that affect the final results should be considered, evaluated, and controlled.

In summary, laboratory data, in quantitative terms, e.g., in milligrams per liter, are reported by the analyst. These values are interpreted by industrial plant engineers to show compliance or noncompliance with permits for discharge, by pollution control agencies to define the need for additional sampling and analysis to confirm violations, or by the environmental authorities to demonstrate that prescribed waste treatment was sufficient to protect the surface waters affected by the discharge.

This training will present the basic factors of air water and wastewater measurements that determine the value of analytical results and provides recommendations for the control of these factors to insure that analytical results are the best possible. Quality assurance programs initiated from, and based upon, these recommendations should increase confidence in the reliability of the reported analytical results.

Primarily, because ultimately a laboratory direct or must assume full responsibility for the reliability of the analytical results submitted, the laboratory director must also assume full responsibility in both design and implementation for the corresponding Quality Assurance/Quality Control program.

SECTION IVA

AIR AND WASTEWATER LABORATORY QUALITY ASSURANCE/QUALITY CONTROL MANAGEMENT TRAINING

AIR AND WASTEWATER LABORATORY MANAGEMENT

Quality Assurance/Quality Control Management Training (QA/QC) Topics

I. Importance of QA/QC in Environmental Monitoring Laboratory Management — 4 weeks

II. Laboratory Services

- Distilled water; automatic stills
- Pure water System
- Demineralized Water
- Compressed Air
- Vacuum Systems
- Hood Systems
- Electrical Services
- Freezer and Refrigeration Systems
- Extractors; oil/fat, soxhlet

III. Water and Wastewater Sampling

- Program Objectives
- Collection of representative samples
- Site Selection
- Basic types of Sampling
- Automatic Samples
- Flow Measurement
- Special Sampling Procedures
- Sample Preservation and Handling
- Chain-of-Custody Procedures
- QA in Field Sampling
- Duplicate, Split and Spiked Samples
- Sample Preservation Blanks
- Precision, Accuracy and Control Charts
- Calibration of Field Equipment
- Sample Preparation for Analysis with or without preliminary treatment or cleanup

IV. Air Emissions Testing and Sampling and Toxic Emissions Monitoring

- Stationary Sources Emissions Testing
- Ambient Air Sampling and Testing
- Air Pollutant Analyses
- Operations and Maintenance of Instrumentation
- Operations of Data Acquisition/Data Support Systems

V. Instrumentation and Analysis

- Operation and Maintenance
- Analytical Balances
- pH/Selective-Ion Meters
- Conductivity Meters
- Turbidimeters (Nephelometers)
- Spectormeters; Atomic Absorption Spectrophotometers
- Organic Carbon Analyzes
- Gas Chromatography
- Centrifuges
- Tritration Systems
- Colorimeters

VI. Glassware

- Types
- Volumetric Analyses
- Cleaning of Glaso and Porcelain
- Special Cleaning Requirements
- Disposable Glassware
- Specialized Glassware
- Fritted Ware

VII. Reagents, Solvents and Gases

- Reagent Quality
- Elimination of Determinate Errors

VIII. Quality Control for Analytical Performance

- Developing and Implementing a comprehensive QA/QC Program

IX. Data Management and Analysis

- Laboratory Record Keeping
- Automated Data Management Systems
- The Analytical Value
- Basic Statistics Glossary
- Report Forms

X. Special Requirements for Trace Organic Analysis

- Sampling and Sample Handling
- Extract Handling
- Supplies and Reagents

XI. Skills and Training Requirements for Environmental Monitoring Laboratory Operations

- Skills
- Training

XII. Microbiology

- Microbiological Samples and Handling

XIII. Laboratory Safety, Storage and Waste Disposal

- Appropriate Egyptian Laws and Authority for Safety and Health
- Laboratory Safety Practice
- Laboratory storage and disposal of chemicals and waste
- Record-keeping
- Report of unsafe or unhealthful conditions

SECTION VA

TRAINING SCHEDULE

TRAINING SCHEDULE

The training as presented in Table 2 presents an approximate time schedule for the personnel to accomplish the training modules on Water and Air Quality as presented in Sections I-IV.

Topics as indicated in Sections I-IV were selected and focused in terms of responding to the interest of the RFP.

Personnel to accomplish the training were selected from Clark Atlanta University, Northern Arizona University and Florida International University. The three universities are members of the HBCU/MI Consortium on Environmental Technology and Waste Management.

TABLE 2
ADVANCED ENVIRONMENTAL LABORATORY TRAINING

Training Team Schedule

Months	January				February				March				April				May				June				
Weeks	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	Pers. Mos.
1 Team Leader							I----I[1]															I---------I			1.5
1 Sr. Research Chemist														I----------			----------				-I				2.0
1 Sr. Research Chemist														I----------			----------				-I				2.0
1 Sr. Microbiologist																	I----------				-I				1.0
1 Sr. Research Chemist																	I----------				-I				1.0
																					Total				7.5

[1]Note: Pretraining Assessment Visit

TRAINING ACTIVITY SCHEDULE

DATE	ACTIVITY
Wednesday, April 15, 1992	Atomic Absorption Spectroscopy (Dr. Richard Foust)
Saturday, May 9, 1992	Special Lecture (Dr. Richard Foust/Dr. Cass Parker)
Saturday, May 16, 1992	Special Lecture (Dr. Richard Foust/Dr. Cass Parker)
Tuesday, May 19, 1992	Laboratory Practicals using World Bank Project
Wednesday, May 20, 1992	Chemical Oxygen Demand Lecture/Biochemical Oxygen Demand (Dr. Harold Speidel)
	Graphite Furnace AA Practical (Dr. Richard Foust)
	COD Lab Practical
Thursday, May 21, 1992	GC-Water Sample Collection, Chain of Custody, Methods for the Determination of Halo carbons, Volatile Organics, Pesticides and PAH's (Dr. Cass Parker)
	Laboratory Demonstration of Separation Techniques and GC (Dr. Cass Parker)
	Analysis of a Water Sample (Dr. Cass Parker)
Saturday, May 23, 1992	Dr. Parker - Lecture Ion Selective Electrode and Report Writing Recap Use of Computer Generated Reporting Forms for Data Reduction
	Special Lecture (Dr. Harold Speidel)
	Laboratory Practical Using Ion Selective Electrodes (Dr. Cass Parker)

Sunday, May 24, 1992	Ambient Air Quality Standards (Dr. Norman Munroe)
	Laboratory Practical (Dr. Harold Speidel)
Monday, May 25, 1992	Lecture - Microbiology (Dr. Harold Speidel)
	Laboratory Practical
	Air Sampling (Dr. Munroe)
	GC Analysis (Dr. Cass Parker)
Tuesday, May 26, 1992	Lecture - (Dr. Norman Munroe) High Volume Methods
	Microbiology Laboratory Practical (Dr. Harold Speidel)
	Gas Chromatography Laboratory Practical (Dr. Cass Parker)
	Air Sampling Laboratory Practical (Dr. Norman Munroe)
Wednesday, May 27, 1992	Lecture - Microbiology (Dr. Harold Speidel)
	Air Sampling Laboratory Practical (Dr. Norman Munroe)
	GC Laboratory Practical (Dr. Cass Parker)
	Microbiology Laboratory Practical (Dr. Harold Speidel)
Thursday, May 28, 1992	Lecture - Total Suspended Particulate (Dr. Norman Munroe)

	Air Sampling Laboratory Practical (Dr. Norman Munroe)
	GC Laboratory Practical (Dr. Cass Parker)
	Microbiology Laboratory Practical (Dr. Harold Speidel)
Saturday, May 30, 1992	Lecture - Microbiology (Dr. Harold Speidel)
	Air Sampling Laboratory Practical (Dr. Norman Munroe)
	Microbiology Laboratory Practical (Dr. Harold Speidel)
Sunday, May 31, 1992	Lecture - Sulfur Dioxide Determination in the Atmosphere (Dr. Norman Munroe)
	Air Sampling Laboratory Practical (Dr. Norman Munroe)
	Microbiology Laboratory Practical (Dr. Harold Speidel)
Monday, June 1, 1992	Lecture - Microbiology (Dr. Harold Speidel)
	Air Sampling Laboratory Practical (Dr. Norman Munroe)
	Microbiology Laboratory Practical (Dr. Harold Speidel)
Tuesday, June 2, 1992	Lecture - Carbon Monoxide and Carbon Dioxide Measurement
	Air Sampling Laboratory Practical (Dr. Norman Munroe)
	Microbiology Laboratory Practical (Dr. Harold Speidel)

Wednesday, June 3, 1992	Expected Delivery of Training Supplies
	Final Fitting of Analytical Work Stations as Described in Report of Week 4
	Laboratory Management Practice of Specific Stations
Thursday, June 4, 1992	Lecture - Ozone Measurements in the Atmosphere (Dr. Norman Munroe)
	Air Sampling Laboratory Practical (Dr. Norman Munroe)
	Microbiology Laboratory Practical (Dr. Harold Speidel)
Saturday, June 6, 1992	Lecture - Nitrogen Dioxide in the Atmosphere and Lead Determination in Particulate (Dr. Norman Munroe)
	Air Sampling Laboratory Practical (Dr. Norman Munroe)
	Microbiology Laboratory Practical (Dr. Harold Speidel)
	Lecture - Microbiology (Dr. Speidel)
	Microbiology Laboratory Practical GC Laboratory Practical with Standards (Dr. Speidel/Dr. Parker)
Monday, June 8, 1992	Lecture - Microbiology (Dr. Speidel)
	Microbiology Laboratory Practical Extraction Test in GC Analysis
Tuesday, June 9, 1992	Lecture - Microbiology (Dr. Speidel)
	Microbiology Laboratory Practical Final Evaluation of Students

June 20-25, 1992	New Equipment and Supplies
	Gas Chromatography Lecture and Laboratory Equipment Check and Installation (Dr. Cass Parker)
June 27-30, 1992	Gas Chromatography Lecture and Laboratory (continued) (Dr. Cass Parker)

SECTION VA

LECTURES/ LABORATORY SESSIONS

LECTURES ON GAS CHROMATOGRAPHY

Chromatographic Analysis

I. Quality Assurance/Quality Control

II. Statistical Treatment of Data

1. Mean
2. Standard deviation
3. Confidence Limits
4. Data Rejection
5. Results Testing
6. Least Squares

III. Laboratory Reporting of Data

- Notebook
- Record Keeping
- Report Writing

IV. Chromatography

1. An Overview of Separation Methods

- Gas, Liquid and Ion Chromatography

a. Gas Chromatography (GC)

i. GC Detectors and Application

- Flame Ionization Detector
- Electron Capture
- Electrolytic Conductivity
- Photoionization Detector
- Electrochemical Detector

ii. Columns

Packed
Macro
Capillary

V. Standard Methods of GC Analysis

1. Purgeable Halocarbons
EPA Method 601, SM 6230B

2. Purgeable Aromatics
   EPA Method 602, SM 6220B

3. Phenols
   EPA method 604, Sm 6420B

4. Acrolein & Acrolonitrile
   EPA Method 603

5. Phthalate Esters
   EPA Method 606

6. Nitrosamine
   EPA Method 607

7. Organochlorine
   EPA Method 608, SM 6630C

8. Polynuclear Aromatic Hydrocarbons
   EPA Method 610, Sm 6440B

9. Haloethers
   EPA Method 611

10. Chlorinated Hydrocarbons
    EPA Method 612

LECTURES ON AIR POLLUTION CONTROL AND MEASUREMENT

Lecture 1 Ambient Air Quality Standards

-Pollution Control Techniques
-Recent Air Pollution Control Technology
-Measurement of Stack and Fugitive Emissions
-Selecting a Control Device

Lecture 2 High Volume Method for the Determination of Particulate Matter in the Atmoshphere

-Principle of Operation
-Air Volume Measurement
-Flow Rate Measurement Devices
-Inherent Sources of Error
-Procedure
-Calibration of High Volume Sampler

Lecture 3 Calculations of Total Suspended Particulate Matter (TSP)

-Calculation of < 10 Micron Size Particulate Matter (PM_{10}) in the Atmosphere
-Test Specifications for PM_{10} Methods
-Operatomg Range
-Accuracy
-Flow Rate Calibration Procedure

Lecture 4 Method for the Determination of Sulfur Dioxide in the Atmosphere

-Principle of Method
-Range
-Decision and Accuracy
-Sampling Apparatus
-Analysis by Spectrophotometer
-Calculations

Lecture 5 Measurement Principle and Calibration Procedure for the Measurement of Carbon Monoxide in the Atmosphere

-Measurement Principle
-Calibration Procedure
-Apparatus

Lecture 6 <u>Measurement Principle and Calibration Procedure for the Measurement of Ozone in the Atmosphere</u>

-Measurement Principle
-Calibration Procedure
-Apparatus
-Procedure

Lecture 7 <u>Measurement Principles and Calibration Procedure for the Measurement of Nitrogen Dioxide in the Atmosphere</u>

-Principle and Applicability
-Sampling
-Calibration
-Apparatus
-Operating Procedure
-Calibration

Lecture 8 <u>Determination of Lead in Suspended Particulate Matter Collected from Ambient Air</u>

-Principle and Applicability
-Range, Sensitivity and Lower Deteutable Limit
-Interference, Precision and Bias
-Apparatus
-Sampling and Analysis

LECTURE/LABORATORY SESSIONS IN MICROBIOLOGY

LECTURE/LAB 1: BIOCHEMICAL OXYGEN DEMAND (BOD)

1. Definition of BOD

2. How BOD is used

3. Procedure (Method 5210, Standard Methods, 1989)
   a. Introduction
      General Discussion
      Sample Collection and Storage Requirements
      Carbonaceous/Nitrogenous BOD
      Dilution Requirements

   b. Five day BOD test
      General discussion
      Apparatus
      Reagents
      Procedure
      Sample Pretreatment
      Dilution Technique
      Calculations Control Limits and Working Range Concepts

LECTURE/LAB 2: CHEMICAL OXYGEN DEMAND (COD)

1. Introduction
2. Discussion of the various methods available
3. Selection of method to use
4. Sample collection and storage requirements
5. Open reflux procedure (Method 5220 B, Standard Methods, 1989)
6. Closed reflux colorimetric procedure (Method S220 D, Std.
7. Methods, 1989)

LECTURES 3-7: MICROBIOLOGY

1. Description of Microbes
2. History of Microbiology
3. Microbial Cell Structure
4. Microbial Metabolism

5. Measurement of Microbes in the Environment
   Sample Collectlon
   Sample Processlng
   Determining Microblal Numbers
   Determining of Microbial Biomass
   Measurement of Microbial Activity

6. Effect of Environmental Factors on Microbes

7. Microbes in the Environment (air, water, and soil)

8. Importance of Microbes in the Environment
   Microbial Role in Biogeochemical Cycling
   Microbes as Disease Agents
   Microbes as Pollution Indicators
   Bioremediation (using microbes to clean the environment)

9. Microbial Treatment of Wastewater
   BOD
   Aeorbic Treatment
   Anaerobic Treatment
   Tertiary Treatment
   Disinfection

10. Treatment and Safety of Water Supplies
    Water Quality Testing

LABORATORY SESSIONS: MICROBIOLOGY LABORATORY

1. Media Making, General Asepsis
2. Isolation and Purification Procedures
3. Gram's Stain Procedure
4. Total and fecal collform - most probable number procedure
5. Total and fecal collform - membrane filter procedure
6. QA/QC requirements for the Microbiology Lab

Special Lecture
"Water Pollution Remediation Processes"

1. Introduction
   A. Advantage of the use of treated wastewater for irrigation
      1. Advantage to the user
      2. Advantage to the supplier
   B. Description of the site
   C. Nature of the complaint

2. Design of the water quality assessment study
   A. Test to be used
      1. Field tests
      2. Laboratory tests
   B. Determination of sample numbers and location
   C. Illustration of the use of equipment

3. Results of assessment

4. Design of Remediation Process
   A. Evaluation of sulfide control methods
   B. Isolation of specific microbes required
   C. Installation of aerators

5. Initiating remediation processes
   A. Pilot tanks studies to evaluate selected processes
   B. Actual lake treatment

6. Results of remediation procedures

Atomic Absorption Analysis of Filter Extracts

Scope of Method: This procedure is to be used on filter extracts which have been prepared from the Filter Extraction Procedure which is provided on a separate sheet. The concentrations and instrumental conditions that are specified are for the digestion of 1/4 of a quartz high volume filter which has been digested in 1:1 nitric acid and diluted to a final volume of 250 ml (or 200 ml). Any change in the filter digestion procedure of in the final volume of the filter extract invalidates this procedure.

Note: The calculations in this procedure are based on a final dilution volume of 250 ml for the filter extract. If a final volume of 200 ml is used (German Volumetric Flasks instead of American Volumetric Flasks), the final calculated numbers should be multiplied by 1.25 to convert them to the correct values.

Experimental Procedure: Use class A volumetric pipets and class A volumetric flasks for all dilutions. Use either double distilled water or a solution of 10%-nitric acid in double distilled water to dilute all samples to final volume.

Prepare the following samples from the filter extract "stock" solution:

a 1:10 diluted stock solution (called "stock A")
a 1:20 diluted stock solution (called "stock B")
a 1:50 diluted stock solution (called "stock C") and,
a 1:100 diluted stock solution (called "stock D")

These solutions are to be prepared before attempting to run any analyses on the atomic absorption spectrophotometer. The stock solution (undiluted filter extract), and stock solutions A, B, C, will be used to analyze for Fe, Mn, Pb, Cu, Zn, Cr, V, Cd and V (depending on concentration). Stock solution b will be used to analyze for Ca and Mg.

Stock Solution A: Pipet 10.0 ml of the filter extract (stock solution) into a 100.0 ml volumetric flask and dilute to final volume with 10% HNO_3.
10% HNO_3.

Stock Solution B: Pipet 5.0 ml of the filter extract (stock solution) into a 100.0 ml volumetric flask and dilute to final volume with 10% HNO_3.
10% HNO_3.

Stock Solution C: Pipet 2.0 ml of the filter extract (stock solution) into a 100.0 ml volumetric flask and dilute to final volume with 10% HNO_3.
10% HNO_3.

Stock Solution D: Pipet 500 µl of the filter extract (stock solution) into a 100.0 ml volumetric flask. Add 10.0 ml of 10,000 PPM La solution and 10.0 ml of Cs solution (10,000 PPM) or KCl solution) and dilute to final volume with double distilled water.

AA Analysis: Install the connector lamp in the atomic absorption, and setup the instrument for operation in absorbance mode. Use the sensitivity check solution to insure that the instrument is working properly, and then switch to concentration mode. Enter the standards into the instrument microprocessor (using the multiple standard calibration method) and then the metal concentrations for the samples using the stock (filter extract) solution. Note any samples that have concentration values that are larger than your highest standard (the instrument "over calibration" light will display), and re-run these samples with stock solution A. If the concentration is still too high, re-run the sample with stock solution B, and if it is still too high, re-run the sample with sock solution C. In your laboratory notebook, record both the metal concentration and the solution from which this value was obtained. Use std #1 to prepare 5.00 PPM, 1.00 PPM and 0.50 PPM standards for Mn, Pb, Cu and Zn and use these solutions to prepare the standard curves. Use Std 2-A, 2-B and 2-C to prepare standard curves for Fe. For calcium and magnesium analysis, the standard solutions are prepared from standard solution #3 (5.00 PPM, 1.00 PPM and 0.50 PPM). The analysis for calcium and magnesium is done on stock solution D. Table 1 lists the operating conditions for the spectrometer.

Table 1. Perkin Elmer 2850 Operating Parameters

Metal	λ	Slit	Flame	Nebulizer	Sen Check
Ca	422.7	0.7	Blue, lean, oxidizing*	Flow Spoiler	4.0
Cu	324.8	0.7	Blue, lean, oxidizing*	Flow Spoiler	4.0
Fe	271.9	0.20	Blue, lean, oxidizing*	Flow Spoiler	15.0
Mg	285.3	0.70	Blue, lean, oxidizing*	Flow Spoiler	0.30
Mn	279.5	0.20	Blue, lean, oxidizing*	Flow Spoiler	2.5
Pb	217.0	0.70	Blue, lean, oxidizing*	Flow Spoiler	9.0
Zn	213.9	0.70	Blue, lean, oxidizing*	Flow Spoiler	1.0

*Fuel setting should be 17-18. Oxidant setting should be 45-46.

Graphite Furnace Analysis: Cr, Cd, Ni, V. Prepare 4 clean 10.00 ml volumetric flasks. Into flask A pipet 5.0 ml of the filter extract solution (stock solution), and dilute to final volume with double distilled water. Pipet 5.0 ml of the filter extract solution (stock solution) into flask B, add 100µl of Std #4 and dilute to volume with double distilled water. Into flask C pipet 5.0 ml of 10% nitric acid solution and

dilute to volume with double distilled water. Pipet 5.0 ml of 10% nitric acid solution into flask D, add 100 µl of Std #4 and dilute to volume with double distilled water.

Install the graphite furnace into the atomic absorption spectrophotometer, install the correct lamp and setup the instrument for operation. Use the sensitivity check sample to insure proper operation of the instrument, and then record triplicate (3) absorbencies for the flasks A, B, C and D prepared above. See footnotes for individual element conditions.

Calculations: The calculations for the percentage composition of the material nitric acid extract solution are shown below. Since only 1/4 of the filter was analyzed, this number is multiplied by a correction factor to jive the total mass of element on the filter.

Calculation for Concentrations from the Filter Extract or Stock Solution:

$$\text{(Reported Value)} \times 250 \times \frac{\text{Mass of total Filter}}{\text{Mass of Filter Digested}} = \text{Total mass of metal on filter}$$

Calculation for Concentrations from the Stock Solution A:

$$\text{(Reported Value)} \times 250 \times 10 \times \frac{\text{Mass of total Filter}}{\text{Mass of Filter Digested}} = \text{Total mass of metal on filter}$$

Calculation for Concentrations from the Stock Solution B:

$$\text{(Reported Value)} \times 250 \times 20 \times \frac{\text{Mass of total Filter}}{\text{Mass of Filter Digested}} = \text{Total mass of metal on filter}$$

Calculation for Concentrations from the Stock Solution C:

$$\text{(Reported Value)} \times 250 \times 50 \times \frac{\text{Mass of total Filter}}{\text{Mass of Filter Digested}} = \text{Total mass of metal on filter}$$

Calculation for Concentrations from the Stock Solution D:

$$\text{(Reported Value)} \times 250 \times 200 \times \frac{\text{Mass of total Filter}}{\text{Mass of Filter Digested}} = \text{Total mass of metal on filter}$$

Calculation for Graphite Furnace Data

Use the formula for a single data method of additions (see lecture notes of April 29) to calculate the concentration of the metal in flasks A and C from graphite furnace analysis described above.

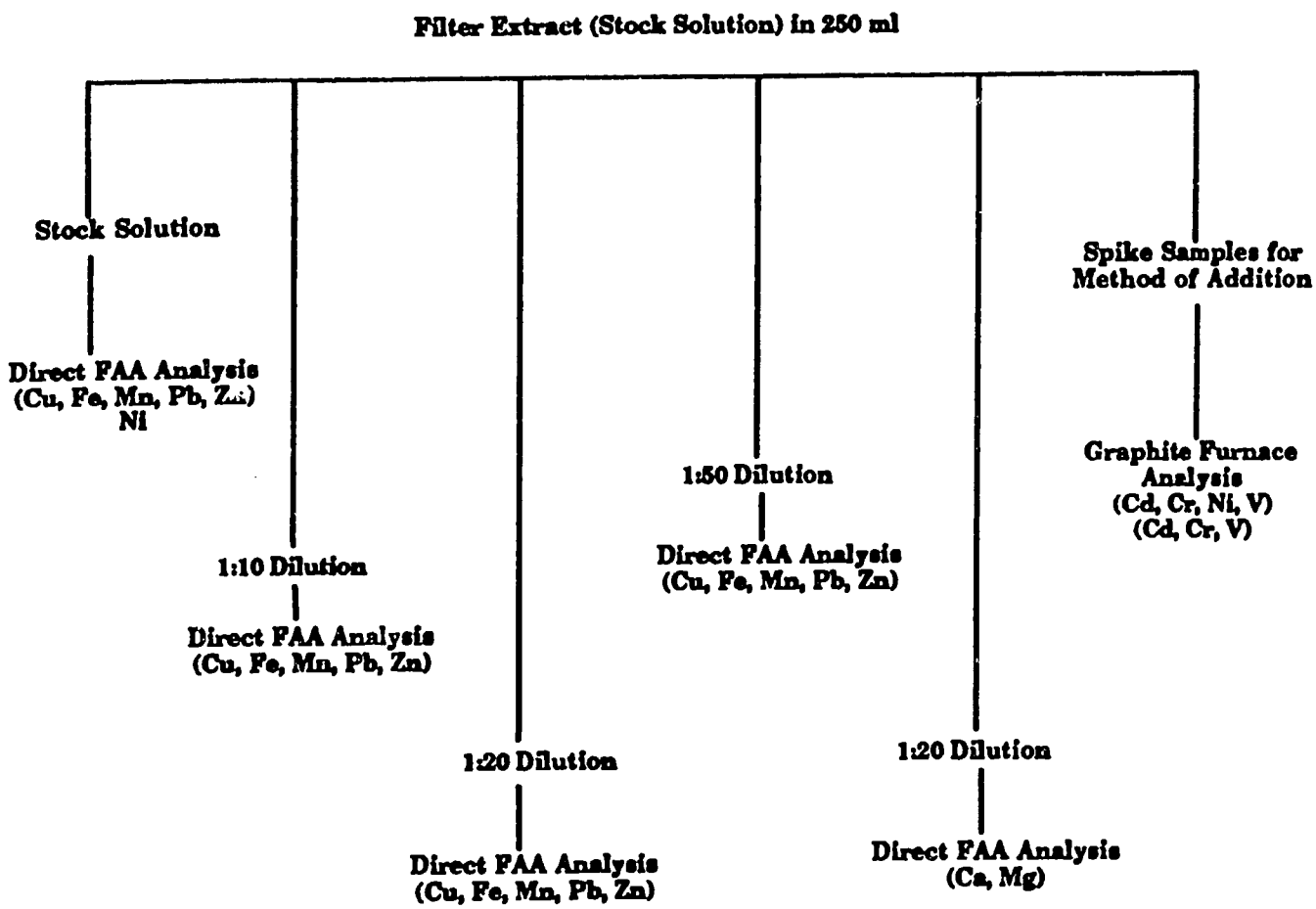
$$C = X \cdot \frac{S}{Y - X}$$

C is the correct concentration in Flask A or C
S is the concentration of added metal (100 PPB)
X is the concentration of metal, as read from a distilled water std curve, in flask A or C
Y is the concentration of metal, as read from a distilled water std curve, in flask B or D (the spiked flasks).

The corrected concentration in flasks A and C will be called C_a and C_c respectively. The total mass of metal on the filter for metal analyzed by graphite furnace is then calculated as follows:

$$(C_a - C_c) \times 250 \times 2 \times \frac{\text{Mass of total Filter}}{\text{Mass of Filter Digested}} = \text{Total mass of metal on filter}$$

Flow Chart of Procedure for Air Filter and Dust Fall Analysis



Note: The direct FAA analyses in the diluted stock solutions are only run on those metals where the observed concentration exceeded the concentration of the highest standard. Most samples should not require any analyses to be run at the 1:20 or the 1:50 dilutions.

Solution to be used for Filter and Dust Fall Analyses

The following list of solutions (100.0 ml) should be prepared and stored on the "Working Standard Shelf."

Std #1 contains the following Metals at 100.0 PPM each: Mn, Pb, Cu and Zn and N:

Std #2-A Fe at 100.0 PPM
Std #2-B Fe at 50.0 PPM
Std #2-C Fe at 10.0 PPM
Std #3 Contains 100.0 PP Ca and Mg
Std #4 Contains the following Metals at 10.0 PPM each: Cr, V, Cd and Ni

Lanthanum Oxide at 10,000 PPM
Cesium Chloride at 10,000 PPM
(Potassium Chloride can be substituted if cesium chloride is not available).

Std #1 100 PPM Mn, Pb, Cu, Zn, Ni from this solution prepare daily:

5.00 PPM
1.00 PPM
0.50 PPM

Std #3 100 PPM Ca & Mg from this solution prepare daily:

5.00 PPM + 1000 PPM La + 1000 PPM Cs
1.00 PPM + 1000 PPM La + 1000 PPM Cs
0.50 PPM + 1000 PPM La + 1000 PPM Cs
0.00 PPM (Cs + La Blank)

Std #4 10.0 PPM Cr, V, Cd, Ni from this solution prepare daily:

1:00 PPM solution that will be used as a spoiler sample for Graphite Furnace Method of additions.

Directions for preparing "Working Stock Solutions").

Working Stock Solution #1: Pipet 10.0 ml of 1000 PPM Mn, 10.00 ml of 1000 PPM Pb, 10.00 ml of 1000 PPM Ni, 10.00 ml of 1000 PPM Cu and 1000 PPM Zn into a 100.0 ml volumetric flask and dilute to volume with 10% nitric acid solution. Store in a 100 ml polyethylene bottle.

Working Stock Solution 2-A: Pipet 10.0 ml of 1000 PPM Fe into a 100.0 ml volumetric flask and dilute to volume with 10% nitric acid solution.

Working Stock Solution 2-B: Pipet 5.00 ml of 1000 PPM Fe into a 100.0 ml volumetric flask and dilute to volume with 10% nitric acid solution.

Working Stock Solution 2-C: Pipet 1000 µl of 1000 PPM Fe into a 100.0 ml volumetric flask and dilute to volume with 10% nitric acid solution.

Working Stock Solution 3: Pipet 1000 µl of 1000 PPM Ca and 1000 PPM Mg into a 100 ml volumetric flask and dilute to volume with double distilled water. Store in a 100.0 ml polyethylene bottle.

Working Stock Solution 4: Pipet 1000 µl of 1000 PPM Cr, 1000 µl of 1000 V, 1000 µl of 1000 PPM Ni into a 100.0 ml volumetric flask and dilute to volume with 10% HNO_3 solution. Store in a polyethylene bottles.

10,000 PPM Lanthanum: Place 1.173g La_2O_3 in a 100.0 ml volumetric flask and add double distilled water to cover the white powder. Add concentrated nitric acid drop-wise until the powder is dissolved, add one extra ml of HNO_3, dilute to volume with double distilled water and store in a polyethylene bottle.

10,000 PPM Cesium: Place 1267g of CsCl in a 100.0 ml volumetric flask and dilute to volume with double distilled water (use 1.907g KCl for potassium solution).

Prepare the following solutions the day the analysis is to be run:

5.00 PPM Std #1 (pipet 500 µl of 100 PPM stock into a 10.00 ml volumetric flask and dilute to volume with a solution of 10% HNO_3 in double distilled water).

1.00 PPM Std #1 (pipet 100 µl of 100 PPM stock into a 10.00 ml volumetric flask and dilute to volume with a solution of 10% HNO_3 in double distilled water).

0.500 PPM Std #1 (pipet 50 µl of 100 PPM stock into a 10.00 ml volumetric flask and dilute to volume with a solution of 10% HNO_3 in double distilled water).

5.00 PPM Std #3 (pipet 500 µl of 100 PPM stock into a 10.00 ml volumetric flask, add 1,000 µl of 10,000 PPM La solution and 1,000 µl of 10,000 PPM Cs solution (or potassium substitute) and dilute to volume with double distilled water.

1.00 PPM Std #3 (pipet 100 µl of 100 PPM stock into a 10.00 ml volumetric flask, add 1,000 µl of 10,000 PPM La solution and 1,000 µl of 10,000 PPM Cs solution and dilute to volume .

0.500 PPM Std #3 (pipet 50 µl of 100 PPM stock into a 10.00 ml volumetric flask, add

1,000 µl of 10,000 PPM La solution and 1,000 µl of 10,000 PPM Cs (or potassium) solution and dilute to volume .

Blank for Std #3 (pipet 1,000 µl of 10,000 PPM La solution and 1,000 µl of Cs solution (or potassium) into 10.00 ml volumetric flask and dilute to volume.

1.00 PPM Std #4 (pipet 1,000 µl of 10.0 PPM stock solution into a 10.0 ml volumetric flask and dilute to volume with 10% HNO_3 solution.

Laboratory Quality Assurance/Quality Control

Notebook

The laboratory notebook should be a permanently bound, ruled notebook. Preferably with pages numbered, with an entry line for date and signature. Numbered page encourage data entry in a sequential manner. Numbered pages also encourage referencing of results to earlier pages, dates and samples. Final results should be validated by requairing the analyst to date and sign each analsyis upon compeltion. A co-signature by the laboratory supervisor or manage strrengthens validation of data.

Reports

Final reports are best handled using preprinted forms or internal standard format. Forms will differ depending on analytical methodology. Forms should indicate report date, sample number, sample source, sample volume, sample collection and analysis date, and results for analyzed species. Internal reports will also reference laboratory notebook number and data pages. Full reports will also reference method, abbreviated procedure, calibration results and quality control/quality assurance protocol.

QA/QC Protocol

Quality Control is assured by adhering to stringent guidelines with regard to

-instrumentation
-glassware
-reagents
-solvents
-gases
-sample collection
-approved methodology
-data reporting

Quality Assurance is assured by adhering to strict laboratory procedures and practices.

-Standard curves
-QC check: Blanks, midpoint standards. spikes and duplicate analysis
-Interlaboratory QC
    -Reference Standards analysis

-Performance evaluation and method studies

A full quality assurance plan will cover

-sample control and documentation

-standard operating procedures (SOP)

-training requirements (minimum for analytical expertises)

-equipment maintenance

-analytical methodology

calibration procedures

correction procedures

quality control

performance audits

data assesment

bias and precision

-report writing

data reduction

validation

reporting

Chromatography

A method of separation due to two mutually immiscible phases that are brought into contact with each other: a mobile phase and a stationary phase. When the two phases are properly chosen separation of the components in the mobile phase occurs.

Strictly speaking, physical and chemical properties of the mobile phase and stationary phase determines the degree of separation.

-size (molecular)

-polarity

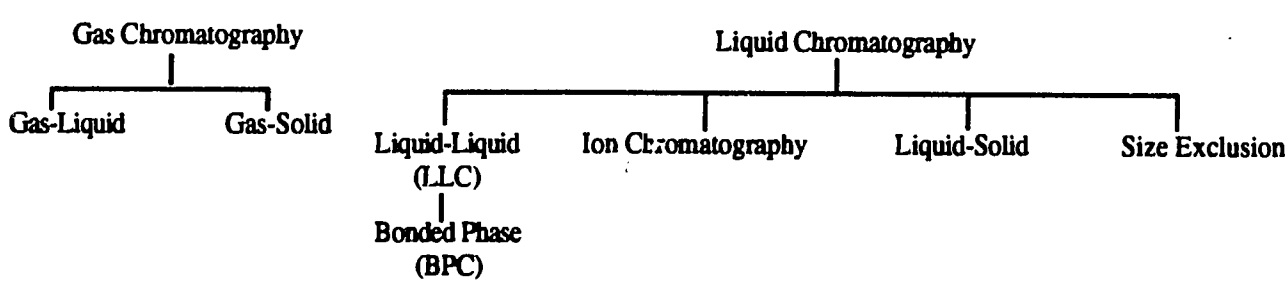
-molecular interaction

Dipole-Dipole

van der Waals

London Dispersion Forces

Chromatography methods



Familiar Terms

Retention Volume-V_r-the volume of mobile phase necessary to convey a solute band from point of injection to detection.

$V_R = t_r F_C$ where t_r is retention time and F_c is the volumetric flow rate. The volumetric flow rate is determined by

$$F_C = (\pi d^2_c/4)\ (\varepsilon_{tot})\ (L/t_M) = V_{col}\, \varepsilon_{tot} / t_M$$

i.e F_C = (cross section) (porosity) (linear velocity)

In the above equation, ε is the ratio of the interstitial volume of packing to the volume of its total mass. Normal values for ε are .35 to .45 for solid packing and .70 to .90 for porous packing. t_M is the transit time of a non-retained solute (no material should elute prior to) and t_M is directly proportional to V_M, the dead volume. Therefore, $V'_R = V_R - V_M$ or $t'_r = t_r - t_M$.

Partition Coefficient-K = C_s/C_M- equilibrium distribution of the solute concentration in the stationary phase and mobile phase. Realize that an ideal case at the detector K = 1, equal partitioning.

$$V_R C_M = V_M C_M + V_S C_S$$

rearranging this equation, and subbing K, we obtain the first equation of chromatography

$$V_R = V_M + KV_S \text{ or } V_R - V_M = KV_S$$

This equation will hold true for liquid partition columns, but V_S should be replace by A_S (surface area of adsorbent) for adsorption columns.

Capacity factor or partition ratio = k'_A - describe the migration rates of analytes on a column.

$k'_A = K_A V_S/V_M$ $\qquad$ $k'_A = t_r - t_M/t_M$

when $k'_A < 1$ elution occurs too fast

when $k'_A > 20$ elution takes too long

ideal k'_A ranges from 1 to 5

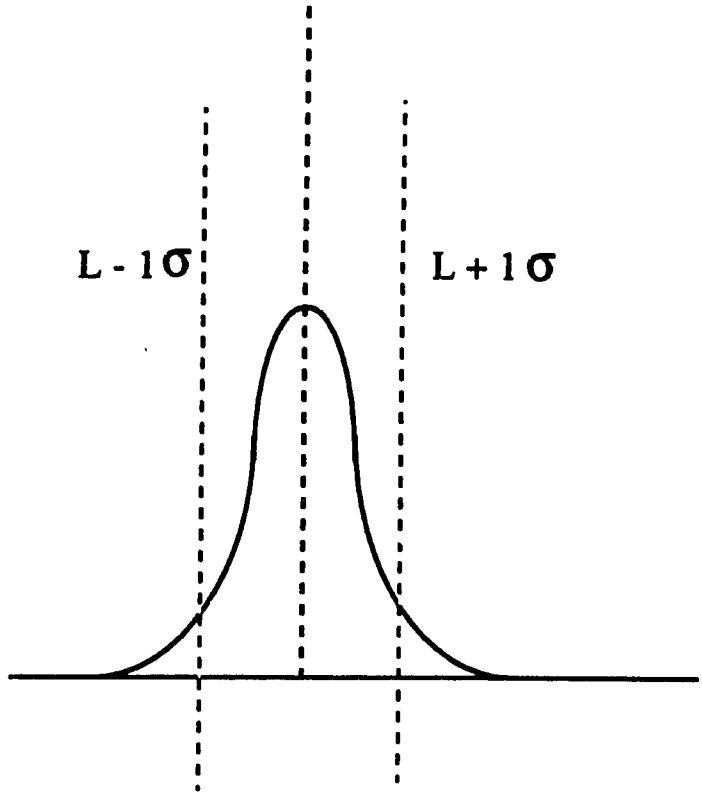
Selectivity factor - α - or relative retention

$\alpha = k'_2/k'_1 = K_2/K_1 = V_{R2}/V_{R1} = t_{r2}/t_{r1}$

Column Efficiency

Plate Height - H or theoretical plates = N

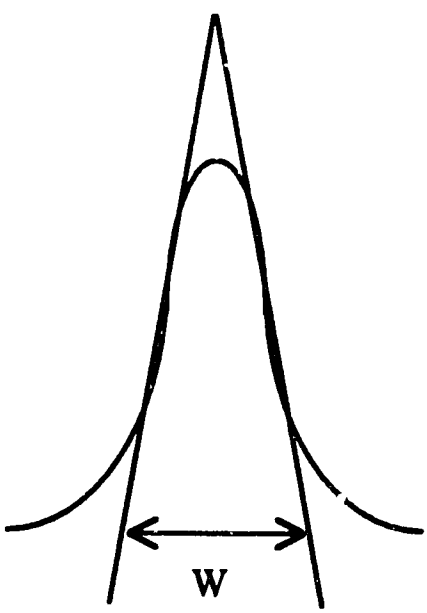
The two terms are related by N = L/H, plate height is related to the elution of a band from a column, i.e. it is a Gaussian distribution in which



$$H = \frac{\sigma^2}{L}$$

σ (sigma) the variance of the concentration in unit length of the column.

Plate height is determine experimentally from a chromatogram.



$H = LW^2/16t^2_R$

and W is the base width of a resolved peak

From this one can derive a simple relationship for the number of theoretical plates,

$N = 16(t_R/W)^{1/2}$ or $N = 5.54(t_R/W_{1/2})^2$, where $W_{1/2}$ = FWHM

Variables that Affect Column Efficiency

linear velocity of mobile phase	μ	$cm\ s^{-1}$
diffusion coefficient in mobile phase	D_M	$cm^2 s^{-1}$
diffusion coefficient in stationary phase	D_S	$cm^2\ s^{-1}$
capacity factor	k^{-1}	unitless
diameter of packing particles	d_p	
thickness of liquid coating on stationary phase	d_f	

Column efficiency can be approximated by $H = B/u + C_S\mu + C_M\mu$, the van Deemter equation. B/μ is the longitudinal diffusion, which is dependent on the analytes diffusion coefficient, D_M

$$B/\mu = 2k_D D_M/\mu$$

k_D is an obstruction factor of the column packing, for packed columns k_D is approximately .6 and for capillary columns $k_D = 1$.

C_S and C_M are mass transfer coefficients

$C_S\mu$ (liquid stationary phase) $= qk'\ d^2_f\ \mu\ /\ (1+k')^2\ D_S$

$C_S\mu$ (solid stationary phase) $= 2t_d\ k^i\ \mu\ /\ (1+k')^2$

$C_M\mu$ (mass transfer) $= f\ (d^2_f\ d^2_c\ \mu)\ \mu\ /\ D_M$

Bottom line is that to control column efficiency, one can reduce the diameter of the column particles and reduce the diameter of the column. In GC, operate at lower temperatures to reduce longitudinal diffusion.

Resolution

The ability to separate two analytes

$R_S = 2\Delta Z/W_A + W_B$, where ΔZ is the peak separation and $W_A + W_B$ are the peak widths. An ideal case occurs when $W_A = W_B$. In such a case the resolution becomes

$R_S = \Delta Z/W = (t_R)_B - (t_R)_A/W$

which can be modified by substitution of other equations to give

$R_S = \sqrt{N}/4\ ((\alpha - 1)/\alpha)\ (k'_B/1 + k'_B)$

The above equation shows that resolution is dependent of several terms;

= selectivity factor = K_B/K_A

k'_B = capacity factor = $K_B V_S/V_M$

and $\sqrt{N}$, the number of theoretical plates = L/H

To improve resolution we can adjust the variable a, k' and N (or H) to improve resolution.

If N is increased, H is reduced.

k' is improved by an increase in T in GC or change in solvent composition in LC.

α is improved (increased) by several factors

1. Change in the mobile phase
2. Change in temperature
3. Change in the stationary phase
4. Chemical effects

Remember k' optimum value is 1 to 10 and that k' and q are closely related.

Analysis

Qualitative analysis is by the retention time only, which requires extensive standards for a particular analysis.

Quantitative analysis by chromatography has very good accuracy and precision. Quantitatively one can use peak height or peak area. Peak height has very good precision, but is very dependent on column temperature, flow rate and rate of injection. Errors can be 5 -10% due to injection alone. Peak area measurement has higher precision and accuracy. However, it is more time consuming, and requires electronic data handling.

Calibration

Calibration is achieve through the use of standards. A simple plot of concentration versus peak height or peak area will give a calibration curve for a set of standards in order to determine the concentration of the unknown. However, since there are errors due to inconsistencies in injection volume, we must modify our analysis. Use of an internal standard avoids the problem of inconsistent volume injections. With an internal standard the sample is spiked with an uninterferring known (retention and concentration) and the relative response factors are calculated for the calibration standards.

$$RF = (A_s)(C_{is})/(C_s)(A_{is})$$

Where superscript $_s$ refers to the sample and superscript $_{is}$ is the internal standard. The internal standard is also added to the unknown and the concentration is determine from:

$$C_{UK} = (A_{uk})(C_{is})/(A_{is})(RF)$$

AIR POLLUTION CONTROL

BY

NORMAN D. H. MUNROE

SPECIAL LECTURE PRESENTED AT THE
TABBIN INSTITUTE OF METALLURGICAL STUDIES
(TIMS)
CAIRO, EGYPT

May 16, 1992

ABSTRACT

Federal, state and local laws have been enacted to limit releases during the past two decades, but only recently has there been a sense of urgency. The U.S. Clean Air Act Amendments of 1990 have mandated the installation of maximum achievable control technology at industries considered by EPA to be major emitters of toxic chemicals. Emissions from stacks or "point" sources have been difficult to measure and "non-point" source emissions have proven to be even more difficult to measure. The techniques utilized in controlling air pollutants are summarized and recent air pollution control technologies are discussed. Some process parameters that should be evaluated before emissions can be characterized and considerations that should be taken in selecting a pollution control device are presented.

Introduction

In the wake of the Emergency Planning and Community Right-to-Know Act of 1986, U.S. companies have been required to report chemical releases annually The U.S. Clean Air Act Amendments of 1990 have mandated the installation of Maximum Achievable Control Technology (MACT) at industries considered by EPA to be major emitters of about 190 specified toxic chemicals. Emission reductions of 10 million tons/year of sulfur oxides (SO_x) and 3 million tons/year of nitrogen oxides (NO_x) were planned for the main components of acid rain. Approximately 79% of all SO_x emissions (20.7 million metric tons) resulted from fuel combustion and 16% were from industrial processes. 55% of NO_x emissions resulted from non-transportation fuel combustion (19.8 million metric tons) and 3% from other industrial processes [1,2].

The air pollutants for which National Ambient Air Quality Standards (NAAQS) have been established are SO_x , NO_x, carbon monoxide (CO), ozone (O_3), lead (Pb) and particulate matter (PM_{10}). Table 1 illustrates the National Primary and Secondary Ambient Air Quality standards for these air pollutants.

Table 1 National Primary and Secondary Ambient Air Quality Standards for Some Air Pollutants [3].

Air Pollutant	Annual Arithmetic Mean		24-Hour Av.		Hourly Av.	
	ug/m^3	ppm	ug/m^3	ppm	ug/m^3	ppm
SO_2	80	0.03	365	0.14	-	-
NO_2	-	-	-	-	100	0.053
CO*	-	-	-	-	10	9
O_3	-	-	-	-	235	0.12
Pb**	1.5	-	-	-	-	-
PM_{10}	50	-	-	-	150	-

* 8 - Hour TWA
** Calendar quarter

Pollution Control Techniques

As regulatory requirements for cleaner air increases, so will the need for emission control technologies. The techniques that are most commonly employed in controlling air pollutants [4] are summarized in Table 2.

Table 2 Technologies for Controlling Air Pollutants

	ORGANIC VAPORS	INORGANIC VAPORS	PARTICULATE MATTER	SO_x NO_x
Incineration	x			
Adsorption	x			
Condensation	x			
Absorption	x	x		
Filtration (Baghouse)			x	x
Electrostatic Precipitation			x	x
Wet Scrubbing		x	x	x
Combustion Modification				x
Chemical Reduction				x

Scrubbing is the most common means of fluegas cleanup for incineration processes. Most plants handle air emissions in one of three ways: an electrostatic precipitator, followed by wet scrubber; a semidry scrubber, wherein lime is atomized with flue gas, followed by a bag filter; or dry scrubbing, using calcium hydroxide. In West Germany, if a unit is out of compliance for 30 minutes, it must reduce its throughput; if it stays out for 1 hour, it must shut down.

Recent Air Pollution Control Technology

The latest trend coming out of West Germany is to employ enhanced wet scrubbing using lime solution in the first scrubbing stage and sodium hydroxide in the second stage as a means to further reduce SO_2 emissions [5].

Dry scrubbing with bicarbonate of soda is being used to remove acid gases from combustion processes [6]. The new system treats off gases from combustion processes that contain heavy loadings of halogen acids and sub micron metal oxide fumes. Bicarbonate powder sprayed into the off gasses neutralizes the acid gases and the dry reagent provides for the baghouse a filter coating that effectively removes metal oxide fumes. Discharge from the baghouse is nontoxic and suitable for landfill disposal.

In-Duct scrubbing [6] for the removal of sulfur from flue gas involves the atomization of calcium hydroxide slurry to droplets < 100 um in diameter. The high surface area of $Ca(OH)_2$ enhances the neutralization of SO_x in less than 2 seconds, producing calcium sulfate and calcium sulfite granules that can be removed by baghouse or electrostatic precipitator.

Still in its testing stage is calcium silicate injection [6] for the removal of sulfur from flue gas. Portland Cement is injected as a powder or mixed with coal/water slurry so that the stoichiometric amount of calcium is twice that of sulfur. Burning of the cement/fuel in a cyclonic burner, results in over 90% of the sulfur reacting with calcium silicate to form calcium sulfate silicate. The waste product - a mixture of calcium sulfate silicate, calcium silicate and ash could be used as a low-cost cement.

Measurement of Stack and Fugitive Emissions

Emissions from stacks or "point sources" are hard to measure for a variety of reasons. Those from "non-point" sources, such as valves, seals and flanges are even more difficult. Estimating models [2] have been developed to quantify emissions from "area" sources such as lagoons and waste water treatment plants. The best way, is to measure emissions directly. Few firms however, have the capabilities to measure emissions directly, because the complex and time consuming procedures require personnel trained to use specialized chemical analysis and flow-measurement instruments.

Companies are therefore resorting to estimation techniques for determining releases, such as mass balances and emission factors. In the emission factors method, standard leakage rates for different equipment in various process conditions and applications are established and set.

Selecting a Control Technology

There are numerous control options for NO_x and SO_x as shown in Table 2. In choosing the most cost-effective control device, the following steps should be taken: clearly define the problem; gather relevant data on emissions; gather relevant data on process parameters; research alternative control approaches; assemble costs of alternatives; determine energy and environmental effects. Some emission data and process parameters used to characterize air pollution are illustrated in Table 3.

Cost

One of the most important criteria on which a control device is judged is cost. This may be expressed in terms of initial capital cost, annual operating expense and cost effectiveness. The latter is defined as follows:

$$\text{Cost Effectiveness} = \frac{\text{Annual Operating Expense}}{\text{Total Tonnage of Emission}}$$

and is a convenient way to reduce control issues to a common denominator that allows comparison of options.

Table 3 Emission Data and Process Parameters for Characterization of Pollution Problem

EXHAUST STREAM	POLLUTANT
Pollutant Concentration	Particle size distribution
Flow rate	Molecular weight
Temperature	Vapor Pressure
Pressure	Solubility
Moisture content	Adsorptive properties
Oxygen content	Lower Explosive Limit (LEL)
Heat content	Reactivity
Corrosiveness	
Explosivity	

Energy Impact

The process fuel equivalent (PFE) which is a measure of all the energy inputs that are required by a device to reduce a unit of pollutant. The PFE is defined as follows:

$$PFE = F + E + S - B$$

where:

- F = Energy content of fuel (GJ/Ton)
- E = Electricity requirement(1 kwh = 11.1EE6 J/hr)
- S = Supplies, Eg. $Ca(OH)_2$, $NaHCO_3$ etc.
- B = By-product energy, Eg. Steam

Secondary Environmental Impacts

The type of solid or liquid waste that are generated by the proposed control device must be taken into consideration so as not to pose a disposal problem. Furthermore, if additional air emissions are generated by the device itself such as from an incinerator, emission characteristics outlined in Table 3 must be determined.

HIGH VOLUME METHOD: DETERMINATION OF SUSPENDED PARTICULATE MATTER

Principle: A measured quantity of ambient air is drown into a covered housing and through a filter during a 24 hour (nominal) sampling period (1440 Mins) aerodynamic diameter (25-50 µm).

- dependent on wind speed and direction
- minimum collection efficiency = 99% for 0.3 µm
- weigh filter after to find mass (moisture equilibration before and after sample)
- convert volume if air sample to EPA Standard Conditions (25°C, 76°mm Hg (101kPa) volume time sampling (v x t).

$$\text{Total suspended particulate matter} = \frac{\text{Mass Collection}}{\text{Volume of Sampled Air}}$$

Expressed as micrograms per standard cubic meter (µg/std. m^3)

Range: Concentration range of method. (2-750 µg/std m^3)

Upper Limit: The point at which flow rate due to increased pressure drop across the loaded filter becomes appreciable due to:

Particle Size Distribution

- Particle size distribution
- Moisture content of particles
- Variability of filter

Lower Limit: Sensitivity of Balance.

Sources of Error:
- Air Flow Variation
- Air Volume Measurement

N.B. (a) minimized by using a constant air flow controlling mechanism
(b) use a calibrated continuos flow rate recording device
(c) continuos flow recorder

Loss of Volatiles

- During sampling, storage and shipment

Formation of Artifact Particular Matter

- Due to oxidation of acid gases in the sampled air resulting in higher than true TSP

Humidity

Use glass fiber filters - insensitive to changes in relative humidity.

- Particles could be hydroscopic
- Moistures conditioning procedure minimizes error

Filter Size

8" X 10" (20.3 cm X 25.4 cm) = 515.62 cm^2
Nominal exposed area 406.5 cm^2

Material

Glass fiber or relatively inert non-hydroscopic material. Recommended pressure drop at 1.5 std m^3/min ≈ 50 SCFM
42 - 54 mm Hg or (5.6 – 7.2 Kpa) 15" – 20" H_2O

Filter Conditioning Environment

15 - 30°C < 3°C Variation
Humidity < 50% RH ± 5%
Leave at Least 24-Hours

(i) Weigh after equilibration
(ii) DO NOT BEND FILTER OR FOLD

DETERMINATION OF AMBIENT PARTICULATE MATTER

Introduction

On July 1, 1987, the U.S. Environmental Agency (USEPA) promulgated a new size-specific air quality standard [1], for ambient particulate matter. This new primary standard applied only to particles with aerodynamic diameters, less than, or equal to, 10 micrometers PM10), and replaces the original rules for total suspended particulate matter (TSP). This new primary standard of 150 μg per standard cubic meter (μg/std. m^3) during a 24-hour period was adopted to protect human health. It is believed that particles of aerodynamic diameter of ≤ 10μm are able to reach the lower regions of the human respiratory track, and thus be responsible for most of the adverse health effects associated with suspended particulate pollution. The secondary standard used to assess the impact of particulate pollution on public welfare has also been established at 150 μg/std m^3.

In order to measure concentrations of PM10 particles, the EPA also promulgates a Federal Reference Method (FRM). The FRM describes the performance requirements for all PM10 samples, which must meet some specified requirements [2]. Only particles ≤ 10μm are drawn through the inlet, and a constant, controlled flow rate is maintained by either a mass flow controller (MFC) or volumetric flow controller (VFC). Particles are collected on a micro-quartz fiber filter that is equilibrated and weighed before (tare) and after good sampling to determine the weight (net mass) gain of the sample. Sampling duration is either controlled by a times accurate to ±15 mins over a 24-hour sample period or measured by an elapsed time indicator.

The operational flow rate (sample volume) must be corrected to USEPA reference conditions (298 K, 760 mm Hg). Figure I shows a schematic diagram of the basic elements of model 1200 VFC HVPM sampler. As ambient air is drawn into the inlet, it is evacuated from the buffer chamber through nine acceleration nozzles into the impaction chamber where particles larger than 10μm are impacted onto a greased collection shim. The acceleration nozzles have critical diameters calculated and performance tested to provide the necessary velocity to effect correct particle size fractionation within the impaction chamber.

As a result, the air velocity of ambient air passing through the device is critical in order to maintain a PM10 cut point within the inlet. It is therefore essential to maintain the correct design flow rate of 1-13 m^3/min ±10% at actual conditions.

The air flow rate can be controlled and maintained by a volumetric flow controller (VFC). Flow control is accomplished by occluding, and accelerating the air flow through the venturi. Critical flow is achieved when the air velocity equals the acoustic velocity so that small changes in the down stream flow do

not adversely affect the actual flow rate of 1-13 m^3/min over a sample period; i.e. critical flow through the venturi is not greatly affected by changes in filter loading, ambient temperature or barometric pressure.

Calibration

In order to determine the sampler's operational flow rate (as required by the FRM) a calibration must be conducted with the motor disconnected from the mass flow controller, and without a filter on the filter cartridge in the sampler.

Check for Leaks

The inlet of the orifice unit is covered with duct tape, and the manometer valves are fully closed. With the motor of the blower running, the orifice plate is wiggled. The absence of a whistling sound and no response on the recorder are indications that the sampler system is leak-free.

The sampler is equipped with two calibration relationships: a) Actual Qa and seasonally adjusted sQa. The calculated flow rates are obtained by using the Sampler Calibrator Parameters in m and b (orifice calibration relationship slope and interrupt) and the relationship:

$$Q_a = \left[\sqrt{(\Delta H_2O\, T_a/P_a)} - b\right]/m$$

Where Ta = Actual Temp. ambient, K); Pa = Actual barometric pressure mm Hg
m = Orifice Calibration Slope
b = Orifice Calibration Intercept

The sampler's response I (which could be any event recorder of arbitrary units) is noted and the actual corrected valve Ic is noted for each calibration point. The letter can be changed by rotating the plate in the orifice calibration unit into different positions.

$$Ic = I\,[(Ta/Pa)^{1/2}]$$

Where Ic = Actual Correction
I = Recorder response, arbitrary units (1)

A plot of the sampler corrected recorder units Ic(Y-axis) versus the corresponding calculated orifice flow rates Qa (X-axis) provides a visual calibration curve and an indication a of the calibration linearity. A five point calibration should yield a regression equation with correlation coefficient of r > 0.990. Since the determination of a Qa flow rate requires the addition of an ambient average temperature and pressure correction, it is not recommended to use a graphic plot of the calibration relationship for subsequent data deduction.

Each sampler therefore must have its own mathematical expression that indicates the slope, interrupt and the linearity of the calibration relationship. The best-fit straight line by the method of least squares obtained, where the equation is:

$$Ic = M(Q_a) + b$$

Where m = Sampler's Calibration Slope (ambient)
b = Sampler's Calibration Interrupt (ambient) (2)

The slope m and the interrupt b, are then calculated to determine the sampler's actual flow rate (Q_a) from:

$$Q_a = 1/m\ [(T_a/P_a)^{1/2}] - b \qquad (3)$$

Corrections for daily temperature and pressure fluctuations are avoided by determining the sampler's operational flow rate by adjusting the sampler's slope and interrupt to seasonal average conditions:

$$m_s = m/\left[\sqrt{(T_s/P_s)}\right] \qquad (4)$$

$$b_s = b/\left[\sqrt{(T_s/P_s)}\right] \qquad (5)$$

Where m_s = Seasonally adjusted sample calibration slope
b_s = Seasonally adjusted sample calibration interrupt
T_s = Seasonal average temperature, K.
P_s = Seasonal average barometric pressure, mm Hg

$$SQ_a = 1/m_s\ [I\ (T_s/P_s)^{1/2}] - b_s \qquad (6)$$

Where SQ_a = Sampler's seasonally adjusted flow rate, m^3/m

The set point flow rate (SFR) is the calculated and recorded:

$$SFR = 1.13\ (P_s/P_a)\ (T_a/T_s) \qquad (7)$$

Where SFR = Sampler's seasonally adjusted set point flow rate m^3/min

The sampler's seasonally adjusted set point recorder response is determined by the following relationship:

$$SSP = \{[m(SFR) - b](/P_a/T_a)^{1/2}\} \qquad (8)$$

Where SSP = Sampler's seasonally adjusted set point, recorder response.

ADJUSTING THE SET POINT OF THE MASS FLOW CONTROLLER

The specific mass flow rate at which the sampler should be set depends upon local conditions of temperature and barometric pressure. The model 321-B SSI is designed to maintain a 10 ± 0.5 µm cut point over a flow rate range of 1.02 to 1.24 m^3/min at actual conditions. Therefore, it is imperative that a set-point be chosen.

Which will "center" the flow rate with respect to fluctuating run of the day temperature and barometric pressure conditions.

The actual mass flow rate of the sampled air is controlled with a reference/sensing flow probe mounted in the throat section of the filter holder. The electrical output of the flow probe and other associated solid state circuitry is used as the control signal to adjust the motor speed. Thus, as ambient conditions or filter loading change, the controller increases or decreases the electrical power to the motor in such a manner that the mass flow rate is maintained at a constant velocity.

The desired samples flow rate is adjusted by a potentiometer following the sample's calibration by reconnecting the motor to the mass flow controller.

A clean filter is installed within the filter cartridge of the sampler which is allowed to warm up to operating temperature. The potentiometer on the mass flow controller is then adjusted until the recorder's response indicates the sampler's seasonally adjusted set point (SSP) as was previously calculated from equation (8). It should be verified that the flow controller maintains this flow rate for at least 10 minutes.

MEASUREMENT OF PARTICULATE MATTER IN THE FIELD

The minimum requirements when measuring particulate matter (3) are as follows:

1. Sampler should be at least 20 meters (m) from trees, buildings or other large obstacles. A general placement rule is that the sampler should be located at least twice as far away from the obstacle as the height of the obstacle.

2. Sampler inlet should be 2 to 7 m above the ground.

3. Sampler must have unrestricted air flow.

4. Sampler inlet should be at least 2 m from any other high-volume sampler inlet. For collocated samplers, the linkets must be within 4 m of each other.

5. Samples must not be placed directly on the ground or gravel roof top.

6. Samples must not be placed near exhaust flues or vents.

7. If samples art to be chemically analyzed, (e.g. mass spec; A.A., etc.) evaluate the site for potential contamination.

The samples installation procedure as outlined by the manufacturer (4) must be observed. Air electrical surge suppresser and a ground fault interrupts (GFI) are recommended to protect the system from transient voltage spikes and for safety. For samplers equipped with MFCs an independent AC power circuit is suggested; alternating current voltage (VAC) at the samples cannot be below 90 vac for the 115 vac system; or below 200 volts for 220 vac systems.

The USEPA protocol [5] is followed. The quartz filter is loaded on the cartridge support screen with the "up" side on which the particulate matter will be deposited facing upwards. For GQMA filters, this is the rough side. The lower side of the filter should have an ID number so that when the sample is folded (post-sampling) it can be identified.

The samples is warmed up for 3-5 minutes. If necessary, adjust the potentiometer on the MFC until the correct set point is indicated. Proceed with sampling noting the time interval.

CALCULATIONS

The samples operational flow is calculated using:

$$SQ_a = 1/m_s[I(T_s/P_s)] - b_s$$

Where SQ_a = Sampler's seasonally adjusted flow rate
I = Continuos records response, arbitrary units
bs = Seasonally adjusted samples calibration intercept
ms = Seasonally adjusted samples calibration slope
Ts = Seasonal average temp, K
Ps = Seasonal average barcmetric pressure, mm Hg

For routine operation however, an approximation can be employed.

$$SQ_a = (I-b_s)/m_s \qquad (9)$$

ACCURACY

To determine whether the operational flow vote is within design specification requirements of the HVPM10 samples.

$$\% \text{ Difference} = [(SQ_a - 1.13/1.13] (100) \qquad (10)$$

Where SQ_a = Sampler's seasonally adjusted flow rate, m^3/min
1.13 = Sampler's design flow rate, m^3/min

This difference must be <10%. If exceeded, recalibrate the samples before the next sample period. The sample duration must be1440 min ±60 min and the samples must have been turned on and off within 30 min of midnight.

PERFORMANCE CHARACTERISTICS OF AUTOMATED METHODS SO_2, CO_2, O_3, NO_2,

A test analyzer must exhibit performance better than, or equal to, the specified valve for each specification except range, to satisfy the requirements of EPA (40 CFR, Chapter 1, 7-1-91 Edition) Subpart B and 53.20

Performance Specifications for Automated Methods

Performance Parameters	Units	SO_2	Photo Chemical Oxidants	CO	NO_2
Range	ppm	0-0.5	0-0.5	0-50	0-0.5
Noise	ppm	005	.005	.50	.005
Lower Detectable Unit	ppm	.01	.01	1.0	.01
Interference Equivalent					
Each Interferent	ppm	±02	±02	±1.0	±.02
Total Interferent	ppm	.06	.06	1.5	.04
Zero Drift, 12 and 24 Hour	ppm	±02	±0.2	±1.0	±.02
Span Drift, 24 Hour					
20% of Upper Range Limit	Percent	±20.0	±20.0	±10.0	±20.0
80% of Upper Range Limit	Percent	±5.0	±5.0	± 2.5	±5.0
Lag Time	Minutes	20	20	10	20
Rise Time	Minutes	15	15	5	15
Fail Time	Minutes	15	15	5	15
Precision (20% µRL)	ppm	.01	.01	.5	02
(80% µRL)	ppm	.015	.01	.5	03

N.B. To convert from PPM to $\mu g/m^3$ at 298 K and 760 mm Hg, multiply by M/0.02447, where M is the molecular weight of the gas.

MONITOR LABS, INC. MODEL 850 FLUORESCENT SO_2 ANALYZER

Range of applicability 0 - 10 ppm - an equivalent method to the Pararosaniline Method

Principles SO_2 molecules are irradiated by uv light far region
- Flourescent of SO_2 occurs
- Secondary emission in the uv region occurs
- Secondary emission yields a light output and
- Sample gas is continuously drawn into a cylinder Teflon-coated Reaction cell at near ambient pressure

- The atmospheric gas is also irradiated by uv light modulated mechanically and filtered to 214 nm.

- The flourescent secondary emission of the SO_2 molecules is measured by a photo-multiplies take (PMT) outputs the signal through an amplifier to a synchronous demodulation.

- The uv light source constancy is measured by a reference photo-detector tube located directly across the reaction cell from the lamp; traveling down and being processed by a nearly identical synchronous demodulator. The mixer board electronious used this signal to compensate for any variation in the uv light source.

Temp range	20 - 30 °C
Humidity Range	0 - 95% RH.
Flow Rate	500 ± 50 cc/mm

Continuous Hydrocarbon Removal System

Called the "kicker" used when there are high levels of hydrocarbon interferents.

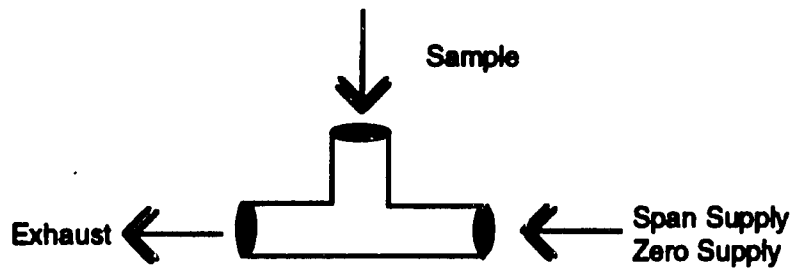
- by a permeable membrane technique
- eliminates the need of replacing catalyst tubes
- operates similar to a catalyst tube to continuously remove hydrocarbons

Sample Gas Connection

- Metal fittings may increase instrument response time
- Sample live length should be kept to a minimum for short residence or reaction time
- Contact of sample gas with non-teflon or non-gas surfaces and fittings must be minimized
- Never use brass or Al fittings; may be clean stainless steel
  If sample is obtained from a closed or pressurized system-install a vent line

N.B. Sample pressure at the rear panel of the analyzer must not exceed 1" of H_2O above or below atmospheric pressure.

Using a TEE as a vent manifold



- Always pull sample from the "brench" of the TEE exhaust span and zero supply from the "run" legs of the TEE.

Permeation Device

A temperature-controlled oven or chamber.

Operating Procedure

All panel controls must be at these settings:

Operation Mode:	Monitor
Range Switch	0.5 ppm
Time Const	55 seconds
Auto Switch	Off (out)
Zero Pot	Refer to Final Test Data
Span Pot	Refer to Final Text Data

1. Turn on Power
2. Start up external pump - 500 ± 50cc/min flow
3. Warm up for 30 min before proceeding
4. Observe red LED at top rear of mixer assembly. This PMT cooler LED should cycle on and off during normal operation
5 Observe neon light for 1ZS and reaction call heaters. Neon lights should flicker when assemblies are at their correct operating temperatures.
6. Switch MODE from Monitor to TI position
   - UV lamp reference channel test
7. Switch Mode to T_2
   - HVPS (high voltage power supply) check
8. Switch mode to T_3
   - Tests for proper chopper assembly operation
9. Switch Mode to ZERO and the Range to Optic Test
   - Apply zero air
10. Switch mode to zero and the range to optic test
   - Supply zero air

N.B. Electric and optic test (70% full scale deflection) in 2 mins are for trouble-shooting purposes only and are not a substitute for calibration, zero/span, or precision check.

Calibration of the Model 8850 SO_2 Analyzer

A calibration system containing a temperature regulated permeation tube chamber and/or cylinder gas delivery system along with a variable source of clean zero air for both zeroing the instrument and dilution of the span gas source is necessary. This system must be able to provide enough clean dilution air to make up five (5) calibration standard concentrations (10, 20, 40, 60, 0% of URL).*

Dirt or the presence of materials other than TFE, FEP, or glass will absorb small amounts of SO_2 and hence offset calibration results.

The calibration system should deliver a minimum of 75 cc/min and a maximum of 2,500 cc/min to ensure good results.

If a permeation device type calibration system is utilized, allow at least 24 hours for warm-up and equalization to the devices certification temperature before proceeding with calibration of the analyzer.

Zero air should contain $\leq$ 0.0005 ppm SO_2 an activated charcoal filter column connected between the outside ambient air being sampled (at sample manifold) and the sample inlet of the analyzer may be used for zeroing the analyzer. The dilution air used to prepare the SO_2 concentrations should be treated similarly.

Charcoal filtering devices used to provide zero and/or span gas dilution air must be charged on a regular basis to ensure SO_2 covers $\leq$ 0.005 PPM.

* URL = Upper Range Limit

OUT-PUT SIGNAL MONITORING

Connections best made with #20 to #26 shielded twin lead cable.

- Length minimum _____, maximum 50 feet
- Connect the cable shield to the signal low of analyzer
- Set recorder zero and span according to manufacturer's instructions

For best accuracy, a digital volt meter (DVM) should be used. The DVM may be hooked to the terminals provided for DAS output.

Employ Formula: Output Scaling Factor (ppm/Vac) = $\frac{\text{Selected Range (ppm)}}{\text{Maximum Voltage Output}}$

RANGE (ppm)	OUTPUT SCALE FACTOR (DAS/RECORDER) ppm/volt
0.25	0.025
0.5	0.05
1	0.1
5	0.5
10	1

This formula yields the results for the five standard Model 8850 ranges and a standard 10 vdc DAS or recorder voltage output.

Multiply the applicable scaling factor by the instruments output voltage results in the corresponding SO_2 concentration in ppm.

CALIBRATION FREQUENCY

- At least once per each monitoring quarter (3 months)
- Whenever, analyzes is moved to a new sampling location
- If the zero response deviates >±0.025 ppm from zero
- If the span (at 80% URL) > ±15% from true concentration

STACK SAMPLING

Velocity and Volumetric Flow Rate:

Applicability for flowing gas streams in ducts, stacks, and flues.

N.B. Cannot be used for (1) cyclonic or swirling; (2) if stack is less then 0.3 m (12 in) in diameter, (3) 0.071 m^2 (113 in^2) cross-sectional area; (4) measurement site is less than 2 X stock or duct diameter downstream or less than 1/2 diameters upstream from a flow disturbance.

Procedure
(1) Selection of measurement site
(a) at least 8 stack or duct diameters downstream
(b) at least 2 stack or duct diameters upstream from any flow disturbance (bends, expansion, contraction, visible flame

Equivalent Diameter

$$De = \frac{2LW}{(L+W)}$$

Where L = Length, W = Width

Minimum Number of Traverse Points for Particulates

Dimension of Stack or Dust	Minimum No of Traverses
Circular, Rectangular (D_e>24")	12
Circular (D_e12" - 24")	8
Rectangular (D_e=12" - 24")	9

When the 8-2 diameter criteria cannot be met the minimum number of traverse points is determined from figure (1-1) (40 CFR Ch., 1) (7-1-91 ED) Pt. 60, App A, Method 1, pp. 690.

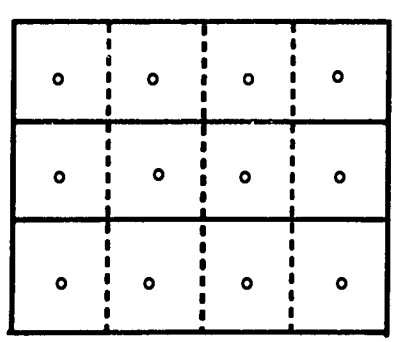
Class-Section Layout for Rectangular Stacks

NUMBER OF TRAVERSE POINTS	MATRIX LAYOUT (D_e)
9	3 x 3
12	4 x 3
16	4 x 4
20	5 x 4
25	5 x 5
30	6 x 5
36	6 x 6

<u>VELOCITY</u> (Non-Particulate)

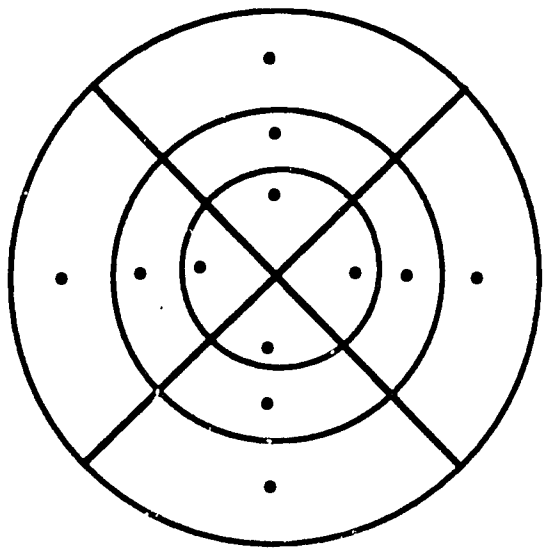
Same procedure as that for particulate is followed, but use instead fig 1-2.

Stack Diameter > 0.61m (24")	No traverse point, must be with 1" from the wall
Stack Diameter ≤ 0.61m (24")	No traverse point, must be with 0.5" from wall



Example of Traverse Points in a rectangular stack cross section divided into 12 equal area.

N.B. Distance from wall = to nozzle inside diameter whichever is longer.



Example of Traverse Points in a circular stack cross section divided into 12 equal areas with locations of Traverse Points.

Verification of Absence of Cyclonic Flow

Gas flow in stacks are usually parallel to the stack walls. However, cyclonic flow may exist after:

(1) Devices such as cyclones, inertial demisters following venturi scrubbers.
(2) Stacks having tangential inlets.

TYPE S PITOT TUBE MANOMETER ASSEMBLY

S-Stausscheibe or Reverse Type

- Used for measurement of average velocity of a gas stream
- Cannot be used for direct measurement in cyclonic flow or swirling flow conditions
- Measure velocity head pressure temp
- Measure static pressure
- Measure atmospheric pressure

(a) Dry Mol. wt. air = 290
(b) CO_2, O_2, CO, N_2 - find dry mol. wt. of gas

(1) Measurement of Gas Volume through P, PES and DUCTS.

$$Q_s = \frac{Vms}{O}$$

Where Q_s = Gas flow rate, m^3/min, standard conditions
Vm_s = Meter volume reading, m^3
O = Elasped time, min

(2) Measurement of gases cone

(a) Sample transport line
(b) Moisture removal system
(c) Particulate filters
(d) Sample pump
(e) Sample flow rate control
(f) Sample gas manifold
(g) Data recording

PARTICULATE EMISSIONS

Probe Nozzle Stainless steel (type 316) or glass

- Button-hook or elbow design, angle tape ≤ 30°
- Range of nozzle size for isokinetic sampling
  1/8" - 1/2" greater for higher volumns

Probe Line Borosiicate or quart with heating system capabe of maintaining a gas temp at the exit end during samping of 120 ± 14°C. (248 ± 25°F)

Pitot Tube Types - for measuring stack gas velocity.

$$V_s = K_p C_p \left(\sqrt{\Delta P}\right)_{avg} \sqrt{\frac{T_{s(avg)}}{P_s M_s}}$$

V_s = Average stack gas velocity, m/sec. (ft/sec)

K_p = Pitot Tube Const.

C = Pitot Tube coefficient, 1 dimensionless

ΔP = Velocity head of stack gas, mm H_20 (in H_20)

T_s = Absolute stack temp °K, (°R)

P_s = Asbsolute stack pressure, mm Hg (in Hg) = $P_{bar} \pm P_3$

P = Stack static pressure, mm Hg (in Hg)

M_s = Mol. nt. of stack gas, wet basis, g/g-mole

= M_d $(1-B_{ws}) \pm 18.0\ B_{ws}$
= B_{ws} = Water vapor
= M_d = Mol. wt. of air any basis

Condensor: 4 Impinges with Greensburg - Smith design with leak-free ground glass fittings

Filters: Glass fier, without organic birdes.
- 99.95% efficiency on 0.3-micron particles

Silica Gel: 6-16 Meek particle size previously dried out 175°C for 2 hours

Desiccant: Anhydrous $CaSO_4$ - indicating type
- desicate the filters at 20 ± 5.6°C and ambient pressure at least 24 hours

Preparation of Collection Train

- Place 100 ml of H_2O in each of the first-two impingers leave third impinges empty.
- Put 200-300 g of pre-weighed silica gel in fourth.
- Place crusehd ice around impinges + pace if necessary.
- Leak check
- Set filter and probe heating systems at desired operating temperatures.
- Allow time for temperature to stabilize.

Maintain Isokinetic Sampling Rate

± 10% of true isokenitic

$$I = \frac{100\, T_s\, [K_3\, V_{lc} + (V_m Y/Tm)\, (P_{bar} + \Delta H/13.6)]}{60\, SV_s P_s\, Am}$$

Ref. 40 CFR CH. 1(7-1-91 Ed.,) Pp. 759, or

$$I = \frac{100\, T_s V_m\, (std)\, P\, std}{60\, T_{std} V_s\, O\, A_n\, P_s\, (1\text{-}B_{ws})} = \frac{K_4\, T_s V_m\, (std)}{P_s\, V_s\, A_n\, S\, (1\text{-}B_{ws})}$$

QUALITY CONTROL

(1) Meter Orifice Check

$$\Delta H_e = 0.0319\, \Delta H\, \frac{TM}{P_{bar}}\, \frac{O_2}{Y^2\, V_m^2}$$

ΔH_e is the orifice pressure differential in units of in. H_2O that correlates to 0.75 cfm of air at 52%°R and 29.92 in. Hg.

(2) Check probe nozzle I.D.'s using a micrometer
- Reshape, sharpen and recalibrate if necessary

(3) Recalibrate pitot tube (type S)

DETERMINATION OF SO_2 EMISSIONS FROM STACKS

Principle: Gas sample is extracted from the stack into 3% H_2O_2 solution at 1.0< pm for 20 minutes. SO_2/SO_3 determined by titration (Barium-thorin titration method)

Minimum Detectable Limit: 3.4 mg. SO_2/m^3
(2.12×10^{-7} (6/cu. ft.)

No Real Upper Limit

Upper limit in a 20 liter sample ≈ 93,3000 mg/m^3

Possible Interferents

(1) Free Ammonia
(2) Water soluble cations } can be romoved by glass wool filters and an isopropanol bubbler
(3) Fluorides

N.B. Use a high efficiency glass fiber filter in place of glass wool plug when very fine mitallic fumes are expected.

SECTION VB

REFERENCES

REFERENCES

Air Quality and Emissions

1. EPA National Air Quality and Emission Trends Report, 1988
2. EPA Toxic Release Inventory Report, Chemical Engineering, May 1990, pp. 27.
3. 40 CFR Part 50 subchapter C.
4. "Wiping Out Air Pollution", Chemical Engineering, September 1990, pp. 106-107.
5. Chemical Engineering, September 26, 1988, pp. 31-38.
6. Chemical Engineering, February 16, 1987, pp. 155-165.

Microbiology

1. NPDES Manual, 1971

2. EPA Microbiological Manual, 1978

REFERENCE MATERIALS FOR GAS CHROMATOGRAPHY

I. Quality Assurance/Quality Control
Handbook for Analytical Quality Control in Water and Wastewater Laboratories-Chapters 1, 4 & 6
QA/QC of Different Methods
Standard Methods for the Examination of Water and Wastewater, Part 1000

II. Statistical Treatment of Data
Handbook for Analytical Quality Control in Water and Wastewater Laboratories-Chapter 7
Fundamentals of Analytical Chemistry, Sixth Edition
Skoog, West and Holler
Standard Methods for the Examination of Water and Wastewater, Part 1000

Software Demonstration and Use
Kaleidagraph
Quattro-Pro

III. Laboratory Reporting of Data
Handbook for Analytical Quality Control in Water and Wastewater Laboratories
Fundamentals of Analytical Chemistry, Sixth Edition, Skoog, West and Holler

Software Demonstration and Use
Kaleidagraph
Quattro-Pro

IV. Chromatography
Principles of Instrumental Analysis, Skoog and Leary
Instrumental Methods of Analysis, Williard, Merritt, Jr., Dean, and Settle, Jr.

V. Standard Methods of GC Analysis
Sampling and Preservation
Code of Federal Regulations, 40, Parts 260-261
Code of Federal Regulations, 40, Parts 136
SW-846, Test Methods for Evaluating Solid Waste, and Updates

Methods for the Determination of Organic Compounds in Drinking Water, EPA/600/4-88/039
Methods for the Determination of Organic Compounds in Drinking Water, Supplement I, EPA/600/4-88/039
Standard Methods for the Examination of Water and Wastewater, 17th Edition, APHA, Part 6000
Methods for Organic Chemical Analysis of Municipal and Industrial Wastewater, EPA/600/4-82-057

VI. Other Topics
Ion Selective Electrodes-Practical
Cyanide, Chlorine, Fluoride, Silver and Sulfide

References
Standard Methods for the Examination of Water and Wastewater, 17th Edition, APHA
Methods for Chemical Analysis of Water and Wastewater

The above materials were used in the program for the wet chemistry and gas chromatography training. All of the above referenced materials have been or are being provided to Tabbin Institute for Metallurgical Studies. Reference books and materials that were left with TIMS include:

Standard Methods for the Examination of Water and Wastewater, 17th Edition, APHA
Code of Federal Regulations, 40, Parts 136
Principles of Instrumental Analysis, Skoog and Leary
Instrumental Methods of Analysis, Williard, Merritt, Jr., Dean, and Settle, Jr.
Fundamentals of Analytical Chemistry, Sixth Edition, Skoog, West and Holler
The Merck Index, Eleventh Edition
CRC, Handbook of Chemistry and Physics, 72nd Edition
Quattro-Pro, Version 4.0, Database Software
Methods for Chemical Analysis of Water and Wastewater
Methods for the Determination of Organic Compounds in Drinking Water, Supplement I, EPA/600/4-88/039

Columns at Tabbin

Type	Method used for
10% Dionyl Phthalate on 80/100 Chromosorb WHP, 1/8 x 4 meters (2)	
10% Dionyl Phthalate on 80/100 Chromosorb WHP, 1/8 x 2 meters (2)	
15% FFAP on 80/100 Chromosorb WAW 1/8 x 2 meters (2)	Phenol, Volatile Organics, i.e 601 or 602
80% Propack N, 1/8 x 2 meters (2)	
80/100 Chromosorb 102, 1/8 x 2 meters (2)	
60/80 Activated Alumina, 1/8 x 2 meters (2)	
80/100 Chromosorb 101, 1/8 x 2 meters (2)	
3% OV-17, 80/100 Chromosorb WHP 1/8 x 2 meters (2)	
10% OV-101, 80/100 Chromosorb WHP 1/8 x 2 meters (2)	
10% Carbowax 20m, on 80/100 Chromosorb WHP, 1/8 x 2 meters (2)	
15% Chromosorb W-NAW on 80/100 K2OM 1/8 x 6 ft	

Columns for Water and Wastewater
Analysis by EPA Standard Methods

1% SP-1000 on Carbopack B (60/80) 3 mm x 2.4 m	EPA-601 **Purgeable Halocarbons** SM 6010B.1
n-Octane on Porasil-C (100/120) 3 mm x 1.8 m	**Hall Detector**
5% SP-1200 and 1.75% Bentone-34 on Supelcoport (100/120) 1/8 in x 6 ft	EPA-602 **Purgeable Aromatics**
5% 1,2,3 Tris(2-cyanoethoxy)propane on Chromosorb W-AW (60/80) 1/8 in x 8 ft	**PID Detector**
Porapak-QS (100/120) 2 mm x 10 ft	EPA-603 **Acrolein & Acrylonitrile**
Chromosorb 101 (60/80) .10 in x 6 ft	**FID**
1% SP-1240DA on Supelcoport 2 mm ID x 1.8 m	EPA-604 **Underivatized Phenols-FID**
5% 0V-17 on Chromosorb W-AW-DMCS (80/100)	EPA-604 **Derivatized Phenols-ECD**
1.5% SP-2250/1.95% SP-2401 Supelcoport (100/120) 4mm ID x 1.8 m (glass)	EPA-606 **Phthalate Ester**
3% OV-1 on Supelcoport (100/120) 4 mm ID x 1.8 m (glass)	**ECD**
10% Carbowax 20 M/2% KOH on Chromosorb W-AW (8/100) 4 mm ID x 1.8 m	EPA-607 **Nitrosamines**
10% SP-2250 on Supelcoport (100/120) 4 mm ID x 1.8 m	**NPD or Hall**
1.5% SP-2250/1.95% SP-2401 Supelcoport (100/120) 4mm ID x 1.8 m	EPA-608 **Organochlorine Pesticides & PCBs**
3% OV-1 on Supelcoport (100/120) 4 mm ID x 1.8 m	**ECD**
1.95% QF-1/1.5% OV-17 on Gas Chrom Q (80/100 mesh) 2 or 4 mm ID x 1.2 m	EPA-609 **Nitroaromatics and Isophorone**
3% OV-101 on Gas Chrom Q (80/100) 2 or 4 mm x 3.0 m (glass)	**FID** (NB)/**ECD** (DNT)

3% 0V-17 on Chromosorb W-AW-DMCS (100/120) 2mm ID x 1.8 m	EPA-610 **Polynuclear Aromatic Hydrocarbons** **FID**
3% SP-1000 on Supelcoport (100/120) 2 mm ID x 1.8 m	EPA-611 **Haloethers**
2,6 diphenylene oxide polymer (60/80) or Tenax, 2 mm ID x 1.8 m	**Hall Detector**
1% SO-1000 on Supelcoport (100/120) 2 mm ID x 1.8 m (glass)	EPA-612 **Chlorinated Hydrocarbons**
1.5% OV-1/2.4% OV-225 on Supelcoport (80/100) 2 mm Id x 1.8 m (glass)	**ECD**

Columns and Detectors for Potable Water

1% SP-1000 on Carbopack B (60/80) 3 mm x 2.4 m n-Octane on Porasil-C (100/120) 3 mm x 1.8 m	EPA-502.1 Purgeable Halocarbons **Hall Detector**
VOCOL, 1.5 um film(Supelco) .75 mm x 60 m DB-624, 3 um film (J&W Scientific) .53 mm x 30 m DB-5, 1 um film (J&W Scientific) .32 x 30 m	EPA 524.2 Purgeable Hydrocarbons **Mass Spectrometer**
DB-1, 1 um film (J&W Scientific) .32 mm x 30 m DB-210, .5 um film (J&W Scientific) SP-2401, .5 um film (Supelco) .32 mm x 30 m	EPA-551 Extractable Halocarbons **ECD Detector**
DB-210, .25 um (J&W Scientific) .32 mm x 30 m confirmation column DB-210, .50 um film (J&W Scientific) .32 mm x 30 m	EPA-552 Extractable Haloacetic acids **ECD Detector**
VOCOL, 1.5 um film(Supelco) .75 mm x 60 m RTX-502.2, 3 um film (RESTEK Corp.) .53 mm x 105 m DB-62, 3 um film (J&W Scientific) .53 x 30 m	EPA-502.2 Purgeable Organics **PID & Hall in Series** **PID & ELCD** **PID & ELCD**
5% SP-1200/1.75% Bentone 34 on Supelcoport (80/100), .085 in x 2.5 m 5% 1,2,3 tris(2-cyanoethoxy)propane on Chromosorb W (60/80), .085 in x 2.5 m	EPA-503.1 Purgeable Aromatic **PID**

SECTION VI

WORKSTATION REARRANGEMENT

PLANS FOR WORKSTATION REARRANGEMENT

A preliminary outline of the laboratory workstations (areas) was developed. Drs. Foust and Parker currently have suggested in a broad sense that the air laboratory become the Remote Site Assessment Laboratory. All equipment, air and water, that is to be used in remote site measurements are being placed in this laboratory.

The Wet Chemistry Lab is to be partitioned for organic and inorganic recovery work. The rough draft of the laboratory reorganization plans are attached. Areas for digestion/extraction of samples are to delineated to prevent contamination and to provide ultimate QA/QC.

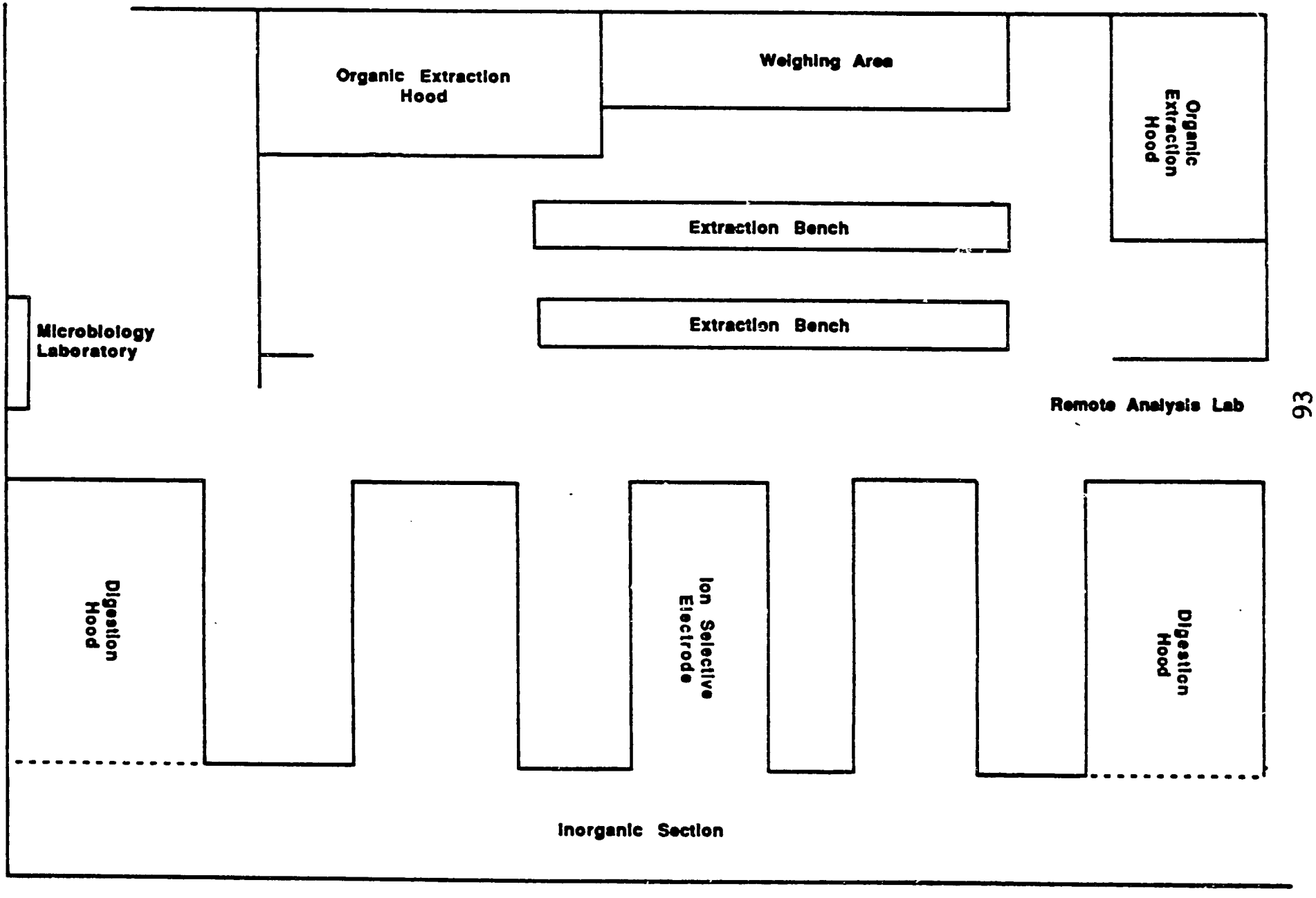
These workstations will be equipped with all necessary glassware, equipment and provided instructions for particular analytical procedures; thus, maximizing efficiency of laboratory operations.

The request for equipment (glassware, etc.) of the Tabbin Institute suggested that there are some supplies available but they are in limited supply. Much of the glassware originally ordered is carefully stored at Tabbin but indicated here and previously, available stores are limited for optimum operation of the laboratory facilities.

Lectures on Gas Chromatography (GC) were focused on quality assurance/quality control with respect to problems faced in gas chromatographic analyses, water and air analyses. The students proved receptive and a lively discussion ensued. Each student was given a copy of a standard QA/QC planning manual and were being asked to consider a QA/QC plan for the laboratory. The focus will quickly shift to the standard methods for sample collection, extraction, analysis and data reduction and handling in GC and other instrumentation; e.g. Atomic Absorption Spectroscopy, etc.

As a result of discussions with the air group at TIMS a new protocol has been developed for use at the Tabbin Institute. The new protocol was put in place and the results were evaluated.

WET LABORATORY



PART II

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS AND RECOMMENDATIONS

Laboratory Management

The single most significant problem in the TIMS Environmental Monitoring Laboratory was laboratory management. Several meetings were held during the training with Dr. Barakat and Mr. Ataf El-Sabby to develop a laboratory management plan. These meetings were serious and productive in terms of addressing the issues with the expectation that as a result of these discussions the following actions by TIMS will be taken:

1. A laboratory management plan will be developed that will permit this laboratory to operate effectively.

2. A list of job responsibilities will be developed that will make clear to all laboratory personnel both their individual and group commitments.

3. A work scheduling plan will be developed so that work can be completed in a timely manner; and

4. A list of TIMS immediate training needs will be prioritized so we can help the TIMS staff meet their commitments to the World Bank and other Project.s.

Our initial suggestions on laboratory management were met with some resistance, but the pressure of meeting deadlines for the World Bank Project resulted in an awareness among TIMS personnel (Dr. Yousey and Mr. Ataf) that these procedures need to be implemented. The pressure of the World Bank Project has been the stimulus needed to make progress on the point of laboratory management.

We believe that while these issues were discussed and apparently agreed upon by the TIMS senior staff we still have yet to see affirmative action. The CAU/HBCU/MI team summarizes the TIMS Laboratory Management issues and makes the recommendations below:

TIMS LABORATORY MANAGEMENT PLAN

OVERVIEW

Tabbin Institute for Metallurgical Studies (TIMS) currently houses two chemical analysis laboratories with a staff of one laboratory manager, 10 analystS and additional supporting staff of approximately 10 people. One laboratory concentrates its efforts on metals analysis only and the second laboratory focuses on environmental analysis. Both laboratories could operate more effectively by adopting a unified laboratory management plan.

The Current Situation

Although job titles have been defined, position descriptions with responsibilities and lines of authority have not been assigned. The result is an inability to assign tasks so that work can be completed in an efficient and timely manner. Additional problems that results from the administrative structure is a failure to establish long term goals, inadequate planning for routine operational goals of the laboratory as a whole. The final factor that has impacted on laboratory operations at TIMS is the commitment to provide data for an environmentally study in the Helwan area. Work for this environmental study, funded by the World Bank, is being done by personnel from both laboratories, and the facilities of both laboratories are being used for the experimental work.

Statement of the Problem

During the training period, we discussed laboratory management with the TIMS technical staff and management personnel (Dr. Yousry Barakat and Mr. Atef El-Sabby). Our discussions touched on all aspects of laboratory at practices, and many of our suggestions were greeted with the response, **"But you don't understand, this is Egypt"**. The view of the training team experts is that we do understand. Instead of the response we received, we would have liked to hear or be adopted as their motto, **"This may be Egypt, but the TIMS Environmental Monitoring Laboratory operates like a certified EPA Laboratory in the United States."**

Current management practices in the Environmental Pollution Laboratory lead to some specific problems that impact on the laboratory's ability to function effectively. These individual problems are discussed separately below:

Lack of an Operational Budget: The lack of an operating budget has resulted in an extreme shortage of routine laboratory supplies. This include chemicals, glassware and reagents (consumables and disposables). The results is an inability to perform sample preparation. An additional problem that has developed at TIMS, because of the shortage of operating supplies, is the hoarding of glassware and chemicals by individual technicians. The result is that an analyst may not be able to conduct an assigned experiment because a chemical or piece of glassware is not available. Whereas in reality it was locked up in a cabinet in the laboratory. This problem is extremely evident in the Air Monitoring Laboratory, in which the "expert" was not present for the first six weeks of training and has many swagelok tube fittings which would be useful for gas line connections on the GC or the AA. This problem is also particularly severe in the competition between the Environmental Pollution Laboratory and the Chemical Analysis Laboratory.

Lack of Defined Responsibility: Although there are approximately 20 individuals working in the two analytical chemical laboratories, specific job responsibilities are not clearly defined and many of the routine tasks of running a laboratory are done only sporadically or do not get done at all. There is no central inventory of

supplies that can be checked for the availability of chemicals and glassware, and therefore, supplies cannot be ordered ahead of time-before they run out. Until recently, nobody assumed responsibility for cleaning glassware and laboratory space. The result was dirty glassware collecting on the benchtops, and many unlabeled beakers and bottles containing samples of unknown origin. Another result of this policy is that some individuals end up doing more than their share of the work while others do less than their share.

Absence of an Administrative Structure: Although Mr. Atef holds the title of Laboratory Manager, implying responsibility for daily operations, the position of Laboratory Director is apparently vacant. The Laboratory Director would have the responsibility of long-term planning and providing the resources for the Laboratory Manager to do his job effectively. Although Dr. Yousry Barakat has assumed the title of Project Director for the World Bank Project, he stated very emphatically that he had no responsibility for any aspects of the laboratory operation. In many respects, it seems that the laboratory technicians have the greatest responsibility of all personnel. They control keys to locked supply cabinets and Mr. Atef must ask their permission if he needs access to equipment in the locked cabinets. This is particularly troublesome if a technician does not come to the laboratory on a given day. Apparently, there are not duplicate keys for any locked cabinets. The functions of Chief Analyst and Quality Assurance Officer are not addressed by any of the currently laboratory personnel.

Lack of Mission Statement and Long-Term Plans: The implied missions of the two analytical chemical laboratories (from their titles) are: 1) Chemical analysis of metallurgical samples for industry and research, and; 2) Analysis of Environmental Samples to Monitor Industrial Pollution. In practice, the missions seem to merge into one common goal of providing chemical analysis for the sample at hand-with available equipment and personnel. By not having a well defined mission and goal statement it is not possible to adopt long-term plans. This situation leads to a lack of purpose and commitment among laboratory personnel, negatively impacting on all laboratory operations.

RECOMMENDATIONS

The first step in improving the laboratory performance at TIMS would be to do a total resource inventory that would include an evaluation of:

1. Available Personnel and Training
2. Available Laboratory Facilities, (including space, fume hoods, instrumentation, chemicals and glassware.
3. Determine monetary support available through TIMS, the Ministry of Industry or the Metallurgical Industries Corporation
4. Determine potential revenue sources from outside TIMS

The issue of two separate or one combined chemical laboratory needs to be addressed and a single mission statement (or two separate mission statements) written clearly to define the role of these laboratories at TIMS. An appropriate administrative structure should then be chosen that will best support the mission

statement(s) and help meet the long term goals for the analytical laboratories and finally, a budget must be adopted that will provide for personnel costs, operations, capital and overhead expenses.

Although, it would be easy for the training team to provide specific recommendations, we believe that the issues are of sufficient importance that all levels of the TIMS staff and administration should be involved in the discussions. Completing the process as outlined will result in a broader support base if our recommendations were adopted. The final reason for following this process is that our perspective is based on only a few weeks of observations and individuals more involved with TIMS will be able to add a perspective to the situation that only experience can provide.

Microbiology Training and Recommendations

The wastewater microbiological training included the following subjects which were covered extensively:

Biological Oxygen Demand,
Chemical Oxygen Demand and
Oil and Grease Determination.

This material was received by the students quite well and we feel that the training goals in these areas were met.

However, the microbiology portion of the training was another matter. Problems encountered in this area were reported in our weekly reports and may be reemphasized. It was evident that there was not enough time to adequately train the students in the area of microbiology. Their background was not adequate enough and the time allotted for the training had to be shortened due to the World Bank Project. Materials necessary for the training were not all present and the delay in ordering the materials (and the necessity for limiting the ordering budget) contributed to the problem.

We are convinced of the need for additional training in microbiology. The microbiology training will need to start with the basic concepts and progress to the practical aspects of the laboratory. It should include provisions for making sure that all supplies and equipment necessary for microbiology are in place. It should also include field trips for sampling various sites and pressures for obtaining data on a timely and accurate basis. In addition to the water microbiology (fecal coliform and total coliform), topics should be extended into the areas of bacterial and viral pathogens for the selected students (specialists) who will deal with these problems in the future. Some aspects of food microbiology and public health microbiology could be addressed. Both the students and the Tabbin staff supported the need for this additional training in microbiology.

SPECIFIC RECOMMENDATIONS

Lead Abatement

After visiting General Metal Works, Inc. where lead is recycled, it is our opinion that there is extreme lead contamination of the entire area surrounding this company. We would therefore recommend training in the awareness of lead poisoning, especially of young children and pregnant mothers. Training should also be provided in the measurement of lead in paint, soil and water and the abatement of lead from these media. This situation is compounded by the fact that leaded gasoline is still being used in Egypt.

Occupational Health and Safety

Based upon our observations of the TIMS technicians while they were conducting air sampling at the Tourah Cement Factory at Corniche on the Nile, we noticed that some of the technicians were not adequately protected with respirators and goggles. We would, therefore, recommend further training, especially for field technicians in "Occupational Respiratory Protection". A training module on Worker Exposure and Safety was proposed in our initial RFP.

Asbestos

Personnel at General Metal Works, Inc. had indicated to me that asbestos containing-material was being removed from factories by untrained personnel who are not aware of the dangers of being exposed to asbestos fibers and also wear no protective clothing nor respirator. TIMS has expressed a desire to be trained in the identification and measurement of asbestos containing-materials and to procure the necessary equipment to acquire this analytical capability.

Asbestos Management training , including the measurement of airborne asbestos fibers and the identification and quantification of asbestos in bulk samples are therefore recommended, particularly in the light of the fact that most Egyptian males smoke cigarettes. Because of the synergistic effect, a smoker who inhales asbestos fibers is 60 to 80 times more likely to die from lung cancer as compared to a non-smoker not exposed to asbestos. Also, please note that some of the training in Occupational Respiratory Protection overlap with some portions of the curriculum for Asbestos Inspection and Abatement.

Our staff qualifications in asbestos management include the following:

- Certification by the USEPA, New York City and Florida States as Asbestos Inspector, Management Planner and Contractor

- Four years of experience as Technical Director of an asbestos training laboratory

- Fours years as Assistant Chief of Inspectors at the Environmental Health and Safety Department, City of New York, where the AHERA (Asbestos Hazardous Emergency Response Act) survey was supervised, and

- The implementation of asbestos abatement response actions for the New York City Public Schools, also

- NIOSH 582 Certification in the measurement of airborne fibers employing Phase Contrast Microscopy

Air Pollution

As a result of a visit to the Nasser Automobile Company, we were approached to investigate the cause and effect of air pollution on the stress-corrosion induced longitudinal cracking of reinforced concrete arches that support the roof over 8 hangers with a total floor space of 180,000 square meters.

We were also approached by one of the engineers from the Egyptian Company of Aluminum Products (Alumisr) after a special lecture in Air Pollution Control. The company is concerned about sulfuric acid and hydrogen vaporization from the tanks.

Another engineer was concerned about the measurement of the efficiency of electrostatic precipitators, especially those at the cement factories. This is of major importance, as would be attested to by anyone who has visited Cairo.

PART IIA

TIMS TECHNICAL STAFF ASSESSMENTS

TIMS TECHNICAL STAFF EVALUATIONS AND ASSESSMENTS

Student Evaluations In Instrumental Analysis

Mrs. Elham is an exceptionally bright and capable student who demonstrated the highest level of natural ability in the class. She has a good grasp of all the fundamentals of environmental analysis that she combines with an initiative to do things on her own. She is fully competent in sample preparation and analysis methods, flame and graphite furnace atomic spectroscopy. She demonstrated unusual independence and competence by connecting the Perkin-Elmer data to the AA spectrophotometry on her own, and working through much of the software problems.

Mrs. Nabamia is the most organized and the best "leader" in the class. She is a very serious student with exceptional organization skills. A very hard worker who demonstrated good initiative by being the first student to do six sample digestion's simultaneously. She is fully competent in sample preparation and analysis methods, flame and graphite furnace spectroscopy. Mrs. Nabamia is our choice for a Quality Assurance Officer, when the position is established.

Mr. Sameir is the best all-around analyst in the group. He is the hardest worker and the most serious about meeting deadlines and getting the job done correctly and on time. He is fully competent in sample preparation and analysis methods, flame and graphite furnace atomic spectroscopy. Mr. Sameir is our choice for Chief Analyst when the position is established.

Mr. Khalid is the most improved student in the groups. He has improved his laboratory skills and shown a sincere desire to learn, especially about atomic spectroscopy. He demonstrated good initiative by finding three difference procedures for eliminating sulfide interference with the cyanide test and good judgment by selecting the best procedure to use for his samples. He is fully competent in sample preparation and analysis methods, flame and graphite furnace AA spectrophotometry. He needs to be watched carefully in the laboratory so he does not slip back into his former sloppy laboratory habits.

Mr. Hassan is a fully competent laboratory technician. The individual with the greatest breadth of laboratory experience. Mr. Hassan is best able to help develop the laboratory by sharing his experience with the younger, less experienced individuals. He is fully competent in sample preparation and analysis methods and the best person in the group with flame AA spectrophotometry. I would judge him "competent" with Graphite Furnace AA, but he needs additional instrument experience to be fully competent.

Mr. Reda is competent in sample preparation and analysis methods, but would benefit from additional supervised experience. His competence with the flame and graphite furnace atomic absorption spectrophotometer is marginal. He should only work with this instrumentation under close supervision.

Student Evaluations in Microbiology

The HBCU/MI Consortium Training Team met with Dr. Barakat during an exit interview and expressed the following opinions on the students who attended training in the areas of BOD, COD, Oil and Grease and Microbiology attended sessions on a regular basis (Madam Elham, Madam Nabawia, Mr. Samir and Mr. Khaled. The other students attended sessions on a very sporadic basis and did not seem as committed as the previously mentioned four students. Of the four students, Madam Elham and Mr. Samir appeared to have had the most potential as far as developing into specialists in various areas. Mr. Samir appeared to be the most well rounded of the students with knowledge and enthusiasm in all the areas of training. Madam Elham had a very good depth of knowledge but was able to put in less time training due to personal commitments. She had to leave by 2PM every day. Madam Nabawia has a very good aptitude for attention to details and as previously assessed, she would be a good candidate for a quality assurance officer for the laboratory. Mr. Khaled appeared to be very interested in training but had a more difficult time picking up some of the material. This may be due to a language problem. Mr. Khaled appeared to be less proficient in English (the language of training) than did the other students. The other students (Mr. Reda and Mr. Hassan) did not really attend enough training session for me to have formed an opinion on them.

Overall Assessment in Microbiology

The overall assessment of the training in the areas of BOD, COD, and Oil and Grease went very well. The microbiology training was not as successful for several reasons. First, some of the necessary supplies were not at TIMS and had to be ordered. This order was delayed and did not arrive prior to the training teams departure from Egypt. The second and most important reason was the lack of biological information in the students background. This made it very difficult to teach basic principles in microbiology in the limited time available. If in the future the students are to become more proficient in this area, they must receive additional training.

STUDENT AND LABORATORY EVALUATIONS IN GAS CHROMATOGRAPHY - JUNE

The June training session involved the installation of capillary columns to upgrade the gas chromatography units at TIMS. This was completed sucessfully by Dr. Parker with the help of Mr. Khaled and Mr. Samir. The installation of the capillary columns were explained and demonstrated to Madams Elham and Nabawia, Misters Khaled, Samir and Atef. Mr. Reda was absent during the installation of the capillary columns. Mr. Khaled worked diligently with Dr. Parker in ensuring that materials necessary for the operation of the GC were in place when the installation was complete. The third column can be easily installed by Mr. Khaled or one of the other trainees.

Mr. Khaled has improved tremendously over the training period and it is the opinion of Dr. Parker that he is the most improved trainee of group. Dr. Parker strongly recommends additional training for Mr. Khaled and Mr. Samir. They can provide the cornerstone to keeping the laboratory abreast of current and developing methods.

The training session continued with lectures on quantitative and qualitative analysis of organic materials using EPA methods. Demonstrations were conducted on the use of Kuderna-Danish Concentrators, clean up columns and analysis of a water sample using the purge and trap. Due to processes which were not under our control the materials necessary for the hands on training were delayed at the airport until two days prior to our departure.

All equipment ordered was delivered to TIMS on June 28, and was immediately placed into the laboratory under the directions of Dr. Parker. Laboratory management was against the idea of placing the equipment into the laboratory directly because they could not track it readily. However, they finally agreed that it was the most logical edict to follow.

PART III

SUMMARY

SUMMARY

The CAU/HBCU/MI team believes that the environmental pollution laboratory at TIMS is capable of operating at a level equivalent to that of a good American laboratory. TIMS has good equipment and a competent, well-trained technical staff. The biggest problem at TIMS is laboratory management, and we feel that the laboratory management should be addressed before any additional funds are committed to the environmental pollution laboratory. A laboratory director with some knowledge of environmental analytical methodology, instrumentation and quality assurance/quality control should be appointed, and this individual must have an administrative organizational structure with an adequate financial base with which to operate. Once these conditions have been met, the next step would be for the laboratory to work as co-partners with some experienced personnel (like the CAU/HBCU/MI training team) to provide additional training and experience. Additional training after the completion of the current project, without a requirement to produce a product (usable data), would be an unwise use of funds. This type of "co-partnership" would involve the issues of planning, human resources and money management (in addition to further advanced technical training). Ideally, this would be a project of approximately one year duration, and again, there must be a requirement to produce a usable product. With the right co-partners, the TIMS environmental pollution laboratory would be able to reach a functional level where they could begin writing environmental technical proposals, conducting necessary research projects and managing the laboratory, monitoring and enforcing Egyptian Laws and Regulations on their own. They are not able to do this and will probably not mature to this stage of independence without additional help and quidance. The CAU/HBCU/MI staff will be glad to help USAID "flesh-out" these ideas and submit a proposal for further work. It is a project we would all like to be involved with, if the opportunity arises.

The CAU/HBCU/MI Consortium training team further believes that the immediate training needs of the TIMS staff have been met as indicated in the individual student assessments. However, progression to a fully functional laboratory as a national resource to effectively monitor and enforce existing regulation requirements for Waste Water Effluents under Egyptian Law 92 (1962) and Law 48 (1984) and Air Emission under their current laws that sets standards and limits for particulate, air toxic and ambient air constituents requires restructuring of the Egyptian Environmental Agencies (role, responsibilities and funding) and setting a national agenda for the environment in the 5-year plan in the Prime Minister's office including the redefining of roles and responsibilities in Ministries of International Cooperation, Economy, Industry, Health and Irrigation where there are overlapping and confused environmental responsibilities.

PART IV

SUPPLIES, REAGENTS AND EQUIPMENT

FISHER SUPPLY LIST
Supply List for Atomic Spectroscopy

NO.	DESCRIPTION	QUAN	ITEM NO.	UNIT COST
1	100 ml Polyethylene Bottles	3 cs	02-893-5C	173.49
2	Total Ionic Strength Adjusting Buffer (TISAB)	1 ea	SB175-4	46.23
3	Glossive Weighing Papers 9 cm x 9 cm or	2 pkg	09-898-12B	18.00
4	Small stainless steel spatulas	2 pkg	14-373	126.00
5	Beakers, Griffin, Pyrex or Kimax, 500 ml	4 pkg	02-539M	67.88
6	Beakers, Griffin, Pyrex or Kimax, 100 ml	2 pkg	02-539H	48.16
7	Beakers, Griffin, Pyrex or Kimax, 50 ml	2 cs	02-539G	138.24
8	Beakers, Griffin, Pyrex or Kimax, 10 ml	2 cs	02-539C	162.00
9	Glass Stirring Rods - 10 cm 1mg x 4mm	4 pkg	11-380-B	39.00
10	Volumetric Flask, 100 ml, Class A	2 cs	10-2105C	266.40
11	Volumetric Pipet, 5.0 ml, Class A	2 cs	13-6502F	115.20
12	Boiling Flask, 250 ml, 24/40 Ground Glass	1 cs	10-067E	117.18
13	Antimony Metal, 99.9+%	1 ea	A845-500	73.47
14	Barium Chloride Dihydrate ($BaCl_2 \cdot 2H_2O$) 99.9+%	1 ea	B34-100	11.01
15	Cadmium Metal, 99.9%+	1 ea	C3-500	44.52
16	Copper Metal Powder, 99.9+%	1 ea	C430-500	30.84
17	Lanthanum Oxide, La_2O_3, for AA Spectroscopy	1 ea	C3357-250	57.42
18	Lithium Carbonate, Li_2CO_3, 99.9+%	1 ea	L119-500	43.05
19	Ammonium Molybdate, $(NH_4)_2MoO_3$, 99.9%	1 ea	A674-500	74.34
20	Nickel Metal Powder, 99.9+%	1 ea	N40-500	48.48
21	Potassium Chloride, 99.9+%	1 ea	P217-500	12.54
22	Selenium Dioxide, SeO_2, 99.9+%	1 ea	S5-464-500	22.98

23	Sodium Chloride, NaC1, 99.9+ %	1 ea	S271-500	11.43
24	1000 ml Polyethylene Bottles	2 pkg	02-893-5F	36.60
25	MM Tubes 6x50	1 cs	14 958A	22.13
26	MM Tubes 16x150	1 cs	14 958H	45.00
27	Bacticapa V Stoppers	1 cs	14 127 28B	42.75
28	EC Medium	1 lb	DF 034010	39.42
29	Gelman Glass Filter 47 mm	1 pkg	09 730 78	19.88
30	Pipet, 1 ml, Class A	2 cs	13-650B	91.80
31	Pipets, 10.0 ml	2 cs	13-650L	110.70
32	Pipet, 50-200 ul	2 ea	21 185 14	288.00
33	Pipets, 200-1000 ul	2 ea	21 185 16	288.00
34	Pipet Tips, Ultra Micro	1 pkg	21 197 2F	27.23
35	Pipet Tips, Rainin Small	1 pkg	21 197 8G	25.50
36	Tips, 1-200 ul	1 pkg	21 244 1	39.00
37	Tips, 201-1000 ul	1 pkg	21 244 2	39.75
38	Screwcap Vials 3 Dr	2 pkg	03 338D	73.50
39	Screwcap Vials, 6 Tall	2 pkg	03-338H	96.76
40	Screwcap Vials Sep-Grdl(Clear)	2 cs	05 719 121	291.98
41	Vial EPA W/O Closure	1 pkg	03-339 5A	43.89
42	Vial/TEF Cap 1/2 Dram	2 pkg	03-339	75.00
43	Screw Cap Test Tubes, 15 ml - PTFE Lined Caps	2pkg	14 933 1A	248.40
44	Tube W/Cap 13x100	3 pkg	14 930 10A	198.72
45	Disposable Pasteur Pipets, 1.0 ml	2 cs	13-678-20A	55.50
46	Btl WM Amber	1 cs	03 320 8F	51.75
47	Borosilicate Sample Bottles and Caps, 1.0 L	1 cs	06414-1D	60.68
48	Borosilicate Sample Bottles and Caps, 500 ml	1 cs	06414-1C	49.58

49	Separatory funnels (Teflon Stopcocks), 250 ml	2 cs	10-437-2C	360.18
50	Separatory funnels (Teflon Stopcocks), 500 ml	2 cs	10-437-2D	413.64
51	Alumina (80-200 Mesh)	1 cs	A540 3	269.66
52	L-Ascorbic Acid	5 ea	A61 100	76.15
54	Florisil (60/100 Mesh)	5 ea	F100-500	176.30
56	Isooctane	1 cs	0298-1	24.67
55	Potassium Carbonate	2 ea	P179-500	47.46
56	Sodium Sulfate	2 ea	S421-500	24.24
57	Sodium Thiosulfate	2 ea	S441-500	86.28
58	2 ml Bulb for Pasteur Pipet	1 pkg	14-065B	24.75
59	30 ml Bulb for use with Volumetric Pipet	6 ea	14-070C	22.50
			TOTAL	$ 5,692.63
			Export	448.00
			Air Freight	838.00
			Insurance	80.00
		TOTAL		$ 7,058.63

PERKIN-ELMER SUPPLY ORDER LIST

Item	Description	Parts number	Quantity	Unit Cost	Total Cost
1	Capillary Columns Adapter	3320146	3	240.00	720.00
2	Detector Receiver	N6020024	3	96.00	288.00
3	Flow Accessory Controller	N6023031	3	165.00	495.00
4	Pressure Regulator	N6023010	3	490.00	1,470.00
	Sub-Total		12		$2,973.00
	Shipping Cost				60.00
	Total				$3,033.00

SUPELCO SUPPLY ORDER LIST

Item	Description	Parts number	quantity	Unit Cost	Total Cost
1	Pesticides, chlorinated	4-2846	0	81.00	0.00
2	Volatiles (VOA-1)	4-2841	2	81.00	162.00
3	Volatiles (VOA-2)	4-2842	2	81.00	162.00
4	VOC Mix 1	4-8775	2	58.00	116.00
5	VOC Mix 2	4-8777	2	58.00	116.00
6	VOC Mix 3	4-8779	2	58.00	116.00
7	VOC Mix 4	4-8786	2	58.00	116.00
8	Purgeable Halocarbon 601-S	4-8769	0	547.00	0.00
9	Purgeable Halocarbon 601 M2	4-8747	2	35.00	70.00
10	Purgeable Aromatics 602M	4-8740	2	35.00	70.00
11	Purgeable Aromatics 602N	4-8760	2	83.00	166.00
12	Phenols 604S	4-8785	1	202.00	202.00
13	Phenols 604N	4-8761	1	202.00	202.00
14	Organochlorine Pesticides PCB 608 S	4-8753	1	506.00	506.00
15	Organochlorine Pesticides PCB 608N	4-8763	1	685.00	685.00
16	PAH 610S	4-8755	1	460.00	460.00
17	PAH 610N	4-8765	1	285.00	285.00
18	5 ul Micro Pippette	5-8618	2	113.00	226.00
19	10 ul Micro Pippete	5-8600	2	120.00	240.00
20	20 ul Micro Pippette	5-8601	2	120.00	240.00
21	Separatory Column 400 X 19 X 22	6-4753	2	49.00	98.00
22	Separatory Column 300 X 22 X 25	6-4754	2	51.00	102.00
23	Separatory Column 400 X 14 X 22	6-4757	2	65.00	130.00

24	Separatory Column 300 X 22 X 25	6-4758	2	71.00	142.00
25	Kuderna-Danish Concentrator Kits	6-4685	5	133.00	665.00
26	Kurderna-Danish, 2 Ball Column	6-4727	5	64.00	320.00
27	Kurdena-Danish, 3 Ball Column	6-4720	5	61.00	305.00
28					

Sub-Total	53	$5,902.00
Shipping Cost (Estimated)		300.00
Total		$6,202.00

SUPELCO SUPPLY ORDER LIST

Item	Description	Parts number	Quantity	Unit Cost	Total Cost
1	Capillary Columns	2-5358	1	1145.00	1,145.00
2	Capillary Columns	2-5341	1	558.00	558.00
3	Capillary Columns	2-5343	1	558.00	558.00
	Sub-Total		3		$2,261.00
	Shipping Cost				9.00
	Total				$2,270.00

BODMAN CHEMICALS

SUPPLY LIST/CAIRO, EGYPT

NO.	DESCRIPTION	QUAN	ITEM NO.	UNIT COST
1	Capillary Column	1 ea	DB-624	925.00
			Shipping Cost	14.00
	TOTAL			939.00

FINAL REPORT

Advanced Training for Tabbin Institute of Metallurgical Studies (TIMS), Cairo, Egypt

USAID CONTRACT #263-010-C-00-2038-00
PROJECT #263-0101

VOLUME II

APPENDIX

ATOMIC ABSORPTION SPECTROSCOPY

Prepared by
A. Curley,
Team Leader
Office of Research and Sponsored Programs
Clark Atlanta University
J.P. Brawley Drive at Fair Street, SW
Atlanta, GA 30314

116

TABLE OF CONTENTS

VOLUME II

HANDOUTS FOR ATOMIC ABSORPTION SPECTROSCOPY

APPENDIX	**Volumes 2 - 4**
Volume 2	Handouts for Atomic Absorption Spectroscopy

177

HANDOUTS

FOR

ATOMIC ABSORPTION SPECTROSCOPY

8K

METALS

(Atomic Absorption Methods)

1. Scope and Application

1.1 Metals in solution may be readily determined by atomic absorption spectroscopy. The method is simple, rapid, and applicable to a large number of metals in drinking, surface, and saline waters, and domestic and industrial wastes. While drinking waters free of particulate matter may be analyzed directly, domestic and industrial wastes require processing to solubilize suspended material. Sludges, sediments and other solid type samples may also be analyzed after proper pretreatment.

1.2 Detection limits, sensitivity and optimum ranges of the metals will vary with the various makes and models of satisfactory atomic absorption spectrophotometers. The data shown in Table 1, however, provide some indication of the actual concentration ranges measurable by direct aspiration and using furnace techniques. In the majority of instances the concentration range shown in the table by direct aspiration may be extended much lower with scale expansion and conversely extended upwards by using a less sensitive wavelength or by rotating the burner head. Detection limits by direct aspiration may also be extended through concentration of the sample and/or through solvent extraction techniques. Lower concentrations may also be determined using the furnace techniques. The concentration ranges given in Table 1 are somewhat dependent on equipment such as the type of spectrophotometer and furnace accessory, the energy source and the degree of electrical expansion of the output signal. When using furnace techniques, however, the analyst should be cautioned as to possible chemical reactions occurring at elevated temperatures which may result in either suppression or enhancement of the analysis element. To insure valid data with furnace techniques, the analyst must examine each matrix for interference effects (see 5.2.1) and if detected, treat accordingly using either successive dilution, matrix modification or method of standard additions (see 8.5).

1.3 Where direct aspiration atomic absorption techniques do not provide adequate sensitivity, in addition to the furnace procedure, reference is made to specialized procedures such as the gaseous hydride method for arsenic and selenium, the cold vapor technique for mercury, and the chelation–extraction procedure for selected metals. Reference to approved colorimetric methods is also made.

1.4 Atomic absorption procedures are provided as the methods of choice; however, other instrumental methods have also been shown to be capable of producing precise and accurate analytical data. These instrumental techniques include emission spectroscopy, X-ray fluorescence, spark source mass spectroscopy, and anodic stripping to name but a few. The analyst should be cautioned that these methods are highly specialized techniques requiring a high degree of skill to interpret results and obtain valid data.

Approved for NPDES and SDWA
Issued 1969
Editorial revision 1974 and 1978

These above mentioned techniques are presently considered as alternate test procedures and approval must be obtained prior to their use.

2. Summary of Method

2.1 In direct aspiration atomic absorption spectroscopy a sample is aspirated and atomized in a flame. A light beam from a hollow cathode lamp whose cathode is made of the element to be determined is directed through the flame into a monochromator, and onto a detector that measures the amount of light absorbed. Absorption depends upon the presence of free unexcited ground state atoms in the flame. Since the wavelength of the light beam is characteristic of only the metal being determined, the light energy absorbed by the flame is a measure of the concentration of that metal in the sample. This principle is the basis of atomic absorption spectroscopy.

2.2 Although methods have been reported for the analysis of solids by atomic absorption spectroscopy (Spectrochim Acta, *24B* 53, 1969) the technique generally is limited to metals in solution or solubilized through some form of sample processing.

2.2.1 Preliminary treatment of wastewater and/or industrial effluents is usually necessary because of the complexity and variability of the sample matrix. Suspended material must be subjected to a solubilization process before analysis. This process may vary because of the metals to be determined and the nature of the sample being analyzed. When the breakdown of organic material is necessitated, the process should include a wet digestion with nitric acid.

2.2.2 In those instances where complete characterization of a sample is desired, the suspended material must be analyzed separately. This may be accomplished by filtration and acid digestion of the suspended material. Metallic constituents in this acid digest are subsequently determined and the sum of the dissolved plus suspended concentrations will then provide the total concentrations present. The sample should be filtered as soon as possible after collection and the filtrate acidified immediately.

2.2.3 The total sample may also be treated with acid without prior filtration to measure what may be termed "total recoverable" concentrations.

2.3 When using the furnace technique in conjunction with an atomic absorption spectrophotometer, a representative aliquot of a sample is placed in the graphite tube in the furnace, evaporated to dryness, charred, and atomized. As a greater percentage of available analyte atoms are vaporized and dissociated for absorption in the tube than the flame, the use of small sample volumes or detection of low concentrations of elements is possible. The principle is essentially the same as with direct aspiration atomic absorption except a furnace, rather than a flame, is used to atomize the sample. Radiation from a given excited element is passed through the vapor containing ground state atoms of that element. The intensity of the transmitted radiation decreases in proportion to the amount of the ground state element in the vapor.

TABLE 1

Atomic Absorption Concentration Ranges[1]

Metal	Direct Aspiration: Detection Limit mg/l	Direct Aspiration: Sensitivity mg/l	Direct Aspiration: Optimum Concentration Range mg/l	Furnace Procedure[4,5]: Detection Limit ug/l	Furnace Procedure[4,5]: Optimum Concentration Range ug/l
Aluminum	0.1	1	5 - 50	3	20 - 200
Antimony	0.2	0.5	1 - 40	3	20 - 300
Arsenic[2,3]	0.002	-	0.002 - 0.02	1	5 - 100
Barium(p)	0.1	0.4	1 - 20	2	10 - 200
Beryllium	0.005	0.025	0.05 - 2	0.2	1 - 30
Cadmium	0.005	0.025	0.05 - 2	0.1	0.5 - 10
Calcium	0.01	0.08	0.2 - 7	-	- -
Chromium	0.05	0.25	0.5 - 10	1	5 - 100
Cobalt	0.05	0.2	0.5 - 5	1	5 - 100
Copper	0.02	0.1	0.2 - 5	1	5 - 100
Gold	0.1	0.25	0.5 - 20	1	5 - 100
Indium(p)	3	8	20 - 500	30	100 - 1500
Iron	0.03	0.12	0.3 - 5	1	5 - 100
Lead	0.1	0.5	1 - 20	1	5 - 100
Magnesium	0.001	0.007	0.02 - 0.5	-	- -
Manganese	0.01	0.05	0.1 - 3	0.2	1 - 30
Mercury[3]	0.0002	-	0.0002 - 0.01	-	- -
Molybdenum(p)	0.1	0.4	1 - 40	1	3 - 60
Nickel(p)	0.04	0.15	0.3 - 5	1	5 - 50
Osmium	0.3	1	2 - 100	20	50 - 500
Palladium(p)	0.1	0.25	0.5 - 15	5	20 - 400
Platinum(p)	0.2	2	5 - 75	20	100 - 2000
Potassium	0.01	0.04	0.1 - 2	-	-
Rhenium(p)	5	15	50 - 1000	200	500 - 5000
Rhodium(p)	0.05	0.3	1 - 30	5	20 - 400
Ruthenium	0.2	0.5	1 - 50	20	100 - 2000
Selenium[2,3]	0.002	-	0.002 - 0.02	2	5 - 100
Silver	0.01	0.06	0.1 - 4	0.2	1 - 25
Sodium	0.002	0.015	0.03 - 1	-	- -
Thallium	0.1	0.5	1 - 20	1	5 - 100
Tin	0.8	4	10 - 300	5	20 - 300
Titanium (p)	0.4	2	5 - 100	10	50 - 500
Vanadium (p)	0.2	0.8	2 - 100	4	10 - 200
Zinc	0.005	0.02	0.05 - 1	0.05	0.2 - 4

(1) The concentrations shown are not contrived values and should be obtainable with any satisfactory atomic absorption spectrophotometer.
(2) Gaseous hydride method.
(3) Cold vapor technique.
(4) For furnace sensitivity values consult instrument operating manual.
(5) The listed furnace values are those expected when using a 20 ul injection and normal gas flow except in the case of arsenic and selenium where gas interrupt is used. The symbol (p) indicates the use of pyrolytic graphite with the furnace procedure.

The metal atoms to be measured are placed in the beam of radiation by increasing the temperature of the furnace thereby causing the injected specimen to be volatilized. A monochromator isolates the characteristic radiation from the hollow cathode lamp and a photosensitive device measures the attenuated transmitted radiation.

3. Definition of Terms

3.1 Optimum Concentration Range: A range, defined by limits expressed in concentration, below which scale expansion must be used and above which curve correction should be considered. This range will vary with the sensitivity of the instrument and the operating condition employed.

3.2 Sensitivity: The concentration in milligrams of metal per liter that produces an absorption of 1%.

3.3 Detection Limit: Detection limits can be expressed as either an instrumental or method parameter. The limiting factor of the former using acid water standards would be the signal to noise ratio and degree of scale expansion used; while the latter would be more affected by the sample matrix and preparation procedure used. The Scientific Apparatus Makers Association (SAMA) has approved the following definition for detection limit: that concentration of an element which would yield an absorbance equal to twice the standard deviation of a series of measurements of a solution, the concentraton of which is distinctly detectable above, but close to blank absorbance measurement. The detection limit values listed in Table I and on the individual analysis sheets are to be considered minimum working limits achievable with the procedures given in this manual. These values may differ from the optimum detection limit reported by the various instrument manufacturers.

3.4 Dissolved Metals: Those constituents (metals) which will pass through a 0.45 u membrane filter.

3.5 Suspended Metals: Those constituents (metals) which are retained by a 0.45 u membrane filter.

3.6 Total Metals: The concentration of metals determined on an unfiltered sample following vigorous digestion (Section 4.1.3), or the sum of the concentrations of metals in both the dissolved and suspended fractions.

3.7 Total Recoverable Metals: The concentration of metals in an unfiltered sample following treatment with hot dilute mineral acid (Section 4.1.4).

4. Sample Handling and Preservation

4.1 For the determination of trace metals, contamination and loss are of prime concern. Dust in the laboratory environment, impurities in reagents and impurities on laboratory apparatus which the sample contacts are all sources of potential contamination. For liquid samples, containers can introduce either positive or negative errors in the measurement of trace metals by (a) contributing contaminants through leaching or surface desorption and (b) by depleting concentrations through adsorption. Thus the collection and treatment of the sample prior to analysis requires particular attention. The sample bottle whether borosilicate glass, polyethylene polypropylene or Teflon should be thoroughly washed with detergent and tap water; rinsed with 1:1 nitric acid, tap

water, 1:1 hydrochloric acid, tap water and finally deionized distilled water in that order.

NOTE 1: Chromic acid may be useful to remove organic deposits from glassware; however, the analyst should be cautioned that the glassware must be thoroughly rinsed with water to remove the last traces of chromium. This is especially important if chromium is to be included in the analytical scheme. A commercial product—NOCHROMIX—available from Godax Laboratories, 6 Varick St. New York, N.Y. 10013, may be used in place of chromic acid. [Chromic acid should not be used with plastic bottles.]

NOTE 2: If it can be documented through an active analytical quality control program using spiked samples, reagent and sample blanks, that certain steps in the cleaning procedure are not required for routine samples, those steps may be eliminated from the procedure.

Before collection of the sample a decision must be made as to the type of data desired, i.e., dissolved, suspended, total or total recoverable. For container preference, maximum holding time and sample preservation at time of collection see Table 1 in the front part of this manual. Drinking water samples containing suspended and settleable material should be prepared using the total recoverable metal procedure (section 4.1.4).

4.1.1 For the determination of dissolved constituents the sample must be filtered through a 0.45 u membrane filter as soon as practical after collection. (Glass or plastic filtering apparatus using plain, non-grid marked, membrane filters are recommended to avoid possible contamination.) Use the first 50–100 ml to rinse the filter flask. Discard this portion and collect the required volume of filtrate. Acidify the filtrate with 1:1 redistilled HNO_3 to a pH of <2. Normally, 3 ml of (1:1) acid per liter should be sufficient to preserve the sample (See Note 3). If hexavalent chromium is to be included in the analytical scheme, a portion of the filtrate should be transferred before acidification to a separate container and analyzed as soon as possible using Method 218.4. Analyses performed on a sample so treated shall be reported as "dissolved" concentrations.

NOTE 3: If a precipitate is formed upon acidification, the filtrate should be digested using 4.1.3. Also, it has been suggested (International Biological Program, Symposium on Analytical Methods, Amsterdam, Oct. 1966) that additional acid, as much as 25 ml of conc. HCl/liter, may be required to stabilize certain types of highly buffered samples if they are to be stored for any length of time. Therefore, special precautions should be observed for preservation and storage of unusual samples intended for metal analysis.

4.1.2 For the determination of suspended metals a representative volume of unpreserved sample must be filtered through a 0.45 u membrane filter. When considerable suspended material is present, as little as 100 ml of a well mixed sample is filtered. Record the volume filtered and transfer the membrane filter containing the insoluble material to a 250 ml Griffin beaker and add 3 ml conc. redistilled HNO_3. Cover the beaker with a watch glass and heat gently. The warm acid will soon dissolve the membrane. Increase the temperature of the hot plate and digest the material. When the acid has nearly evaporated, cool the beaker and watch glass and add another 3 ml of conc. redistilled HNO_3. Cover and continue heating until

the digestion is complete, generally indicated by a light colored digestate. Evaporate to near dryness (DO NOT BAKE), add 5 ml distilled HCl (1:1) and warm the beaker gently to dissolve any soluble material. (If the sample is to be analyzed by the furnace procedure, 1 ml of 1:1 distilled HNO_3 per 100 ml dilution should be substituted for the distilled 1:1 HCl.) Wash down the watch glass and beaker walls with deionized distilled water and filter the sample to remove silicates and other insoluble material that could clog the atomizer. Adjust the volume to some predetermined value based on the expected concentrations of metals present. This volume will vary depending on the metal to be determined. The sample is now ready for analysis. Concentrations so determined shall be reported as "suspended" (See Note 4.)

NOTE 4: Certain metals such as antimony arsenic, gold, iridium, mercury, osmium, palladium, platinium, rhenium, rhodium, ruthenium, selenium, silver, thallium, tin and titanium require modification of the digestion procedure and the individual sheets for these metals should be consulted.

4.1.3 For the determination of total metals the sample is acidified with 1:1 redistilled HNO_3 to a pH of less than 2 at the time of collection. The sample is not filtered before processing. Choose a volume of sample appropriate for the expected level of metals. If much suspended material is present, as little as 50–100 ml of well mixed sample will most probably be sufficient. (The sample volume required may also vary proportionally with the number of metals to be determined.)

Transfer a representative aliquot of the well mixed sample to a Griffin beaker and add 3 ml of conc. redistilled HNO_3. Place the beaker on a hot plate and evaporate to near dryness cautiously, making certain that the sample does not boil. (DO NOT BAKE.) Cool the beaker and add another 3 ml portion of conc. redistilled HNO_3. Cover the beaker with a watch glass and return to the hot plate. Increase the temperature of the hot plate so that a gentle reflux action occurs. Continue heating, adding additional acid as necessary, until the digestion is complete (generally indicated when the digestate is light in color or does not change in appearance with continued refluxing). Again, evaporate to near dryness and cool the beaker. Add a small quantity of redistilled 1:1 HCl (5 ml/100 ml of final solution) and warm the beaker to dissolve any precipitate or residue resulting from evaporation. (If the sample is to be analyzed by the furnace procedure, substitute distilled HNO_3 for 1:1 HCl so that the final dilution contains 0.5% (v/v) HNO_3.) Wash down the beaker walls and watch glass with distilled water and filter the sample to remove silicates and other insoluble material that could clog the atomizer. Adjust the volume to some predetermined value based on the expected metal concentrations. The sample is now ready for analysis. Concentrations so determined shall be reported as "total" (see Note 4).

4.1.4 To determine total recoverable metals, acidify the entire sample at the time of collection with conc. redistilled HNO_3, 5 ml/1. At the time of analysis a 100 ml aliquot of well mixed sample is transferred to a beaker or flask. Five ml of distilled HCl (1:1) is added and the sample heated on a steam bath or hot plate until the

volume has been reduced to 15-20 ml making certain the samples do not boil. (If the sample is being prepared for furnace analysis, the same process should be followed except HCl should be omitted.) After this treatment the sample is filtered to remove silicates and other insoluble material that could clog the atomizer and the volume adjusted to 100 ml. The sample is then ready for analysis. Concentrations so determined shall be reported as "total". (See Notes 4, 5, and 6.)

NOTE 5: The analyst should be cautioned that this digestion procedure may not be sufficiently vigorous to destroy certain metal complexes if a colorimetric procedure is to be employed for the final determination. When this is suspect, the more vigorous digestion given in 4.1.3 should be followed.

NOTE 6: For drinking water analyses by direct aspiration, the final volume may be reduced to effect up to a 10X concentration of the sample, provided the total dissolved solids in the original sample do not exceed 500 mg/l, the determination is corrected for any non-specific absorbance and there is no loss by precipitation.

5. Interferences

5.1 Direct Aspiration

5.1.1 The most troublesome type of interference in atomic absorption spectrophotometry is usually termed "chemical" and is caused by lack of absorption of atoms bound in molecular combination in the flame. This phenomenon can occur when the flame is not sufficiently hot to dissociate the molecule, as in the case of phosphate interference with magnesium, or because the dissociated atom is immediately oxidized to a compound that will not dissociate further at the temperature of the flame. The addition of lanthanum will overcome the phosphate interference in the magnesium, calcium and barium determinations. Similarly, silica interference in the determination of manganese can be eliminated by the addition of calcium.

5.1.2 Chemical interferences may also be eliminated by separating the metal from the interfering material. While complexing agents are primarily employed to increase the sensitivity of the analysis, they may also be used to eliminate or reduce interferences.

5.1.3 The presence of high dissolved solids in the sample may result in an interference from non-atomic absorbance such as light scattering. If background correction is not available, a non-absorbing wavelength should be checked. Preferably, high solids type samples should be extracted (see 5.1.1 and 9.2).

5.1.4 Ionization interferences occur where the flame temperature is sufficiently high to generate the removal of an electron from a neutral atom, giving a positive charged ion. This type of interference can generally be controlled by the addition, to both standard and sample solutions, of a large excess of an easily ionized element.

5.1.5 Although quite rare, spectral interference can occur when an absorbing wavelength of an element present in the sample but not being determined falls within the width of the absorption line of the element of interest. The results of the determination will then be erroneously high, due to the contribution of the interfering element to the atomic absorption signal. Also, interference can occur

when resonant energy from another element in a multi-element lamp or a metal impurity in the lamp cathode falls within the bandpass of the slit setting and that metal is present in the sample. This type of interference may sometimes be reduced by narrowing the slit width.

5.2 Flameless Atomization

5.2.1 Although the problem of oxide formation is greatly reduced with furnace procedures because atomization occurs in an inert atmosphere, the technique is still subject to chemical and matrix interferences. The composition of the sample matrix can have a major effect on the analysis. It is those effects which must be determined and taken into consideration in the analysis of each different matrix encountered. To help verify the absence of matrix or chemical interference use the following procedure. Withdraw from the sample two equal aliquots. To one of the aliquots add a known amount of analyte and dilute both aliquots to the same predetermined volume. [The dilution volume should be based on the analysis of the undiluted sample. Preferably, the dilution should be 1:4 while keeping in mind the optimum concentration range of the analysis. Under no circumstances should the dilution be less than 1:1]. The diluted aliquots should then be analyzed and the unspiked results multiplied by the dilution factor should be compared to the original determination. Agreement of the results (within ±10%) indicates the absence of interference. Comparison of the actual signal from the spike to the expected response from the analyte in an aqueous standard should help confirm the finding from the dilution analysis. Those samples which indicate the presence of interference, should be treated in one or more of the following ways.

a. The samples should be successively diluted and reanalyzed to determine if the interference can be eliminated.

b. The matrix of the sample should be modified in the furnace. Examples are the addition of ammonium nitrate to remove alkali chlorides, ammonium phosphate to retain cadmium, and nickel nitrate for arsenic and selenium analyses [ATOMIC ABSORPTION NEWSLETTER Vol. 14, No. 5, p 127, Sept-Oct 1975]. The mixing of hydrogen with the inert purge gas has also been used to suppress chemical interference. The hydrogen acts as a reducing agent and aids in molecular dissociation.

c. Analyze the sample by method of standard additions while noting the precautions and limitations of its use (See 8.5).

5.2.2 Gases generated in the furnace during atomization may have molecular absorption bands encompassing the analytical wavelength. When this occurs, either the use of background correction or choosing an alternate wavelength outside the absorption band should eliminate this interference. Non-specific broad band absorption interference can also be compensated for with background correction.

5.2.3 Interference from a smoke-producing sample matrix can sometimes be reduced by extending the charring time at a higher temperature or utilizing an ashing cycle in

the presence of air. Care must be taken, however, to prevent loss of the analysis element.

5.2.4 Samples containing large amounts of organic materials should be oxidized by conventional acid digestion prior to being placed in the furnace. In this way broad band absorption will be minimized.

5.2.5 From anion interference studies in the graphite furnace it is generally accepted that nitrate is the preferred anion. Therefore nitric acid is preferable for any digestion or solubilization step. If another acid in addition to HNO_3 is required a minimum amount should be used. This applies particularly to hydrochloric and to a lesser extent to sulfuric and phosphoric acids.

5.2.6 Carbide formation resulting from the chemical environment of the furnace has been observed with certain elements that form carbides at high temperatures. Molybdenum may be cited as an example When this takes place, the metal will be released very slowly from the carbide as atomization continues. For molybdenum, one may be required to atomize for 30 seconds or more before the signal returns to baseline levels. This problem is greatly reduced and the sensitivity increased with the use of pyrolytically-coated graphite.

5.2.7 Ionization interferences have to date not been reported with furnace techniques.

5.2.8 For comments on spectral interference see section 5.1.5.

5.2.9 Contamination of the sample can be a major source of error because of the extreme sensitivities achieved with the furnace. The sample preparation work area should be kept scrupulously clean. All glassware should be cleaned as directed in part 6.9 of the Atomic Absorption Methods section of this manual. Pipet tips have been known to be a source of contamination. If suspected, they should be acid soaked with 1:5 HNO_3 and rinsed thoroughly with tap and deionized water. The use of a better grade pipet tip can greatly reduce this problem. It is very important that special attention be given to reagent blanks in both analysis and the correction of analytical results. Lastly, pyrolytic graphite because of the production process and handling can become contaminated. As many as five to possibly ten high temperature burns may be required to clean the tube before use.

6. Apparatus

6.1 Atomic absorption spectrophotometer: Single or dual channel, single-or double-beam instrument having a grating monochromator, photomultiplier detector, adjustable slits, a wavelength range of 190 to 800 nm, and provisions for interfacing with a strip chart recorder.

6.2 Burner: The burner recommended by the particular instrument manufacturer should be used. For certain elements the nitrous oxide burner is required.

6.3 Hollow cathode lamps: Single element lamps are to be preferred but multi-element lamps may be used. Electrodeless discharge lamps may also be used when available.

6.4 Graphite furnace: Any furnace device capable of reaching the specified temperatures is satisfactory.

6.5 Strip chart recorder: A recorder is strongly recommended for furnace work so that there will be a permanent record and any problems with the analysis such as drift, incomplete atomization, losses during charring, changes in sensitivity, etc., can be easily recognized.

6.6 Pipets: Microliter with disposable tips. Sizes can range from 5 to 100 microliters as required. NOTE 7: Pipet tips which are white in color, do not contain CdS, and have been found suitable for research work are available from Ulster Scientific, Inc. 53 Main St. Highland, NY 12528 (914) 691-7500.

6.7 Pressure-reducing valves: The supplies of fuel and oxidant shall be maintained at pressures somewhat higher than the controlled operating pressure of the instrument by suitable valves.

6.8 Separatory flasks: 250 ml, or larger, for extraction with organic solvents.

6.9 Glassware: All glassware, linear polyethylene, polyproplyene or Teflon containers, including sample bottles, should be washed with detergent, rinsed with tap water, 1:1 nitric acid, tap water, 1:1 hydrochloric acid, tap water and deionized distilled water in that order [See Notes 1 and 2 under (4.1) concerning the use of chromic acid and the cleaning procedure.]

6.10 Borosilicate glass distillation apparatus.

7. Reagents

7.1 Deionized distilled water: Prepare by passing distilled water through a mixed bed of cation and anion exchange resins. Use deionized distilled water for the preparation of all reagents, calibration standards, and as dilution water.

7.2 Nitric acid (conc.): If metal impurities are found to be present, distill reagent grade nitric acid in a borosilicate glass distillation apparatus or use a spectrograde acid.
Caution: Distillation should be performed in hood with protective sash in place.

7.2.1 Nitric Acid (1:1): Prepare a 1:1 dilution with deionized, distilled water by adding the conc. acid to an equal volume of water.

7.3 Hydrochloric acid (1:1): Prepare a 1:1 solution of reagent grade hydrochloric acid and deionized distilled water. If metal impurities are found to be present, distill this mixture from a borosilicate glass distillation apparatus or use a spectrograde acid.

7.4 Stock standard metal solutions: Prepare as directed in (8.1) and under the individual metal procedures. Commercially available stock standard solutions may also be used.

7.5 Calibration standards: Prepare a series of standards of the metal by dilution of the appropriate stock metal solution to cover the concentration range desired.

7.6 Fuel and oxidant: Commercial grade acetylene is generally acceptable. Air may be supplied from a compressed air line, a laboratory compressor, or from a cylinder of compressed air. Reagent grade nitrous oxide is also required for certain determinations. Standard, commercially available argon and nitrogen are required for furnace work.

7.7 Special reagents for the extraction procedure.

7.7.1 Pyrrolidine dithiocarbamic acid (PDCA) "see footnote": Prepare by adding 18 ml of analytical reagent grade pyrrolidine to 500 ml of chloroform in a liter flask.

The name pyrrolidine dithiocarbamic acid (PDCA), although commonly referenced in the scientific literature is ambiguous. From the chemical reaction of pyrrolidine and carbon disulfide a more proper name would be 1-pyrrolidine carbodithioic acid, PCDA (CAS Registry No. 25769-03-3).

(See Note 8) Cool and add 15 ml of carbon disulfide in small portions and with swirling. Dilute to 1 liter with chloroform. The solution can be used for several months if stored in a brown bottle in a refrigerator.

NOTE 8: An acceptable grade of pyrrolidine may be obtained from the Aldrich Chemical Co., 940 West St. Paul Ave., Milwaukee, WI. 53233 (414, 273-3850).

7.7.2 Ammonium hydroxide, 2N: Dilute 13 ml conc. NH_4OH to 100 ml with deionized distilled water.

7.7.3 Bromophenol blue indicator (1 g/liter): Dissolve 0.1 g bromophenol blue in 100 ml of 50 percent ethanol or isopropanol.

7.7.4 HCl, 2.5% v/v: Dilute 2 ml redistilled HCl (6N) to 40 ml with deionized distilled water.

8. Preparation of Standards and Calibration

8.1 Stock standard solutions are prepared from high purity metals, oxides or nonhygroscopic reagent grade salts using deionized distilled water and redistilled nitric or hydrochloric acids. (See individual analysis sheets for specific instruction.) Sulfuric or phosphoric acids should be avoided as they produce an adverse effect on many elements. The stock solutions are prepared at concentrations of 1000 mg of the metal per liter. Commercially available standard solutions may also be used.

8.2 Calibration standards are prepared by diluting the stock metal solutions at the time of analysis. For best results, calibration standards should be prepared fresh each time an analysis is to be made and discarded after use. Prepare a blank and at least four calibration standards in graduated amounts in the appropriate range. The calibration standards should be prepared using the same type of acid or combination of acids and at the same concentration as will result in the samples following processing. As filtered water samples are preserved with 1:1 redistilled HNO_3 (3 ml per liter), calibration standards for these analyses should be similarly prepared with HNO_3. Beginning with the blank and working toward the highest standard, aspirate the solutions and record the readings. Repeat the operation with both the calibration standards and the samples a sufficient number of times to secure a reliable average reading for each solution. Calibration standards for furnace procedures should be prepared as described on the individual sheets for that metal.

8.3 Where the sample matrix is so complex that viscosity, surface tension and components cannot be accurately matched with standards, the method of standard addition must be used. This technique relies on the addition of small, known amounts of the analysis element to portions of the sample—the absorbance difference between those and the original solution giving the slope of the calibration curve. The method of standard addition is described in greater detail in (8.5).

65

19

8.4 For those instruments which do not read out directly in concentration, a calibration curve is prepared to cover the appropriate concentration range. Usually, this means the preparation of standards which produce an absorption of 0 to 80 percent. The correct method is to convert the percent absorption readings to absorbance and plot that value against concentration. The following relationship is used to convert absorption values to absorbance:

$$\text{absorbance} = \log(100/\%T) = 2 - \log \%T$$
$$\text{where } \%T = 100 - \%\ \text{absorption}$$

As the curves are frequently nonlinear, especially at high absorption values, the number of standards should be increased in that portion of the curve.

8.5 Method of Standard Additions: In this method, equal volumes of sample are added to a deionized distilled water blank and to three standards containing different known amounts of the test element. The volume of the blank and the standards must be the same. The absorbance of each solution is determined and then plotted on the vertical axis of a graph, with the concentrations of the known standards plotted on the horizontal axis. When the resulting line is extrapolated back to zero absorbance, the point of interception of the abscissa is the concentration of the unknown. The abscissa on the left of the ordinate is scaled the same as on the right side, but in the opposite direction from the ordinate. An example of a plot so obtained is shown in Fig. 1.

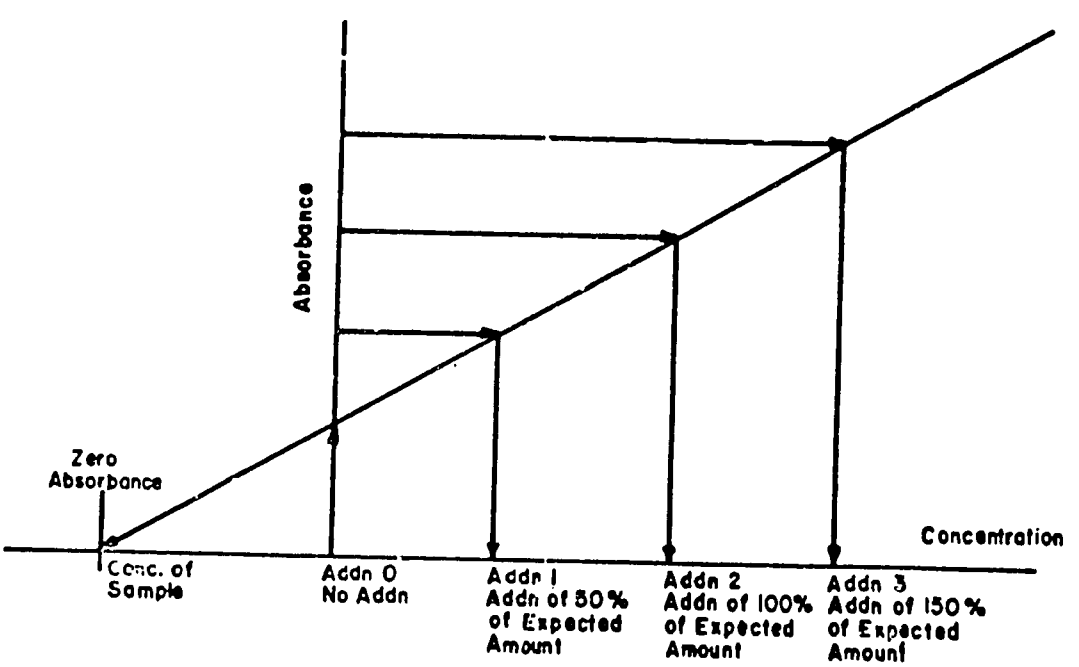


FIGURE 1. STANDARD ADDITION PLOT

The method of standard additions can be very useful, however, for the results to be valid the following limitations must be taken into consideration:

a) the absorbance plot of sample and standards must be linear over the concentration range of concern. For best results the slope of the plot should be nearly the same as the slope of the aqueous standard curve. If the slope is significantly different (more than 20%) caution should be exercised.

b) the effect of the interference should not vary as the ratio of analyte concentration to sample matrix changes and the standard addition should respond in a similar manner as the analyte.

c) the determination must be free of spectral interference and corrected for non-specific background interference.

9. General Procedure for Analysis by Atomic Absorption

9.1 Direct Aspiration: Differences between the various makes and models of satisfactory atomic absorption spectrophotometers prevent the formulation of detailed instructions applicable to every instrument. The analyst should follow the manufacturer's operating instructions for his particular instrument. In general, after choosing the proper hollow cathode lamp for the analysis, the lamp should be allowed to warm up for a minimum of 15 minutes unless operated in a double beam mode. During this period, align the instrument, position the monochromator at the correct wavelength, select the proper monochromator slit width, and adjust the hollow cathode current according to the manufactuerer's recommendation. Subsequently, light the flame and regulate the flow of fuel and oxidant, adjust the burner and nebulizer flow rate for maximum percent absorption and stability, and balance the photometer. Run a series of standards of the element under analysis and construct a calibration curve by plotting the concentrations of the standards against the absorbance. For those instruments which read directly in concentration set the curve corrector to read out the proper concentration. Aspirate the samples and determine the concentrations either directly or from the calibration curve. Standards must be run each time a sample or series of samples are run.

9.1.1 Calculation - Direct determination of liquid samples: Read the metal value in mg/l from the calibration curve or directly from the readout system of the instrument.

9.1.1.1 If dilution of sample was required:

$$\text{mg/l metal in sample} = A \left(\frac{C + B}{C}\right)$$

where:

A = mg/l of metal indicated aliquot from calibration curve

B = ml of deionized distilled water used for dilution

C = ml of sample aliquot

Aluminum, beryllium, barium and strontium also do not react with APDC. While the **APDC-MIBK** chelating-solvent system can be used satisfactorily, it is possible to experience difficulties. (See Note 9.)

NOTE 9: Certain metal chelates, manganese-APDC in particular, are not stable in MIBK and will redissolve into the aqueous phase on standing. The extraction of other metals is sensitive to both shaking rate and time. As with cadmium, prolonged extraction beyond 1 minute, will reduce the extraction efficiency, whereas 3 minutes of vigorous shaking is required for chromium.

Also, when multiple metals are to be determined either larger sample volumes must be extracted or individual extractions made for each metal being determined. The acid form of APDC-pyrrolidine dithiocarbamic acid prepared directly in chloroform as described by Lakanen, [Atomic Absorption Newsletter *5*, p 17 (1966)], (see 7.7.1) has been found to be most advantageous. In this procedure the more dense chloroform layer allows for easy combination of multiple extractions which are carried out over a broader pH range favorable to multielement extractions. Pyrrolidine dithiocarbamic acid in chloroform is very stable and may be stored in a brown bottle in the refrigerator for months. Because chloroform is used as the solvent, it may not be aspirated into the flame. The following procedure is suggested.

9.2.1 Extraction procedure with pyrrolidine dithiocarbamic acid (PDCA) in chloroform.

9.2.1.1 Transfer 200 ml of sample into a 250 ml separatory funnel, add 2 drops bromphenol blue indicator solution (7.7.3) and mix.

9.2.1.2 Prepare a blank and sufficient standards in the same manner and adjust the volume of each to approximately 200 ml with deionized distilled water. All of the metals to be determined may be combined into single solutions at the appropriate concentration levels.

9.2.1.3 Adjust the pH by addition of 2N NH_4OH solution (7.7.2) until a blue color persists. Add HCl (7.7.4) dropwise until the blue color just disappears; then add 2.0 ml HCl (7.7.4) in excess. The pH at this point should be 2.3. (The pH adjustment may be made with a pH meter instead of using indicator.)

9.2.1.4 Add 5 ml of PDCA-chloroform reagent (7.7.1) and shake vigorously for 2 minutes. Allow the phases to separate and drain the chloroform layer into a 100 ml beaker. (See NOTE 10.)

NOTE 10: If hexavalent chromium is to be extracted, the aqueous phase must be readjusted back to a pH of 2.3 after the addition of PDCA–chloroform and maintained at that pH throughout the extraction. For multielement extraction, the pH may adjusted upward [illegible] been extracted

9.2.1.5 Add a second portion of 5 ml PDCA-chloroform reagent (7.7.1) and shake vigorously for 2 minutes. Allow the phases to separate and combine the chloroform phase with that obtained in step (9.2.1.4).

9.2.1.6 Determine the pH of the aqueous phase and adjust to 4.5.

9.2.1.7 Repeat step (9.2.1.4) again combining the solvent extracts.

9.2.1.8 R adjust the pH to 5.5, and extract a fourth time. Combine all extracts and evaporate to dryness on a steam bath.

9.2.1.9 Hold the beaker at a 45 degree angle, and slowly add 2 ml of conc. distilled nitric acid, rotating the beaker to effect thorough contact of the acid with the residue.

9.2.1.10 Place the beaker on a low temperature hotplate or steam bath and evaporate just to dryness.

9.2.1.11 Add 2 ml of nitric acid (1:1) to the beaker and heat for 1 minute. Cool, quantitatively transfer the solution to a 10 ml volumetric flask and bring to volume with distilled water. The sample is now ready for analysis.

9.2.2 Prepare a calibration curve by plotting absorbance versus the concentration of the metal standard (*ug*/l) in the 200 ml extracted standard solution. To calculate sample concentration read the metal value in *ug*/l from the calibration curve or directly from the readout system of the instrument. If dilution of the sample was required use the following equation:

$$\text{mg/l metal in sample} = Z\left(\frac{C + B}{C}\right)$$

where:

Z = *ug*/l of metal in diluted aliquot from calibration curve
B = ml of deionized distilled water used for dilution
C = ml of sample aliquot

9.3 Furnace Procedure: Furnace devices (flameless atomization) are a most useful means of extending detection limits. Because of differences between various makes and models of satisfactory instruments, no detailed operating instuctions can be given for each instrument. Instead, the analyst should follow the instructions provided by the manufacturer of his particular instrument and use as a guide the temperature settings and other instrument conditions listed on the individual analysis sheets which are recommended for the Perkin-Elmer HGA-2100. In addition, the following points may be helpful.

9.3.1 With flameless atomization, background correction becomes of high importance

beam. If no correction is made, sample absorbance will be greater than it should be, and the analytical result will be erroneously high.

9.3.2 If during atomization all the analyte is not volatilized and removed from the furnace, memory effects will occur. This condition is dependent on several factors such as the volatility of the element and its chemical form, whether pyrolytic graphite is used, the rate of atomization and furnace design. If this situation is detected through blank burns, the tube should be cleaned by operating the furnace at full power for the required time period as needed at regular intervals in the analytical scheme.

9.3.3 Some of the smaller size furnace devices, or newer furnaces equipped with feedback temperature control (Instrumentation Laboratories MODEL 555, Perkin-Elmer MODELS HGA 2200 and HGA 76B, and Varian MODEL CRA-90) employing faster rates of atomization, can be operated using lower atomization temperatures for shorter time periods than those listed in this manual.

9.3.4 Although prior digestion of the sample in many cases is not required providing a representative aliquot of sample can be pipeted into the furnace, it will provide for a more uniform matrix and possibly lessen matrix effects.

9.3.5 Inject a measured microliter aliquot of sample into the furnace and atomize. If the concentration found is greater than the highest standard, the sample should be diluted in the same acid matrix and reanalyzed. The use of multiple injections can improve accuracy and help detect furnace pipetting errors.

9.3.6 To verify the absence of interference, follow the procedure as given in part 5.2.1.

9.3.7 A check standard should be run approximately after every 10 sample injections. Standards are run in part to monitor the life and performance of the graphite tube. Lack of reproducibility or significant change in the signal for the standard indicates that the tube should be replaced. Even though tube life depends on sample matrix and atomization temperature, a conservative estimate would be that a tube will last at least 50 firings. A pyrolytic-coating would extend that estimate by a factor of 3.

9.3.8 Calculation–For determination of metal concentration by the furnace: Read the metal value in ug/l from the calibration curve or directly from the readout system of the instrument.

9.3.8.1 If different size furnace injection volumes are used for samples than for standards:

ug/l of metal in sample = (S)

9.3.8.2 If dilution of sample was required but sample injection volume same as for standard:

$$\mu g/l \text{ of metal in sample} = Z \left(\frac{C + B}{C}\right)$$

where:

Z = $\mu g/l$ metal in diluted aliquot from calibration curve
B = ml of deionized distilled water used for dilution
C = ml of sample aliquot

9.3.9 For sample conta g particulates:

$$\mu g/l \text{ of metal in sample} = Z \left(\frac{V}{C}\right)$$

where:

Z = $\mu g/l$ of metal in processed sample from calibration curve (See 9.3.8.1)
V = final volume of processed sample in ml
C = ml of sample aliquot processed

9.3.10 For solid samples: Report all concentrations as mg/kg dry weight

9.3.10.1 Dry sample:

$$\text{mg metal/kg sample} = \frac{\left(\frac{Z}{1,000}\right) V}{D}$$

where:

Z = $\mu g/l$ of metal in processed sample from calibration curve (See 9.3.8.1)
V = final volume of processed sample in ml
D = weight of dry sample in grams

9.3.10.2 Wet sample:

$$\text{mg metal/kg sample} = \frac{\left(\frac{Z}{1,000}\right) V}{[illegible]}$$

10 Quality Control For Drinking Water Analysis

10.1 Minimum requirements

10.1.1 All quality control data should be maintained and available for easy reference or inspection.

10.1.2 An unknown performance sample (when available) must be analyzed once per year for the metals measured. Results must be within the control limit established by EPA. If problems arise, they should be corrected, and a follow-up performance sample should be analyzed.

10.2 Minimum Daily control

10.2.1 After a calibration curve composed of a minimum of a reagent blank and three standards has been prepared, subsequent calibration curves must be verified by use of at least a reagent blank and one standard at or near the MCL. Daily checks must be within ± 10 percent of original curve.

10.2.2 If 20 or more samples per day are analyzed, the working standard curve must be verified by running an additional standard at or near the MCL every 20 samples Checks must be within ± 10 percent of original curve.

10.3 Optional Requirements

10.3.1 A current service contract should be in effect on balances and the atomic absorption spectrophotometer.

10.3.2 Class S weights should be available to make periodic checks on balances.

10.3.3 Chemicals should be dated upon receipt of shipment and replaced as needed or before shelf life has been exceeded.

10.3.4 A known reference sample (when available) should be analyzed once per quarter for the metals measured. The measured value should be within the control limits established by EPA.

10.3.5 At least one duplicate sample should be run every 10 samples, or with each set of samples to verify precision of the method. Checks should be within the control limit established by EPA.

10.3.6 Standard deviation should be obtained and documented for all measurements being conducted.

10.3.7 Quality Control charts or a tabulation of mean and standard deviation should be used to document validity of data on a daily basis.

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Inductively Coupled Plasma—Atomic Emission Spectrometric Method for Trace Element Analysis of Water and Wastes—Method 200.7

1. Scope and Application

1.1 This method may be used for the determination of dissolved, suspended, or total elements in drinking water, surface water, domestic and industrial wastewaters.

1.2 Dissolved elements are determined in filtered and acidified samples. Appropriate steps must be taken in all analyses to ensure that potential interference are taken into account. This is especially true when dissolved solids exceed 1500 mg/L (See 5.)

1.3 Total elements are determined after appropriate digestion procedures are performed. Since digestion techniques increase the dissolved solids content of the samples, appropriate steps *must* be taken to correct for potential interference effects. (See 5.)

1.4 Table 1 lists elements for which this method applies along with recommended wavelengths and typical estimated instrumental detection limits using conventional pneumatic nebulization. Actual working detection limits are sample dependent and as the sample matrix varies, these concentrations may also vary. In time, other elements may be added as more information becomes available and as required.

1.5 Because of the differences between various makes and models of satisfactory instruments, no detailed instrumental operating instructions can be provided. Instead, the analyst is referred to the instructions provided by the manufacturer of the particular instrument.

2. Summary of Method

2.1 The method describes a technique for the simultaneous or sequential multielement determination of trace elements in solution. The basis of the method is the measurement of atomic emission by an optical spectroscopic technique. Samples are nebulized and the aerosol that is produced is transported to the plasma torch where excitation occurs. Characteristic atomic-line emission spectra are produced by a radio-frequency inductively coupled plasma (ICP). The spectra are dispersed by a grating spectrometer and the intensities of the lines are monitored by photomultiplier tubes. The photocurrents from the photomultiplier tubes are processed and controlled by a computer system. A background correction technique is required to compensate for variable background contribution to the

74

137

NITROGEN, NITRATE

Method 352.1 (Colorimetric, Brucine)

STORET NO. Total 00620

1. Scope and Application
   1.1 This method is applicable to the analysis of drinking, surface and saline waters, domestic and industrial wastes. Modification can be made to remove or correct for turbidity, color, salinity, or dissolved organic compounds in the sample.
   1.2 The applicable range of concentrations is 0.1 to 2 mg NO_3-N/liter.
2. Summary of Method
   2.1 This method is based upon the reaction of the nitrate ion with brucine sulfate in a 13 N H_2SO_4 solution at a temperature of 100°C. The color of the resulting complex is measured at 410 nm. Temperature control of the color reaction is extremely critical.
3. Sample Handling and Preservation
   3.1 Analysis should be made as soon as possible. If analysis can be made within 24 hours, the sample should be preserved by refrigeration at 4°C. When samples must be stored for more than 24 hours, they should be preserved with sulfuric acid (2 ml conc. H_2SO_4 per liter) and refrigeration.
4. Interferences
   4.1 Dissolved organic matter will cause an off color in 13 N H_2SO_4 and must be compensated for by additions of all reagents except the brucine-sulfanilic acid reagent. This also applies to natural color present not due to dissolved organics.
   4.2 The effect of salinity is eliminated by addition of sodium chloride to the blanks, standards and samples.
   4.3 All strong oxidizing or reducing agents interfere. The presence of oxidizing agents may be determined with a total residual chlorine test kit.
   4.4 Residual chlorine interference is eliminated by the addition of sodium arsenite.
   4.5 Ferrous and ferric iron and quadrivalent manganese give slight positive interferences, but in concentrations less than 1 mg/l these are negligible.
   4.6 Uneven heating of the samples and standards during the reaction time will result in erratic values. The necessity for absolute control of temperature during the critical color development period cannot be too strongly emphasized.
5. Apparatus
   5.1 Spectrophotometer or filter photometer suitable for measuring absorbance at 410 nm.
   5.2 Sufficient number of 40–50 ml glass sample tubes for reagent blanks, standards and samples.
   5.3 Neoprene coated wire racks to hold sample tubes.
   5.4 Water bath suitable for use at 100°C. This bath should contain a stirring mechanism so that all tubes are at the same temperature and should be of sufficient capacity to accept

Approved for NPDES and SDWA
Issued 1971

the required number of tubes without significant drop in temperature when the tubes are immersed.

5.5 Water bath suitable for use at 10–15°C.

6. Reagents

6.1 Distilled water free of nitrite and nitrate is to be used in preparation of all reagents and standards.

6.2 Sodium chloride solution (30%): Dissolve 300 g NaCl in distilled water and dilute to 1 liter

6.3 Sulfuric acid solution: Carefully add 500 ml conc. H_2SO_4 to 125 ml distilled water. Cool and keep tightly stoppered to prevent absorption of atmospheric moisture.

6.4 Brucine-sulfanilic acid reagent: Dissolve 1 g brucine sulfate $[(C_{23}H_{26}N_2O_4)_2 \cdot H_2SO_4 \cdot 7H_2O]$ and 0.1 g sulfanilic acid $(NH_2C_6H_4SO_3H \cdot H_2O)$ in 70 ml hot distilled water. Add 3 ml conc. HCl, cool, mix and dilute to 100 ml with distilled water. Store in a dark bottle at 5°C. This solution is stable for several months; the pink color that develops slowly does not effect its usefulness. Mark bottle with warning CAUTION: Brucine Sulfate is toxic; take care to avoid ingestion.

6.5 Potassium nitrate stock solution: 1.0 ml = 0.1 mg NO_3–N. Dissolve 0.7218 g anhydrous potassium nitrate (KNO_3) in distilled water and dilute to 1 liter in a volumetric flask. Preserve with 2 ml chloroform per liter. This solution is stable for at least 6 months.

6.6 Potassium nitrate standard solution: 1.0 ml = 0.001 mg NO_3–N. Dilute 10.0 ml of the stock solution (6.5) to 1 liter in a volumetric flask. This standard solution should be prepared fresh weekly.

6.7 Acetic acid (1 + 3): Dilute 1 volume glacial acetic acid (CH_3COOH) with 3 volumes of distilled water.

6.8 Sodium hydroxide (1N): Dissolve 40 g of NaOH in distilled water. Cool and dilute to 1 liter.

7. Procedure

7.1 Adjust the pH of the samples to approximately 7 with acetic acid (6.7) or sodium hydroxide (6.8). If necessary, filter to remove turbidity.

7.2 Set up the required number of sample tubes in the rack to handle reagent blank, standards and samples. Space tubes evenly throughout the rack to allow for even flow of bath water between the tubes. This should assist in achieving uniform heating of all tubes.

7.3 If it is necessary to correct for color or dissolved organic matter which will cause color on heating, a set of duplicate samples must be run to which all reagents except the brucine-sulfanilic acid have been added.

7.4 Pipette 10.0 ml of standards and samples or an aliquot of the samples diluted to 10.0 ml into the sample tubes.

7.5 If the samples are saline, add 2 ml of the 30% sodium chloride solution (6.2) to the reagent blank, standards and samples. For fresh water samples, sodium chloride solution may be omitted. Mix contents of tubes by swirling and place rack in cold water bath (0–10°C).

7.6 Pipette 10.0 ml of sulfuric acid solution (6.3) into each tube and mix by swirling. Allow tubes to come to thermal equilibrium in the cold bath. Be sure that temperatures have equilibrated in all tubes before continuing.

7.7 Add 0.5 ml brucine-sulfanilic acid reagent (6.4) to each tube (except the interference control tubes, 7.3) and carefully mix by swirling, then place the rack of tubes in the 100°C water bath for exactly 25 minutes.

Caution: Immersion of the tube rack into the bath should not decrease the temperature of the bath more than 1 to 2°C. In order to keep this temperature decrease to an absolute minimum, flow of bath water between the tubes should not be restricted by crowding too many tubes into the rack. If color development in the standards reveals discrepancies in the procedure, the operator should repeat the procedure after reviewing the temperature control steps.

7.8 Remove rack of tubes from the hot water bath and immerse in the cold water bath and allow to reach thermal equilibrium (20–25°C).

7.9 Read absorbance against the reagent blank at 410 nm using a 1 cm or longer cell.

8. Calculation

8.1 Obtain a standard curve by plotting the absorbance of standards run by the above procedure against mg NO_3–N/l. (The color reaction does not always follow Beer's law).

8.2 Subtract the absorbance of the sample without the brucine-sulfanilic reagent from the absorbance of the sample containing brucine-sulfanilic acid and determine mg NO_3–N/l. Multiply by an appropriate dilution factor if less than 10 ml of sample is taken.

9. Precision and Accuracy

9.1 Twenty-seven analysts in fifteen laboratories analyzed natural water samples containing exact increments of inorganic nitrate, with the following results:

Increment as Nitrogen, Nitrate mg N/liter	Precision as Standard Deviation mg N/liter	Accuracy as Bias, %	Accuracy as Bias, mg N/liter
0.16	0.092	–6.79	–0.01
0.19	0.083	+8.30	+0.02
1.08	0.245	+4.12	+0.04
1.24	0.214	+2.82	+0.04

(FWPCA Method Study 2, Nutrient Analyses).

Bibliography

1. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 427, Method 419D (1975).
2. Annual Book of ASTM Standards, Part 31, "Water", Standard D 992–71, p 363 (1976).
3. Jenkins, D., and Medsken, L., "A Brucine Method for the Determination of Nitrate in Ocean, Estuarine, and Fresh Waters", Anal Chem., 36, p 610, (1964).

NITROGEN, NITRATE-NITRITE

Method 353.1 (Colorimetric, Automated, Hydrazine Reduction)

STORET NO. Total 00630

1. Scope and Application
   1.1 This method is applicable to drinking and surface water, and domestic and industrial wastes. The applicable range of this method is 0.01–10 mg/l nitrate-nitrite nitrogen. Approximately 20 samples per hour can be analyzed.
2. Summary of Method
   2.1 Nitrate is reduced to nitrite with hydrazine sulfate and the nitrite (that originally present plus reduced nitrate) is determined by diazotizing with sulfanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly colored azo dye which is measured colorimetrically.
3. Sample Handling and Preservation
   3.1 Analysis should be made as soon as possible. If analysis can be made within 24 hours, samples should be preserved by refrigeration at 4°C. When samples must be stored for more than 24 hours, they should be preserved with 2 ml of sulfuric acid (H_2SO_4) per liter and refrigerated.
4. Interferences
   4.1 Sample color that absorbs in the photometric range used for analysis will interfere.
   4.2 The apparent NO_3 and NO_2 concentrations varied ±10 percent with concentrations of sulfide ion up to 10 mg/l.
5. Apparatus
   5.1 Sampler
   5.2 Manifold AAI or AAII
   5.3 Proportioning Pump
   5.4 Heating bath 32°C AAI or 37°C AAII
   5.5 Continuous filter
   5.6 Colorimeter equipped with an 8 mm, 15 mm or 50 mm flow cell and 529 nm filters.
6. Reagents
   6.1 Color developing reagent: To approximately 500 ml of distilled water add 200 ml concentrated phosphoric acid (sp. gr. 1.834), 10 g sulfanilamide ($H_2NC_6H_4SO_2NH_2$) followed by 0.8 g N (1-Naphthyl) ethylenediamine dihydrochloride. Dilute the solution to 1 liter with distilled water and store in a dark bottle in the refrigerator. This solution is stable for approximately 1 month.
   6.2 Copper sulfate stock solution: Dissolve 2.5 g of copper sulfate ($CuSO_4 \cdot 5H_2O$) in distilled water and dilute to 1 liter.

Approved for NPDES and SDWA
Issued 1971
Reissued with revision 1978

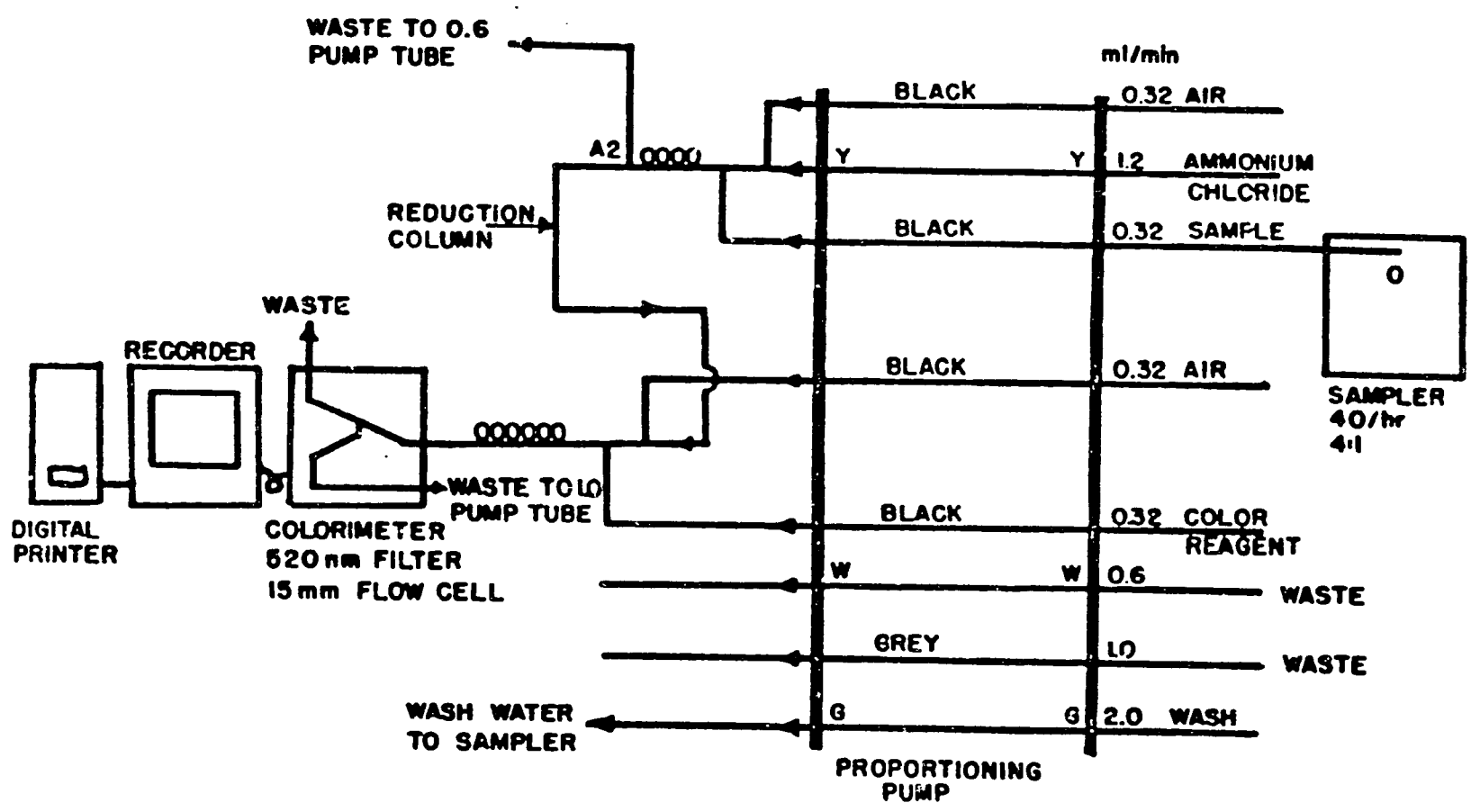


FIGURE 3 NITRATE-NITRITE MANIFOLD AA II

NITROGEN, NITRATE-NITRITE

Method 353.3 (Spectrophotometric, Cadmium Reduction)

STORET NO. Total 00630

1. Scope and Application
   1.1 This method is applicable to the determination of nitrite singly, or nitrite and nitrate combined in drinking, surface and saline waters, domestic and industrial wastes. The applicable range of this method is 0.01 to 1.0 mg/l nitrate-nitrite nitrogen. The range may be extended with sample dilution.
2. Summary of Method
   2.1 A filtered sample is passed through a column containing granulated copper-cadmium to reduce nitrate to nitrite. The nitrite (that originally present plus reduced nitrate) is determined by diazotizing with sulfanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly colored azo dye which is measured spectrophotometrically. Separate, rather than combined nitrate-nitrite, values are readily obtained by carrying out the procedure first with, and then without, the Cu-Cd reduction step.
3. Sample Handling and Preservation
   3.1 Analysis should be made as soon as possible. If analysis can be made within 24 hours, the sample should be preserved by refrigeration at 4°C. When samples must be stored for more than 24 hours, they should be preserved with sulfuric acid (2 ml H_2SO_4 per liter) and refrigeration.

   Caution: Samples for reduction column must not be preserved with mercuric chloride.
4. Interferences
   4.1 Build up of suspended matter in the reduction column will restrict sample flow. Since nitrate-nitrogen is found in a soluble state, the sample may be pre-filtered through a glass fiber filter or a 0.45u membrane filter. Highly turbid samples may be pretreated with zinc sulfate before filtration to remove the bulk of particulate matter present in the sample.
   4.2 Low results might be obtained for samples that contain high concentrations of iron, copper or other metals. EDTA is added to the samples to eliminate this interference.
   4.3 Samples that contain large concentrations of oil and grease will coat the surface of the cadmium. This interference is eliminated by pre-extracting the sample with an organic solvent.
   4.4 This procedure determines both nitrate and nitrite. If only nitrate is desired, a separate determination must be made for nitrite and subsequent corrections made. The nitrite may be determined by the procedure below without the reduction step.

Approved for NPDES and SDWA
Issued 1974

5. Apparatus

5.1 Reduction column: The column in Figure 1 was constructed from a 100 ml pipet by removing the top portion. This column may also be constructed from two pieces of tubing joined end to end. A 10 mm length of 3 cm I.D. tubing is joined to a 25 cm length of 3.5 mm I.D. tubing.

5.2 Spectrophotometer for use at 540 nm, providing a light path of 1 cm or longer.

6. Reagents

6.1 Granulated cadmium: 40-60 mesh (MCB Reagents).

6.2 Copper-Cadmium: The cadmium granules (new or used) are cleaned with dilute HCl and copperized with 2% solution of copper sulfate in the following manner:

6.2.1 Wash the cadmium with dilute HCl (6.10) and rinse with distilled water. The color of the cadmium should be silver.

6.2.2 Swirl 25 g cadmium in 100 ml portions of a 2% solution of copper sulfate (6.11) for 5 minutes or until blue color partially fades, decant and repeat with fresh copper sulfate until a brown colloidal precipitate forms.

6.2.3 Wash the copper-cadmium with distilled water (at least 10 times) to remove all the precipitated copper. The color of the cadmium so treated should be black.

6.3 Preparation of reaction column: Insert a glass wool plug into the bottom of the reduction column and fill with distilled water. Add sufficient copper-cadmium granules to produce a column 18.5 cm in length. Maintain a level of distilled water above the copper-cadmium granules to eliminate entrapment of air. Wash the column with 200 ml of dilute ammonium chloride solution (6.5). The column is then activated by passing through the column 100 ml of a solution composed of 25 ml of a 1.0 mg/l NO_3-N standard and 75 ml of ammonium chloride - EDTA solution (6.4). Use a flow rate between 7 and 10 ml per minute.

6.4 Ammonium chloride - EDTA solution: Dissolve 13 g ammonium chloride and 1.7 g disodium ethylenediamine tetracetate in 900 ml of distilled water. Adjust the pH to 8.5 with conc. ammonium hydroxide (6.9) and dilute to 1 liter.

6.5 Dilute ammonium chloride-EDTA solution: Dilute 300 ml of ammonium chloride-EDTA solution (6.4) to 500 ml with distilled water.

6.6 Color reagent: Dissolve 10 g sulfanilamide and 1 g N(1-naphthyl)-ethylene-diamine dihydrochloride in a mixture of 100 ml conc. phosphoric acid and 800 ml of distilled water and dilute to 1 liter with distilled water.

6.7 Zinc sulfate solution: Dissolve 100 g $ZnSO_4 \cdot 7H_2O$ in distilled water and dilute to 1 liter.

6.8 Sodium hydroxide solution, 6N: Dissolve 240 g NaOH in 500 ml distilled water, cool and dilute to 1 liter.

6.9 Ammonium hydroxide, conc.

6.10 Dilute hydrochloric acid, 6N: Dilute 50 ml of conc. HCl to 100 ml with distilled water.

6.11 Copper sulfate solution, 2%: Dissolve 20 g of $CuSO_4 \cdot 5H_2O$ in 500 ml of distilled water and dilute to 1 liter.

6.12 Stock nitrate solution: Dissolve 7.218 g KNO_3 in distilled water and dilute to 1000 ml. Preserve with 2 ml of chloroform per liter. This solution is stable for at least 6 months. 1.0 ml = 1.00 mg NO_3-N.

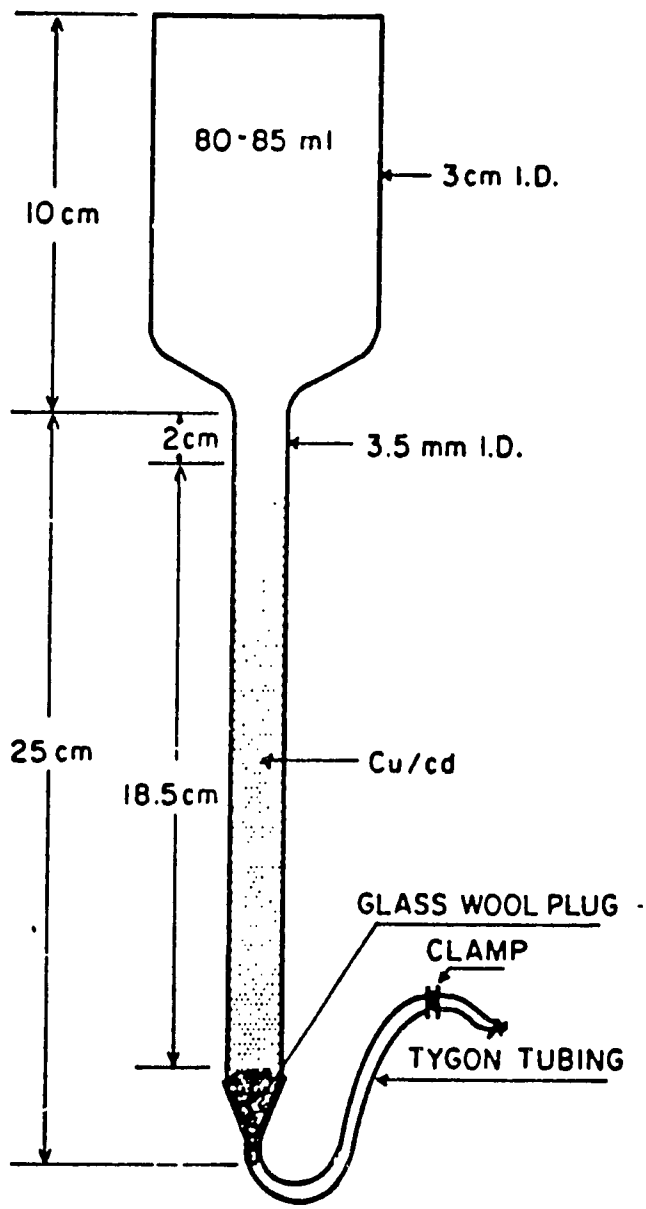


FIGURE 1. REDUCTION COLUMN

6.13 Standard nitrate solution: Dilute 10.0 ml of nitrate stock solution (6.12) to 1000 ml with distilled water. 1.0 ml = 0.01 mg NO_3-N.

6.14 Stock nitrite solution: Dissolve 6.072 g KNO_2 in 500 ml of distilled water and dilute to 1000 ml. Preserve with 2 ml of chloroform and keep under refrigeration. Stable for approximately 3 months. 1.0 ml = 1.00 mg NO_2-N.

6.15 Standard nitrite solution: Dilute 10.0 ml of stock nitrite solution (6.14) to 1000 ml with distilled water. 1.0 ml = 0.01 mg NO_2-N.

6.16 Using standard nitrate solution (6.13) prepare the following standards in 100 ml volumetric flasks:

Conc., mg-NO_3-N/l	ml of Standard Solution/100.0 ml
0.00	0.0
0.05	0.5
0.10	1.0
0.20	2.0
0.50	5.0
1.00	10.0

7. Procedure

7.1 Turbidity removal: One of the following methods may be used to remove suspended matter.

7.1.1 Filter sample through a glass fiber filter or a 0.45*u* membrane filter.

7.1.2 Add 1 ml zinc sulfate solution (6.7) to 100 ml of sample and mix thoroughly. Add 0.4–0.5 ml sodium hydroxide solution (6.8) to obtain a pH of 10.5 as determined with a pH meter. Let the treated sample stand a few minutes to allow the heavy flocculent precipitate to settle. Clarify by filtering through a glass fiber filter or a 0.45*u* membrane filter.

7.2 Oil and grease removal: Adjust the pH of 100 ml of filtered sample to 2 by addition of conc. HCl. Extract the oil and grease from the aqueous solution with two 25 ml portions of a non-polar solvent (Freon, chloroform or equivalent).

7.3 If the pH of the sample is below 5 or above 9, adjust to between 5 and 9 with either conc. HCl or conc. NH_4OH. This is done to insure a sample pH of 8.5 after step 7.4.

7.4 To 25.0 ml of sample or an aliquot diluted to 25.0 ml, add 75 ml of ammonium chloride-EDTA solution (6.4) and mix.

7.5 Pour sample into column and collect sample at a rate of 7–10 ml per minute.

7.6 Discard the first 25 ml, collect the rest of the sample (approximately 70 ml) in the original sample flask. Reduced samples should not be allowed to stand longer than 15 minutes before addition of color reagent, step 7.7.

7.7 Add 2.0 ml of color reagent (6.6) to 50.0 ml of sample. Allow 10 minutes for color development. Within 2 hours measure the absorbance at 540 nm against a reagent blank. **NOTE:** If the concentration of sample exceeds 1.0 mg NO_3-N/l, the remainder of the reduced sample may be used to make an appropriate dilution before proceeding with step 7.7.

7.8 Standards: Carry out the reduction of standards exactly as described for the samples. At least one nitrite standard should be compared to a reduced nitrate standard at the same concentration to verify the efficiency of the reduction column.

8. Calculation

8.1 Obtain a standard curve by plotting the absorbance of standards run by the above procedure against NO_3–N mg/l. Compute concentration of samples by comparing sample absorbance with standard curve.

8.2 If less than 25 ml of sample is used for the analysis the following equation should be used:

$$\text{mg}NO_2 + NO_3 - N/l = \frac{A \times 25}{\text{ml sample used}}$$

where:

A = Concentration of nitrate from standard curve.

9. Precision and Accuracy

9.1 In a single laboratory (EMSL), using sewage samples at concentrations of 0.04, 0.24, 0.55 and 1.04 mg NO_3 + NO_2-N/l, the standard deviations were ±0.005, ±0.004, ±0.005 and ±0.01, respectively.

9.2 In a single laboratory (EMSL), using sewage samples at concentrations of 0.24, 0.55, and 1.05 mg NO_3 + NO_2–N/l, the recoveries were 100%, 102% and 100%, respectively.

Bibliography

1. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 423, Method 419C (1975).
2. Henrikson, A., and Selmer-Olsen, "Automatic Methods for Determining Nitrate and Nitrite in Water and Soil Extracts". Analyst, May 1970, Vol. 95, p 514–518.
3. Grasshoff, K., "A Simultaneous Multiple Channel System for Nutrient Analysis in Sea Water with Analog and Digital Data Record", "A ances in Automated Analysis", Technicon International Congress, 1969, Vol. 11, p 133–145.
4. Brewer, P. G., Riley, J. P., "The Automatic Determination of Nitrate in Sea Water", Deep Sea Research, 1965, Vol. 12, p 765–772.

147

NITROGEN, NITRITE

Method 354.1 (Spectrophotometric)

STORET NO. Total 00615

1. Scope and Application
   1.1 This method is applicable to the determination of nitrite in drinking, surface and saline waters, domestic and industrial wastes.
   1.2 The method is applicable in the range from 0.01 to 1.0 mg NO_2-N/l.
2. Summary of Method
   2.1 The diazonium compound formed by diazotation of sulfanilamide by nitrite in water under acid conditions is coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride to produce a reddish-purple color which is read in a spectrophotometer at 540 nm.
3. Sample Handling and Preservation
   3.1 Samples should be analyzed as soon as possible. They may be stored for 24 to 48 hours at 4°C.
4. Interferences
   4.1 There are very few known interferences at concentrations less than 1,000 times that of the nitrite; however, the presence of strong oxidants or reductants in the samples will readily affect the nitrite concentrations. High alkalinity (> 600 mg/l) will give low results due to a shift in pH.
5. Apparatus
   5.1 Spectrophotometer equipped with 1 cm or larger cells for use at 540 nm.
   5.2 Nessler tubes, 50 ml or volumetric flasks, 50 ml.
6. Reagents
   6.1 Distilled water free of nitrite and nitrate is to be used in preparation of all reagents and standards.
   6.2 Buffer-color reagent: To 250 ml of distilled water, add 105 ml conc. hydrochloric acid, 5.0 g sulfanilamide and 0.5 g N-(1-naphthyl) ethylenediamine dihydrochloride. Stir until dissolved. Add 136 g of sodium acetate ($CH_3COONa \cdot 3H_2O$) and again stir until dissolved. Dilute to 500 ml with distilled water. This solution is stable for several weeks if stored in the dark.
   6.3 Nitrite stock solution: 1.0 ml = 0.10 mg NO_2-N. Dissolve 0.1493 g of dried anhydrous sodium nitrite (24 hours in desiccator) in distilled water and dilute to 1000 ml. Preserve with 2 ml chloroform per liter.
   6.4 Nitrite standard solution: 1.0 ml = 0.001 mg NO_2-N. Dilute 10.0 ml of the stock solution (6.3) to 1000 ml.
7. Procedure
   7.1 If the sample has a pH greater than 10 or a total alkalinity in excess of 600 mg/l, adjust to approximately pH 6 with 1:3 HCl.

Approved for NPDES
Issued 1971

148

7.2 If necessary, filter the sample through a 0.45 μ pore size filter using the first portion of filtrate to rinse the filter flask.

7.3 Place 50 ml of sample, or an aliquot diluted to 50 ml, in a 50 ml Nessler tube; hold until preparation of standards is completed.

7.4 At the same time prepare a series of standards in 50 ml Nessler tubes as follows:

ml of Standard Solution 1.0 ml = 0.001 mg NO_2-N	Conc., When Diluted to 50 ml, mg/l of NO_2-N
0.0	(Blank)
0.5	0.01
1.0	0.02
1.5	0.03
2.0	0.04
3.0	0.06
4.0	0.08
5.0	0.10
10.0	0.20

7.5 Add 2 ml of buffer-color reagent (6.2) to each standard and sample, mix and allow color to develop for at least 15 minutes. The color reaction medium should be between pH 1.5 and 2.0.

7.6 Read the color in the spectrophotometer at 540 nm against the blank and plot concentration of NO_2-N against absorbance.

8. Calculation

8.1 Read the concentration of NO_2-N directly from the curve.

8.2 If less than 50.0 ml of sample is taken, calculate mg/l as follows:

$$NO_2 - N, \text{ mg/l} = \frac{\text{mg/l from std. curve} \times 50}{\text{ml sample used}}$$

9. Precision and Accuracy

9.1 Precision and Accuracy data are not available at this time.

Bibliography

1. Standard Methods for the Examination for Water and Wastewater, 14th Edition, p 434, Method 420, (1975).

PHOSPHORUS, ALL FORMS

Method 365.2 (Colorimetric, Ascorbic Acid, Single Reagent)

STORET NO. See Section 4

1. Scope and Application
   1.1 These methods cover the determination of specified forms of phosphorus in drinking, surface and saline waters, domestic and industrial wastes.
   1.2 The methods are based on reactions that are specific for the orthophosphate ion. Thus, depending on the prescribed pre-treatment of the sample, the various forms of phosphorus given in Figure 1 may be determined. These forms are defined in Section 4.
      1.2.1 Except for in-depth and detailed studies, the most commonly measured forms are phosphorus and dissolved phosphorus, and orthophosphate and dissolved orthophosphate. Hydrolyzable phosphorus is normally found only in sewage-type samples and insoluble forms of phosphorus are determined by calculation.
   1.3 The methods are usable in the 0.01 to 0.5 mg P/l range.
2. Summary of Method
   2.1 Ammonium molybdate and antimony potassium tartrate react in an acid medium with dilute solutions of phosphorus to form an antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The color is proportional to the phosphorus concentration.
   2.2 Only orthophosphate forms a blue color in this test. Polyphosphates (and some organic phosphorus compounds) may be converted to the orthophosphate form by sulfuric acid hydrolysis. Organic phosphorus compounds may be converted to the orthophosphate form by persulfate digestion[1].
3. Sample Handling and Preservation
   3.1 If benthic deposits are present in the area being sampled, great care should be taken not to include these deposits.
   3.2 Sample containers may be of plastic material, such as cubitainers, or of Pyrex glass.
   3.3 If the analysis cannot be performed the day of collection, the sample should be preserved by the addition of 2 ml conc. H_2SO_4 per liter and refrigeration at 4°C.
4. Definitions and Storet Numbers
   4.1 Total Phosphorus (P) — all of the phosphorus present in the sample, regardless of form, as measured by the persulfate digestion procedure. (00665)
      4.1.1 Total Orthophosphate (P, ortho) — inorganic phosphorus $[(PO_4)^{-3}]$ in the sample as measured by the direct colorimetric analysis procedure. (70507)
      4.1.2 Total Hydrolyzable Phosphorus (P, hydro) - phosphorus in the sample as measured by the sulfuric acid hydrolysis procedure, and minus pre-determined orthophosphates. This hydrolyzable phosphorus includes polyphosphorus. $[(P_2O_7)^{-4}, (P_3O_{10})^{-5}$, etc.] plus some organic phosphorus. (00669)

Approved for NPDES
Issued 1971

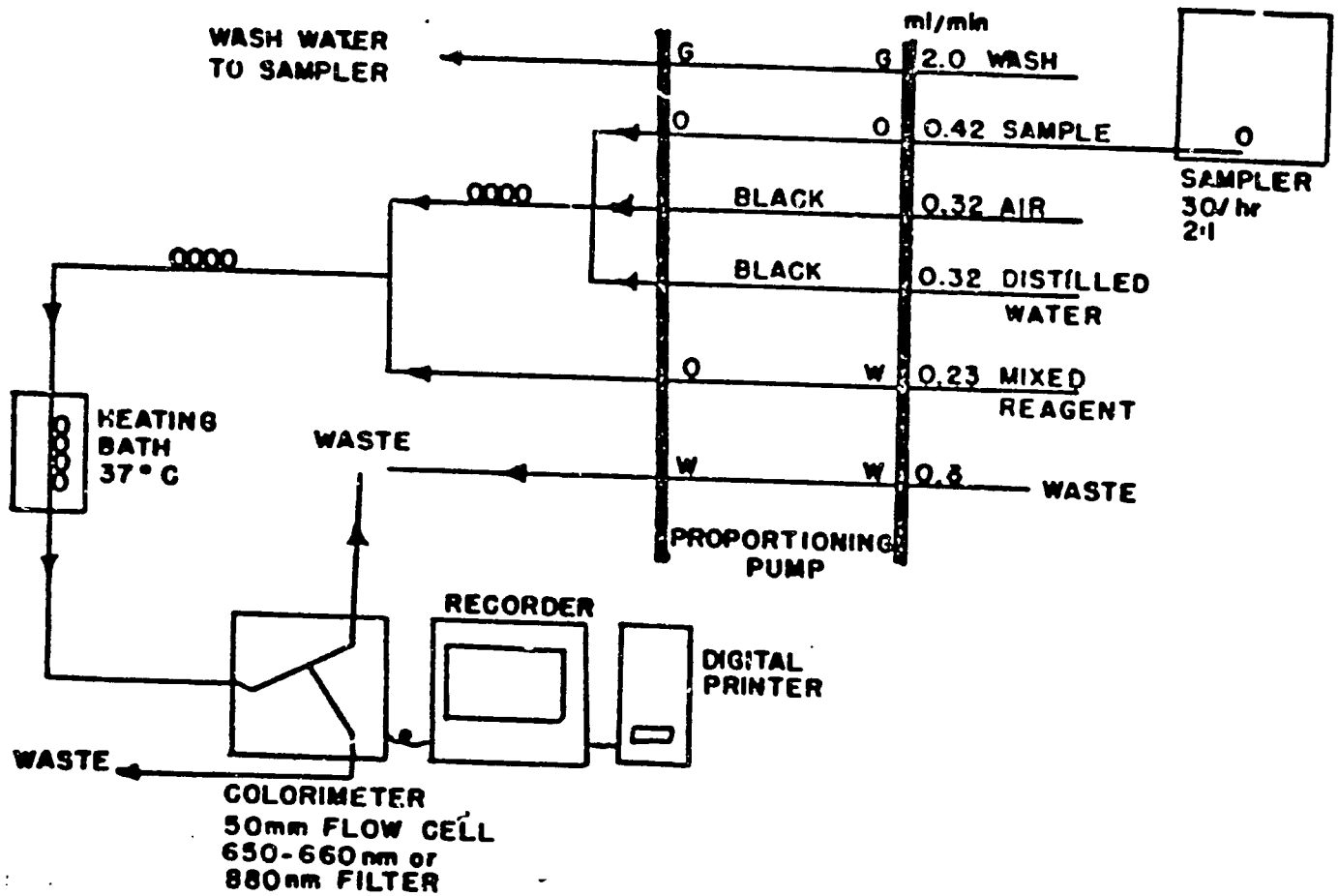


FIGURE 3 PHOSPHORUS MANIFOLD AA II

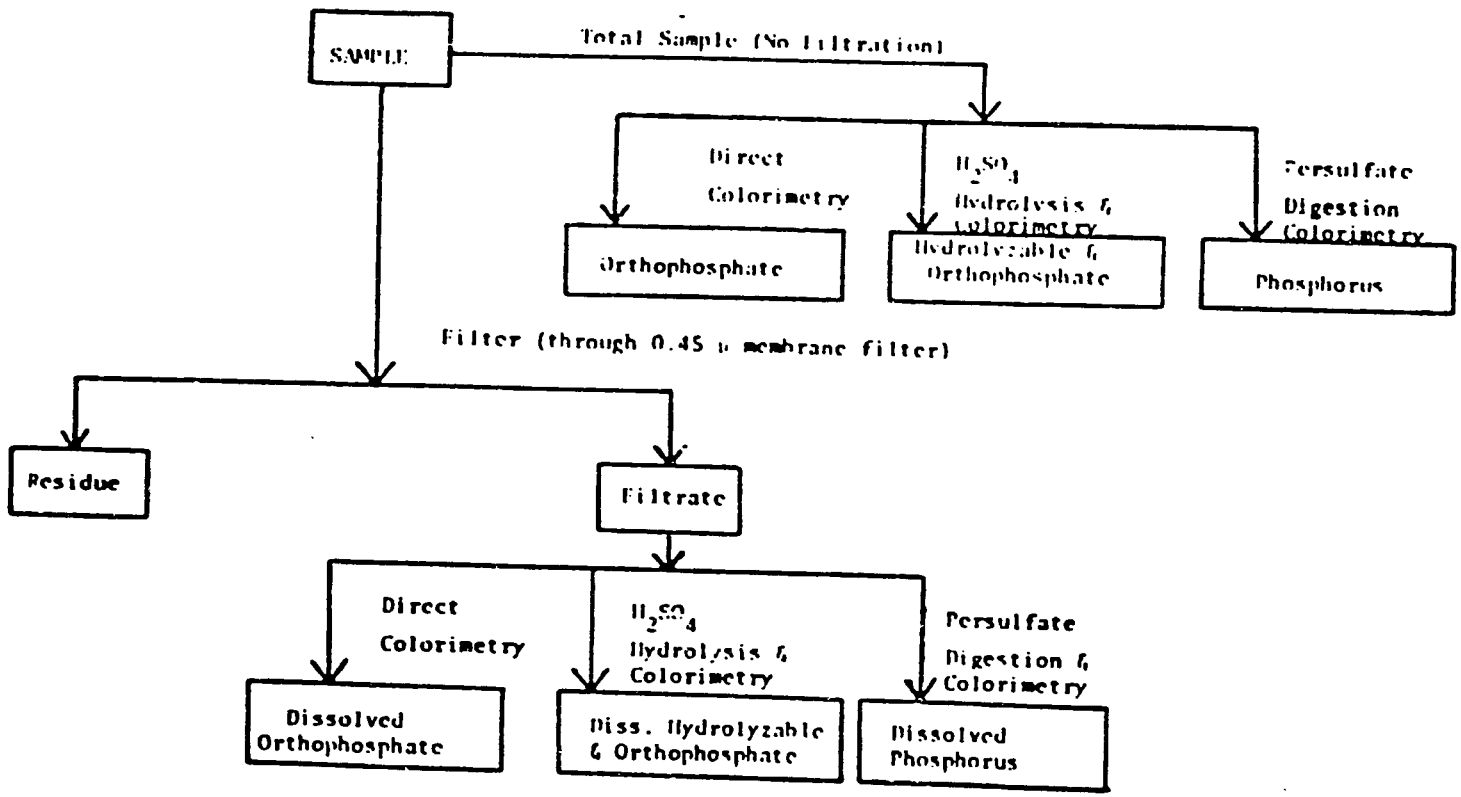


FIGURE 1. ANALYTICAL SCHEME FOR DIFFERENTIATION OF PHOSPHORUS FORMS

4.1.3 Total Organic Phosphorus (P, org) — phosphorus (inorganic plus oxidizable organic) in the sample measured by the persulfate digestion procedure, and minus hydrolyzable phosphorus and orthophosphate. (00670)

4.2 Dissolved Phosphorus (P-D) — all of the phosphorus present in the filtrate of a sample filtered through a phosphorus-free filter of 0.45 micron pore size and measured by the persulfate digestion procedure (00666)

4.2.1 Dissolved Orthophosphate (P-D, ortho) -- as measured by the direct colorimetric analysis procedure. (00671)

4.2.2 Dissolved Hydrolyzable Phosphorus (P-D, hydro) — as measured by the sulfuric acid hydrolysis procedure and minus pre-determined dissolved orthophosphates. (00672)

4.2.3 Dissolved Organic Phosphorus (P-D, org) — as measured by the persulfate digestion procedure, and minus dissolved hydrolyzable phosphorus and orthophosphate. (00673)

4.3 The following forms, when sufficient amounts of phosphorus are present in the sample to warrant such consideration, may be calculated:

4.3.1 Insoluble Phosphorus (P-I) = (P)-(P-D). (00667)

4.3.1.1 Insoluble orthophosphate (P-I, ortho) = (P, ortho)-(P-D, ortho). (00674)

4.3.1.2 Insoluble Hydrolyzable Phosphorus (P-I, hydro) = (P, hydro)-(P-D, hydro). (00675)

4.3.1.3 Insoluble Organic Phosphorus (P-I, org) = (P, org) - (P-D, org). (00676)

4.4 All phosphorus forms shall be reported as P, mg/l, to the third place.

5. Interferences

5.1 No interference is caused by copper, iron, or silicate at concentrations many times greater than their reported concentration in sea water. However, high iron concentrations can cause precipitation of and subsequent loss of phosphorus.

5.2 The salt error for samples ranging from 5 to 20% salt content was found to be less than 1%.

5.3 Arsenate is determined similarly to phosphorus and should be considered when present in concentrations higher than phosphorus. However, at concentrations found in sea water, it does not interfere.

6. Apparatus

6.1 Photometer – A spectrophotometer or filter photometer suitable for measurements at 650 or 880 nm with a light path of 1 cm or longer.

6.2 Acid-washed glassware: All glassware used should be washed with hot 1:1 HCl and rinsed with distilled water. The acid-washed glassware should be filled with distilled water and treated with all the reagents to remove the last traces of phosphorus that might be adsorbed on the glassware. Preferably, this glassware should be used only for the determination of phosphorus and after use it should be rinsed with distilled water and

415

103

kept covered until needed again. If this is done, the treatment with 1:1 HCl and reagents is only required occasionally. Commercial detergents should never be used.

7. Reagents

7.1 Sulfuric acid solution, 5N: Dilute 70 ml of conc. H_2SO_4 with distilled water to 500 ml.

7.2 Antimony potassium tartrate solution: Weigh 1.3715 g $K(SbO)C_4H_4O_6 \cdot 1/2H_2O$, dissolve in 400 ml distilled water in 500 ml volumetric flask, dilute to volume. Store at 4°C in a dark, glass-stoppered bottle.

7.3 Ammonium molybdate solution: Dissolve 20 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 500 ml of distilled water. Store in a plastic bottle at 4°C.

7.4 Ascorbic acid, 0.1M: Dissolve 1.76 g of ascorbic acid in 100 ml of distilled water. The solution is stable for about a week if stored at 4°C.

7.5 Combined reagent: Mix the above reagents in the following proportions for 100 ml of the mixed reagent: 50 ml of 5N H_2SO_4, (7.1), 5 ml of antimony potassium tartrate solution (7.2), 15 ml of ammonium molybdate solution (7.3), and 30 ml of ascorbic acid solution (7.4). Mix after addition of each reagent. All reagents must reach room temperature before they are mixed and must be mixed in the order given. If turbidity forms in the combined reagent, shake and let stand for a few minutes until the turbidity disappears before proceeding. Since the stability of this solution is limited, it must be freshly prepared for each run.

7.6 Sulfuric acid solution, 11 N: Slowly add 310 ml conc. H_2SO_4 to 600 ml distilled water. When cool, dilute to 1 liter.

7.7 Ammonium persulfate.

7.8 Stock phosphorus solution: Dissolve in distilled water 0.2197 g of potassium dihydrogen phosphate, KH_2PO_4, which has been dried in an oven at 105°C. Dilute the solution to 1000 ml; 1.0 ml = 0.05 mg P.

7.9 Standard phosphorus solution: Dilute 10.0 ml of stock phosphorus solution (7.8) to 1000 ml with distilled water; 1.0 ml = 0.5 ug P.

7.9.1 Using standard solution, prepare the following standards in 50.0-ml volumetric flasks:

ml of Standard Phosphorus Solution (7.9)	Conc., mg/l
0	0.00
1.0	0.01
3.0	0.03
5.0	0.05
10.0	0.10
20.0	0.20
30.0	0.30
40.0	0.40
50.0	0.50

7.10 Sodium hydroxide, 1 N: Dissolve 40 g NaOH in 600 ml distilled water. Cool and dilute to 1 liter.

8. Procedure

8.1 Phosphorus

8.1.1 Add 1 ml of H_2SO_4 solution (7.6) to a 50 ml sample in a 125 ml Erlenmeyer flask.

8.1.2 Add 0.4 g of ammonium persulfate.

8.1.3 Boil gently on a pre-heated hot plate for approximately 30–40 minutes or until a final volume of about 10 ml is reached. Do not allow sample to go to dryness. Alternatively, heat for 30 minutes in an autoclave at 121°C (15–20 psi).

8.1.4 Cool and dilute the sample to about 30 ml and adjust the pH of the sample to 7.0 ±0.2 with 1 N NaOH (7.10) using a pH meter. If sample is not clear at this point, add 2–3 drops of acid (7.6) and filter. Dilute to 50 ml.
Alternatively, if autoclaved see NOTE 1.

8.1.5 Determine phosphorus as outlined in 8.3.2 Orthophosphate.

8.2 Hydrolyzable Phosphorus

8.2.1 Add 1 ml of H_2SO_4 solution (7.6) to a 50 ml sample in a 125 ml Erlenmeyer flask.

8.2.2 Boil gently on a pre-heated hot plate for 30–40 minutes or until a final volume of about 10 ml is reached Do not allow sample to go to dryness. Alternatively, heat for 30 minutes in an autoclave at 121°C (15–20 psi).

8.2.3 Cool and dilute the sample to about 30 ml and adjust the pH of the sample to 7.0 ±0.2 with NaOH (7.10) using a pH meter. If sample is not clear at this point, add 2–3 drops of acid (7.6) and filter. Dilute to 50 ml.
Alternatively, if autoclaved see NOTE 1.

8.2.4 The sample is now ready for determination of phosphorus as outlined in 8.3.2 Orthophosphate.

8.3 Orthophosphate

8.3.1 The pH of the sample must be adjusted to 7±0.2 using a pH meter.

8.3.2 Add 8.0 ml of combined reagent (7.5) to sample and mix thoroughly. After a minimum of ten minutes, but no longer than thirty minutes, measure the color absorbance of each sample at 650 or 880 nm with a spectrophotometer, using the reagent blank as the reference solution.
NOTE 1: If the same volume of sodium hydroxide solution is not used to adjust the pH of the standards and samples, a volume correction has to be employed.

9. Calculation

9.1 Prepare a standard curve by plotting the absorbance values of standards versus the corresponding phosphorus concentrations.

9.1.1 Process standards and blank exactly as the samples. Run at least a blank and two standards with each series of samples. If the standards do not agree within ±2% of the true value prepare a new calibration curve.

9.2 Obtain concentration value of sample directly from prepared standard curve. Report results as P, mg/l. SEE NOTE 1.

417

10. Precision and Accuracy

10.1 Thirty-three analysts in nineteen laboratories analyzed natural water samples containing exact increments of organic phosphate, with the following results:

Increment as Total Phosphorus mg P/liter	Precision as Standard Deviation mg P/liter	Accuracy as Bias, %	Accuracy as Bias mg P/liter
0.110	0.033	+3.09	+0.003
0.132	0.051	+11.99	+0.016
0.772	0.130	+2.96	+0.023
0.882	0.128	-0.92	-0.008

(FWPCA Method Study 2, Nutrient Analyses)

10.2 Twenty-six analysts in sixteen laboratories analyzed natural water samples containing exact increments of orthophosphate, with the following results:

Increment as Orthophosphate mg P/liter	Precision as Standard Deviation mg P/liter	Accuracy as Bias, %	Accuracy as Bias, mg P/liter
0.029	0.010	-4.95	-0.001
0.039	0.008	-6.00	-0.002
0.335	0.018	-2.75	-0.009
0.383	0.023	-1.76	-0.007

(FWPCA Method Study 2, Nutrient Analyses)

Bibliography

1. Murphy, J., and Riley, J., "A modified Single Solution for the Determination of Phosphate in Natural Waters", Anal. Chim. Acta., 27, 31 (1962).
2. Gales, M., Jr., Julian, E., and Kroner, R., "Method for Quantitative Determination of Total Phosphorus in Water", Jour. AWWA, 58, No. 10, 1363 (1966).
3. Annual Book of ASTM Standards, Part 31, "Water", Standard D515-72, Method A, p 389 (1976).
4. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 476 and 481, (1975).

41 156

PHOSPHORUS, ALL FORMS

Method 365.3 (Colorimetric, Ascorbic Acid, Two Reagent)

STORET NO. See Section 4

1. Scope and Application
   1.1 These methods cover the determination of specified forms of phosphorus in drinking, surface and saline waters, domestic and industrial wastes.
   1.2 The methods are based on reactions that are specific for the orthophosphate ion. Thus, depending on the prescribed pretreatment of the sample, the various forms may be determined.
      1.2.1 Except for in-depth and detailed studies, the most commonly measured forms are phosphorus and dissolved phosphorus, and orthophosphate and dissolved orthophosphate. Hydrolyzable phosphorus is normally found only in sewage-type samples and insoluble forms of phosphorus are determined by calculation.
   1.3 The methods are usable in the 0.01 to 1.2 mg P/l range.
2. Summary of Method
   2.1 Ammonium molybdate and antimony potassium tartrate react in an acid medium with dilute solutions of phosphorus to form an antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The color is proportional to the phosphorus concentration.
   2.2 Only orthophosphate forms a blue color in this test. Polyphosphates (and some organic phosphorus compounds) may be converted to the orthophosphate form by sulfuric-acid-hydrolysis. Organic phosphorus compounds may be converted to the orthophosphate form by persulfate digestion.
3. Sample Handling and Preservation
   3.1 If benthic deposits are present in the area being sampled, great care should be taken not to include these deposits.
   3.2 Sample containers may be of plastic material, such as cubitainers, or of Pyrex glass.
   3.3 If the analysis cannot be performed the day of collection, the sample should be preserved by the addition of 2 ml conc. H_2SO_4 per liter and refrigeration at 4°C.
4. Definitions and Storet Numbers
   4.1 Total Phosphorus (P) — all of the phosphorus present in the sample, regardless of form, as measured by the persulfate digestion procedure. (00665)
      4.1.1 Total Orthophosphate (P, ortho) — inorganic phosphorus [$(PO_4)^{-3}$] in the sample as measured by the direct colorimetric analysis procedure. (70507)
      4.1.2 Total Hydrolyzable Phosphorus (P, hydro) - phosphorus in the sample as measured by the sulfuric acid hydrolysis procedure, and minus pre-determined orthophosphates. This hydrolyzable phosphorus includes polyphosphorus. [$(P_2O_7)^{-4}$, $(P_3O_{10})^{-5}$, etc.] plus some organic phosphorus. (00669)

Approved for NPDES
Issued 1978

157

4.1.3 Total Organic Phosphorus (P, org) — phosphorus (inorganic plus oxidizable organic) in the sample measured by the persulfate digestion procedure, and minus hydrolyzable phosphorus and orthophosphate. (00670)

4.2 Dissolved Phosphorus (P-D) — all of the phosphorus present in the filtrate of a sample filtered through a phosphorus-free filter of 0.45 micron pore size and measured by the persulfate digestion procedure. (00666)

4.2.1 Dissolved Orthophosphate (P-D, ortho) — as measured by the direct colorimetric analysis procedure. (00671)

4.2.2 Dissolved Hydrolyzable Phosphorus (P-D, hydro) — as measured by the sulfuric acid hydrolysis procedure and minus pre-determined dissolved orthophosphates. (00672)

4.2.3 Dissolved Organic Phosphorus (P-D, org) — as measured by the persulfate digestion procedure, and minus dissolved hydrolyzable phosphorus and orthophosphate. (00673)

4.3 The following forms, when sufficient amounts of phosphorus are present in the sample to warrant such consideration, may be calculated:

4.3.1 Insoluble Phosphorus (P-I) = (P)-(P-D). (00667)

4.3.1.1 Insoluble orthophosphate (P-I, ortho) = (P, ortho) - (P-D, ortho). (00674)

4.3.1.2 Insoluble Hydrolyzable Phosphorus (P-I, hydro) = (P, hydro) - (P-D, hydro). (00675)

4.3.1.3 Insoluble Organic Phosphorus (P-I, org) = (P, org) - (P-D, org). (00676)

4.4 All phosphorus forms shall be reported as P, mg/l, to the third place.

5. Interferences

5.1 Arsenate is determined similarily to phosphorus and should be considered when present. This interference may be eliminated by reducing the arsenic acid to arsenious acid with sodium bisulfite (7.4).

5.2 When high concentrations of iron are present low recovery of phosphorus will be obtained because it will use some of the reducing agent. The bisulfite treatment will also eliminate this interference.

6. Apparatus

6.1 Photometer–A spectrophotometer or filter photometer suitable for measurements at 660 or 880 nm with a light path of 1 cm or longer.

6.2 Acid-washed glassware: All glassware used should be washed with hot 1:1 HCl and rinsed with distilled water. The acid-washed glassware should be filled with distilled water and treated with all the reagents to remove the last traces of phosphorus that might be absorbed on the glassware. Preferably, this glassware should be used only for the determination of phosphorus and after use it should be rinsed with distilled water and kept covered until needed again. If this is done, the treatment with 1:1 HCl and reagents is only required occasionally. Commercial detergents should never be used.

6.3 Water bath, 95°C.

7. Reagents

7.1 Ammonium molybdate-antimony potassium tartrate solution: Dissolve 8 g of ammonium molybdate and 0.2 g antimony potassium tartrate in 800 ml of distilled water and dilute to 1 liter.

7.2 Ascorbic acid solution: Dissolve 60 g of ascorbic acid in 800 ml of distilled water and dilute to 1 liter. Add 2 ml of acetone. This solution is stable for two weeks.

7.3 Sulfuric acid, 11 N: Slowly add 310 ml of conc. H_2SO_4 to approximately 600 ml distilled water. Cool and dilute to 1000 ml.

7.4 Sodium bisulfite ($NaHSO_3$) solution: Dissolve 5.2 g of $NaHSO_3$ in 100 ml of 1.0 N H_2SO_4.

7.5 Ammonium persulfate.

7.6 Stock phosphorus solution: Dissolve 0.4393 g of predried (105°C for one hour) KH_2PO_4 in distilled water and dilute to 1000 ml. 1.0 ml = 0.1 mg P.

7.7 Standard phosphorus solution: Dilute 100 ml of stock phosphorus solution to 1000 ml with distilled water. 1.0 ml = 0.01 mg P. Prepare an appropriate series of standards by diluting suitable volumes of standard or stock solutions to 100 ml with distilled water.

8. Procedure

8.1 Total Phosphorus

8.1.1 Transfer 50 ml of sample or an aliquot diluted to 50 ml into a 125 ml Erlenmeyer flask and add 1 ml of 11 N sulfuric acid (7.3).

8.1.2 Add 0.4 g ammonium persulfate (7.5), mix and boil gently for approximately 30–40 minutes or until a final volume of about 10 ml is reached. Alternatively heat for 30 minutes in an autoclave at 121°C (15–20 psi). Cool, dilute to approximately 40 ml and filter.

8.1.3 For samples containing arsenic or high levels of iron, add 5 ml of sodium bisulfite (7.4), mix and place in a 95°C water bath for 30 minutes (20 minutes after the temperature of the sample reaches 95°C). Cool and dilute to 50 ml.

8.1.4 Determine phosphorus as outlined in (8.3) orthophosphate.

8.2 Hydrolyzable Phosphorus

8.2.1 Add 1 ml of H_2SO_4 solution (8.3) to a 50 ml sample in a 125 ml Erlenmeyer flask.

8.2.2 Boil gently on a pre-heated hot plate for 30–40 minutes or until a final volume of about 10 ml is reached. Do not allow sample to go to dryness. Alternatively, heat for 30 minutes in an autoclave at 121°C (15–20 psi). Cool, dilute to approximately 40 ml and filter.

8.2.3 Treat the samples as in 8.1.3.

8.2.4 Determine phosphorus as outlined in (8.3) orthophosphate.

8.3 Orthophosphate

8.3.1 To 50 ml of sample and/or standards, add 1 ml of 11 N sulfuric acid (7.3) and 4 ml of ammonium molybdate-antimony potassium tartrate (7.1) and mix.
NOTE: If sample has been digested for total or hydrolyzable phosphorus do not add acid.

8.3.2 Add 2 ml of ascorbic acid solution (7.2) and mix.

8.3.3 After 5 minutes, measure the absorbance at 650 nm with a spectrophotometer and determine the phosphorus concentration from the standard curve. The color is stable for at least one hour. For concentrations in the range of 0.01 to 0.3 mg P/l, a 5 cm cell should be used. A one cm cell should be used for concentrations in the range of 0.3 to 1.2 mg P/l.

9. Calculation

9.1 Prepare a standard curve by plotting the absorbance values of standards versus the corresponding phosphorus concentrations on linear graph paper.

9.2 Obtain concentration value of sample directly from prepared standard curve. Report results as P, mg/l.

10. Precision and Accuracy

10.1 Precision data is not available at this time.

10.2 In a single laboratory (EMSL) using industrial waste and sewage samples at concentrations of 7.6 and 0.55 mg P/l, recoveries were 99 and 100%, respectively.

PHOSPHORUS, TOTAL

Method 365.4 (Colorimetric, Automated, Block Digestor AA II)

STORET NO. 00665

1. Scope and Application
   1.1 This method covers the determination of total phosphorus in drinking water, surface water and domestic and industrial wastes. The applicable range of this method is 0.01 to 20 mg P/l.
2. Summary of Method
   2.1 The sample is heated in the presence of sulfuric acid, K_2SO_4 and $HgSO_4$ for two and one half hours. The residue is cooled, diluted to 25 ml and placed on the AutoAnalyzer for phosphorus determination.
3. Sample Handling and Preservation
   3.1 Sample containers may be of plastic material, such as a cubitainer, or of Pyrex glass.
   3.2 If the analysis cannot be performed the day of collection, the sample should be preserved by the addition of 2 ml of conc. H_2SO_4 per liter and refrigeration at 4°C.
4. Apparatus
   4.1 Block Digestor BD-40
   4.2 Technicon Method No. 327-74W for Phosphorus
5. Reagents
   5.1 Mercuric sulfate: Dissolve 8 g red mercuric oxide (HgO) in 50 ml of 1:4 sulfuric acid (10 conc. H_2SO_4: 40 ml distilled water) and dilute to 100 ml with distilled water.
   5.2 Digestion solution: (Sulfuric acid-mercuric sulfate-potassium sulfate solution): Dissolve 133 g of K_2SO_4 in 600 ml of distilled water and 200 ml of conc. H_2SO_4. Add 25 ml of mercuric sulfate solution (5.1) and dilute to 1 liter.
   5.3 Sulfuric acid solution (0.72 N): Add 20 ml of conc. sulfuric acid to 800 of distilled water, mix and dilute to 1 liter.
   5.4 Molybdate/antimony solution: Dissolve 8 g of ammonium molybdate and 0.2 g of antimony potassium tartrate in about 800 ml of distilled water and dilute to 1 liter.
   5.5 Ascorbic acid solution: Dissolve 60 g of ascorbic acid in about 600 ml of distilled water. Add 2 ml of acetone and dilute to 1 liter.
   5.6 Diluent water: Dissolve 40 g of NaCl in about 600 ml of distilled water and dilute to 1 liter.
   5.7 Sulfuric acid solution, 4%: Add 40 ml of conc. sulfuric acid to 800 ml of ammonia-free distilled water, cool and dilute to 1 liter.
6. Procedure

   Digestion

   6.1 To 20 or 25 ml of sample, add 5 ml of digestion solution and mix. (Use a vortex mixer).
   6.2 Add 4–8 Teflon boiling chips. Too many boiling chips will cause the sample to boil over.

Pending approval for NPDES and Section 304(h), CWA
Issued 1974

SILICA, DISSOLVED

Method 370.1 (Colorimetric)

STORET NO. Dissolved 00955

1. Scope and Application
   1.1 This method is applicable to drinking, surface and saline waters, domestic and industrial wastes.
   1.2 The working range of the method is approximately 2 to 25 mg silica/l. The upper range can be extended by taking suitable aliquots; the lower range can be extended by the addition of amino-naphthol-sulfonic acid solution as described in (6.8).
2. Summary of Method
   2.1 A well-mixed sample is filtered through a 0.45 μ membrane filter. The filtrate, upon the addition of molybdate ion in acidic solution, forms a greenish-yellow color complex proportional to the dissolved silica in the sample. The color complex is then measured spectrophotometrically.
   2.2 In the low concentration modification the yellow (410 nm) molybdosilicic acid color is reduced by 1-amino-2-naphthol-4-sulfonic acid to a more intense heteropoly blue (815 nm or 650 nm).
3. Interferences
   3.1 Excessive color and/or turbidity interfere. Correct by running blanks prepared without addition of the ammonium molybdate solution. See (6.7).
   3.2 Tannin interference may be eliminated and phosphate interferences may be decreased with oxalic acid.
   3.3 Large amounts of iron and sulfide interfere.
   3.4 Contact with glass should be minimized, silica free reagents should be used as much as possible. A blank should be run.
4. Apparatus
   4.1 Platinum dishes, 100 ml.
   4.2 Colorimetric equipment–one of the following:
      4.2.1 Spectrophotometer for use at 410 nm, 650 nm and/or 815 nm with a 1 cm or longer cell.
      4.2.2 Filter photometer with a violet filter having maximum transmittance as near 410 nm as possible and a 1 cm or longer cell.
      4.2.3 Nessler tubes, matched, 50 ml, tall form.
5. Reagents
   5.1 Use chemicals low in silica and store in plastic containers.
   5.2 Sodium bicarbonate, $NaHCO_3$, powder.
   5.3 Sulfuric acid, H_2SO_4, 1 N.

Approved for NPDES
Issued 1971
Editorial revision 1978

427 167

5.4 Hydrochloric acid, HCl, 1 + 1.

5.5 Ammonium molybdate reagent: Place 10 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in distilled water in a 100 ml volumetric. Dissolve by stirring and gently warming. Dilute to the mark. Filter if necessary. Adjust to pH 7 to 8 with silica free NH_4OH or NaOH. Store in plastic bottle.

5.6 Oxalic acid solution: Dissolve 10 g $H_2C_2O_4 \cdot 2H_2O$ in distilled water in a 100 ml volumetric flask, dilute to the mark. Store in plastic.

5.7 Stock silica solution: Dissolve 4.73 g sodium metasilicate nonahydrate, $Na_2SiO_3 \cdot 9H_2O$, in recently boiled and cooled distilled water. Dilute to approximately 900 ml. Analyze 100.0 ml portions by gravimetry (ref. 1, p. 484). Adjust concentration to 1.000 mg/l SiO_2. Store in tightly stoppered plastic bottle.

5.8 Standard silica solution: Dilute 10.0 ml stock solution to 1 liter with recently boiled and cooled distilled water. This is 10 mg/l SiO_2 (1.00 ml = 10.0 ug SiO_2). Store in a tightly stoppered plastic bottle.

5.9 Permanent color solutions

5.9.1 Potassium chromate solution: Dissolve 630 mg K_2CrO_4 in distilled water in a 1 liter volumetric flask and dilute to the mark.

5.9.2 Borax solution: Dissolve 10 g sodium borate decahydrate, $(Na_2B_4O_7 \cdot 10H_2O)$ in distilled water in a 1 liter volumetric flask and dilute to the mark.

5.10 Reducing agent: Dissolve 500 mg of 1-amino-2-naphthol-4-sulfonic acid and 1 g Na_2SO_3 in 50 ml distilled water with gentle warming if necessary. Dissolve 30 g $NaHSO_3$ in 150 ml distilled water. Mix these two solutions. Filter into a plastic bottle. Refrigerate and avoid exposure to light. Discard when it darkens. If there is incomplete solubility or immediate darkening of the aminonaphthosulfonic acid solution do not use.

6. Procedure

6.1 Filter sample through a 0.45 u membrane filter.

6.2 Digestion: If molybdate unreactive silica is present and its inclusion in the analysis is desired, include this step, otherwise proceed to 6.3.

6.2.1 Place 50 ml, or a smaller portion diluted to 50 ml, of filtered (6.1) sample in a 100 ml platinum dish.

6.2.2 Add 200 mg silica-free $NaHCO_3$ (5.2) and digest on a steam bath for 1 hour. Cool.

6.2.3 Add slowly and with stirring 2.4 ml H_2SO_4 (5.3).

6.2.4 Immediately transfer to a 50 ml Nessler tube, dilute to the mark with distilled water and proceed to 6.3 without delay.

6.3 Color development

6.3.1 Place 50 ml sample in a Nessler tube.

6.3.2 Add rapidly 1.0 ml of 1 + 1 HCl (5.4) and 2.0 ml ammonium molybdate reagent (5.5).

6.3.3 Mix by inverting at least 5 times.

6.3.4 Let stand 5 to 10 minutes.

6.3.5 Add 1.5 ml oxalic acid solution (5.6) and mix thoroughly.

6.3.6 Read color (spectrophotometrically or visually) after 2 minutes but before 15 minutes from the addition of oxalic acid.

6.4 Preparation of Standards

6.4.1 If digestion (6.2) was used add 200 mg $NaHCO_3$ (5.2) and 2.4 ml H_2SO_4 (5.3) to standards to compensate for silica introduced by these reagents and for effect of the salt on the color intensity.

6.5 Photometric measurement

6.5.1 Prepare a calibration curve using approximately six standards to span the range shown below with the selected light path.

Selection of Light Path Length for Various Silica Concentrations

Light Path cm	Silica in 54.5 ml final volume (ug)
1	200–1300
2	100–700
5	40–250
10	20–130

6.5.2 Carry out the steps in 6.3 using distilled water as the reference. Read a blank.

6.5.3 Plot photometric reading versus ug of silica in the final solution of 54.5 ml. Run a reagent blank and at least one standard with each group of samples.

6.6 Visual Comparison

6.6.1 Prepare a set of permanent artificial color standards according to the table. Use well stoppered, properly labelled 50 ml Nessler tubes.

Silica value mg	Potassium chromate solution (5.9.1) ml	Borax solution (5.9.2) ml	Distilled water ml
0.00	0.0	25	30
0.10	1.0	25	29
0.20	2.0	25	28
0.40	4.0	25	26
0.50	5.0	25	25
0.75	7.5	25	22
1.00	10.0	25	20

6.6.2 Verify permanent standards by comparison to color developed by standard silica solutions.

6.6.3 These permanent artificial color standards are only for color comparison procedure, not for photometric procedure.

6.7 Correction for color or turbidity

6.7.1 A special blank is run using a portion of the sample and carrying out the procedure in 6.1, 6.2 if used, and 6.3 except for the addition of ammonium molybdate (6.3.2).

6.7.2 Zero the photometer with this blank before reading the samples.

6.8 Procedure for low concentration (< 1000 ug/l)

6.8.1 Perform steps 6.1 and 6.2 if needed.

6.8.2 Place 50 ml sample in a Nessler tube.

6.8.3 In rapid succession add 1.0 ml of 1 + 1 HCl (5.4).

6.8.4 Add 2.0 ml ammonium molybdate reagent (5.5).

6.8.5 Mix by inverting at least six times.

6.8.6 Let stand 5 to 10 minutes.

6.8.7 Add 1.5 ml oxalic acid solution (5.6).

6.8.8 Mix thoroughly.

6.8.9 At least 2, but not more than 15 minutes after oxalic acid addition, add 2.0 ml reducing agent (5.10).

6.8.10 Mix thoroughly.

6.8.11 Wait 5 minutes, read photometrically or visually.

6.8.12 If digestion (6.2) was used see (6.4).

6.8.13 Photometric measurement

6.8.13.1 Prepare a calibration curve using approximately 6 standards and a reagent blank to span the range shown below with the selected light path.

Selection of Light Path Length for Various Silica Concentrations

Light Path cm	Silica in 56.5 ml Final volume, ug 650 nm	815 nm
1	40-300	20-100
2	20-150	10-50
5	7-50	4-20
10	4-30	2-10

6.8.13.2 Read versus distilled water.

6.8.13.3 Plot photometric reading at 650 nm or at 815 nm versus ug of silica in 56.5 ml.

6.8.13.4 For turbidity correction use 6.1, 6.2 if used and 6.8.2–6.8.11 omitting 6.8.4 and 6.8.9.

6.8.13.5 Run a reagent blank and at least one standard (to check calibration curve drift) with each group of samples.

6.8.14 Visual comparison

6.8.14.1 Prepare not less than 12 standards covering the range of 0 to 120 ug SiO_2 by placing the calculated volumes of standard silica (5.8) in 50 ml Nessler tubes, diluting to the mark and develop the color as in 6.8.2–6.8.11.

437 165

7. Calculations

7.1 Read ug SiO_2 from calibration curve or by visual comparison

7.2 mg/l SiO_2 =

$$\frac{ug\ SiO_2}{ml\ sample}$$

7.3 Report whether $NaHCO_3$ digestion (6.2) was used

8. Precision and Accuracy

8.1 A synthetic unknown sample containing 5.0 mg/l SiO_2, 10 mg/l chloride, 0.200 mg/l ammonia N, 1.0 mg/l nitrate N, 1.5 mg/l organic N, and 10.0 mg/l phosphate in distilled water was analyzed in 19 laboratories by the molybdosilicate method, with a relative standard deviation of 14.3% and a relative error of 7.8%.

8.2 Another synthetic unknown sample containing 15.0 mg/l SiO_2, 200 mg/l chloride, 0.800 mg/l ammonia N, 1.0 mg/l nitrate N, 0.800 mg/l organic N, and 5.0 mg/l phosphate in distilled water was analyzed in 19 laboratories by the molybdosilicate method, with a relative standard deviation of 8.4% and a relative error of 4.2%.

8.3 A third synthetic unknown sample containing 30.0 mg/l SiO_2, 400 mg/l chloride, 1.50 mg/l ammonia N, 1.0 mg/l nitrate N, 0.200 mg/l organic N, and 0.500 mg/l phosphate in distilled water was analyzed in 20 laboratories by the molybdosilicate method, with a relative standard deviation of 7.7% and a relative error of 9.8%. All results were obtained after sample digestion with $NaHCO_3$.

8.4 Photometric evaluations by the amino-naphthol-sulfonic acid procedure have an estimated precision of ±0.10 mg/l in the range from 0 to 2 mg/l (ASTM).

8.5 Photometric evaluations of the silico-molybdate color in the range from 2 to 50 mg/l have an estimated precision of approximately 4% of the quantity of silica measured (ASTM).

Bibliography

1. Annual Book of ASTM Standards, Part 31, "Water", Standard D859–68, p 401 (1976).
2. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 487, Method 426B, (1975).

SULFATE

Method 375.1 (Colorimetric, Automated, Chloranilate)

STORET NO. Total 00945

1. Scope and Application
   1.1 This automated method is applicable to drinking and surface waters, domestic and industrial wastes, in the range of 10 to 400 mg SO_4/l. Approximately 15 samples per hour can be analyzed.
2. Summary of Method
   2.1 When solid barium chloranilate is added to a solution containing sulfate, barium sulfate is precipitated, releasing the highly colored acid chloranilate ion. The color intensity in the resulting chloranilic acid is proportional to the amount of sulfate present.
3. Sample Handling and Preservation
   3.1 Refrigeration at 4°C.
4. Interferences
   4.1 Cations, such as calcium, aluminum, and iron, interfere by precipitating the chloranilate. These ions are removed automatically by passage through an ion exchange column.
5. Apparatus
   5.1 Technicon AutoAnalyzer consisting of:
      5.1.1 Sampler I.
      5.1.2 Continuous filter.
      5.1.3 Manifold.
      5.1.4 Proportioning pump.
      5.1.5 Colorimeter equipped with 15 mm tubular flow cell and 520 nm filters.
      5.1.6 Recorder.
      5.1.7 Heating bath, 45°C.
   5.2 Magnetic stirrer.
6. Reagents
   6.1 Barium chloranilate: Add 9 g of barium chloranilate ($BaC_6Cl_2O_4$) to 333 ml of ethyl alcohol and dilute to 1 liter with distilled water.
   6.2 Acetate buffer, pH 4.63: Dissolve 13.6 g of sodium acetate in distilled water. Add 6.4 ml of acetic acid and dilute to 1 liter with distilled water. Make fresh weekly.
   6.3 NaOH-EDTA solution: Dissolve 65 g of NaOH and 6 g of EDTA in distilled water and dilute to 1 liter.
   NOTE 1: This solution is also used to clean out manifold system at end of sampling run.
   6.4 Ion exchange resin: Dowex-50 W-X8, ionic form-H^+.
   NOTE 2: Column is prepared by sucking a slurry of the resin into 12 inches of 3/16-inch OD sleeving. This may be conveniently done by using a pipette and a loose-fitting glass wool plug in the sleeve. The column, upon exhaustion, turns red.

Issued 1971

433 167

8. Precision and Accuracy

8.1 A synthetic unknown sample containing 259 mg/l sulfate, 108 mg/l Ca, 82 mg/l Mg, 3.1 mg/l K, 19.9 mg/l Na, 241 mg/l chloride, 250 ug/l nitrite N, 1.1 mg/l nitrate N and 42.5 mg/l alkalinity (contributed by $NaHCO_3$), was analyzed in 32 laboratories by the gravimetric method, with a relative standard deviation of 4.7% and a relative error of 1.9%.

Bibliography

1. Annual Book of ASTM Standards, Part 31, "Water", Standard D516–68, Method A, p 429 (1976).
2. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 493, Method 427A, (1975).

SULFATE

Method 375.4 (Turbidimetric)

STORET NO. Total 00945

1. Scope and Application
   1.1 This method is applicable to drinking and surface waters, domestic and industrial wastes.
   1.2 The method is suitable for all concentration ranges of sulfate; however, in order to obtain reliable readings, use a sample aliquot containing not more than 40 mg SO_4/l.
   1.3 The minimum detectable limit is approximately 1 mg/l sulfate.
2. Summary of Method
   2.1 Sulfate ion is converted to a barium sulfate suspension under controlled conditions. The resulting turbidity is determined by a nephelometer, filter photometer or spectrophotometer and compared to a curve prepared from standard sulfate solutions.
   2.2 Suspended matter and color interfere. Correct by running blanks from which the barium chloride has been omitted.
   2.3 Silica in concentrations over 500 mg/l will interfere.
3. Comments
   3.1 Proprietary reagents, such as Hach Sulfaver or equivalent, are acceptable.
   3.2 Preserve by refrigeration at 4°C.
4. Apparatus
   4.1 Magnetic stirrer, variable speed so that it can be held constant just below splashing. Use identical shape and size magnetic stirring bars.
   4.2 Photometer: one of the following which are given in order of preference.
      4.2.1 Nephelometer
      4.2.2 Spectrophotometer for use at 420 nm with light path of 4 to 5 cm.
      4.2.3 Filter photometer with a violet filter having a maximum near 420 nm and a light path of 4 to 5 cm.
   4.3 Stopwatch, if the magnetic stirrer is not equipped with an accurate timer.
   4.4 Measuring spoon, capacity 0.2 to 0.3 ml.
5. Reagents
   5.1 Conditioning reagent: Place 30 ml conc. HCl, 300 ml distilled water, 100 ml 95% ethanol or isopropanol and 75 g NaCl in solution in a container. Add 50 ml glycerol and mix.
   5.2 Barium chloride, $BaCl_2$, crystals, 20 to 30 mesh.
   5.3 Sodium carbonate solution (approximately 0.05N): Dry 3 to 5 g primary standard Na_2CO_3 at 250°C for 4 hours and cool in a desiccator. Weigh 2.5 ±0.2 g (to the nearest mg), transfer to a 1 liter volumetric flask and fill to the mark with distilled water.

Approved for NPDES
Issued 1971
Editorial revision 1978

205 169

5.4 Standard sulfate solution (1.00 ml = 100 μg SO_4): Prepare by either 5.4.1 or 5.4.2.

5.4.1 Standard sulfate solution from H_2SO_4

5.4.1.1 Standard sulfuric acid, 0.1N: dilute 3.0 ml conc. H_2SO_4 to 1 liter with distilled water. Standardize versus 40.00 ml of 0.05 N Na_2CO_3 solution (5.3) with about 60 ml distilled water by titrating potentiometrically to pH about 5. Lift electrodes and rinse into beaker. Boil gently for 3–5 minutes under a watch glass cover. Cool to room temperature. Rinse cover glass into beaker. Continue titration to the pH inflection point. Calculate normality using

$$N = \frac{A \times B}{53.00 \times C}$$

where:

A = g Na_2CO_3 weighed into 1 liter
B = ml Na_2CO_3 solution
C = ml acid used to inflection point

5.4.1.2 Standard acid, 0.02 N: Dilute appropriate amount of standard acid, 0.1 N (5.4.1.1) to 1 liter (200.00 ml if 0.1000 N). Check by standardization versus 15 ml of 0.05 N Na_2CO_3 solution (5.3).

5.4.1.3 Place 10.41 ml standard sulfuric acid, 0.02 N (5.4.1.2) in a 100 ml volumetric and dilute to the mark.

5.4.2 Standard sulfate solution from Na_2SO_4: Dissolve 147.9 mg anhydrous Na_2SO_4 in distilled water in a 1 liter volumetric flask and dilute to the mark with distilled water.

6. Procedure

6.1 Formation of barium sulfate turbidity

6.1.1 Place 100 ml sample, or a suitable portion diluted to 100 ml, into a 250 Erlenmeyer flask.

6.1.2 Add exactly 5.0 ml conditioning reagent (5.1).

6.1.3 Mix in the stirring apparatus.

6.1.4 While the solution is being stirred, add a measuring spoonful of $BaCl_2$ crystals (5.2) and begin timing immediately.

6.1.5 Stir exactly 1.0 minutes at constant speed.

6.2 Measurement of barium sulfate turbidity

6.2.1 Immediately after the stirring period has ended, pour solution into absorbance cell.

6.2.2 Measure turbidity at 30 second intervals for 4 minutes.

6.2.3 Record the maximum reading obtained in the 4 minute period.

6.3 Preparation of calibration curve.

6.3.1 Prepare calibration curve using standard sulfate solution (5.4).

6.3.2 Space standards at 5 mg/l increments in the 0–40 mg/l sulfate range.

6.3.3 Above 50 mg/l the accuracy decreases and the suspensions lose stability.

6.3.4 Check reliability of calibration curve by running a standard with every 3 or 4 samples.

6.4 Correction for sample color and turbidity.

6.4.1 Run a sample blank using the procedure 6.1 and 6.2 without the addition of barium chloride (6.1.4).

7. Calculations

7.1 Read mg SO_4 from calibration curve

$$\text{mg } SO_4/l = \frac{\text{mg } SO_4 \times 1{,}000}{\text{ml sample}}$$

8. Precision and Accuracy

8.1 Thirty-four analysts in 16 laboratories analyzed six synthetic water samples containing exact increments of inorganic sulfate with the following results:

Increment as Sulfate mg/liter	Precision as Standard Deviation mg/liter	Accuracy as Bias, %	Accuracy as Bias mg/liter
8.6	2.30	-3.72	-0.3
9.2	1.78	-8.26	-0.8
110	7.86	-3.01	-3.3
122	7.50	-3.37	-4.1
188	9.58	+0.04	+0.1
199	11.8	-1.70	-3.4

(FWPCA Method Study 1, Mineral and Physical Analyses).

8.2 A synthetic unknown sample containing 259 mg/l sulfate, 108 mg/l Ca, 82 mg/l Mg, 3.1 mg/l K, 19.9 mg/l Na, 241 mg/l chloride, 0.250 mg/l nitrite N, 1.1 mg/l nitrate N, and 42.5 mg/l total alkalinity (contributed by $NaHCO_3$) was analyzed in 19 laboratories by the turbidimetric method, with a relative standard deviation of 9.1% and a relative error of 1.2%.

Bibliography

1. Annual Book of ASTM Standards, Part 31, "Water", Standard D516-68, Method B, p 430 (1976).
2. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 496, Method 427C, (1975).

SULFIDE

Method 376.1 (Titrimetric, Iodine)

STORET NO. Total 00745
Dissolved 00746

1. Scope and Application
   1.1 This method is applicable to the measurement of total and dissolved sulfides in drinking, surface and saline waters, domestic and industrial wastes.
   1.2 Acid insoluble sulfides are not measured by the use of this test. (Copper sulfide is the only common sulfide in this class).
   1.3 This method is suitable for the measurement of sulfide in concentrations above 1 mg/l.
2. Summary of Method
   2.1 Excess iodine is added to a sample which may or may not have been treated with zinc acetate to produce zinc sulfide. The iodine oxidizes the sulfide to sulfur under acidic conditions. The excess iodine is backtitrated with sodium thiosulfate or phenylarsine oxide.
3. Comments
   3.1 Reduced sulfur compounds, such as sulfite, thiosulfate and hydrosulfite, which decompose in acid may yield erratic results. Also, volatile iodine-consuming substances will give high results.
   3.2 Samples must be taken with a minimum of aeration. Sulfide may be volatilized by aeration and any oxygen inadvertently added to the sample may convert the sulfide to an unmeasurable form.
   3.3 If the sample is not preserved with zinc acetate and NaOH, the analysis must be started immediately. Similarly, the measurement of dissolved sulfides must also be commenced immediately.
4. Apparatus: Ordinary laboratory glassware.
5. Reagents
   5.1 Hydrochloric acid, HCl, 6 N
   5.2 Standard iodine solution, 0.0250 N: Dissolve 20 to 25 g KI in a little water in a liter volumetric and add 3.2 g iodine. Allow to dissolve. Dilute to 1 liter and standardize against 0.0250 N sodium thiosulfate or phenylarsine oxide using a starch indicator.
   5.3 Phenylarsine oxide 0.0250 N: commercially available.
   5.4 Starch indicator: commercially available.
   5.5 Procedure for standardization (see Residual Chlorine–iodometric titration Method 330.3, section 5.15).

Approved for NPDES
Issued 1971
Editorial revision 1978

6. Procedure

6.1 Unprecipitated sample

6.1.1 Place a known amount of standard iodine solution (5.2) into a 500 ml flask. The amount should be estimated to be in excess of the amount of sulfide expected.

6.1.2 Add distilled water, if necessary, to bring the volume to approximately 20 ml.

6.1.3 Add 2 ml of 6 N HCl (5.1).

6.1.4 Pipet 200 ml of sample into the flask, keeping the tip of the pipet below the surface of the sample.

6.1.5 If the iodine color disappears, add more iodine until the color remains. Record the total number of milliliters of standard iodine used in performing steps 6.1.1 and 6.1.5.

6.1.6 Titrate with the reducing solution (0.0250 N sodium thiosulfate or 0.0250 N phenylarsine oxide solution (5.3)) using a starch indicator (5.4) until the blue color disappears. Record the number of milliliters used.

6.2 Precipitated samples

6.2.1 Add the reagents to the sample in the original bottle. Perform steps 6.1.1, 6.1.3, 6.1.5, and 6.1.6.

6.3 Dewatered samples

6.3.1 Return the glass fibre filter paper which contains the sample to the original bottle. Add 200 ml distilled water. Perform steps 6.1.1, 6.1.3, 6.1.5, and 6.1.6.

6.3.2 The calculations (7) should be based on the volume of original sample put through the filter.

7. Calculations

7.1 One ml of 0.0250 N standard iodine solution (5.2) reacts with 0.4 mg of sulfide present in the titration vessel.

7.2 Use the formula

$$\text{mg/l sulfide} = \frac{400\,(A - B)}{\text{ml sample}}$$

where:

A = ml of 0.0250 N standard iodine solution (5.2)

B = ml of 0.0250N standard reducing sodium thosulfate or phenylarsine oxide) solution (5.3).

8. Precision and Accuracy

8.1 Precision and accuracy for this method have not been determined.

Bibliography

1. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 505. Method 428D, (1975).

4.50

SULFIDE

Method 376.2 (Colorimetric, Methylene Blue)

STORET NO. Total 00745
Dissolved 00746

1. Scope and Application
   1.1 This method is applicable to the measurement of total and dissolved sulfides in drinking, surface and saline waters, domestic and industrial wastes.
   1.2 Acid insoluble sulfides are not measured by this method. Copper sulfide is the only common sulfide in this class.
   1.3 The method is suitable for the measurement of sulfide in concentrations up to 20 mg/l.
2. Summary of Method
   2.1 Sulfide reacts with dimethyl-p-phenylenediamine (p-aminodimethyl aniline) in the presence of ferric chloride to produce methylene blue, a dye which is measured at a wavelength maximum of 625 nm.
3. Comments
   3.1 Samples must be taken with a minimum of aeration. Sulfide may be volatilized by aeration and any oxygen inadvertently added to the sample may convert the sulfide to an unmeasurable form. Dissolved oxygen should not be present in any water used to dilute standards.
   3.2 The analysis must be started immediately.
   3.3 Color and turbidity may interfere with observations of color or with photometric readings.
4. Apparatus
   4.1 Matched test tubes, approximately 125 mm long and 15 mm O.D.
   4.2 Droppers, delivering 20 drops/ml. To obtain uniform drops, hold dropper in vertical position and allow drops to form slowly.
   4.3 Photometer, use either 4.3.1 or 4.3.2.
      4.3.1 Spectrophotometer, for use at 625 nm with cells of 1 cm and 10 cm light path.
      4.3.2 Filter photometer, with filter providing transmittance near 625 nm.
5. Reagents
   5.1 Amino-sulfuric acid stock solution: Dissolve 27 g N,N-dimethyl-p-phenylenediamine oxalate (p-aminodimethylaniline) in a cold mixture of 50 ml conc. H_2SO_4 and 20 ml distilled water in a 100 ml volumetric flask. Cool and dilute to the mark. If dark discard and purchase fresh reagent. Store in dark glass bottle.
   5.2 Amino-sulfuric acid reagent: Dissolve 25 ml amino-sulfuric acid stock solution (5.1) with 975 ml of 1 + 1 H_2SO_4 (5.4). Store in a dark glass bottle. This solution should be clear.
   5.3 Ferric chloride solution: Dissolve 100 g $FeCl_3 \cdot 6H_2O$ in 40 ml distilled water.

Approved for NPDES
Issued 1978

5.4 Sulfuric acid solution, H_2SO_4, 1 + 1

5.5 Diammonium hydrogen phosphate solution: Dissolve 400 g $(NH_4)_2HPO_4$ in 800 ml distilled water.

5.6 Methylene blue solution I: Dissolve 1.0 g of methylene blue in distilled water in a 1 liter volumetric flask and dilute to the mark. Use U.S.P. grade or one certified by the Biological Stain Commission. The dye content reported on the label should be 84% or more. Standardize (5.8) against sulfide solutions of known strength and adjust concentration so that 0.05 ml (1 drop) equals 1.0 mg/l sulfide.

5.7 Methylene blue solution II: Dilute 10.00 ml of adjusted methylene blue solution I (5.6) to 100 ml with distilled water in a volumetric flask.

5.8 Standardization of methylene blue I solution:

5.8.1 Place several grams of clean, washed crystals of sodium sulfide $Na_2S \cdot 9H_2O$ in a small beaker.

5.8.2 Add somewhat less than enough water to cover the crystals.

5.8.3 Stir occasionally for a few minutes. Pour the solution into another vessel. This reacts slowly with oxygen but the change is insignificnat over a few hours. Make the solution daily.

5.8.4 To 1 liter of distilled water add 1 drop of solution and mix.

5.8.5 Immediately determine the sulfide concentration by the methylene blue procedure (6) and by the titrimetric iodide procedure (Method 376.1, this manual).

5.8.6 Repeat using more than one drop of sulfide solution or less water until at least five tests have been made in the range of 1 to 8 mg/l sulfide.

5.8.7 Calculate the average percent error of the methylene blue procedure (6) as compared to the titrimetric iodide procedure (Method 376.1).

5.8.8 Adjust by dilution or by adding more dye to methylene blue solution I (5.6).

6. Procedure

6.1 Color development

6.1.1 Transfer 7.5 ml of sample to each of two matched test tubes using a special wide tipped pipet or filling to a mark on the test tubes.

6.1.2 To tube A add 0.5 ml amine-sulfuric acid reagent (5.2) and 0.15 ml (3 drops) $FeCl_3$ solution (5.3).

6.1.3 Mix immediately by inverting the tube only once.

6.1.4 To tube B add 0.5 ml 1 + 1 H_2SO_4 (5.4) and 0.15 ml (3 drops) $FeCl_3$ solution (5.3) and mix.

6.1.5 Color will develop in tube A in the presence of sulfide. Color development is usually complete in about 1 minute, but a longer time is often required for the fading of the initial pink color.

6.1.6 Wait 3 to 5 minutes.

6.1.7 Add 1.6 ml $(NH_4)_2HPO_4$ solution (5.5) to each tube.

6.1.8 Wait 3 to 5 minutes and make color comparisons. If zinc acetate was used wait at least 10 minutes before making comparison.

6.2 Color comparison

6.2.1 Visual

6.2.1.1 Add methylene blue solution I (5.6) and/or II (5.7) (depending on sulfide concentration and accuracy desired) dropwise to tube B (6.1.4) until the color matches that developed in the first tube.

6.2.1.2 If the concentration exceeds 20 mg/l, repeat 6.2.1.1 using a portion of the sample diluted to one tenth.

6.2.2 Photometric

6.2.2.1 Use a 1 cm cell for 0.1 to 2.0 mg/l. Use a 10 cm cell for up to 20 mg/l.

6.2.2.2 Zero instrument with portion of sample from tube B (6.1.4).

6.2.2.3 Prepare calibration curve from data obtained in methylene blue standardization (5.8), plotting concentraton obtained from titrimetric iodide procedure (Method 376.1) versus absorbance. A straight line relationship can be assumed from 0 to 1.0 mg/l.

6.2.2.4 Read the sulfide concentration from the calibration curve.

7. Calculations

7.1 Visual comparison: With methylene blue solution I (5.6), adjusted so that 0.05 ml (1 drop) = 1.0 mg/l sulfide and a 7.5 ml sample

mg/l sulfide = number drops methylene blue solution I (5.6) + 0.1 x [number of drops methylene blue solution II (5.7)].

7.2 Photometric: see 6.2.2.4

8. Precision and Accuracy:

8.1 The precision has not been determined. The accuracy is about ±10%.

Bibliography

1. Standard Methods for he Examination of Water and Wastewater, 14th edition, p. 503, Method 428C (1975).

SULFITE

Method 377.1 (Titrimetric)

STORET NO. 00740

1. Scope and Application
   1.1 This method is applicable to drinking and surface waters, sewage and industrial wastes. The primary application has been to cooling, process and distribution water systems and boiler feedwaters to which sulfide is added in order to reduce dissolved oxygen and eliminate corrosion.
   1.2 The minimum detectable limit is 2–3 mg/l SO_3.
2. Summary of Method
   2.1 An acidified sample containing a starch indicator is titrated with a standard potassium iodide-iodate titrant to a faint permanent blue end point which appears when the reducing power of the sample has been completely exhausted.
3. Interferences
   3.1 The temperature of the sample must be below 50°C.
   3.2 Care must be taken to allow as little contact with air as possible. For example, do not filter the sample. Keep the buret tip below the surface of the sample.
   3.3 Other oxidizable substances, such as organic compounds, ferrous iron and sulfide are positive interferences. Sulfide may be removed by adding 0.5g of zinc acetate and analyzing the supernatant of the settled sample.
   3.4 Nitrite gives a negative interference by oxidizing sulfite when the sample is acidified; this is corrected by either using a proprietary indicator which eliminates nitrite or by adding sulfamic acid.
   3.5 Copper and possibly other heavy metals catalyze the oxidation of sulfite; EDTA is used to complex metals.
   3.6 A blank must be run to correct for interferences present in the reagents.
4. Apparatus
   4.1 Standard laboratory glassware is used.
5. Reagents
   5.1 Sulfuric acid, H_2SO_4, 1 + 1.
   5.2 Starch indicator: Amylose, Mallinckrodt Chemical Works; Thyodene, Magnus Chemical Co. or equivalent.
   5.3 Dual-Purpose Sulfite Indicator Powder: a proprietary formulation containing sulfamic acid to destroy nitrite.
   5.4 Standard potassium iodide-iodate titrant, 0.0125N: Dissolve 445.8 mg anhydrous potassium iodate, KIO_3 (primary standard grade dried for several hours at 120°C), 4.25g

Approved for NPDES
Issued 1974
Editorial revision 1978

455

177

LECTURE/HANDOUT MATERIAL

METALS ANALYSIS BY ATOMIC ABSORPTION SPECTROPHOTOMETRY.

FLAME AND GRAPHITE FURNACE EPA METHODS FOR THE FOLLOWING METALS:

ALUMINUM, ANTIMONY, ARSENIC, BARIUM, CADMIUM, CALCIUM, CHROMIUM, COBALT, COOPER, IRON, LEAD, MAGNESIUM, MANGANESE, MERCURY, MOLYBDENUM, NICKEL, POTASSIUM, SELENIUM, SILVER, SODIUM, TIN, VANADIUM AND ZINC

SPECIAL CASES: GASEOUS HYDRIDE

As, Se, Te, Sb, Bi and Sn: Needs Hydrides Generation System
(See Next Page)

Cold Mercury Vapor
(See Page 3)

5.2 *Arsenic standard solution II*, 1.00 mL= 10.0 μg As: Dilute 5.00 mL arsenic standard solution I and 1 mL concentrated HNO_3 (sp gr 1.41) to 500.0 mL with demineralized water. Discard after 3 months.

5.3 *Arsenic standard solution III*, 1.00 mL= 0.10 μg As: Dilute 5.00 mL arsenic standard solution II and 1 mL concentrated HNO_3 (sp gr 1.41) to 500.0 mL with demineralized water. Prepare fresh weekly.

5.4 *Hydrochloric acid*, concentrated (sp gr 1.19): Use analytical-grade acid with arsenic content not greater than 1×10^{-6} percent.

5.5 *Nitric acid*, concentrated (sp gr 1.41): Use analytical-grade acid with arsenic content not greater than 5×10^{-7} percent.

5.6 *Potassium iodide solution*, 15 g/100 mL: Dissolve 15 g KI in 100 mL demineralized water. This solution is stable when stored in an amber bottle.

5.7 *Sodium borohydride solution*, 4 g/100 mL: Dissolve 4 g $NaBH_4$ and 2 g NaOH in 100 mL demineralized water. Prepare fresh before each use.

5.8 *Stannous chloride solution*, 33.6 g/100mL concentrated HCl: Dissolve 40g $SnCl_2 \cdot 2H_2O$ in 100 mL concentrated HCl. This solution is unstable. Prepare fresh daily.

5.9 *Sulfuric acid, 9M: Cautiously*, and with constant stirring and cooling, add 250 mL concentrated H_2SO_4 (sp gr 1.84) to 250 mL demineralized water.

6. Procedure

6.1 Clean all glassware used in this determination with dilute HCl (1+4) and rinse with demineralized water immediately before each use.

6.2 Follow instructions in paragraph 6.2.1 for water or water-suspended sediment and paragraph 6.2.2 for bottom material.

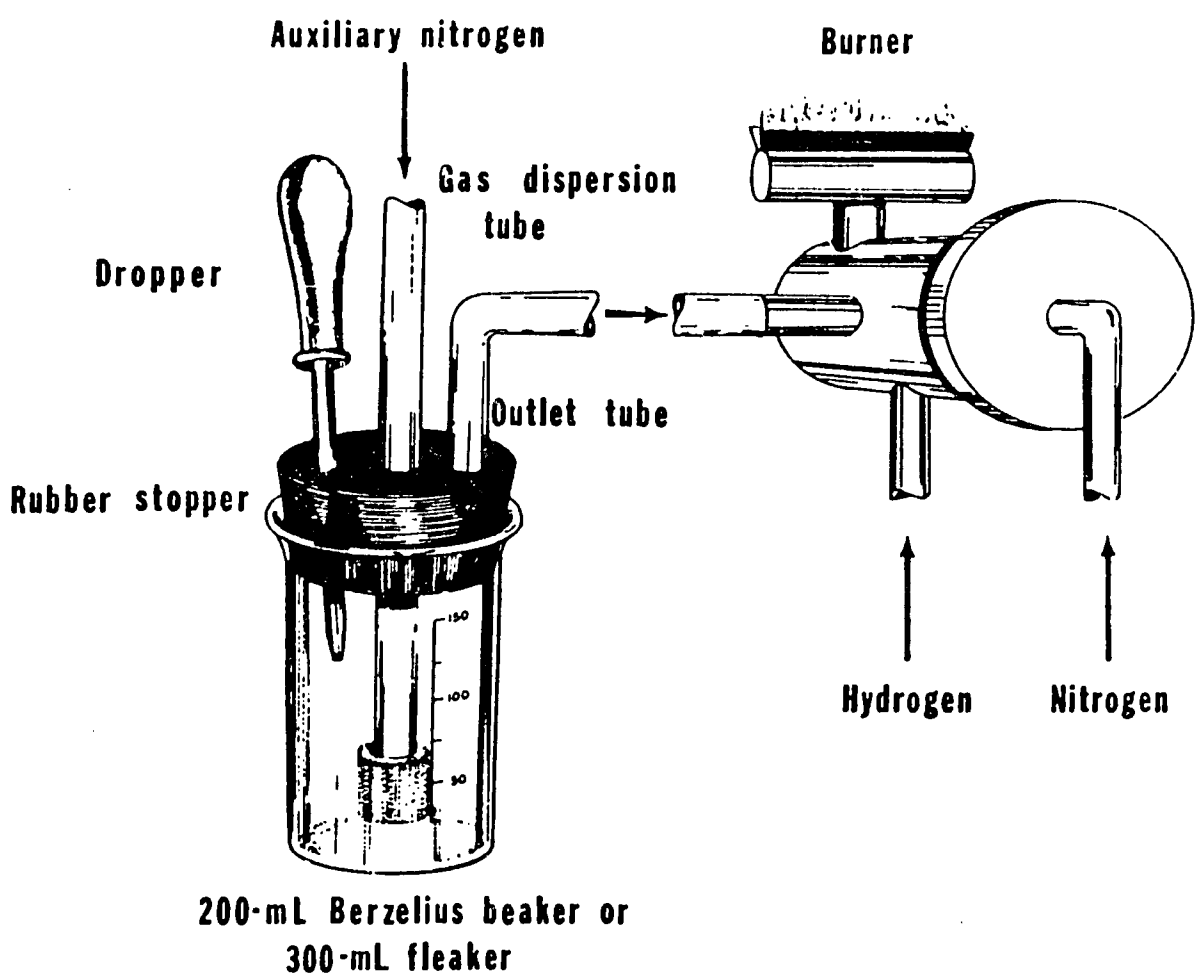


Figure 11.—Arsine vapor analyzer

179

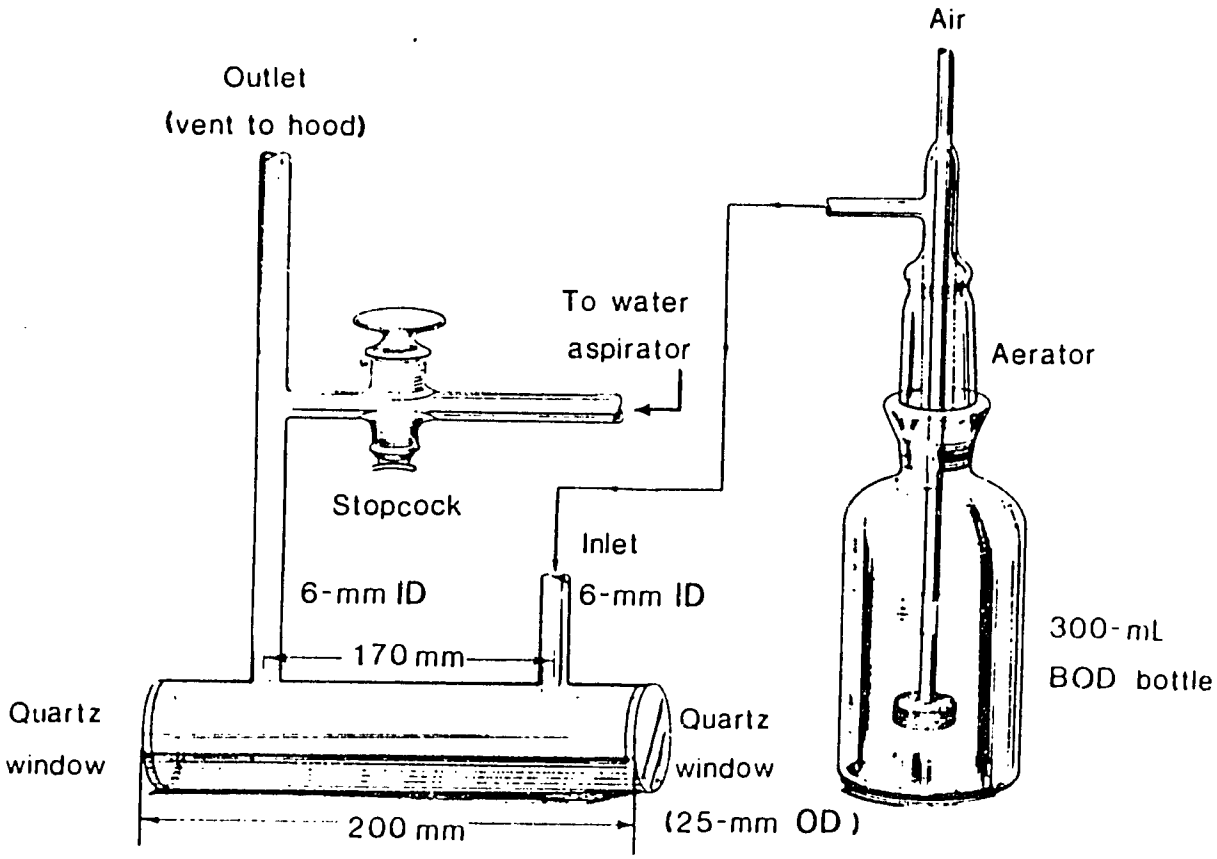


Figure 29.—Absorption cell and aerator

4.3 *Atomic absorption spectrometer and recorder* or a commerical mercury analyzer.

4.4 Refer to the manufacturer's manual to optimize instrument for the following:

Grating	Ultraviolet
Wavelength ..	253.7 nm
Source	Mercury-vapor discharge, hollow-cathode, or electrodeless-discharge lamp

4.5 *BOD bottle*, 300-mL capacity.

4.6 *Water bath* or controlled-temperature oven, 95 °C.

5. Reagents

5.1 *Hydroxylamine hydrochloride-sodium chloride solution*: Dissolve 10 g $NH_2OH{\cdot}HCl$ and 12 g NaCl in demineralized water and dilute to 100 mL (NOTE 2). Prepare fresh daily. Alternatively, 12 g hydroxylamine sulfate may be used instead of the hydroxylamine hydrochloride.

NOTE 2. A larger volume of this reagent can be prepared if it is kept refrigerated.

5.2 *Mercury standard solution I*, 1.00 mL = 100 μg Hg: Dissolve 0.1712 g $Hg(NO_3)_2{\cdot}H_2O$ in demineralized water. Add 1.5 mL concentrated HNO_3 and dilute to 1,000 mL with demineralized water.

5.3 *Mercury standard solution II*, 1.00 mL = 1.00 μg Hg: Dilute 5.00 mL mercury standard solution I and 1.5 mL concentrated HNO_3 to 500 mL with demineralized water. This and the following mercury standard solutions must be prepared fresh daily.

5.4 *Mercury standard solution III*, 1.00 mL = 0.050 μg Hg: Dilute 10.0 mL mercury standard solution II and 1.5 mL concentrated HNO_3 to 200 mL with demineralized water. Use this solution to prepare working standards at the time of analysis.

5.5 *Nitric acid*, concentrated (sp gr 1.41), with low mercury content: duPont, reagent-grade acid has been found satisfactory.

130

TABLE I
INSTRUMENTAL CONDITIONS FOR THE FURNACE

At.	λ (nm)	SBW (nm)	At.	λ (nm)	SBW (nm)
Ag	328.1	0.7	Na	589.6	0.7
Al	309.3	0.7	Nd	492.4	0.2
As	193.7	0.7	Ni	232.0	0.2
Au	242.8	0.7	Os	290.9	0.2
B	249.7	0.7	P	213.6	0.7
Ba	553.6	0.4	Pb	283.3	0.7
Be	234.9	0.7	Pd	247.6	0.7
Bi	223.1	2.	Pr	495.1	0.2
Ca	422.7	0.7	Pt	265.9	0.7
Cd	228.8	0.7	Rb	780.0	0.4
Co	240.7	0.2	Re	346.0	0.2
Cr	357.9	0.7	Rh	348.5	0.2
Cs	852.1	0.7	Ru	349.9	0.2
Cu	324.7	0.7	Sb	217.6	2.
Dy	421.2	0.2	Sc	391.2	0.2
Er	400.8	0.2	Se	196.0	2.
Eu	459.4	0.2	Si	251.6	0.2
Fe	248.3	0.2	Sm	429.7	0.2
Ga	287.4	0.7	Sn	224.6	0.7
Gd	407.9	0.2		286.4	0.7
Ge	265.1	0.2	Sr	460.7	1.4
Hg	253.7	0.7	Ta	432.6	0.2
Ho	410.4	0.2	Te	214.3	2.
In	303.9	0.7	Ti	365.3	0.2
Ir	264.0	0.2	Tl	276.8	0.7
K	766.5	0.4	Tm	371.8	0.2
La	550.0	0.2	U	351.5	0.2
Li	670.8	0.4	V	318.4	0.7
Lu	336.0	0.2	Y	410.2	0.2
Mg	285.2	0.7	Yb	398.8	0.2
Mn	279.5	2.	Zn	213.9	0.7
Mo	313.3	0.7			

TABLE II FURNACE CONDITIONS

At.	W/P[2]	Modifier	Char Temp. (°C)	Atom. Temp. (°C)	m_o[4]	Det. Limit (pg)	Foot Note	Ref.
Ag	P	200 μg PO_4	650	1600	1.4	0.5	5	780j
Al	P	50 μg $Mg(NO_3)_2$	1700	2400	10	4.		780j
As	P	20 μg Ni	1500	2500	17	20		780j
Au	P	50 μg Ni	1000	2200	13	10		780j
B	W	5 μg Ca	1000	2700	700	1000		780j
Ba	W		1200	2700	6.5	10		780j
Be	P	50 μg $Mg(NO_3)_2$	1500	2500	1.2	1		780j
Bi	P	20 μg Ni	900	1900	23	10		780j
Ca	W		1100	2600	0.8	—		780j
Cd	P	200 μg PO_4 - 10 μg $Mg(NO_3)_2$	900	1600	0.35	0.3	5	780j
Co	P	50 μg $Mg(NO_3)_2$	1400	2400	6	2		780j
Cr	P	50 μg $Mg(NO_3)_2$	1650	2500	3.5	1		780j
Cs	P		900	1900	5	5		780j
Cu	P		1200	2300	4	1	6	780j
Dy	W		1500	2700	38	—		305
Er	W		1700	2700	67	—		305
Eu	W		1300	2600	16	—		305
Fe	P	50 μg $Mg(NO_3)_2$	1400	2400	5	2		780j
Ga	P		800	2700	42	—		305
Gd	W		1600	2700	11,000	—		305
Ge	P	50 μg $Mg(NO_3)_2$	950	2500	30	15		101a
Hg	P	$K_2Cr_2O_7$	250	1000		—		413
In	P		800	1400	88	—		305
Ir	W		1000	2500	275	—		305
K	W		950	1500	0.8	—		305
La	W		1600	2700	27,000	—		305
Li	P		900	2600	1.4	2		780j
Mg	P		900	2400	0.3	0.5		780j
Mn	P	50 μg $Mg(NO_3)_2$	1400	2200	2.	1		780j
Mo	W		1800	2700	9	4		780j
Na	W		900	1500	1.0	—		305
Nd	W		1500	2700	1,800	—		305
Ni	P	50 μg $Mg(NO_3)_2$	1400	2400	13	10		780j

TABLE II FURNACE CONDITIONS

At.	W/P[2]	Modifier	Char Temp. (°C)	Atom. Temp. (°C)	m_o[4]	Det. Limit (pg)	Foot Note	Ref.
Os	W		200	2800	1,400	—		305
P	P	50 μg $La(NO_3)_2$	1400	2700	3,000	3000		780j
Pb	P	200 μg PO_4 + 10 μg $Mg(NO_3)_2$	600	1900	11	5	5	780j
Pd	P		1000	2300	24	25		780j
Pt	W		1300	2500	100	50		780j
Rb	P		800	1900	2.3	5		780j
Rh	W		1300	2400	12	—		305
Ru	W		1400	2500	32	—		305
Sb	P	20 μg Ni	1100	2400	38	15		780j
Se	P	20 μg Ni + 25 μg $Mg(NO_3)_2$	900	2000	30	30		780j
Si	P		1400	2700	40	40		780j
Sm	W		1400	2600	240	—		305
Sn	P	200 μg PO_4 + 50 μg $Mg(NO_3)_2$	1000	2100	23	20	3.5	780j
Sr	W		1300	2600	1.4	2		780j
Te	P	20 μg Ni	900	2000	15	10		780j
Ti	W		1400	2700	43	50		780j
Tl	P	1% H_2SO_4	600	1400	10	10		780j
Tm	W	—	1700	2700	13	—		305
V	W		1400	2650	30	20		780j
Y	W	—	1400	2700	13,000	—		305
Yb	W		1300	2500	2.5	—		305
Zn	P	6 μg $Mg(NO_3)_2$	600	1800	0.04	0.1		780j

NOTES

1. Pyrotically coated tubes are recommended for all determinations, as is gas stop. Max power heating is used.
2. W P. W is wall data. P is platform data. Integrated absorbance is used if the platform is used and usually for wall atomization also. The sample size is assumed to be 20 μL. The drying time is 60 s at 250°C with 1 s ramp. For platform char. the hold time is 45 s after 1 s ramp. The tube is cleaned out with a final 6 s firing at 2600°C using 1 s ramp.
3. Assuming use of the 286.4 nm line for Sn for the Zeeman/5000.
4. C is the characteristic mass in pg 0.0044 A·s.
5. PO_4 is either $(NH_4)_2HPO_4$ or $NH_4H_2PO_4$.
6. The characteristic mass, C, for Cu with the Zeeman 5000 is about 8.5 pg 0.0044 A·s.

83

ALUMINUM

Method 202.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 01105
Dissolved 01106
Suspended 01107

Optimum Concentration Range: 5–50 mg/l using a wavelength of 309.3 nm
Sensitivity: 1 mg/l
Detection Limit: 0.1 mg/l

Preparation of Standard Solution

1. Stock Solution: Carefully weigh 1.000 gram of aluminum metal (analytical reagent grade). Add 15 ml of conc. HCl and 5 ml conc. HNO_3 to the metal, cover the beaker and warm gently. When solution is complete, transfer quantitatively to a 1 liter volumetric flask and make up to volume with deionized distilled water. 1 ml = 1 mg Al (1000 mg/l).
2. Potassium Chloride Solution: Dissolve 95 g potassium chloride (KCl) in deionized distilled water and make up to 1 liter.
3. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. The calibration standards should be prepared using the same type of acid and at the same concentration as will result in the sample to be analyzed either directly or after processing. To each 100 ml of standard and sample alike add 2.0 ml potassium chloride solution.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for the preparation of the sample as given in part 4.1.1 through 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.

Instrumental Parameters (General)

1. Aluminum hollow cathode lamp
2. Wavelength: 309.3 nm
3. Fuel: Acetylene
4. Oxidant: Nitrous oxide

Approved for NPDES
Issued 1971
Editorial revision 1974 and 1978

184

5. Type of flame: Fuel rich

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Interferences

1. Aluminum is partially ionized in the nitrous oxide-acetylene flame. This problem may be controlled by the addition of an alkali metal (potassium, 1000 ug/ml) to both sample and standard solutions.

Notes

1. The following lines may also be used:
   308.2 nm Relative Sensitivity 1
   396.2 nm Relative Sensitivity 2
   394.4 nm Relative Sensitivity 2.5
2. Data to be entered into **STORET** must be reported as ug/1.
3. For concentrations of aluminum below 0.3 mg/1, the furnace procedure (Method 202.2) is recommended
4. The Eriochrome cyanine R colorimetric method may also be used. The optimum range for this method lies between 20 and 300 ug/1. (Standard Methods, 14th Edition, p. 171.) In the absence of fluorides and complex phosphates, a detection limit of 6 ug/1 is possible.

Precision and Accuracy

1. An interlaboratory study on trace metal analyses by atomic absorption was conducted by the Quality Assurance and Laboratory Evaluation Branch of EMSL. Six synthetic concentrates containing varying levels of aluminum, cadmium, chromium, copper, iron, manganese, lead and zinc were added to natural water samples. The statistical results for aluminum were as follows:

Number of Labs	True values ug/liter	Mean Value ug/liter	Deviation ug/liter	Accuracy as %Bias
38	1205	1281	299	6.3
38	1004	1003	391	-0.1
37	500	463	202	-7.4
37	625	582	272	-6.8
22	35	96	108	175
21	15	109	168	626

185

ALUMINUM

Method 202.2 (Atomic Absorption, furnace technique)

STORET NO. Total 01105
Dissolved 01106
Suspended 01107

Optimum Concentration Range: 20–200 ug/l
Detection Limit: 3 ug/l

Preparation of Standard Solution

1. Stock solution: Prepare as described under "direct aspiration method".
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. These solutions are also to be used for "standard additions".
3. The calibration standard should be diluted to contain 0.5% (v/v) HNO_3.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. Prepare as described under "direct aspiration method". Sample solutions for analysis should contain 0.5% (v/v) HNO_3.

Instrument Parameters (General)

1. Drying Time and Temp: 30 sec–125°C.
2. Ashing Time and Temp: 30 sec–1300°C.
3. Atomizing Time and Temp: 10 sec–2700°C.
4. Purge Gas Atmmosphere: Argon
5. Wavelength: 309.3 nm
6. Other operating parameters should be set as specified by the particular instrument manufacturer.

Analysis Procedure

1. For the analysis procedure and the caculation, see "Furnace Procedure" part 9.3 of the Atomic Absorption Methods section of this manual.

Approved for NPDES
Issued 1978

87

Notes

1. The above concentration values and instrument conditions are for a Perkin-Elmer HGA-2100, based on the use of a 20 ul injection, continuous flow purge gas and non-pyrolytic graphite.
2. Background correction may be required if the sample contains high dissolved solids.
3. It has been reported that chloride ion and that nitrogen used as a purge gas suppress the aluminum signal. Therefore the use of halide acids and nitrogen as a purge gas should be avoided.
4. For every sample matrix analyzed, verification is necessary to determine that method of standard addition is not required (see part 5.2.1 of the Atomic Absorption Methods section of this manual).
5. If method of standard addition is required, follow the procedure given earlier in part 8.5 of the Atomic Absorption Methods section of this manual.
6. Data to be entered into STORET must be reported as ug/l.

Precision and Accuracy

1. Precision and accuracy data are not available at this time.

ANTIMONY

Method 204.1 (Atomic absorption, direct aspiration)

STORET NO. Total 01097
Dissolved 01095
Suspended 01096

Optimum Concentration Range: 1–40 mg/l using a wavelength of 217.6 nm
Sensitivity: 0.5 mg/l
Detection Limit: 0.2 mg/l

Preparation of Standard Solution

1. Stock Solution: Carefully weigh 2.7426 g of antimony potassium tartrate $K(SbO)C_4H_4O_6 \cdot \frac{1}{2}H_2O$ (analytical reagent grade) and dissolve in deionized distilled water. Dilute to 1 liter with deionized distilled water. 1 ml = 1 mg Sb (1000 mg/l).
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. The calibration standards should be prepared using the same type of acid and at the same concentration as will result in the sample to be analyzed either directly or after processing.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for preparation of the sample as given in parts 4.1.1 through 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.

Instrumental Parameters (General)

1. Antimony hollow cathode lamp
2. Wavelength: 217.6 nm
3. Fuel: Acetylene
4. Oxidant: Air
5. Type of flame: Fuel lean

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Approved for NPDES
Issued 1974
Editorial revision 1978

87 188

Interferences

1. In the presence of lead (1000 mg/l), a spectral interference may occur at the 217.6 nm resonance line. In this case the 231.1 nm antimony line should be used.
2. Increasing acid concentrations decrease antimony absorption. To avoid this effect, the acid concentration in the samples and in the standards should be matched.

Notes

1. Data to be entered into STORET must be reported as ug/l.
2. For concentrations of antimony below 0.35 mg/l, the furnace procedure (Method 204.2) is recommended.

Precision and Accuracy

1. In a single laboratory (EMSL), using a mixed industrial-domestic waste effluent at concentrations of 5.0 and 15 mg Sb/l, the standard deviations were ±0.08 and ±0.1, respectively. Recoveries at these levels were 96% and 97%, respectively.

ANTIMONY

Method 204.2 (Atomic Absorption, furnace technique)

STORET NO. Total 01097
Dissolved 01095
Suspended 01096

Optimum Concentration Range: 20–300 ug/l
Detection Limit: 3 ug/l

Preparation of Standard Solution

1. Stock solution: Prepare as described under "direct aspiration method".
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. These solutions are also to be used for "standard additions".
3. The calibration standard should be diluted to contain 0.2% (v/v) HNO_3.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for preparation of the sample as given in parts 4.1.1 thru 4.1.3 of the Atomic Absorption Methods section of this manual should be followed including the addition of sufficient 1:1 HCl to dissolve the digested residue for the analysis of suspended or total antimony. The sample solutions used for analysis should contain 2% (v/v) HNO_3.

Instrument Parameters (General)

1. Drying Time and Temp: 30 sec–125°C
2. Ashing Time and Temp: 30 sec–800°C.
3. Atomizing Time and Temp: 10 sec–2700°C.
4. Purge Gas Atmosphere: Argon
5. Wavelength: 217.6 nm
6. Other operating parameters should be set as specified by the particular instrument manufacturer.

Analysis Procedure

1. For the analysis procedure and the calculation, see "Furnace Procedure" part 9.3 of the Atomic Absorption Methods section of this manual.

Approved for NPDES
Dated 1978

190

Notes

1. The above concentration values and instrument conditions are for a Perkin-Elmer HGA-2100, based on the use of a 20 ul injection, continuous flow purge gas and non-pyrolytic graphite. Smaller size furnace devices or those employing faster rates of atomization can be operated using lower atomization temperatures for shorter time periods than the above recommended settings.
2. The use of background correction is recommended.
3. Nitrogen may also be used as the purge gas.
4. If chloride concentration presents a matrix problem or causes a loss previous to atomization, add an excess of 5 mg of ammonium nitrate to the furnace and ash using a ramp accessory or with incremental steps until the recommended ashing temperature is reached.
5. For every sample matrix analyzed, verification is necessary to determine that method of standard addition is not required (see part 5.2.1 of the Atomic Absorption Methods section of this manual).
6. If method of standard addition is required, follow the procedure given earlier in part 8.5 of the Atomic Absorption Methods section of this manual.
7. Data to be entered into STORET must be reported as ug/l.

Precision and Accuracy

1. Precision and accuracy data are not available at this time.

ARSENIC

Method 206.2 (Atomic Absorption, furnace technique)

STORET NO. Total 01002
Dissolved 01000
Suspended 01001

Optimum Concentration Range: 5–100 *ug/l*
Detection Limit: 1 *ug/l*

Preparation of Standard Solution

1. Stock solution: Dissolve 1.320 g of arsenic trioxide, As_2O_3 (analytical reagent grade) in 100 ml of deionized distilled water containing 4 g NaOH. Acidify the solution with 20 ml conc. HNO_3 and dilute to 1 liter. 1 ml = 1 mg As (1000 mg/l).

2. Nickel Nitrate Solution, 5%: Dissolve 24.780 g of ACS reagent grade $Ni(NO_3)_2 \cdot 6H_2O$ in deionized distilled water and make up to 100ml.

3. Nickel Nitrate Solution, 1%: Dilute 20 ml of the 5% nickel nitrate to 100 ml with deionized distilled water.

4. Working Arsenic Solution: Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. Withdraw appropriate aliquots of the stock solution, add 1 ml of conc. HNO_3, 2ml of 30% H_2O_2 and 2ml of the 5% nickel nitrate solution. Dilute to 100 ml with deionized distilled water.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. Transfer 100 ml of well-mixed sample to a 250 ml Griffin beaker, add 2 ml of 30% H_2O_2 and sufficient conc. HNO_3 to result in an acid concentration of 1%(v/v). Heat for 1 hour at 95°C or until the volume is slightly less than 50 ml.

2. Cool and bring back to 50 ml with deionized distilled water.

3. Pipet 5 ml of this digested solution into a 10-ml volumetric flask, add 1 ml of the 1% nickel nitrate solution and dilute to 10 ml with deionized distilled water. The sample is now ready for injection into the furnace.

Approved for NPDES and SDWA
Issued 1978

NOTE: If solubilization or digestion is not required, adjust the HNO_3 concentration of the sample to 1% (v/v) and add 2 ml of 30% H_2O_2 and 2 ml of 5% nickel nitrate to each 100 ml of sample. The volume of the calibration standard should be adjusted with deionized distilled water to match the volume change of the sample.

Instrument Parameters (General)

1. Drying Time and Temp: 30 sec-125°C.
2. Ashing Time and Temp: 30 sec-1100°C.
3. Atomizing Time and Temp: 10 sec-2700°C.
4. Purge Gas Atmosphere: Argon
5. Wavelength: 193.7 nm
6. Other operating parameters should be set as specified by the particular instrument manufacturer.

Analysis Procedure

1. For the analysis procedure and the calculation, see "Furnace Procedure" part 9.3 of the Atomic Absorption Methods section of this manual.

Notes

1. The above concentration values and instrument conditions are for a Perkin-Elmer HGA-2100, based on the use of a 20 ul injection, purge gas interrupt and non-pyrolytic graphite. Smaller size furnace devices or those employing faster rates of atomization can be operated using lower atomization temperatures for shorter time periods than the above recommended settings.
2. The use of background correction is recommended.
3. For every sample matrix analyzed, verification is necessary to determine that method of standard addition is not required (see part 5.2.1 of the Atomic Absorption Methods section of this manual).
4. If method of standard addition is required, follow the procedure given earlier in part 8.5 of the Atomic Absorption Methods section of this manual.
5. For quality control requirements and optional recommendations for use in drinking water analyses, see part 10 of the Atomic Absorption Methods section of this manual.
6. Data to be entered into STORET must be reported as *ug*/l.

Precision and Accuracy

1. In a single laboratory (EMSL), using a mixed industrial-domestic waste effluent containing 15 *ug*/l and spiked with concentrations of 2, 10 and 25 *ug*/l, recoveries of 85%, 90% and 88% were obtained respectively. The relative standard deviation at these concentrations levels were ±8.8%, ±6.2%, ±5.4% and ±8.7%, respectively.
2. In a single laboratory (EMSL), using Cincinnati, Ohio tap water spiked at concentrations of 20, 50 and 100 *ug* As/l, the standard deviations were ±0.7, ±1.1 and ±1.6 respectively. Recoveries at these levels were 105%, 106% and 101%, respectively.

ARSENIC

Method 206.3 (Atomic Absorption—gaseous hydride)

STORET NO. Total 01002
Dissolved 01000
Suspended 01001

1. Scope and Application
   1.1 The gaseous hydride method determines inorganic arsenic when present in concentrations at or above 2 ug/l. The method is applicable to drinking water and most fresh and saline waters in the absence of high concentrations of chromium, cobalt, copper, mercury, molybdenum, nickel and silver.
2. Summary of Method
   2.1 Arsenic in the sample is first reduced to the trivalent form using $SnCl_2$ and converted to arsine, AsH_3, using zinc metal. The gaseous hydride is swept into an argon-hydrogen flame of an atomic absorption spectrophotometer. The working range of the method is 2–20 ug/l. The 193.7 nm wavelength line is used.
3. Comments
   3.1 In analyzing drinking water and most surface and ground waters, interferences are rarely encountered. Industrial waste samples should be spiked with a known amount of arsenic to establish adequate recovery.
   3.2 Organic forms of arsenic must be converted to inorganic compounds and organic matter must be oxidized before beginning the analysis. The oxidation procedure given in Method 206.5 (Standard Methods, 14th Edition, Method 404B, p. 285, Procedure 4.a) has been found suitable.
   3.3 For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.
   3.4 For quality control requirements and optional recommendations for use in drinking water analyses, see part 10 of the Atomic Absorption Methods section of this manual.
   3.5 Data to be entered into STORET must be reported as ug/l.
4. Precision and Accuracy
   4.1 Ten replicate solutions of o-arsenilic acid at the 5, 10 and 20 ug/l level were analyzed by a single laboratory. Standard deviations were ±0.3, ±0 9 and ±1.1 with recoveries of 94, 93 and 85%, respectively. (Caldwell, J. S., Lishka, R. J., and McFarren, E. F., "Evaluation of a Low Cost Arsenic and Selenium Determination at Microgram per Liter Levels", JAWWA., vol 65, p 731, Nov., 1973.)

Approved for NPDES and SDWA
Issued 1974

95

190

5. References

5.1 Except for the perchloric acid step, the procedure to be used for this determination is found in: Standard Methods for the Examination of Water and Wastewater, 14th Edition, p159, Method 301A(VII),(1975)

BARIUM

Method 208.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 01007
Dissolved 01005
Suspended 01006

Optimum Concentration Range: 1–20 mg/l using a wavelength of 553.6 nm
Sensitivity: 0.4 mg/l
Detection Limit: 0.1 mg/l

Preparation of Standard Solution

1. Stock Solution: Dissolve 1.7787 g barium chloride ($BaCl_2 \cdot 2H_2O$, analytical reagent grade) in deionized distilled water and dilute to 1 liter. 1 ml = 1 mg Ba (1000 mg/l).
2. Potassium chloride solution: Dissolve 95 g potassium chloride, KCl, in deionized distilled water and make up to 1 liter.
3. Prepare dilutions of the stock barium solution to be used as calibration standards at the time of analysis. To each 100 ml of standard and sample alike add 2.0 ml potassium chloride solution. The calibration standards should be prepared using the same type of acid and the same concentration as will result in the sample to be analyzed either directly or after processing.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for preparation of the sample as given in parts 4.1.1 through 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.

Instrumental Parameters (General)

1. Barium hollow cathode lamp
2. Wavelength: 553.6 nm
3. Fuel: Acetylene
4. Oxidant: Nitrous oxide
5. Type of flame: Fuel rich

Approved for NPDES and SDWA
Issued 1974

196

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Interferences

1. The use of a nitrous oxide-acetylene flame virtually eliminates chemical interference; however, barium is easily ionized in this flame and potassium must be added (1000 mg/l) to standards and samples alike to control this effect.
2. If the nitrous oxide flame is not available and acetylene-air is used, phosphate, silicon and aluminum will severely depress the barium absorbance. This may be overcome by the addition of 2000 mg/l lanthanum.

Notes

1. Data to be entered into **STORET** must be reported as *ug*/1.
2. For concentrations of barium below 0.2 mg/l, the furnace procedure (Method 208.2) is recommended.
3. For quality control requirements and optional recommendations for use in drinking water analyses, see part 10 of the Atomic Absorption Methods section of this manual.

Precision and Accuracy

1. In a single laboratory (EMSL), using a mixed industrial-domestic waste effluent at concentrations of 0.40 and 2.0 mg Ba/l, the standard deviations were ±0.043 and ±0.13, respectively. Recovereis at these levels were 94% and 113%, respectively.
2. In a round-robin study reported by Standard Methods (13th Edition, p215, method 129A, 1971), three synthetic samples containing barium were analyzed by 13 laboratories. At concentrations of 500, 1000 and 5000 *ug* Ba/l, the reported standard deviations were ±50, ±89 and ±185 *ug*, respectively. The relative error at these concentrations was 8.6%, 2.7% and 1.4%, respectively.

Method 208.2 (Atomic Absorption, furnace technique)

STORET NO. 01007
Dissolved 01005
Suspended 01006

Optimum Concentration Range: 10–200 ug/l
Detection Limit: 2 ug/l

Preparation of Standard Solution

1. Stock solution: Prepare as described under "direct aspiration method".
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. These solutions are also to used for "standard additions".
3. The calibration standard should be diluted to contain 0.5% (v/v) HNO_3.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. Prepare as described under "direct aspiration method". Sample solutions for analysis should contain 0.5% (v/v) HNO_3.

Instrument Parameters (General)

1. Drying Time and Temp: 30 sec–125°C.
2. Ashing Time and Temp: 30 sec–1200°C.
3. Atomizing Time and Temp: 10 sec–2800°C.
4. Purge Gas Atmosphere: Argon
5. Wavelength: 553.6 nm
6. Other operating parameters should be set as specified by the particular instrument manufacturer.

Analysis Procedure

1. For the analysis procedure and the calculation, see "Furnace Procedure" part 9.3 of the Atomic Absorption Methods section of this manual.

Notes

1. The above concentration values and instrument conditions are for a Perkin-Elmer HGA-2100, based on the use of a 20 ul injection, continuous flow purge gas and pyrolytic graphite.

Approved for NPDES and SDWA
Issued 1978

1-3 198

2. The use of halide acid should be avoided.
3. Because of possible chemical interaction, nitrogen should not be used as a purge gas.
4. For every sample matrix analyzed, verification is necessary to determine that method of standard addition is not required (see part 5.2.1 of the Atomic Absorption Methods section of this manual).
5. If method of standard addition is required, follow the procedure given earlier in part 8.5 of the Atomic Absorption Methods section of this manual.
6. For quality control requirements and optional recommendations for use in drinking water analyses, see part 10 of the Atomic Absorption Methods section of this manual.
7. Data to be entered into STORET must be reported as ug/l.

Precision and Accuracy

1. In a single laboratory (EMSL), using Cincinnati, Ohio tap water spiked at concentrations of 500 and 1000 ug Ba/l, the standard deviations were ±2.5 and ±2.2 ug, respectively. Recoveries at these levels were 96% and 102%, respectively A dilution of 1:10 was required to bring the spikes within the analytical range of the method.

Method 213.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 01027
Dissolved 01025
Suspended 01026

Optimum Concentration Range: 0.05–2 mg/l using a wavelength of 228.8 nm
Sensitivity: 0.025 mg/l
Detection Limit: 0.005 mg/l

Preparation of Standard Solution

1. Stock Solution: Carefully weigh 2.282 g of cadmium sulfate ($3CdSO_4 \cdot 8H_2O$, analytical reagent grade) and dissolve in deionized distilled water make up to 1 liter with deionized distilled water. 1 ml = 1 mg Cd (1000 mg/l)
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. The calibration standards should be prepared using the same type of acid and at the same concentration as will result in the sample to be analyzed either directly or after processing.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for preparation of the sample as given in parts 4.1.1 through 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.

Instrumental Parameters (General)

1. Cadmium hollow cathode lamp
2. Wavelength: 228.8 nm
3. Fuel: Acetylene
4. Oxidant: Air
5. Type of flame: Oxidizing

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Approved for NPDES and SDWA
Issued 1971
Editorial revision 1974

111

200

Notes

1. For levels of cadmium below 20 ug/l, either the Special Extraction Procedure given in Part 9.2 of the Atomic Absorption methods section as the furnace technique, Method 213.2 is recommended.
2. Data to be entered into STORET must be reported as ug/l.
3. For quality control requirements and optional recommendations for use in drinking water analyses, see part 10 of the Atomic Absorption Methods section of this manual.

Precision and Accuracy

1. An interlaboratory study on trace metal analyses by atomic absorption was conducted by the Quality Assurance and Laboratory Evaluation Branch of EMSL. Six synthetic concentrates containing varying levels of aluminum, cadmium, chromium, copper, iron, manganese, lead and zinc were added to natural water samples. The statistical results for cadmium were as follows:

Number of Labs	True Values ug/liter	Mean Value ug/liter	Standard Deviation ug/liter	Accuracy as % Bias
74	71	70	21	-2.2
73	78	74	18	-5.7
63	14	16 8	11.0	19 8
68	18	18 3	10.3	1.9
55	1 4	3.3	5.0	135
51	2.8	2.9	2.8	4.7

201

CADMIUM

Method 213.2 (Atomic Absorption, furnace technique)

STORET NO. 01027
Dissolved 01025
Suspended 01026

Optimum Concentration Range: 0.5–10 ug/l
Detection Limit: 0.1 ug/l

Preparation of Standard Solution

1. Stock solution: Prepare as described under "direct aspiration method".
2. Ammonium Phosphate solution (40%): Dissolve 40 grams of ammonium phosphate, $(NH_4)_2HPO_4$ (analytical reagent grade) in deionized distilled water and dilute to 100 ml.
3. Prepare dilutions of the stock cadmium solution to be used as calibration standards at the time of analysis. To each 100 ml of standard and sample alike add 2.0 ml of the ammonium phosphate solution. The calibration standards should be prepared to contain 0.5% (v/v) HNO_3.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. Prepare as described under "direct aspiration method". Sample solutions for analysis should contain 0.5% (v/v) HNO_3.

Instrument Parameters (General)

1. Drying Time and Temp: 30 sec–125°C.
2. Ashing Time and Temp: 30 sec–500°C.
3. Atomizing Time and Temp: 10 sec–1900°C.
4. Purge Gas Atmosphere: Argon
5. Wavelength: 228.8 nm
6. Other operating parameters should be set as specified by the particular instrument manufacturer.

Analysis Procedure

1. For the analysis procedure and the calculation, see "Furnace Procedure" part 9.3 of the Atomic Absorption Methods section of this manual.

Approved for NPDES and SDWA
Issued 1978

13

202

Notes

1. The above concentration values and instrument conditions are for a Perkin-Elmer HGA-2100, based on the use of a 20 ul injection, continuous flow purge gas and non-pyrolytic graphite. Smaller size furnace devices or those employing faster rates of atomization can be operated using lower atomization temperatures for shorter time periods than the above recommended settings.
2. The use of background correction is recommended.
3. Contamination from the work area is critical in cadmium analysis. Use of pipet tips which are free of cadmium is of particular importance. (See part 5.2.9 of the Atomic Absorption Methods section of this manual.)
4. For every sample matrix analyzed, verification is necessary to determine that method of standard addition is not required (see part 5.2.1 of the Atomic Absorption Methods section of this manual).
5. If method of standard addition is required, follow the procedure given earlier in part 8.5 of the Atomic Absorption Methods section of this manual.
6. For quality control requirements and optional recommendations for use in drinking water analyses, see part 10 of the Atomic Absorption Methods section of this manual.
7. Data to be entered into STORET must be reported as ug/1.

Precision and Accuracy

1. In a single laboratory (EMSL), using Cincinnati, Ohio tap water spiked at concentrations of 2.5, 5.0 and 10.0 ug Cd/1, the standard deviations were ±0.10, ±0.16 and ±0.33, respectively. Recoveries at these levels were 96%, 99% and 98%, respectively.

Method 215.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 00916
Dissolved 00915

Optimum Concentration Range: 0.2–7 mg/l using a wavelength of 422.7 nm
Sensitivity: 0.08 mg/l
Detection Limit: 0.01 mg/l

Preparation of Standard Solution

1. Stock Solution: Suspend 1.250 g of $CaCO_3$ (analytical reagent grade), dried at 180°C for 1 hour before weighing, in deionized distilled water and dissolve cautiously with a minimum of dilute HCl. Dilute to 1000 ml with deionized distilled water. 1 ml = 0.5 mg Ca (500 mg/l).
2. Lanthanum chloride solution: Dissolve 29 g of La_2O_3, slowly and in small portions, in 250 ml conc. HCl (Caution: Reaction is violent) and dilute to 500 ml with deionized distilled water.
3. Prepare dilutions of the stock calcium solutions to be used as calibration standards at the time of analysis. To each 10 ml volume of calibration standard and sample alike add 1.0 ml of the lanthanum chloride solution, i.e., 20 ml of standard or sample + 2 ml $LaCl_3$ = 22 ml.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. For the analysis of total calcium in domestic and industrial effluents, the procedures for the determination of total metals as given in parts 4.1.3 and 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.
2. For ambient waters, a representative aliquot of a well-mixed sample may be used directly for analysis. If suspended solids are present in sufficient amounts to clog the nebulizer, the sample may be allowed to settle and the supernatant liquid analyzed directly.

Instrumental Parameters (General)

1. Calcium hollow cathode lamp
2. Wavelength: 422.7 nm

Approved for NPDES
Issued 1971
Editorial revision 1974

,15

204

3. Fuel: Acetylene
4. Oxidant: Air
5. Type of flame: Reducing

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Notes

1. Phosphate, sulfate and aluminum interfere but are masked by the addition of lanthanum. Since low calcium values result if the pH of the sample is above 7, both standards and samples are prepared in dilute hydrochloric acid solution. Concentrations of magnesium greater than 1000 mg/l also cause low calcium values. Concentrations of up to 500 mg/l each of sodium, potassium and nitrate cause no interference.
2. Anionic chemical interferences can be expected if lanthanum is not used in samples and standards.
3. The nitrous oxide-acetylene flame will provide two to five times greater sensitivity and freedom from chemical interferences. Ionization interferences should be controlled by adding a large amount of alkali to the sample and standards. The analysis appears to be free from chemical suppressions in the nitrous oxide-acetylene flame. (Atomic Absorption Newsletter 14, 29 [1975]).
4. The 239.9 nm line may also be used. This line has a relative sensitivity of 120.
5. Data to be entered into **STORET** must be reported as mg/l.
6. The EDTA titrimetric method may also be used (Standard Methods, 14th Edition, p 189).

Precision and Accuracy

1. In a single laboratory (EMSL), using distilled water spiked at concentrations of 9.0 and 36 mgCa/l, the standard deviations were ±0.3 and ±0.6, respectively. Recoveries at both these levels were 99%.

CHROMIUM

Method 218.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 01034
Dissolved 01030
Suspended 01031

Optimum Concentration Range: 0.5–10 mg/l using a wavelength of 357.9 nm
Sensitivity: 0.25 mg/l
Detection Limit: 0.05 mg/l

Preparation of Standard Solution

1. Stock Solution: Dissolve 1.923 g of chromium trioxide (CrO_3, reagent grade) in deionized distilled water. When solution is complete, acidify with redistilled HNO_3 and dilute to 1 liter with deionized distilled water. 1 ml = 1 mg Cr (1000 mg/l).
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. The calibration standards should be prepared using the same type of acid and at the same concentration as will result in the sample to be analyzed either directly or after processing.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for preparation of the sample as given in parts 4.1.1 thru 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.

Instrumental Parameters (General)

1. Chromium hollow cathode lamp
2. Wavelength: 357.9 nm
3. Fuel: Acetylene
4. Oxidant: Nitrous oxide
5. Type of flame: Fuel rich

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Approved for NPDES and SDWA
Issued 1971
Editorial revision 1974 and 1978

206

Notes

1. The following wavelengths may also be used:
   359.3 nm Relative Sensitivity 1.4
   425.4 nm Relative Sensitivity 2
   427.5 nm Relative Sensitivity 3
   428.9 nm Relative Sensitivity 4

2. The fuel rich air–acetylene flame provides greater sensitivity but is subject to chemical and matrix interference from iron, nickel, and other metals. If the analysis is performed in a lean flame the interference can be lessened but the sensitivity will also be reduced.
3. The suppression of both Cr (III) and Cr (VI) absorption by most interfering ions in fuel rich air-acetylene flames is reportedly controlled by the addition of 1% ammonium bifluoride in 0.2% sodium sulfate [Talanta 20, 631 (1973)]. A 1% oxine solution is also reported to be useful.
4. For levels of chromium between 50 and 200 ug/l where the air-acetylene flame can not be used or for levels below 50 ug/l, either the furnace procedure or the extraction procedure is recommended. See Method 218.2 for the furnace procedure and Method 218.3 for the chelation–extraction procedure.
5. For quality control requirements and optional recommendations for use in drinking water analyses, see part 10 of the Atomic Absorption Methods section of this manual.
6. Data to be entered into **STORET** must be reported as ug/l.

Precision and Accuracy

1. An interlaboratory study on trace metal analyses by atomic absorption was conducted by the Quality Assurance and Laboratory Evaluation Branch of EMSL. Six synthetic concentrates containing varying levels of aluminum, cadmium, chromium, copper, iron, manganese, lead and zinc were added to natural water samples. The statistical results for chromium were as follows:

Number of Labs	True Values ug/liter	Mean Value ug/liter	Standard Deviation ug/liter	Accuracy as % Bias
74	370	353	105	-4.5
76	407	380	128	-6.5
72	74	72	29	-3.1
70	93	84	35	-10.2
47	7.4	10.2	7.8	37.7
47	15.0	16.0	9.0	6.8

CHROMIUM

Method 218.2 (Atomic Absorption, furnace technique)

STORET NO. 01034
Dissolved 01030
Suspended 01031

Optimum Concentration Range: 5–100 ug/l
Detection Limit: 1 ug/l

Preparation of Standard Solution

1. Stock solution: Prepare as described under "direct aspiration method".
2. Calcium Nitrate Solution: Dissolve 11.8 grams of calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$ (analytical reagent grade) in deionized distilled water and dilute to 100 ml. 1 ml = 20 mg Ca.
3. Prepare dilutions of the stock chromium solution to be used as calibration standards at the time of analysis. The calibration standards should be prepared to contain 0.5% (v/v) HNO_3. To each 100 ml of standard and sample alike, add 1 ml of 30% H_2O_2 and 1 ml of the calcium nitrate solution.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. Prepare as described under "direct aspiration method". Sample solutions for analysis should contain 0.5% v/v HNO_3.

Instrument Parameters (General)

1. Drying Time and Temp: 30 sec–125°C.
2. Ashing Time and Temp: 30 sec–1000°C.
3. Atomizing Time and Temp: 10 sec–2700°C.
4. Purge Gas Atmosphere: Argon
5. Wavelength: 357.9 nm
6. Other operating parameters should be set as specified by the particular instrument manufacturer.

Analysis Procedure

1. For the analysis procedure and the calculation, see "Furnace Procedure" part 9.3 of the Atomic Absorption Methods section of this manual.

Approved for NPDES and SDWA
Issued 1978

205

Notes

1. The above concentration values and instrument conditions are for a Perkin-Elmer HGA-2100, based on the use of a 20 ul injecton, continuous flow purge gas and non-pyrolytic graphite.
2. Hydrogen peroxide is added to the acidified solution to convert all chromium to the trivalent state. Calcium is added to a level above 200 mg/l where its suppressive effect becomes constant up to 1000 mg/l.
3. Background correction may be required if the sample contains high dissolved solids.
4. Nitrogen should not be used as a purge gas because of possible CN band interference.
5. Pipet tips have been reported to be a possible source of contamination. (See part 5.2.9 of the Atomic Absorption Methods section of this manual.)
6. For every sample matrix analyzed, verification is necessary to determine that method of standard addition is not required (see part 5.2.1 of the Atomic Absorption Methods section of this manual).
7. If method of standard addition is required, follow the procedure given earlier in part 8.5 of the Atomic Absorption Methods section of this manual.
8. For quality control requirements and optional recommendations for use in drinking water analyses, see part 10 of the Atomic Absorption Methods section of this manual.
9. Data to be entered into STORET must be reported as ug/l.

Precision and Accuracy

1. In a single laboratory (EMSL), using Cincinnati, Ohio tap water spiked at concentrations of 19, 48, and 77 ug Cr/l, the standard deviations were ±0.1, ±0.2, and ±0.8, respectively. Recoveries at these levels were 97%, 101%, and 102%, respectively.

COBALT

Method 219.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 01037
Dissolved 01035
Suspended 01036

Optimum Concentration Range: 0.5–5 mg/l using a wavelength of 240.7 nm
Sensitivity: 0.2 mg/l
Detection Limit: 0.05 mg/l

Preparation of Standard Solution

1. Stock Solution: Dissolve 4.307 g of cobaltous chloride, $CoCl_2 \cdot 6H_2O$ (analytical reagent grade), in deionized distilled water. Add 10 ml of concentrated nitric acid and dilute to 1 liter with deionized distilled water. 1 ml = 1 mg Co (1000 mg/l).
2. Prepare dilutions of the stock cobalt solution to be used as calibration standards at the time of analysis. The calibration standards should be prepared using the same type of acid and at the same concentration as will result in the sample to be analyzed either directly or after processing.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for preparation of the sample as given in parts 4.1.1 thru 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.

Instrumental Parameters (General)

1. Cobalt hollow cathode lamp
2. Wavelength: 240.7 nm
3. Fuel: Acetylene
4. Oxidant: Air
5. Type of Flame: Oxidizing

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Approved for NPDES
Issued 1974
Editorial revision 1978

210

Notes

1. For levels of cobalt below 100 *ug*/1, either the Special Extraction Procedure, given in part 9.2 of the Atomic Absorption Methods section or the furnace technique, Method 219.2 is recommended.
2. Data to be entered into **STORET** must be reported as *ug*/1.

Precision and Accuracy

1. In a single laboratory (EMSL), using a mixed industrial-domestic waste effluent at concentrations of 0.20, 1.0 and 5.0mg Co/1, the standard deviations were ±0.013, ±0.01 and ±0.05, respectively. Recoveries at these levels were 98%, 98%, and 97%, respectively.

COBALT

Method 219.2 (Atomic Absorption, furnace technique)

STORET NO. Total 01037
Dissolved 01035
Suspended 01036

Optimum Concentration Range: 5–100 ug/l
Detection Limit: 1 ug/l

Preparation of Standard Solution

1. Stock solution: Prepare as described under "direct aspiration method".
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. These solutions are also to be used for "standard additions".
3. The calibration standard should be diluted to contain 0.5% (v/v) HNO_3.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. Prepare as described under "direct aspiration method". Sample solutions for analysis should contain 0.5% (v/v) HNO_3.

Instrument Parameters (General)

1. Drying Time and Temp: 30 sec–125°C.
2. Ashing Time and Temp: 30 sec–900°C.
3. Atomizing Time and Temp: 10 sec–2700°C.
4. Purge Gas Atmosphere: Argon
5. Wavelength: 240.7 nm
6. Other operating parameters should be set as specified by the particular instrument manufacturer.

Analysis Procedure

1. For the analysis procedure and the calculation, see "Furnace Procedure" part 9.3 of the Atomic Absorption Methods section of this manual.

Notes

1. The above concentration values and instrument conditions are for a Perkin-Elmer HGA-2100, based on the use of a 20 ul injection, continuous flow purge gas and non-pyrolytic

Approved for NPDES
Issued 1978

graphite. Smaller size furnace devices or those employing faster rates of atomization can be operated using lower atomization temperatures for shorter time periods than the above recommended settings.

2. The use of background correction is recommended.
3. Nitrogen may also be used as the purge gas but with reported lower sensitivity.
4. For every sample matrix analyzed, verification is necessary to determine that method of standard addition is not required (see part 5.2.1 of the Atomic Absorption Methods section of this manual).
5. If method of standard addition is required, follow the procedure given earlier in part 8.5 of the Atomic Absorption Methods section of this manual.
6. Data to be entered into STORET must be reported as ug/l.

Precision and Accuracy

1. Precision and accuracy data are not available at this time.

COPPER

Method 220.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 01042
Dissolved 01040
Suspended 01041

Optimum Concentration Range: 0.2–5 mg/l using a wavelength of 324.7 nm
Sensitivity: 0.1 mg/l
Detection Limit: 0.02 mg/l

Preparation of Standard Solution

1. Stock Solution: Carefully weigh 1.00 g of electrolyte copper (analytical reagent grade). Dissolve in 5 ml redistilled HNO_3 and make up to 1 liter with deionized distilled water. Final concentration is 1 mg Cu per ml (1000 mg/l).
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. The calibration standards should be prepared using the same type of acid and at the same concentration as will result in the sample to be analyzed either directly or after processing.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for preparation of the sample as given in parts 4.1.1 thru 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.

Instrumental Parameters (General)

1. Copper hollow cathode lamp
2. Wavelength: 324.7 nm
3. Fuel: Acetylene
4. Oxidant: Air
5. Type of flame: Oxidizing

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Approved for NPDES
Issued 1971
Editorial revision 1974 and 1978

139 214

Notes

1. For levels of copper below 50 ug/l, either the Special Extraction Procedure, given in par 9.2 of the Atomic Absorption Methods section or the furnace technique, Method 220.2 is recommended.
2. Numerous absorption lines are available for the determination of copper. By selecting suitable absorption wavelength, copper samples may be analyzed over a very wide ran of concentration. The following lines may be used:
   327.4 nm Relative Sensitivity 2
   216.5 nm Relative Sensitivity 7
   222.5 nm Relative Sensitivity 20
3. Data to be entered into **STORET** must be reported as ug/l.
4. The 2,9-dimethyl-1, 10-phenanthroline colorimetric method may also be used (Standa Methods, 14th Edition, p. 196).

Precision and Accuracy

1. An interlaboratory study on trace metal analyses by atomic absorption was conducted the Quality Assurance and Laboratory Evaluation Branch of EMSL. Six synthe concentrates containing varying levels of aluminum, cadmium, chromium, copper, irc manganese, lead and zinc were added to natural water samples. The statistical results f copper were as follows:

Number of Labs	True Values ug/liter	Mean Value ug/liter	Standard Deviation ug/liter	Accuracy as % Bias
91	302	305	56	0.9
92	332	324	56	-2.4
86	60	64	23	7.0
84	75	76	22	1.3
66	7.5	9.7	6.1	29.7
66	12.0	13.9	9.7	15.5

215

COPPER

Method 220.2 (Atomic Absorption, furnace technique)

STORET NO. 01042
Dissolved 01040
Suspended 01041

Optimum Concentration Range: 5–100 ug/l
Detection Limit: 1 ug/l

Preparation of Standard Solution

1. Stock solution: Prepare as described under "direct aspiration method".
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. These solutions are also to be used for "standard additions".
3. The calibration standard should be diluted to contain 0.5% (v/v) HNO_3.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. Prepare as described under "direct aspiration method". Sample solutions for analysis should contain 0.5% (v/v) HNO_3.

Instrument Parameters (General)

1. Drying Time and Temp: 30 sec–125°C.
2. Ashing Time and Temp: 30 sec–900°C.
3. Atomizing Time and Temp: 10 sec–2700°C.
4. Purge Gas Atmosphere: Argon
5. Wavelength: 324.7 nm
6. Other operating parameters should be set as specified by the particular instrument manufacturer.

Analysis Procedure

1. For the analysis procedure and the calculation, see "Furnace Procedure" part 9.3 of the Atomic Absorption Methods section of this manual.

Notes

1. The above concentration values and instrument conditions are for a Perkin-Elmer HGA-2100, based on the use of a 20 ul injection, continuous flow purge gas and non-pyrolytic

Approved for NPDES
Issued 1978

141

216

graphite. Smaller size furnace devices or those employing faster rates of atomization can be operated using lower atomization temperatures for shorter time periods than the above recommended settings.

2. Background correction may be required if the sample contains high dissolved solids.
3. Nitrogen may also be used as the purge gas.
4. For every sample matrix analyzed, verification is necessary to determine that method of standard addition is not required (see part 5.2.1 of the Atomic Absorption Methods section of this manual).
5. If method of standard addition is required, follow the procedure given earlier in part 8.5 of the Atomic Absorption Methods section of this manual.
6. Data to be entered into STORET must be reported as ug/1.

Precision and Accuracy

1. Precision and accuracy data are not available at this time.

IRON

Method 236.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 01045
Dissolved 01046
Suspended 01044

Optimum Concentration Range: 0.3–5 mg/l using a wavelength of 248.3 nm
Sensitivity: 0.12 mg/l
Detection Limit: 0.03 mg/l

Preparation of Standard Solution

1. Stock Solution: Carefully weigh 1.000 g of pure iron wire (analytical reagent grade) and dissolve in 5 ml redistilled HNO_3, warming if necessary. When solution is complete make up to 1 liter with deionized distilled water. 1 ml = 1 mg Fe (1000 mg/l).
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. The calibration standards should be prepared using the same type of acid and at the same concentration as will result in the sample to be analyzed either directly or after processing.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for preparation of the sample as given in parts 4.1.1 thru 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.

Instrumental Parameters (General)

1. Iron hollow cathode lamp
2. Wavelength: 248.3 nm
3. Fuel: Acetylene
4. Oxidant: Air
5. Type of flame: Oxidizing

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Approved for NPDES
Issued 1971
Editorial revision 1974 and 1978

151

215

Notes

1. The following lines may also be used:
   248.8 nm Relative Sensitivity 2
   271.9 nm Relative Sensitivity 4
   302.1 nm Relative Sensitivity 5
   252.7 nm Relative Sensitivity 6
   372.0 nm Relative Sensitivity 10
2. Data to be reported into **STORET** must be reported as ug/l.
3. The 1,10-phenanthroline colorimetric method may also be used (Standard Methods, 14th Edition, p. 208).
4. For concentrations of iron below 0.05 mg/l, either the Special Extraction Procedure given in part 9.2 of the Atomic Absorption Methods section or the furnace procedure, Method 236.2, is recommended.

Precision and Accuracy

1. An interlaboratory study on trace metal analyses by atomic absorption was conducted by the Quality Assurance and Laboratory Evaluation Branch of EMSL. Six synthetic concentrates containing varying levels of aluminum, cadmium, chromium, copper, iron, manganese, lead and zinc were added to natural water samples. The statistical results for iron were as follows:

Number Of Labs	True Values ug/liter	Mean Value ug/liter	Standard Deviation ug/liter	Accuracy as % Bias
82	840	855	173	1.8
85	700	680	178	-2.8
78	350	348	131	-0.5
79	438	435	183	-0.7
57	24	58	69	141
54	10	48	69	382

219

IRON

Method 236.2 (Atomic Absorption, furnace technique)

STORET NO. Total 01045
Dissolved 01046
Suspended 01044

Optimum Concentration Range: 5–100 ug/l
Detection Limit: 1 ug/l

Preparation of Standard Solution

1. Stock Solution: Prepare as described under "direct aspiration method".
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. These solutions are also to be used for "standard additions".
3. The calibration standard should be diluted to contain 0.5% (v/v) HNO_3.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. Prepare as described under "direct aspiration method". Sample solutions for analysis should contain 0.5% (v/v) HNO_3.

Instrument Parameters (General)

1. Drying Time and Temp: 30 sec–125°C.
2. Ashing Time and Temp: 30 sec–1000°C.
3. Atomizing Time and Temp: 10 sec–2700°C.
4. Purge Gas Atmosphere: Argon
5. Wavelength: 248.3 nm
6. Other operating parameters should be set as specified by the particular instrument manufacturer.

Analysis Procedure

1. For the analysis procedure and the calculation, see "Furnace Procedure" part 9.3 of the Atomic Absorption Methods section of this manual.

Notes

1. The above concentration values and instrument conditions are for a Perkin-Elmer HGA-2100, based on the use of a 20 ul injection, continuous flow purge gas and non-pyrolytic

Approved for NPDES
Issued 1978

153 220

graphite. Smaller size furnace devices or those employing faster rates of atomization can be operated using lower atomization temperatures for shorter time periods than the above recommended settings.

2. The use of background correction is recommended.
3. Nitrogen may also be used as the purge gas.
4. For every sample matrix analyzed, verification is necessary to determine that method of standard addition is not required (see part 5.2.1 of the Atomic Absorption Methods section of this manual).
5. If method of standard addition is required, follow the procedure given earlier in part 8.5 of the Atomic Absorption Methods section of this manual.
6. Data to be entered into STORET must be reported as ug/l.

Precision and Accuracy

1. Precision and accuracy data are not available at this time.

LEAD

Method 239.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 01051
Dissolved 01049
Suspended 01050

Optimum Concentration Range: 1–20 mg/l using a wavelength of 283.3 nm
Sensitivity: 0.5 mg/l
Detection Limit: 0.1 mg/l

Preparation of Standard Solution

1. Stock Solution: Carefully weigh 1.599 g of lead nitrate, $Pb(NO_3)_2$ (analytical reagent grade), and dissolve in deionized distilled water. When solution is complete acidify with 10 ml redistilled HNO_3 and dilute to 1 liter with deionized distilled water. 1 ml = 1 mg Pb (1000 mg/l).
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. The calibration standards should be prepared using the same type of acid and at the same concentration as will result in the sample to be analyzed either directly or after processing.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for preparation of the sample as given in parts 4.1.1 thru 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.

Instrumental Parameters (General)

1. Lead hollow cathode lamp
2. Wavelength: 283.3 nm
3. Fuel: Acetylene
4. Oxidant: Air
5. Type of flame: Oxidizing

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Approved for NPDES and SDWA
Issued 1971
Editorial revision 1974 and 1978

155

Notes

1. The analysis of this metal is exceptionally sensitive to turbulence and absorption bands in the flame. Therefore, some care should be taken to position the light beam in the most stable, center portion of the flame. To do this, first adjust the burner to maximize the absorbance reading with a lead standard. Then, aspirate a water blank and make minute adjustments in the burner alignment to minimize the signal.
2. For levels of lead below 200 ug/l, either the Special Extraction Procedure given in part 9.2 of the Atomic Absorption Methods section or the furnace technique, Method 239.2, is recommended.
3. The following lines may also be used:
   217.0 nm Relative Sensitivity 0.4
   261.4 nm Relative Sensitivity 10
4. For quality control requirements and optional recommendations for use in drinking water analyses, see part 10 of the Atomic Absorption Methods section of this manual.
5. Data to be entered into STORET must be reported as ug/l.

Precision and Accuracy

1. An interlaboratory study on trace metal analyses by atomic absorption was conducted by the Quality Assurance and Laboratory Evaluation Branch of EMSL. Six synthetic concentrates containing varying levels of aluminum, cadmium, chromium, copper, iron, manganese, lead and zinc were added to natural water samples. The statistical results for lead were as follows:

Number of Labs	True Values ug/liter	Mean Value ug/liter	Standard Deviation ug/liter	Accuracy as % Bias
74	367	377	128	2.9
74	334	340	111	1.8
64	101	101	46	-0.2
64	84	85	40	1.1
61	37	41	25	9.6
60	25	31	22	25.7

222

LEAD

Method 239.2 (Atomic Absorption, furnace technique)

STORET NO. Total 01051
Dissolved 01049
Suspended 01050

Optimum Concentration Range: 5–100 ug/l
Detection Limit: 1 ug/l

Preparation of Standard Solution

1. Stock solution: Prepare as described under "direct aspiration method".
2. Lanthanum Nitrate Solution: Dissolve 58.64 g of ACS reagent grade La_2O_3 in 100 ml conc. HNO_3 and dilute to 1000 ml with deionized distilled water. 1 ml = 50 mg La.
3. Working Lead Solution: Prepare dilutions of the stock lead solution to be used as calibration standards at the time of analysis. Each calibration standard should contain 0.5% (v/v) HNO_3. To each 100 ml of diluted standard add 10 ml of the lanthanum nitrate solution.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. Prepare as described under "direct aspiration method". Sample solutions for analysis should contain 0.5% (v/v) HNO_3.
2. To each 100 ml of prepared sample solution add 10 ml of the lanthanum nitrate solution.

Instrument Parameters (General)

1. Drying Time and Temp: 30 sec–125°C.
2. Ashing Time and Temp: 30 sec–500°C.
3. Atomizing Time and Temp: 10 sec–2700°C.
4. Purge Gas Atmosphere: Argon
5. Wavelength: 283.3 nm
6. Other operating parameters should be set as specified by the particular instrument manufacturer.

Analysis Procedure

1. For the analysis procedure in the calculation see "Furnace Procedure", part 9.3 of the Atomic Absorption Methods section of this manual.

Approved for NPDES and SDWA
Issued 1978

159

Notes

1. The above concentration values and instrument conditions are for a Perkin-Elmer HGA-2100, based on the use of a 20 *ul* injection, continuous flow purge gas and non-pyrolytic graphite. Smaller size furnace devices or those employing faster rates of atomization can be operated using lower atomization temperatures for shorter time periods than the above recommended settings.
2. The use of background correction is recommended.
3. Greater sensitivity can be achieved using the 217.0 nm line, but the optimum concentration range is reduced. The use of a lead electrodeless discharge lamp at this lower wavelength has been found to be advantageous. Also a lower atomization temperature (2400°C) may be preferred.
4. To suppress sulfate interference (up to 1500 ppm) lanthanum is added as the nitrate to both samples and calibration standards. (Atomic Absorption Newsletter Vol. 15, No. 3, p 71, May-June 1976.)
5. Since glassware contamination is a severe problem in lead analysis, all glassware should be cleaned immediately prior to use, and once cleaned, should not be open to the atmosphere except when necessary.
6. For every sample matrix analyzed, verification is necessary to determine that method of standard addition is not required (see part 5.2.1 of the Atomic Absorption Methods section of this manual).
7. For quality control requirements and optional recommendations for use in drinking water analyses, see part 10 of the Atomic Absorption Methods section of this manual.
8. If method of standard addition is required, follow the procedure given earlier in part 8.5 of the Atomic Absorption Methods section of this manual.
9. Data to be entered into STORET must be reported as *ug*/l.

Precision and Accuracy

1. In a single laboratory (EMSL), using Cincinnati, Ohio tap water spiked at concentration of 25, 50, and 100 *ug* Pb/l, the standard deviations were ±1.3, ±1.6, and ±3.7 respectively. Recoveries at these levels were 88%, 92%, and 95% respectively.

MAGNESIUM

Method 242.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 00927
Dissolved 00925
Suspended 00926

Optimum Concentration Range: 0.02–0.5 mg/l using a wavelength of 285.2 nm
Sensitivity: 0.007 mg/l
Detection Limit: 0.001 mg/l

Preparation of Standard Solution

1. Stock Solution: Dissolve 0.829 g of magnesium oxide, MgO (analytical reagent grade), in 10 ml of redistilled HNO_3 and dilute to 1 liter with deionized distilled water. 1 ml = 0.50 mg Mg (500 mg/l).
2. Lanthanum chloride solution: Dissolve 29 g of La_2O_3, slowly and in small portions in 250 ml conc. HCl, (Caution: Reaction is violent), and dilute to 500 ml with deionized distilled water.
3. Prepare dilutions of the stock magnesium solution to be used as calibration standards at the time of analysis. To each 10 ml volume of calibration standard and sample alike add 1.0 ml of the lanthanum chloride solution, i.e., 20 ml of standard or sample + 2 ml $LaCl_3$ = 22 ml.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. For the analysis of total magnesium in domestic and industrial effluents, the procedures for the determination of total metals as given in parts 4.1.3 and 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.
2. For ambient waters, a representative aliquot of a well-mixed sample may be used directly for analysis. If suspended solids are present in sufficient amounts to clog the nebulizer, the sample may be allowed to settle and the supernatant liquid analyzed directly.
3. Samples should be preserved with (1:1) nitric acid to a pH of 2 at the time of collection.

Instrumental Parameters (General)

1. Magnesium hollow cathode lamp
2. Wavelength: 285.2 nm

Approved for NPDES
Issued 1971
Editorial revision 1974 and 1978

3. Fuel: Acetylene
4. Oxidant: Air
5. Type of flame: Oxidizing

Notes

1. The interference caused by aluminum at concentrations greater than 2 mg/l is masked by addition of lanthanum. Sodium, potassium and calcium cause no interference at concentrations less than 400 mg/l.
2. The following line may also be used:
   202.5 nm Relative Sensitivity 25
3. To cover the range of magnesium values normally observed in surface waters (0.1–20 mg/l), it is suggested that either the 202.5 nm line be used or the burner head be rotated. A 90° rotation of the burner head will produce approximately one-eighth the normal sensitivity.
4. Data to be entered into STORET must be reported as mg/l.
5. The gravimetric method may also be used (Standard Methods, 14th Edition, p 221).

Precision and Accuracy

1. In a single laboratory (EMSL), using distilled water spiked at concentrations of 2.1 and 8.2 mg Mg/l the standard deviations were ±0.1 and ±0.2, respectively. Recoveries at both of these levels were 100%.

227

MANGANESE
Method 243.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 01055
Dissolved 01056
Suspended 01054

Optimum Concentration Range: 0.1–3 mg/l using a wavelength of 279.5 nm
Sensitivity: 0.05 mg/l
Detection Limit: 0.01 mg/l

Preparation of Standard Solution

1. Stock Solution: Carefully weigh 1.000 g of manganese metal (analytical reagent grade) and dissolve in 10 ml of redistilled HNO_3. When solution is complete, dilute to 1 liter with 1% (V/V) HCl. 1 ml = 1 mg Mn (1000 mg/l).
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. The calibration standards should be prepared using the same type of acid and at the same concentration as will result in the sample to be analyzed either directly or after processing.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for preparation of the sample as given in parts 4.1.1 thru 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.

Instrumental Parameters (General)

1. Manganese hollow cathode lamp
2. Wavelength: 279.5 nm
3. Fuel: Acetylene
4. Oxidant: Air
5. Type of flame: Oxidizing

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Approved for NPDES
Issued 1971
Editorial revision 1974 and 1978

Notes

1. For levels of manganese below 25 ug/l, either the furnace procedure, Method 243.2, or the Special Extraction Procedure given in part 9.2 of the Atomic Absorption Methods section is recommended. The extraction is carried out at a pH of 4.5 to 5. The manganese chelate is very unstable and the analysis must be made without delay to prevent its re-solution in the aqueous phase.
2. The following line may also be used:
   403.1 nm Relative Sensitivity 10.
3. Data to be entered into STORET must be reported as ug/l.
4. The persulfate colorimetric method may also be used (Standard Methods, 14th Edition, p 225).

Precision and Accuracy

1. An interlaboratory study on trace metal analyses by atomic absorption was conducted by the Quality Assurance and Laboratory Evaluation Branch of EMSL. Six synthetic concentrates containing varying levels of aluminum, cadmium, chromium, copper, iron, manganese, lead and zinc were added to natural water samples. The statistical results for manganese were as follows:

Number of Labs	True Values ug/liter	Mean Value ug/liter	Standard Deviation ug/liter	Accuracy as % Bias
77	426	432	70	1.5
78	469	474	97	1.2
71	84	86	26	2.1
70	106	104	31	-2.1
55	11	21	27	93
55	17	21	20	22

229

MANGANESE

Method 243.2 (Atomic Absorption, furnace technique)

STORET NO. Total 01055
Dissolved 01056
Suspended 01054

Optimum Concentration Range: 1–30 ug/l
Detection Limit: 0.2 ug/l

Preparation of Standard Solution

1. Stock solution: Prepare as described under "direct aspiration method".
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. These solutions are also to be used for "standard additions".
3. The calibration standard should be diluted to contain 0.5% (v/v) HNO_3.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. Prepare as described under "direct aspiration method". Sample solutions for analysis should contain 0.5% (v/v) HNO_3.

Instrument Parameters (General)

1. Drying Time and Temp: 30 sec–125°C.
2. Ashing Time and Temp: 30 sec–1000°C.
3. Atomizing Time and Temp: 10 sec–2700°C.
4. Purge Gas Atmosphere: Argon
5. Wavelength: 279.5 nm
6. Other operating parameters should be set as specified by the particular instrument manufacturer.

Analysis Procedure

1. For the analysis procedure and the calculation, see "Furnace Procedure" part 9.3 of the Atomic Absorption Methods section of this manual.

Notes

1. The above concentration values and instrument conditions are for a Perkin-Elmer HGA-2100, based on the use of a 20 ul injection, continuous flow purge gas and non-pyrolytic

Approved for NPDES
Issued 1978

2,30 12

FINAL REPORT

Advanced Training for Tabbin Institute of Metallurgical Studies (TIMS), Cairo, Egypt

USAID CONTRACT #263-010-C-00-2038-00
PROJECT #263-0101

VOLUME III

APPENDIX

GAS CHROMATOGRAPHY

Prepared by
A. Curley,
Team Leader
Office of Research and Sponsored Programs
Clark Atlanta University
J.P. Brawley Drive at Fair Street, SW
Atlanta, GA 30314

731

TABLE OF CONTENTS

VOLUME III

HANDOUTS FOR GAS CHROMATOGRAPHY

APPENDIX	**Volumes 2 - 4**
Volume 3	Methods for Organic Chemical Analysis of Municipal and Industrial Wastewater
	Purgeable Aromatics - Method 602
	Benzidines - Method 605
	Phthalate Esters - Method 606
	Nitrosamines - Method 607
	Organochlorine Pesticides and PCBs - Method 608
	Nitroaromatics and Isophorone - Method 609
	Polynuclear Aromatic Hydrocarbons - Method 610
	Haloethers - Method 611
	Chlorinated Hydrocarbons - Metthod 612
	2, 3, 7, 8 - Tetrachlorodibenzo-p-Dioxin - Method 613
	Purgeables - Method 624
	Base/Neutrals and Acids - Method 625
	Handbook for Analytical QC in Water and Wastewater Labs

HANDOUTS

FOR

GAS CHROMATOGRAPHY

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA-600/4-82-057 July 1982

PB 83-201-798

$19.00

1-800-553-6847

Test Methods

Methods for Organic Chemical Analysis of Municipal and Industrial Wastewater

James E. Longbottom and James J. Lichtenberg, Editors

$24

Distribution Record for Methods for Organic Chemical Analysis of Municipal and Industrial Wastewater

To receive future additions to this mannual and to aid us in establishing a mailing list, please fill out the information below, affix postage where indicated on reverse side, and mail Any future change in name address should also be forwarded to us.

Last Name *First* *Middle Initial*

Professional Affiliation

Address, Street

City *State* *Zip Code*

231

Disclaimer

This report has been reviewed by the Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Place
Stamp
Here

U.S Environmental Protection Agency
Environmental Monitoring and Support Laboratory
26 W St Clair Street
Cincinnati, OH 45268

ATTN MOCAW Distribution Record

235

Contents

Foreword iii
Acknowledgments iv
Introduction vi
Approved Test Procedures for Organic Chemicals ix

Purgeable Halocarbons ..... Method 601
Purgeable Aromatics ..... Method 602
Acrolein and Acrylonitrile ..... Method 603
Phenols ..... Method 604
Benzidines ..... Method 605
Phthalate Esters ..... Method 606
Nitrosamines ..... Method 607
Pesticides and PCBs ..... Method 608
Nitroaromatics and Isophorone ..... Method 609
Polynuclear Aromatic Hydrocarbons ..... Method 610
Haloethers ..... Method 611
Chlorinated Hydrocarbons ..... Method 612
2 3,7,8-Tetrachlorodibenzo-p-dioxin ..... Method 613
Purgeables ..... Method 624
Base/Neutrals, Acids, and Pesticides ..... Method 625

Appendix A A-1

236

Introduction

In 1976, the U.S. Environmental Protection Agency entered into a Settlement Agreement requiring it to study and, if necessary, regulate 65 "priority" pollutants and classes of pollutants (1). This list was later defined to include 129 specific "priority pollutants." In December 1977, Congress passed the Clean Water Act of 1977 (PL 95-217) declaring the 65 pollutants and classes of pollutants to be "toxic" under Section 307(a) of the Act.

The Settlement Agreement included a rigid time schedule for the completion of industrial wastewater analytical surveys and promulgation of effluent guidelines. To provide the Effluent Guidelines Division of USEPA with a means of measuring the concentration of pollutants in these wastewaters, the USEPA's Environmental Monitoring and Support Laboratory in Cincinnati, and the Environmental Research Laboratory in Athens, Georgia, collaborated on a research project that resulted in an analytical protocol (2) that was successfully applied to a variety of wastewaters. The gas chromatography/mass spectrometry (GC/MS) procedures in that protocol for the measurement of organic pollutants were the forerunners of methods 624 and 625 that appear in this manual.

In a parallel research project, the Environmental Monitoring and Support Laboratory in Cincinnati, through a series of research contract and in-house activities, undertook a systematic study of the analytical behavior of each of the individual classes of organic compounds to identify or develop non-MS approaches that could be used for routine monitoring of regulated discharges. This approach was pursued to minimize the requirements for expensive mass spectrometer equipment and the skilled operators required to use it. The resulting test procedures were identified as methods 601 through 612 and were first published in early 1979, along with a GC/MS method for the measurement of TCDD (3,4).

All fifteen of the test procedures discussed above were proposed as amendments to the "Guidelines Establishing Test Procedures for the Analyses of Pollutants" (40 CFR, Part 136) in December 3, 1979 (5). These guidelines are required by Section 304(h) of the Clean Water Act. As a result of public comment from over 200 respondents, extensive editorial revisions were made to the methods. The majority of the revisions were made either for clarification or to add additional flexibility for the analyst. These revised methods constitute the body of this methods manual.

The methods contained herein represent an effort to provide procedures that are as uniform and cost-effective as practical for a wide cross-section of chemical compound classes. Due to the variable chemical and physical properties of the parameters under study, some compromises were made. Therefore, in some of the methods, the extraction procedures, cleanup procedures, and determinative steps are not optimum for all parameters.

A distribution list will be established for this manual. The list will be prepared from the distribution record cards that accompany this package. Future revisions of existing methods and the addition of new methods will only be sent to individuals who return those record cards. Correspondence on these methods is invited.

References

1. Natural Resources Defense Council, Inc., et al., v. Train, 8 ERC 2120 (D.D.C. 1976), modified 12 ERC 1883 (D.D.C. 1979).
2. "Sampling and Analysis Procedures for Screening of Industrial Effluents for Priority Pollutants," March 1977 (revised, April 1977). U.S. Environmental Protection Agency, Effluent Guidelines Division, Washington, D.C. 20460.
3. "Methods for Organic Compounds in Municipal and Industrial Wastewater," March 1979, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.
4. "Methods for Organic Compounds in Municipal and Industrial Wastewater," April 1979, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.
5. Guidelines Establishing Test Procedures for the Analysis of Pollutants; Proposed Regulations, 40 Code of Federal Regulations (CFR), Part 136, Published in *Federal Register, 44*, 69464.

231

Approved Test Procedures for Organic Chemicals

Section 136 3(a) of 40 Code of Federal Regulations cites approved test procedures through the use of five subtables:

Table 1A - Biological parameters
Table 1B - Inorganic and physical parameters
Table 1C - Organic chemical parameters
Table 1D - Pesticide parameters
Table 1E - Radiological parameters

The test procedures in this manual are cited in Tables 1C and 1D. These tables have been reproduced here for the convenience of the user.

138

Table 1C. *List of approved test procedures for organic compounds*

Parameter[1]	EPA Method Number[2] GC	GC/MS	HPLC	Other
1 Acenaphthene	610	625	610	- -
2. Acenaphthalene	610	625	610	- -
3 Acrolein	603	624[4]	- -	- -
4. Acrylonitrile	603	624[4]	- -	- -
5. Anthracene	610	625	610	- -
6. Benzene	602	624	- -	- -
7. Benzidine	- -	625[5]	605	Note 3, p. 1; Note 6, p. S48
8. Benzo(a)anthracene	610	625	610	- -
9. Benzo(a)pyrene	610	625	610	- -
10. Benzo(b)fluoranthene	610	625	610	- -
11 Benzo(ghi)perylene	610	625	610	- -
12. Benzo(k)fluoranthene	610	625	610	- -
13. Benzyl Chloride	- -	- -	- -	Note 3, p. 130; Note 6, p.S102
14. Benzyl Butyl Phthalate	606	625	- -	- -
15. Bis(2-chloroethyl) ether	611	625	- -	- -
16. Bis(2-chloroethoxy) methane	611	625	- -	- -
17 Bis(2-chloroisopropyl) ether	611	625	- -	- -
18 Bis(2-ethylhexyl) phthalate	606	625	- -	- -
19 Bromodichloromethane	601	624	- -	- -
20. Bromoform	601	624	- -	- -
21. Bromoethane	601	624	- -	- -
22. 4-Bromophenylphenyl ether	611	625	- -	- -
23. Carbon tetrachloride	601	624	- -	Note 3, p. 130; Note 6, p.S102
24. 4-Chloro-3-methylphenol	604	625	- -	- -
25. Chlorobenzene	601,602	624	- -	Note 3, p.130; Note 6, p. S102
26. Chloroethane	601	624	- -	- -
27. 2-Chloroethylvinyl ether	601	624	- -	- -
28. Chloroform	601	624	- -	Note 3, p. 130; Note 6, p.S102
29. Chloromethane	601	624	- -	- -
30. 2-Chloronaphthalene	612	625	- -	- -
31. 2-Chlorophenol	604	625	- -	- -
32. 4-Chlorophenylphenyl ether	611	625	- -	- -
33. Chrysene	610	625	610	- -
34. Dibenzo(a,h)anthracene	610	625	610	- -
35. Dibromochloromethane	601	624	- -	- -
36. 1,2-Dichlorobenzene	601, 602, 612	624, 625	- -	- -
37. 1,3-Dichlorobenzene	601, 602, 612	624, 625	- -	- -
38. 1,4-Dichlorobenzene	601, 601, 612	624, 625	- -	- -
39. 3,3'-Dichlorobenzidine	- -	625	605	- -
40. Dichlorodifluoromethane	601	- -	- -	- -
41. 1,1-Dichloroethane	601	624	- -	- -
42. 1,2-Dichloroethane	601	624	- -	- -
43. Dichloroethene	601	624	- -	- -
44. trans-1,2-Dichloroethene	601	624	- -	- -
45. 2,4-Dichlorophenol	604	625	- -	- -
46. 1,2-Dichloropropane	601	624	- -	
47. cis-1,3-Dichloropropene	601	624	- -	- -
48. trans-1,3-Dichoropropene	601	624	- -	- -
49. Diethyl phthalate	606	625	- -	- -
50. 2,4-Dimethylphenol	604	625	- -	- -
51. Dimethyl phthalate	606	625	- -	- -
52. Di-n-butyl phthalate	606	625	- -	- -
53. Di-n-octyl phthalate	606	625	- -	- -
54. 2,4-Dinitrophenol	604	625	- -	- -
55. 2,4-Dinitrotoluene	609	625	- -	- -
56. 2,6-Dinitrotoluene	609	625	- -	- -

239

Table 1C. *Continued*

Parameter[1]	EPA Method Number[2] GC	GC/MS	HPLC	Other
57 Epichlorohydrin	. .	. .	. .	Note 3, p.130; Note 6, p.S102
58. Ethylbenzene	602	624	. .	. .
59. Fluoranthene	610	625	610	. .
60. Fluorene	610	625	610	. .
61. Hexachlorobenzene	612	625	. .	. .
62. Hexachlorobutadiene	612	625	. .	. .
63. Hexachlorocyclopentadiene	612	625[5]	. .	. .
64 Hexachloroethane	612	625	. .	. .
65. Ideno(1,2,3-cd)pyrene	610	625	610	. .
66. Isophorone	609	625	. .	. .
67. Methylene chloride	601	624	. .	Note 3, p. 130; Note 6, p.S102
68 2-Methyl-4,6-dinitrophenol	604	625	. .	. .
69. Naphthalene	610	625	. .	. .
70. Nitrobenzene	609	625	. .	. .
71. 2-Nitrophenol	604	625	. .	. .
72. 4-Nitrophenol	604	625	. .	. .
73. N-Nitrosodimethylamine	607	625[5]	. .	. .
74. N-Nitrosodi-n-propylamine	607	625	. .	. .
75. N-Nitrosodiphenylamine	607	625[5]	. .	. .
76. PCB-1016	608	625	. .	Note 3, p. 43; Note 6, p. S78
77. PCB-1221	608	625	. .	Note 3, p. 43; Note 6, p. S78
78. PCB-1232	608	625	. .	Note 3, p. 43; Note 6, p.S78
79. PCB-1242	608	625	. .	Note 3, p. 43; Note 6, p. S78
80. PCB-1248	608	625	. .	Note 3, p. 43; Note 6, p. S78
81. PCB-1254	608	625	. .	Note 3, p.43; Note 6, p. S78
82. PCB-1260	608	625	. .	Note 3, p. 43; Note 6, p. S78
83. Pentachlorophenol	604	625	. .	Note 3, p. 140; Note 6, p. S50
84. Phenanthrene	610	625	610	. .
85. Phenol	604	625	. .	. .
86. Pyrene	610	625	610	. .
87. 2,3,7,8-Tetrachloro-dibenzo-p-dioxin	. .	613	. .	. .
88. 1,1,2,2-Tetrachloroethane	601	624	. .	Note 3, p. 130; Note 6, p. S102
89. Tetrachloroethene	601	624	. .	Note 3, p. 130; Note 6, p. S102
90. Toluene	602	624	. .	. .
91. 1,2,4-Trichlorobenzene	612	625	. .	Note 3, p. 130; Note 6, p. S102
92. 1,1,1-Trichloroethane	601	624	. .	. .
93. 1,1,2-Trichloroethane	601	624	. .	Note 3, p. 130; Note 6, p.S102
94. Trichloroethene	601	624	. .	. .
95. Trichlorofluoromethane	601	624	. .	. .
96. 2,4,6-Trichlorophenol	604	625	. .	. .
97. Vinyl Chloride	601	624	. .	. .

240

Table 1C. *Notes*

[1] *All parameter concentrations are expressed in micrograms per liter (µg/L).*
[2] *"Methods for Organic Chemical Analysis of Municipal and Industrial Wastewater," USEPA, July 1982.*
[3] *"Methods for Benzidine, Chlorinated Organic Compounds, Pentachlorophenol and Pesticides in Water and Wastewater," USEPA, September 1978.*
[4] *Method 624 may be extended to screen samples for acrolein and acrylonitrile. However, when they are known to be present, the preferred method for these two compounds is Method 603.*
[5] *Method 625 may be extended to include benzidine, hexachlorocyclopentadiene, N-nitrosodimethylamine, and N-nitrosodiphenylamine. However, when they are known to be present, Method 605, 612, 607, and 607, respectively, are the preferred methods for these compound.*
[6] *"Selected Analytical Methods Approved and Cited by the United States Environmental Protection Agency," Supplement to the Fifteenth Edition of Standard Methods for the Examination of Water and Wastewater (1981).*

40 C.F.R. Part 136, § 136.3

Table 1D. *List of approved test procedures for pesticides.*[1]

Parameter (µg/L)	Method	EPA[2]	Std. Methods 15th Ed.	ASTM	Other
1. Aldrin	GC	608	509A	D3086	Note 3, p. 7;
	GC/MS	625	----	----	Note 4, p. 30
2. Ametryn	GC	---	----	----	Note 3, p. 83; Note 6, p. S68
3. Aminocarb	TLC	---	----	----	Note 3, p. 94; Note 6, p. S16
4. Atraton	GC	---	----	----	Note 3, p. 83; Note 6, p. S68
5. Atrazine	GC	---	----	----	Note 3, p. 83; Note 6, p. S68
6. Azinphos methyl	gc	---	----	----	Note 3, p. 25;
				----	Note 6, p.S51
7. Barban	TLC	---	----	----	znote 3, p. 104; Note 6, p. S64
8. α-BHC	GC	608	509A	D3086	Note 3, p. 7
	GC/MS	625[5]	----	----	----
9. β-BHC	GC	608	----	D3086	---
	GC/MS	625	---	----	----
10. δ-BHC	GC	608	----	D3086	----
	GC/MS	625[5]	----	----	----
11 δ-BHC (Lindane)	GC	608	509A	D3086	Note 3, p. 7; Note 4, p. 30. Note 6, p. S73
	GC/MS	625	----	----	----
12. Captan	GC	---	509A	----	Note 3, p. 7
13. Carbaryl	TLC	---	----	----	Note 3, p. 94; Note 6, p. S73
14. Carbophenothion	GC	---	----	----	Note 4, p. 30; Note 6, p. S73
15. Chlordane	GC	608	509A	D3086	Note 3, p. 7
	GC/MS	625	----	----	----
16. Chlorpropham	TLC	---	----	----	Note 3, p. 104, Note 6, p.S64
17. 2,4-D	GC	---	509B	----	Note 3, p. 115; Note 4, p. 35
18. 4,4'-DDD	GC	608	509A	D3086	Note 3, p. 7; Note 4, p. 30
	GC/MS	625	----	----	----
19. 4,4'-DDE	GC	608	509A	D3086	Note 3, p. 7; Note 4, p. 30
	GC/MS	625	----	----	----
20. 4,4'-DDT	GC	608	509A	D3086	Note 3, p. 7; Note 4, p. 30
	GC/MS	625	----	----	----
21. Demeton-O	GC	---	----	----	Note 3, p. 25; Note 6, p. S51

Table 1D. *Continued*

Parameter (µg/L)	Method	EPA[7]	Std. Methods 15th Ed	ASTM	Other
22. Demeton-S	GC	---	----	----	Note 3, p. 25; Note 6, p. S51
23. Diazinon	GC	---	----	----	Note 3, p. 25; Note 4, p. 30; Note 6, p. S51
24. Dicamba	GC	---	----	----	Note 3, p. 115
25. Dichlofenthion	GC	---	----	----	Note 4, p. 30; Note 6, p. S73
26. Dichloran	GC	---	509A	----	Note 3, p. 7
27. Dicofol	GC	---	----	D3086	----
28. Dieldrin	GC	608	509A	----	Note 3, p. 7; Note 4, p. 30 Note 6, p. S73
	GC/MS	625	----	----	----
29. Dioxathion	GC	---	----	----	Note 4, p. 30; Note 6, p. S73
30 Disulfoton	GC	---	----	----	Note 3, p. 25; Note 6, p. S51
31. Diuron	TLC	---	----	----	Note 3, p. 104; Note 6, p.S64
32. Endosulfan I	GC	608	509A	D3086	Note 3, p. 7
	GC/MS	625[5]	----	----	----
33. Endosulfan II	GC	608	509A	D3086	Note 3, p. 7
	GC/MS	625[5]	----	----	----
34. Endosulfan sulfate	GC	608	----	----	----
	GC/MS	625	----	----	----
35. Endrin	GC	608	509A	D3086	Note 3, p. 7; Note 4, p. 30
	GC/MS	625[5]	----	----	----
36. Endrin aldehyde	GC	608	----	----	----
	GC/MS	625	----	----	----
37. Ethion	GC	---	----	----	Note 4, p. 30; Note 6, p. S64
38. Fenuron	TLC	---	----	----	Note 3, p. 104; Note 6, p. S64
39. Fenuron-TCA	TLC	---	----	----	Note 3, p. 104; Note 6, p. S64
40. Heptachlor	GC	608	509A	D3086	Note 3, p. 7; Note 4, p. 40
	GC/MS	625	----	----	----
41. Heptachlor epoxide	GC	608	509A	D3086	Note 3, p. 7; Note 4, p. 30; Note 6, p. S73
	GC/MS	625	----	----	----
42. Isodrin	GC	---	----	----	Note 4, p. 30; Note 6, p. S73
43. Linuron	TLC	---	----	----	Note 3, p. 104; Note 6, p. S64
44. Malathion	GC	---	509A	----	Note 3, p. 25; Note 4, p. 30; Note 6, p. S51
45. Methiocarb	TLC	---	----	----	Note 3, p. 94; Note 6, p. S60
46. Methoxychlor	GC	---	509A	D3086	Note 3, p. 7; Note 4, p. 30
47. Mexacarbate	TLC	---	----	----	Note 3, p. 94; Note 6, p. S60
48. Mirex	GC	---	509A	----	Note 3, p. 7
49. Monuron	TLC	---	----	----	Note 3, p. 104; Note 6, p. S64
40. Monuron-TCA	TLC	---	----	----	Note 3, p. 104; Note 6, p. S64

242

Table 1D. *Continued*

Parameter (µg/L)	Method	EPA[2]	Std Methods 15th Ed	ASTM	Other
51. Neburon	TLC	---	----	----	Note 3, p. 104; Note 6, p. S64
52. Parathion methyl	GC	---	509A	----	Note 3, p. 25; Note 4, p. 30
53. Parathion ethyl	GC	---	509A	----	Note 3, p. 25
54. PCNB	GC	---	509A	----	Note 3, p. 7
55. Perthane	GC	---	----	D3086	----
56. Prometon	GC	---	----	----	Note 3, p. 83; Note 6, p. S68
57. Prometryn	GC	---	----	----	Note 3, p. 83; Note 6, p. S68
58. Propazine	GC	---	----	----	Note 3, p. 83; Note 6, p. S68
59. Propham	TLC	---	----	----	Note 3, p. 104; Note 6, p. S64
60. Propoxur	TLC	---	----	----	Note 3, p. 94; Note 6, p. S60
61. Secbumeton	TLC	---	----	----	Note 3, p. 83; Note 6, p. S68
62. Siduron	TLC	---	----	----	Note 3, p. 104; Note 6, p. S64
63. Simazine	GC	---	----	----	Note 3, p. 83; Note 6, p. S68
64. Strobane	GC	---	509A	----	Note 3, p. 7
65. Swep	TLC	---	----	----	Note 3, p. 104; Note 6, p. S64
66. 2,4,5-T	GC	---	509B	----	Note 3, p. 115; Note 4, p. 35
67. 2,4,5-tp (Silvex)	GC	---	509B	----	Note 3, p. 83; Note 6, p. S68
68. Terbuthylazine	GC	---	----	----	Note 3, p. 83; Note 6, p. S68
69. Toxaphene	GC	608	509A	D3086	Note 3, p. 7; Note 4, p. 30
	GC/MS	625	----	----	----
70. Trifluralin	GC	---	----	----	Note 3, p. 7

[1] Pesticides are listed in this table by common name for the convenience of the reader. Additional pesticides may be found under Table 1C, where entries are listed by chemical name.

[2] "Methods for Organic Chemical Analysis of Municipal and Industrial Wastewater." USEPA, July 1982.

[3] "Methods for Benzidine, Chlorinated Organic Compounds, Pentachlorophenol and Pesticides in Water and Wastewater." USEPA, September 1978.

[4] Methods for Analysis of Organic Substances in Water, U.S. Geological Survey Techniques of Water Resources Inv., Book 5, Ch. A3 (1972), p. 30.

[5] The method may be extended to screen samples for α-BHC, and δ-BHC, endosulfan I, endosulfan II, and endrin. However, when they are known to be present, the referenced gas chromatographic procedures are the preferred methods.

[6] "Selected Analytical Methods Approved and Cited by the United States Environmental Protection Agency." Supplement to the Fifteenth Edition of Standard Methods for the Examination of Water and Wastewater (1981).

243

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Purgeable Halocarbons—Method 601

1. Scope and Application

1.1 This method covers the determination of 29 purgeable halocarbons. The following parameters may be determined by this method:

Parameter	STORET No.	CAS No.
Bromodichloromethane	32101	75-27-4
Bromoform	32104	75-25-2
Bromomethane	34413	74-83-9
Carbon tetrachloride	32102	56-23-5
Chlorobenzene	34301	108-90-7
Chloroethane	34311	75-00-3
2-Chloroethylvinyl ether	34576	100-75-8
Chloroform	32106	67-66-3
Chloromethane	34418	74-87-3
Dibromochloromethane	32105	124-48-1
1,2-Dichlorobenzene	34536	95-50-1
1,3-Dichlorobenzene	34566	541-73-1
1,4-Dichlorobenzene	34571	106-46-7
Dichlorodifluoromethane	34668	75-71-8
1,1-Dichloroethane	34496	75-34-3
1,2-Dichloroethane	34531	107-06-2
1,1-Dichloroethene	34501	75-35-4
trans-1,2-Dichloroethene	34546	156-60-5
1,2-Dichloropropane	34541	78-87-5
cis-1,3-Dichloropropene	34704	10061-01-5
trans-1,3-Dichloropropene	34699	10061-02-6
Methylene chloride	34423	75-09-2
1,1,2,2-Tetrachloroethane	34516	79-34-5
Tetrachloroethene	34475	127-18-4
1,1,1-Trichloroethane	34506	71-55-6
1,1,2-Trichloroethane	34511	79-00-5
Trichloroethene	39180	79-01-6
Trichlorofluoromethane	34488	75-69-4
Vinyl chloride	39175	75-01-4

1.2 This is a purge and trap gas chromatographic method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for any or all of the compounds above, compound identification should be supported by at least one additional qualitative

244

technique. This method describes analytical conditions for a second gas chromatographic column that can be used to confirm measurements made with the primary column. Method 624 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results for most of the parameters listed above.

1.3 The method detection limit (MDL, defined in Section 12.1)[1] for each parameter is listed in Table 1. The MDL for a specific wastewater may differ from those listed, depending upon the nature of interferences in the sample matrix.

1.4 Any modification of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval of alternate test procedures under 40 CFR 136.4 and 136.5.

1.5 This method is restricted to use by or under the supervision of analysts experienced in the operation of a purge and trap system and a gas chromatograph and in the interpretation of chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 An inert gas is bubbled through a 5-mL water sample contained in a specially-designed purging chamber at ambient temperature. The halocarbons are efficiently transferred from the aqueous phase to the vapor phase. The vapor is swept through a sorbent trap where the halocarbons are trapped. After purging is completed, the trap is heated and backflushed with the inert gas to desorb the halocarbons onto a gas chromatographic column. The gas chromatograph is temperature programmed to separate the halocarbons which are then detected with a halide-specific detector.[2,3]

2.2 The method provides an optional gas chromatographic column that may be helpful in resolving the compounds of interest from interferences that may occur.

3. Interferences

3.1 Impurities in the purge gas and organic compounds out-gassing from the plumbing ahead of the trap account for the majority of contamination problems. The analytical system must be demonstrated to be free from contamination under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5. The use of non-TFE plastic tubing, non-TFE thread sealants, or flow controllers with rubber components in the purging device should be avoided.

3.2 Samples can be contaminated by diffusion of volatile organics (particularly fluorocarbons and methylene chloride) through the septum seal into the sample during shipment and storage. A field reagent blank prepared from reagent water and carried through the sampling and handling protocol can serve as a check on such contamination.

3.3 Contamination by carry-over can occur whenever high level and low level samples are sequentially analyzed. To reduce carry-over, the purging device and sample syringe must be rinsed with reagent water between sample analyses. Whenever an unusually concentrated sample is encountered, it should be followed by an analysis of reagent water to check for cross contamination. For samples containing large amounts of water-soluble materials, suspended solids, high boiling compounds or high organohalide levels, it may be necessary to wash out the purging device with a detergent solution, rinse it with distilled water, and then dry it in a 105 °C oven between analyses. The trap and other parts of the system are also subject to contamination; therefore, frequent bakeout and purging of the entire system may be required.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[4-6] for the information of the analyst.

4.2 The following parameters covered by this method have been tentatively classified as known or suspected, human or mammalian carcinogens: carbon tetrachloride, chloroform, 1,4-dichlorobenzene, and vinyl chloride. Primary standards of these toxic compounds should be prepared in a hood. A NIOSH/MESA approved toxic gas respirator should be worn when the analyst handles high concentrations of these toxic compounds.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete sampling.

5.1.1 Vial—25-mL capacity or larger, equipped with a screw cap with hole in center (Pierce #13075 or equivalent). Detergent wash, rinse cap with tap and distilled water, and dry at 105 °C before use.

5.1.2 Septum—Teflon-faced silicone (Pierce #12722 or equivalent). Detergent wash, rinse with tap and distilled water, and dry at 105 °C for one hour before use.

5.2 Purge and trap device—The purge and trap device consists of three separate pieces of equipment: the sample purger, trap, and the desorber. Several complete devices are now commercially available.

5.2.1 The sample purger must be designed to accept 5-mL samples with a water column at least 3 cm deep. The gaseous head space between the water column and the trap must have a total volume of less than 15-mL. The purge gas must pass through the water column as finely divided bubbles with a diameter of less than 3 mm at the origin. The purge gas must be introduced no more than 5 mm from the base of the water column. The sample purger, illustrated in Figure 1, meets these design criteria.

5.2.2 The trap must be at least 25 cm long and have an inside diameter of at least 0.105 inch. The trap must be packed to contain the following minimum lengths of adsorbents: 1.0 cm of methyl silicone coated backing (Section 6.3.3), 7.7 cm of 2,6-diphenylene oxide polymer (Section 6.3.2), 7.7 cm of silica gel, 7.7 gm of coconut charcoal (Section 6.3.1). If it is not necessary to analyze for dichlorodifluoromethane, the charcoal can be eliminated, and the polymer section lengthened to 15 cm. The minimum specifications for the trap are illustrated in Figure 2.

5.2.3 The desorber must be capable of rapidly heating the trap to 180 °C. The polymer section of the trap should

245

not be heated higher than 180 °C and the remaining sections should not exceed 220 °C. The desorber design, illustrated in Figure 2, meets these criteria.

5.2.4 The purge and trap device may be assembled as a separate unit or be coupled to a gas chromatograph as illustrated in Figures 3 and 4.

5.3 Gas chromatograph—An analytical system complete with a temperature programmable gas chromatograph suitable for on-column injection and all required accessories including syringes, analytical columns, gases, detector, and strip-chart recorder. A data system is recommended for measuring peak areas.

5.3.1 Column 1—8 ft long × 0.1 in ID stainless steel or glass, packed with 1% SP-1000 on Carbopack B (60/80 mesh) or equivalent. This column was used to develop the method performance statements in Section 12. Guidelines for the use of alternate column packings are provided in Section 10.1.

5.3.2 Column 2—6 ft long × 0.1 in ID stainless steel or glass, packed with chemically bonded n-octane on Porasil-C (100/120) mesh or equivalent.

5.3.3 Detector—Electrolytic conductivity or microcoulometric. These types of detectors have proven effective in the analysis of wastewaters for the parameters listed in the scope. The electrolytic conductivity detector was used to develop the method performance statements and MDL listed in Tables 1 and 2. Guidelines for the use of alternate detectors are provided in Section 10.1.

5.4 Syringes—5-mL glass hypodermic with Luerlok tip (two each), if applicable to the purging device.

5.5 Micro syringes—25 µL, 0.006 in ID needle.

5.6 Syringe valve—2-way, with Luer ends (three each).

5.7 Syringe—5-mL, gas-tight with shut-off valve.

5.8 Bottle—15-mL, screw cap, with Teflon cap liner.

5.9 Balance—Analytical, capable of accurately weighing 0.0001 g.

6. Reagents

6.1 Reagent water—Reagent water is defined as a water in which an interferent is not observed at the MDL of the parameters of interest.

6.1.1 Reagent water can be generated by passing tap water through a carbon filter bed containing about 1 lb of activated carbon (Filtrasorb-300 or equivalent (Calgon Corp.)).

6.1.2 A water purification system (Millipore Super-Q or equivalent) may be used to generate reagent water.

6.1.3 Reagent water may also be prepared by boiling water for 15 minutes. Subsequently, while maintaining the temperature at 90 °C, bubble a contaminant-free inert gas through the water for one hour. While still hot, transfer the water to a narrow mouth screw-cap bottle and seal with a Teflon-lined septum and cap.

6.2 Sodium thiosulfate—(ACS) Granular.

6.3 Trap Materials

6.3.1 Coconut charcoal (6/10 mesh sieved to 26 mesh), (Barnaby Chaney, CA-580-26 lot # M-2649 or equivalent).

6.3.2 2,6-Diphenylene oxide polymer—Tenax, (60/80 mesh), chromatographic grade or equivalent.

6.3.3 Methyl silicone packing—3% OV-1 on 60/80 mesh Chromosorb-W or equivalent.

6.3.4 Silica gel—35/60 mesh, Davison, grade-15 or equivalent.

6.4 Methyl Alcohol—Pesticide quality or equivalent.

6.5 Stock standard solutions—Stock standard solutions may be prepared from pure standard materials or purchased as certified solutions. Prepare stock standard solutions in methyl alcohol using assayed liquids or gas cylinders as appropriate. Because of the toxicity of some of the organohalides, primary dilutions of these materials should be prepared in a hood. A NIOSH/MESA approved toxic gas respirator should be used when the analyst handles high concentrations of such materials.

6.5.1 Place about 9.8 mL of methyl alcohol into a 10-mL ground glass stoppered volumetric flask. Allow the flask to stand, unstoppered, for about 10 minutes or until all alcohol wetted surfaces have dried. Weigh the flask to the nearest 0.1 mg.

6.5.2 Add the assayed reference material:

6.5.2.1 Liquids—Using a 100-µL syringe, immediately add two or more drops of assayed reference material to the flask, then reweigh. Be sure that the drops fall directly into the alcohol without contacting the neck of the flask.

6.5.2.2 Gases—To prepare standards for any of the six halocarbons that boil below 30 °C (bromomethane, chloroethane, chloromethane, dichlorodifluoromethane, trichlorofluoromethane, vinyl chloride), fill a 5-mL valved gas-tight syringe with the reference standard to the 5.0-mL mark. Lower the needle to 5 mm above the methyl alcohol meniscus. Slowly introduce the reference standard above the surface of the liquid (the heavy gas will rapidly dissolve into the methyl alcohol).

6.5.3 Reweigh, dilute to volume, stopper, then mix by inverting the flask several times. Calculate the concentration in micrograms per microliter from the net gain in weight. When compound purity is assayed to be 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.5.4 Transfer the stock standard solution into a Teflon-sealed screw-cap bottle. Store, with minimal headspace, at −10 to −20 °C and protect from light.

6.5.5 Prepare fresh standards weekly for the six gases and 2-chloroethylvinyl ether. All other standards must be replaced after one month, or sooner if comparison with check standards indicate a problem.

6.6 Secondary dilution standards—Using stock standard solutions, prepare secondary dilution standards in methyl alcohol that contain the compounds of interest, either singly or mixed together. The secondary dilution standards should be prepared at concentrations such that the aqueous calibration standards prepared in Sections 7.3.1 or 7.4.1 will bracket the working range of the analytical system. Secondary dilution standards should be stored with minimal headspace and should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check standards that can be used to determine the accuracy of calibration standards will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, in Cincinnati, Ohio.

7. Calibration

7.1 Assemble a purge and trap device that meets the specifications in Section 5.2. Condition the trap overnight at 180 °C by backflushing with an inert gas flow of at least 20 mL/min. Prior to use, daily condition traps 10 minutes while backflushing at 180 °C.

7.2 Connect the purge and trap device to a gas chromatograph. The gas chromatograph must be operated using temperature and flow rate parameters equivalent to those in Table 1. Calibrate the purge and trap-gas chromatographic system using either the external standard technique (Section 7.3) or the internal standard technique (Section 7.4).

7.3 External standard calibration procedure:

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter by carefully adding 20.0 µL of one or more secondary dilution standards to 100, 500, or 1000 mL of reagent water. A 25-µL syringe with a 0.006 inch ID needle should be used for this operation. One of the external standards should be at a concentration near, but above, the method detection limit (See Table 1) and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector. These aqueous standards can be stored up to 24 hours, if held in sealed vials with zero headspace as described in Section 9.2. If not so stored, they must be discarded after one hour.

7.3.2 Analyze each calibration standard according to Section 10, and tabulate peak height or area responses versus the concentration in the standard. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to concentration (calibration factor) is a constant over the working range (<10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.3.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ± 10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that parameter.

7.4 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples. The compounds recommended for use as surrogate spikes in Section 8.7 have been used successfully as internal standards, because of their generally unique retention times.

7.4.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest as described in Section 7.3.1.

7.4.2 Prepare a spiking solution containing each of the internal standards using the procedures described in Sections 6.5 and 6.6. It is recommended that the secondary dilution standard be prepared at a concentration of 15 µg/mL of each internal standard compound. The addition of 10 µL of this standard to 5.0 mL of sample or calibration standard would be equivalent to 30 µg/L.

7.4.3 Analyze each calibration standard, according to Section 10, adding 10 µL of internal standard spiking solution directly to the syringe (Section 10.4). Tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1.

$$\text{Eq. 1} \quad RF = (A_s C_{is})/(A_{is} C_s)$$

where:

A_s = Response for the parameter to be measured.

A_{is} = Response for the internal standard.

C_{is} = Concentration of the internal standard.

C_s = Concentration of the parameter to be measured.

If the RF value over the working range is a constant (<10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.4.4 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ± 10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in methyl alcohol 500 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a syringe, add 10 µL of the check sample concentrate to each of a minimum of four 5-mL aliquots of reagent water. A representative waste-

water may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 2, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > 2p$ or $|X - R| > 2p$, review potential problem areas and repeat the test.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3.1 Calculate upper and lower control limts for method performance:

Upper Control Limit (UCL) = R + 3s
Lower Control Limit (LCL) = R − 3s

where R and s are calculated as in Section 8.2.3. The UCL and LCL can be used to construct control charts[7] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly.[7]

8.4 The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 11.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Each day, the analyst must demonstrate through the analysis of reagent water, that interferences from the analytical system are under control.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as gas chromatography with a dissimilar column, specific element detector, or mass spectrometer must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

8.7 The analyst should maintain constant surveillance of both the performance of the analytical system and the effectiveness of the method in dealing with each sample matrix by spiking each sample, standard and blank with surrogate halocarbons. A combination of bromochloromethane, 2-bromo-1-chloropropane, and 1,4-dichlorobutane is recommended to encompass the range of the temperature program used in this method. From stock standard solutions prepared as above, add a volume to give 7500 µg of each surrogate to 45 mL of reagent water contained in a 50-mL volumetric flask, mix and dilute to volume (15 ng/µL). If the internal standard calibration procedure is being used, the surrogate compounds may be added directly to the internal standard spiking solution (Section 7.4.2). Add 10 µL of this surrogate spiking solution directly into the 5-mL syringe with every sample and reference standard analyzed. Prepare a fresh surrogate spiking solution on a weekly basis.

9. Sample Collection, Preservation, and Handling

9.1 All samples must be iced or refrigerated from the time of collection until extraction. If the sample contains free or combined chlorine, add sodium thiosulfate preservative (10 mg/40 mL is sufficient for up to 5 ppm Cl_2) to the empty sample bottle just prior to shipping to the sampling site. USEPA methods 330.4 and 330.5 may be used for measurement of residual chlorine.[8] Field test kits are available for this purpose.

9.2 Grab samples must be collected in glass containers having a total volume of at least 25 mL. Fill the sample bottle just to overflowing in such a manner that no air bubbles pass through the sample as the bottle is being filled. Seal the bottle so that no air bubbles are entrapped in it. If preservative has been added, shake vigorously for one minute. Maintain the hermetic seal on the sample bottle until time of analysis.

9.3 All samples must be analyzed within 14 days of collection.

10. Sample Extraction and Gas Chromatography

10.1 Table 1 summarizes the recommended operating conditions for the gas chromatograph. Included in this Table are estimated retention times and method detection limits that can be achieved by this method. An example of the separations achieved by Column 1 is shown in Figure 5. Other packed columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met.

10.2 Calibrate the system daily as described in Section 7.

10.3 Adjust the purge gas (nitrogen or helium) flow rate to 40 mL/min. Attach the trap inlet to the purging device, and set the device to purge. Open the syringe valve located on the purging device sample introduction needle.

10.4 Allow sample to come to ambient temperature prior to introducing it to the syringe. Remove the plunger from a 5-mL syringe and attach a closed syringe valve. Open the sample bottle (or standard) and carefully pour the sample into the syringe barrel to just short of overflowing. Replace the

1/8

syringe plunger and compress the sample. Open the syringe valve and vent any residual air while adjusting the sample volume to 5.0 mL. Since this process of taking an aliquot destroys the validity of the sample for future analysis, the analyst should fill a second syringe at this time to protect against possible loss of data. Add 10.0 μL of the surrogate spiking solution (8.7) and 10.0 μL of the internal standard spiking solution (Section 7.4.2), if applicable, through the valve bore, then close the valve.

10.5 Attach the syringe-syringe valve assembly to the syringe valve on the purging device. Open the syringe valves and inject the sample into the purging chamber.

10.6 Close both valves and purge the sample for 11.0 ± .1 minutes at ambient temperature.

10.7 After the 11-minute purge time, attach the trap to the chromatograph, adjust the device to the desorb mode, and begin to temperature program the gas chromatograph. Introduce the trapped materials to the GC column by rapidly heating the trap to 180 °C while backflushing the trap with an inert gas between 20 and 60 mL/min for four minutes. If rapid heating of the trap cannot be achieved, the gas chromatographic column must be used as a secondary trap by cooling it to 30 °C (subambient temperature, if poor peak geometry or random retention time problems persist) instead of the initial program temperature of 45 °C.

10.8 While the trap is being desorbed into the gas chromatograph, empty the purging chamber using the sample introduction syringe. Wash the chamber with two 5-mL flushes of reagent water.

10.9 After desorbing the sample for four minutes recondition the trap by returning the purge and trap device to the purge mode. Wait 15 seconds then close the syringe valve on the purging device to begin gas flow through the trap. The trap temperature should be maintained at 180 °C. After approximately seven minutes turn off the trap heater and open the syringe valve to stop the gas flow through the trap. When cool the trap is ready for the next sample.

10.10 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size; however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

10.11 If the response for the peak exceeds the working range of the system, prepare a dilution of the sample with reagent water from the aliquot in the second syringe and reanalyze.

11. Calculations

11.1 Determine the concentration of individual compounds in the sample.

11.1.1 If the external standard calibration procedure is used, calculate the concentration of material from the peak response using the calibration curve or calibration factor determined in Section 7.3.2.

11.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.4.3 and equation 2.

Eq. 2.

Concentration μg/L = $(A_s C_{is})/(A_{is})(RF)$

where:

A_s = Response for the parameter to be measured.

A_{is} = Response for the internal standard.

C_s = Concentration of the internal standard.

11.2 Report results in micrograms per liter. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

11.3 For samples processed as part of a set where the spiked sample recovery falls outside of the control limits which were established according to Section 8.3, data for the affected parameters must be labeled as suspect.

12. Method Performance

12.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero.[1] The MDL concentrations listed in Table 1 were obtained using reagent water.[9] Similar results were achieved using representative wastewaters. The MDL actually achieved in a given analysis will vary depending on instrument sensitivity and matrix effects.

12.2 This method is recommended for use in the concentration range from the MDL up to 1000 × MDL. Direct aqueous injection techniques should be used to measure concentration levels above 1000 × MDL.

12.3 In a single laboratory (Monsanto Research), using reagent water and wastewaters spiked at or near background levels, the average recoveries presented in Table 2 were obtained[9]. The standard deviation of the measurement in percent recovery is also included in Table 2[9].

12.4 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. See Appendix A.
2. Bellar, T.A., and Lichtenberg, J.J. *Journal American Water Works Association, 66,* 739, (1974).
3. Bellar, T.A., and Lichtenberg, J.J. "Semi-Automated Headspace Analysis of Drinking Waters and Industrial Waters for Purgeable Volatile Organic Compounds," Proceedings from Symposium on Measurement of Organic Pollutants in Water and Wastewater, American Society for Testing and Materials, STP 686, C.E. Van Hall, editor, 1978.
4. "Carcinogens—Working With Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.
5. "OSHA Safety and Health Standards, General Industry," (29 CFR 1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
6. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.
7. "Handbook for Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory—Cincinnati, Ohio 45268, March 1979.
8. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600/4-79-020, U.S. Environmental Protection Agency,

749

Environmental Monitoring and Support Laboratory—Cincinnati, Ohio 45268, March 1979.
9. "EPA Method Validation Study 23, Method 601 (Purgeable Halocarbons)," Report for EPA Contract 68-03-2856 (In preparation).

Table 1. Chromatographic Conditions and Method Detection Limits

Parameter	Retention Time (min.) Column 1	Retention Time (min.) Column 2	Method Detection Limit µg/L
Chloromethane	1.50	5.28	0.08
Bromomethane	2.17	7.05	1.18
Dichlorodifluoromethane	2.62	nd	1.81
Vinyl chloride	2.67	5.28	0.18
Chloroethane	3.33	8.68	0.52
Methylene chloride	5.25	10.1	0.25
Trichlorofluoromethane	7.18	nd	nd
1,1-Dichloroethene	7.93	7.72	0.13
1,1-Dichloroethane	9.30	12.6	0.07
trans-1,2-Dichloroethene	10.1	9.38	0.10
Chloroform	10.7	12.1	0.05
1,2-Dichloroethane	11.4	15.4	0.03
1,1,1-Trichloroethane	12.6	13.1	0.03
Carbon tetrachloride	13.0	14.4	0.12
Bromodichloromethane	13.7	14.6	0.10
1,2-Dichloropropane	14.9	16.6	0.04
trans-1,3-Dichloropropene	15.2	16.6	0.34
Trichloroethene	15.8	13.1	0.12
Dibromochloromethane	16.5	16.6	0.09
1,1,2-Trichloroethane	16.5	18.1	0.02
cis-1,3-Dichloropropene	16.5	18.0	0.20
2-Chloroethylvinyl ether	18.0	nd	0.13
Bromoform	19.2	19.2	0.20
1,1,2,2-Tetrachloroethane	21.6	nd	0.03
Tetrachloroethene	21.7	15.0	0.03
Chlorobenzene	24.2	18.8	0.25
1,3-Dichlorobenzene	34.0	22.4	0.32
1,2-Dichlorobenzene	34.9	23.5	0.15
1,4-Dichlorobenzene	35.4	22.3	0.24

nd = not determined

Column 1 conditions: Carbopack B 60/80 mesh coated with 1% SP-1000 packed in an 8 ft × 0.1 in ID stainless steel or glass column with helium carrier gas at 40 mL/min flow rate. Column temperature held at 45 °C for 3 min. then programmed at 8 °C/min. to 220° and held for 15 min.

Column 2 conditions: Porasil-C 100/120 mesh coated with n-octane packed in a 6 ft × 0.1 in ID stainless steel or glass column with helium carrier gas at 40 mL/min flow rate. Column temperature held at 50 °C for 3 min then programmed at 6 °C/min to 170° and held for 4 min.

250

Table 2. Single Operator Accuracy and Precision

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
Bromodichloromethane	100.9	5.0	0.43-46.7	21	3
Bromoform	89.5	9.0	1.45-50	20	3
Bromomethane	105.0	17.3	3.39-49.2	21	3
Carbon tetrachloride	82.5	25.6	0.55-50	19	3
Chlorobenzene	93.9	8.9	2.21-50	20	3
Chloroethane	91.5	22.4	3.95-50	21	3
2-Chloroethylvinyl ether	96.3	9.9	4.39-133	20	3
Chloroform	101.7	20.6	0.44-50	20	3
Chloromethane	91.4	13.4	0.55-23.9	21	3
Dibromochloromethane	98.3	6.5	0.75-93.0	21	3
1,2-Dichlorobenzene	10.20	2.0	4.89-154	21	3
1,3-Dichlorobenzene	91.6	4.3	2.94-46.7	21	3
1,4-Dichlorobenzene	97.5	9.3	2.99-51.6	21	3
Dichlorodifluoromethane	87.8	18.0	2.18-43.4	21	3
1,1-Dichloroethane	102.3	5.5	0.44-46.7	21	3
1,2-Dichloroethane	97.8	4.8	0.44-46.7	21	3
1,1-Dichloroethene	101.1	21.7	0.37-50	19	3
trans-1,2-Dichloroethene	91.0	19.3	0.44-98.0	20	3
1,2-Dichloropropane	97.7	8.8	0.29-39.0	21	3
cis-1,3-Dichloropropene	86.7	6.0	0.44-46.7	21	3
trans-1,3-Dichloropropene	73.5	17.2	0.43-50	20	3
Methylene chloride	97.9	2.6	0.73-46.7	21	3
1,1,2,2-Tetrachloroethane	91.9	15.0	0.46-46.7	21	3
Tetrachloroethene	94.1	18.1	0.50-35.0	21	3
1,1,1-Trichloroethane	75.1	12.5	0.37-29.0	21	3
1,1,2-Trichloroethane	91.0	25.1	0.45-50	21	3
Trichloroethene	106.1	7.4	0.38-46.7	21	3
Trichlorofluoromethane	89.3	13.9	149	14	2
Vinyl chloride	101.9	11.4	0.82-32.3	21	3

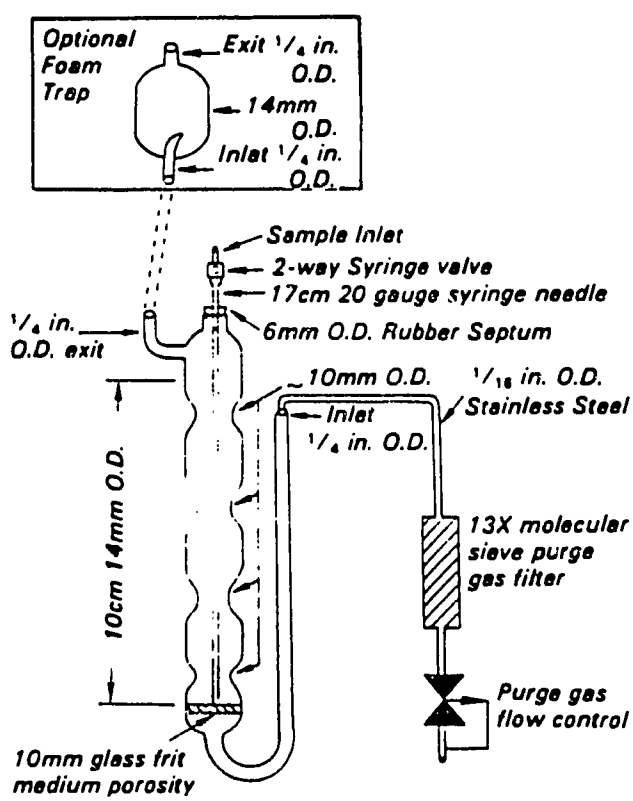


Figure 1. *Purging device*

751

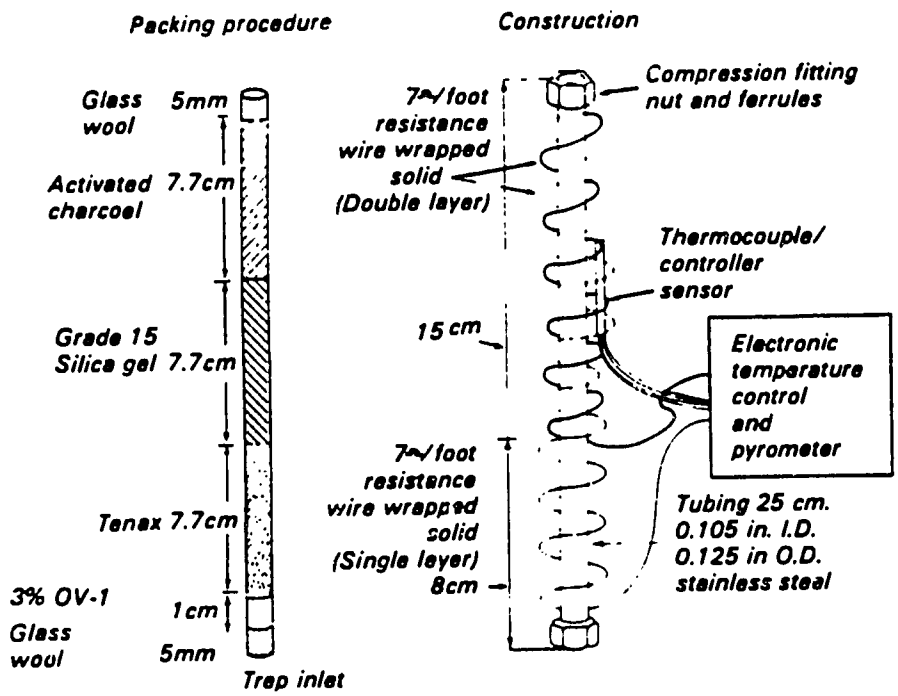


Figure 2. *Trap packings and construction to include desorb capability*

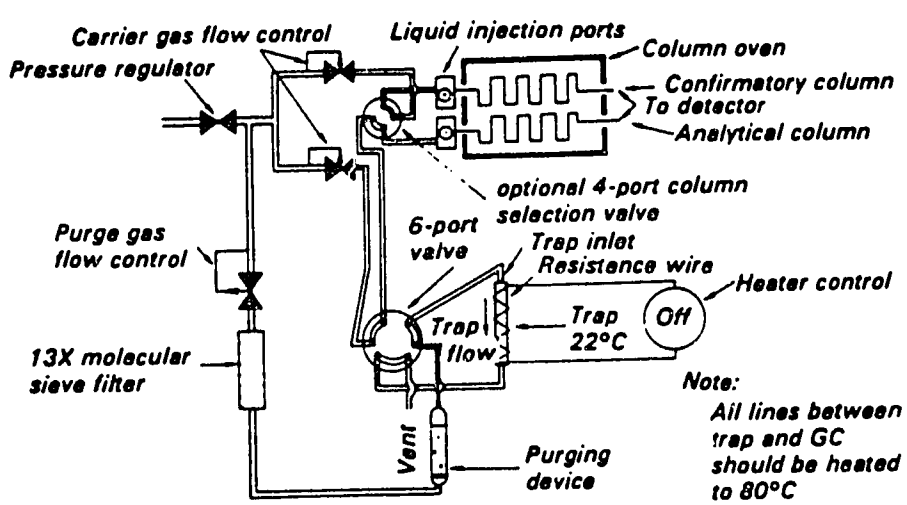


Figure 3. *Schematic of purge and trap device — purge mode*

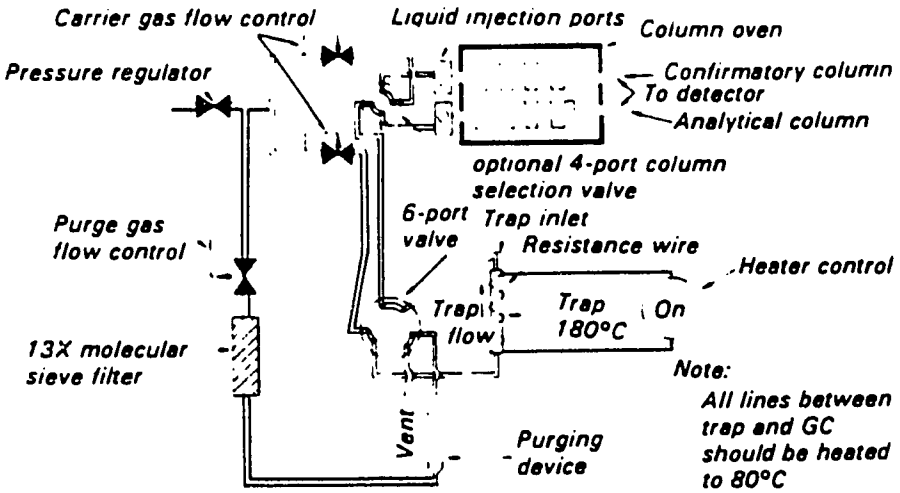


Figure 4. *Schematic of purge and trap device — desorb mode*

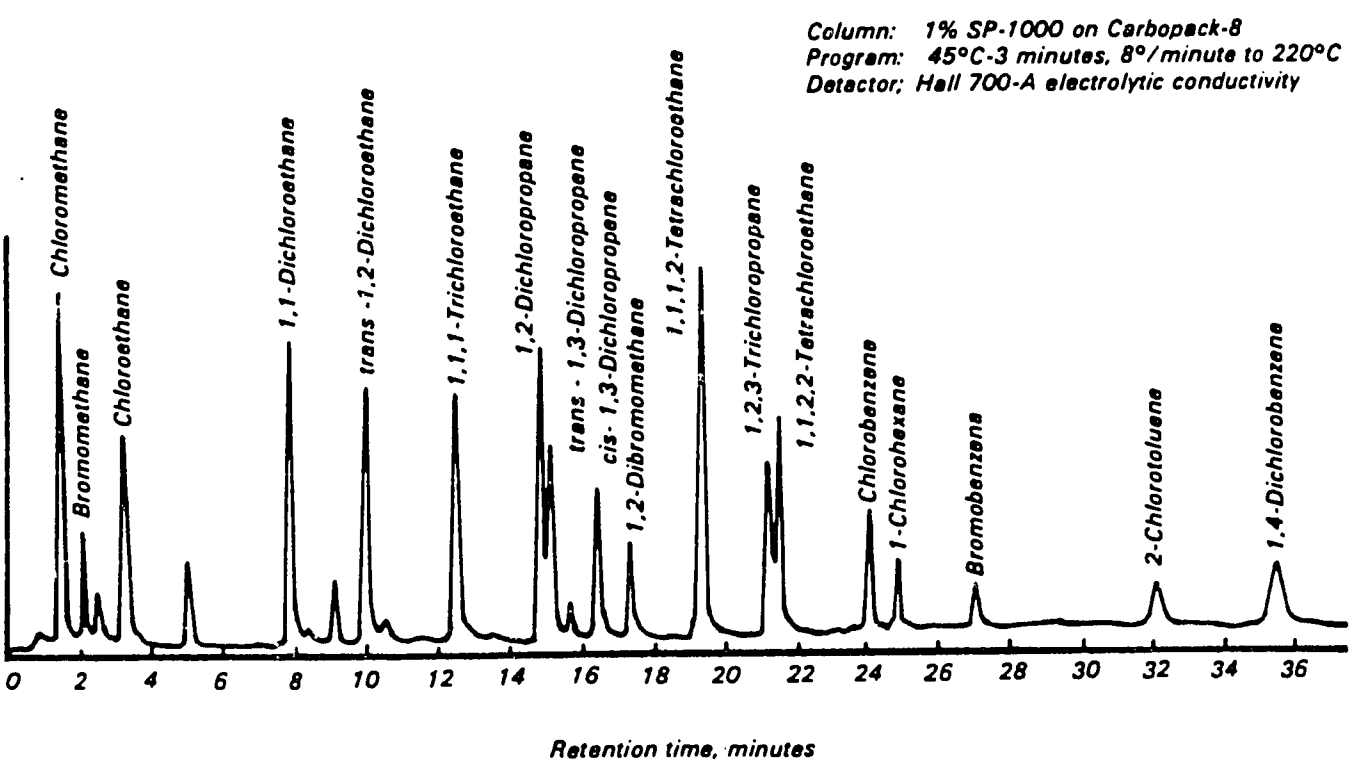


Figure 5. *Gas chrometogram of purgeable halocarbons*

252

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Purgeable Aromatics—Method 602

1. Scope and Application

1.1 This method covers the determination of various purgeable aromatics. The following parameters may be determined by this method:

Parameter	STORET No.	CAS No.
Benzene	34030	71-43-2
Chlorobenzene	34301	108-90-7
1,2-Dichlorobenzene	34536	95-50-1
1,3-Dichlorobenzene	34566	541-73-1
1,4-Dichlorobenzene	34571	106-46-7
Ethylbenzene	34371	100-41-4
Toluene	34010	108-88-3

1.2 This is a purge and trap gas chromatographic method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for any or all of the compounds above, compound identifications should be supported by at least one additional qualitative technique. This method describes analytical conditions for a second gas chromatographic column that can be used to confirm measurements made with the primary column. Method 624 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results for all of the parameters listed above.

1.3 The method detection limit (MDL, defined in Section 12.1[1]) for each parameter is listed in Table 1. The MDL for a specific wastewater may differ from these listed depending upon the nature of interferences in the sample matrix.

1.4 Any modification of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval for alternate test procedures under 40 CFR 136.4 and 136.5

1.5 This method is restricted to use by or under the supervision of analysts experienced in the operation of a purge and trap system and a gas chromatograph and in the interpretation of chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 An inert gas is bubbled through a 5-mL water sample contained in a specially-designed purging chamber at ambient temperature. The aromatics are efficiently transferred from the aqueous phase to the vapor phase. The vapor is swept through a sorbent trap where the aromatics are trapped. After

254

purging is completed, the trap is heated and backflushed with the inert gas to desorb the aromatics onto a gas chromatographic column. The gas chromatograph is temperature programmed to separate the aromatics which are then detected with a photoionization detector[2,3].

2.2 The method provides an optional gas chromatographic column that may be helpful in resolving the compounds of interest from interferences that may occur.

3. Interferences

3.1 Impurities in the purge gas and organic compounds out-gassing from the plumbing ahead of the trap account for the majority of contamination problems. The analytical system must be demonstrated to be free from contamination under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5. The use of non-TFE plastic tubing, non-TFE thread sealants, or flow controllers with rubber components in the purging device should be avoided.

3.2 Samples can be contaminated by diffusion of volatile organics through the septum seal into the sample during shipment and storage. A field reagent blank prepared from reagent water and carried through the sampling and handling protocol can serve as a check on such contamination.

3.3 Contamination by carry-over can occur whenever high level and low level samples are sequentially analyzed. To reduce carry-over, the purging device and sample syringe must be rinsed with reagent water between sample analyses. Whenever an unusually concentrated sample is encountered, it should be followed by an analysis of reagent water to check for cross contamination. For samples containing large amounts of water-soluble materials, suspended solids, high boiling compounds or high aromatic levels, it may be necessary to wash out the purging device with a detergent solution, rinse it with distilled water, and then dry it in an oven at 105 °C between analyses. The trap and other parts of the system are also subject to contamination; therefore, frequent bakeout and purging of the entire system may be required.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[4-6] for the information of the analyst.

4.2 The following parameters covered by this method have been tentatively classified as known or suspected, human or mammalian carcinogens: benzene and 1,4-dichlorobenzene. Primary standards of these toxic compounds should be prepared in a hood. An NIOSH/MESA approved toxic gas respirator should be worn when the analyst handles high concentrations of these toxic compounds.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete sampling.

5.1.1 Vial—25-mL capacity or larger, equipped with a screw cap with hole in center (Pierce #13075 or equivalent). Detergent wash, rinse with tap and distilled water, and dry at 105 °C before use.

5.1.2 Septum—Teflon-faced silicone (Pierce #12722 or equivalent). Detergent wash, rinse with tap and distilled water, and dry at 105 °C for one hour before use.

5.2 Purge and trap device—The purge and trap device consists of three separate pieces of equipment: the sample purger, trap, and the desorber. Several complete devices are now commercially available.

5.2.1 The sample purger must be designed to accept 5-mL samples with a water column at least 3 cm deep. The gaseous head space between the water column and the trap must have a total volume of less than 15 mL. The purge gas must pass through the water column as finely divided bubbles with a diameter of less than 3 mm at the origin. The purge gas must be introduced no more than 5 mm from the base of the water column. The sample purger, illustrated in Figure 1, meets these design criteria.

5.2.2 The trap must be at least 25 cm long and have an inside diameter of at least 0.105 inch.

5.2.2.1 The trap is packed with 1 cm of methyl silicone and 23 cm 2,6-diphenylene oxide polymer as shown in Figure 2. This trap was used to develop the method performance statements in Section 12.

5.2.2.2 Alternatively, either of the two traps described in Method 601 may be used, although water vapor will preclude the measurement of low concentrations of benzene.

5.2.3 The desorber must be capable of rapidly heating the trap to 180 °C. The polymer section of the trap should not be heated higher than 180 °C and the remaining sections should not exceed 200 °C. The desorber design, illustrated in Figure 2, meets these criteria.

5.2.4 The purge and trap device may be assembled as a separate unit or be coupled to a gas chromatograph as illustrated in Figures 3, 4, and 5.

5.3 Gas chromatograph—Analytical system complete with a temperature programmable gas chromatograph suitable for on-column injection and all required accessories including syringes, analytical columns, gases, detector, and stripchart recorder. A data system is recommended for measuring peak areas.

5.3.1 Column 1—6 ft long × 0.082 in ID stainless steel or glass, packed with 5% SP-1200 and 1.75% Bentone-34 on Supelcoport (100/120 mesh) or equivalent. This column was used to develop the method performance statements and the MDLs listed in Tables 1 and 2. Guidelines for the use of alternate column packings are provided in Section 10.1. .

5.3.2 Column 2—8 ft long × 0.1 in ID stainless steel or glass, packed with 5% 1,2,3-Tris(2-cyanoethoxy)propane on Chromosorb W-AW (60/80 mesh) or equivalent.

5.3.3 Detector—Photoionization detector (h-nu Systems, Inc. Model PI-51-02 or equivalent). This type of detector has been proven effective in the analysis of wastewaters for the parameters listed in the scope, and was used to develop the performance statements in Section 12. Guidelines for the use of alternate detectors are provided in Section 10.1.

5.4 Syringes—5-mL glass hypodermic with Luerlok tip (two each), if applicable to the purge device.

255

5.5 Micro syringes—25 µL, 0.006 in ID needle.

5.6 Syringe valve—2-way, with Luer ends (three each).

5.7 Bottle—15-mL screw-cap with Teflon cap liner.

5.8 Balance—Analytical, capable of accurately weighing 0.0001 g.

6. Reagents

6.1 Reagent water—Reagent water is defined as a water in which an interferent is not observed at the MDL of the parameters of interest.

6.1.1 Reagent water can be generated by passing tap water through a carbon filter bed containing about 1 lb. of activated carbon. (Filtrasorb-300 or equivalent (Calgon Corp.)).

6.1.2 A water purification system (Millipore Super-Q or equivalent) may be used to generate reagent water.

6.1.3 Reagent water may also be prepared by boiling water for 15 minutes. Subsequently, while maintaining the temperature at 90 °C, bubble a contaminant-free inert gas through the water for one hour. While still hot, transfer the water to a narrow mouth screw-cap bottle and seal with a Teflon-lined septum and cap.

6.2 Sodium thiosulfate—(ACS) Granular.

6.3 Hydrochloric acid (1 + 1)—Add 50 mL of concentrated HCl to 50 mL of reagent water.

6.4 Trap Materials

6.4.1 2,6-Diphenylene oxide polymer-Tenax, (60/80 mesh) chromatographic grade or equivalent.

6.4.2 Methyl silicone—3% OV-1 on Chromosorb-W (60/80 mesh) or equivalent.

6.5 Methyl alcohol—Pesticide quality or equivalent.

6.6 Stock standard solutions—Stock standard solutions may be prepared from pure standard materials or purchased as certified solutions. Prepare stock standard solutions in methyl alcohol using assayed liquids. Because benzene and 1,4-dichlorobenzene are suspected carcinogens, primary dilutions of these materials should be prepared in a hood.

6.6.1 Place about 9.8 mL of methyl alcohol into a 10-mL ground glass stoppered volumetric flask. Allow the flask to stand, unstoppered, for about 10 minutes or until all alcohol wetted surfaces have dried. Weigh the flask to the nearest 0.1 mg.

6.6.2 Using a 100-µL syringe, immediately add two or more drops of assayed reference material to the flask, then reweigh. Be sure that the drops fall directly into the alcohol without contacting the neck of the flask.

6.6.3 Reweigh, dilute to volume, stopper, then mix by inverting the flask several times. Calculate the concentration in micrograms per microliter from the net gain in weight. When compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used, at any concentration, if they are certified by the manufacturer or by an independent source.

6.6.4 Transfer the stock standard solution into a Teflon-sealed screw-cap bottle. Store at 4 °C and protect from light.

6.6.5 All standards must be replaced after one month, or sooner if comparison with check standards indicate a problem.

6.7 Secondary dilution standards—Using stock standard solutions, prepare secondary dilution standards in methyl alcohol that contain the compounds of interest, either singly or mixed together. The secondary dilution standards should be prepared at concentrations such that the aqueous calibration standards prepared in Sections 7.3.1 or 7.4.1 will bracket the working range of the analytical system. Secondary solution standards must be stored with zero headspace and should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check standards that can be used to determine the accuracy of calibration standards will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, in Cincinnati, Ohio.

7. Calibration

7.1 Assemble a purge and trap device that meets the specifications in Section 5.2. Condition the trap overnight at 180 °C by backflushing with an inert gas flow of at least 20 mL/min. Prior to use, daily condition traps 10 minutes while backflushing at 180 °C.

7.2 Connect the purge and trap device to a gas chromatograph. The gas chromatograph must be operated using temperature and flow rate parameters equivalent to those in Table 1. Calibrate the purge and trap-gas chromatographic system using either the external standard technique (Section 7.3) or the internal standard technique (Section 7.4.).

7.3 External standard calibration procedure:

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter by carefully adding 20.0 µL of one or more secondary dilution standards to 100, 500, or 1000 mL of reagent water. A 25-µL syringe with a 0.006 inch iD needle should be used for this operation. One of the external standards should be at a concentration near, but above, the MDL (see Table 1) and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector. These aqueous standards must be prepared fresh daily.

7.3.2 Analyze each calibration standard according to Section 10, and tabulate peak height or area responses versus the concentration in the standard. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to concentration (calibration factor) is a constant over the working range ($\leq$10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.3.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that parameter.

7.4 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that

256

is applicable to all samples. The compound, α,α,α-trifluorotoluene, recommended as a surrogate spiking compound in Section 8.7 has been used successfully as an internal standard.

7.4.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest as described in Section 7.3.1.

7.4.2 Prepare a spiking solution containing each of the internal standards using the procedures described in Sections 6.6 and 6.7. It is recommended that the secondary dilution standard be prepared at a concentration of 15 µg/mL of each internal standard compound. The addition of 10 µL of this standard to 5.0 mL of sample or calibration standard would be equivalent to 30 µg/L.

7.4.3 Analyze each calibration standard, according to Section 10, adding 10 µL of internal standard spiking solution directly to the syringe as indicated in Section 10.4. Tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1.

$$\text{Eq. 1} \quad RF = (A_s C_{is})/(A_{is} C_s)$$

where:

A_s = Response for the parameter to be measured.
A_{is} = Response for the internal standard.
C_{is} = Concentration of the internal standard.
C_s = Concentration of the parameter to be measured.

If the RF value over the working range is a constant (<10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.4.4 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in methyl alcohol 500 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a syringe, add 10 µL of the check sample concentrate to each of a minimum of four 5-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 2, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > 2p$ or $|X - R| > 2p$, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance:

$$\text{Upper Control Limit (UCL)} = R + 3s$$
$$\text{Lower Control Limit (LCL)} = R - 3s$$

where R and s are calculated as in Section 8.2.3

The UCL and LCL can be used to construct control charts[7] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[7].

8.4 The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results

257

reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 11.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Each day, the analyst must demonstrate through the analysis of reagent water, that interferences from the analytical system are under control.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as gas chromatography with a dissimilar column, specific element detector, or mass spectrometer must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

8.7 The analyst should maintain constant surveillance of both the performence of the analytical system and the effectiveness of the method in dealing with each sample matrix by spiking each sample, standard and blank with surrogate compounds (e.g. α,α,α,-trifluorotoluene). From stock standard solutions prepared as above, add a volume to give 7500 μg of each surrogate to 45 mL of organic-free water contained in a 50-mL volumetric flask, mix and dilute to volume (15 ng/μL). If the internal standard calibration procedure is being used, the surrogate compounds may be added directly to the internal standard spiking solution (Section 7.4.2). Dose 10 μL of this surrogate spiking solution directly into the 5-mL syringe with every sample and reference standard analyzed. Prepare a fresh surrogate spiking solution on a weekly basis.

9. Sample Collection, Preservation, and Handling

9.1 The samples must be iced or refrigerated from the time of collection until extraction. If the sample contains free or combined chlorine, add sodium thiosulfate preservative (10 mg/40 mL is sufficient for up to 5 ppm Cl_2) to the empty sample bottles just prior to shipping to the sampling site. USEPA Methods 330.4 or 330.5 may be used to measure residual chlorine[8]. Field Test Kits are available for this purpose.

9.2 Collect about 500 mL sample in a clean container. Adjust the pH of the sample to about 2 by adding 1 + 1 HCl while stirring gently. Fill the sample bottle in such a manner that no air bubbles pass through the sample as the bottle is being filled. Seal the bottle so that no air bubbles are entrapped in it. Maintain the hermetic seal on the sample bottle until time of analysis.

9.3 All samples must be analyzed within 14 days of collection.[3]

10. Sample Extraction and Gas Chromatography

10.1 Table 1 summarizes the recommended operating conditions for the gas chromatograph. Included in this table are estimated retention times and method detection limits that can be achieved by this method. An example of the separations achieved by Column 1 is shown in Figure 6. Other packed columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met.

10.2 Calibrate the system daily as described in Section 7.

10.3 Adjust the purge gas (nitrogen or helium) flow rate to 40 mL/min. Attach the trap inlet to the purging device, and set the device to purge. Open the syringe valve located on the purging device sample introduction needle.

10.4 Allow sample to come to ambient temperature prior to introducing it into the syringe. Remove the plunger from a 5-mL syringe and attach a closed syringe valve. Open the sample bottle (or standard) and carefully pour the sample into the syringe barrel to just short of overflowing. Replace the syringe plunger and compress the sample. Open the syringe valve and vent any residual air while adjusting the sample volume to 5.0 mL. Since this process of taking an aliquot destroys the validity of the sample for future analysis, the analyst should fill a second syringe at this time to protect against possible loss of data. Add 10.0 μL of the surrogate spiking solution (Section 8.7) and 10.0 μL of the internal standard spiking solution (Section 7.4.2), if applicable, through the valve bore, then close the valve.

10.5 Attach the syringe-syringe valve assembly to the syringe valve on the purging device. Open the syringe valves and inject the sample into the purging chamber.

10.6 Close both valves and purge the sample for 12.0 ± 0.1 minutes at ambient temperature.

10.7 After the 12-minute purge time, disconnect the purge chamber from the trap. Dry the trap by maintaining a flow of 40 mL/min of dry purge gas through it for six minutes. See Figure 4. A dry purger should be inserted into the device to minimize moisture in the gas. Attach the trap to the chromatograph, adjust the device to the desorb mode, and begin to temperature program the gas chromatograph. Introduce the trapped materials to the GC column by rapidly heating the trap to 180 °C while backflushing the trap with an inert gas between 20 and 60 mL/min for four minutes. If rapid heating cannot be achieved, the gas chromatographic column must be used as a secondary trap by cooling it to 30 °C (subambient temperature, if poor peak geometry and random retention time problems persist) instead of the initial program temperature of 50 °C.

10.8 While the trap is being desorbed onto the GC column, empty the purging chamber using the sample introduction syringe. Wash the chamber with two 5-mL flushes of reagent water.

10.9 After desorbing the sample for four minutes, recondition the trap by returning the purge and trap device to the purge mode. Wait 15 seconds then close the syringe valve on the purging device to begin gas flow through the trap. The trap temperature should be maintained at 180 °C. After approximately seven minutes, turn off the trap heater and open the syringe valve to stop the gas flow through the trap. When cool, the trap is ready for the next sample.

10.10 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size; however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

10.11 If the response for the peak exceeds the working range of the system, prepare a dilution of the sample with reagent water from the aliquot in the second syringe and reanalyze.

11. Calculations

11.1 Determine the concentration of individual compounds in the sample.

11.1.1 If the external standard calibration procedure is used, calculate the concentration of material from the peak response using the calibration curve or calibration factor determined in Section 7.3.2.

11.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.4.3 and equation 2.

Eq. 2.

Concentration $\mu g/L = (A_s C_{is})/(A_{is})(RF)$

where:

A_s = Response for the parameter to be measured.

A_{is} = Response for the internal standard.

C_{is} = Concentration of the internal standard.

11.2 Report results in micrograms per liter. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

11.3 For samples processed as part of a set where the spiked sample recovery falls outside of the control limits which were described in Section 8.3, data for the affected parameters must be labeled as suspect.

12. Method Performance

12.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[1]. The MDL concentrations listed in Table 1 were obtained using reagent water[9]. Similar results were achieved using representative wastewaters.

12.2 This method has been demonstrated to be applicable for the concentration range from the MDL up to 1000 × MDL[9]. Direct aqueous injection techniques should be used to measure concentration levels above 1000 × MDL.

12.3 In a single laboratory (Monsanto Research), using reagent water and wastewaters spiked at or near background levels, the average recoveries presented in Table 2 were obtained[9]. The standard deviation of the measurement in percent recovery is also included in Table 2.

12.4 The Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. See Appendix A.
2. Bellar, T.A., and Lichtenberg, J.J. *Journal American Water Works Association, 66,* 739, (1974).
3. Bellar, T.A., and Lichtenberg, J.J. "Semi-Automated Headspace Analysis of Drinking Waters and Industrial Waters for Purgeable Volatile Organic Compunds," Proceedings of Symposium on Measurement of Organic Pollutants in Water and Wastewater. American Society for Testing and Materials, STP 686, C.E. Van Hall, editor, 1978.
4. "Carcinogens—Working with Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health. Publication No. 77-206, August 1977.
5. "OSHA Safety and Health Standards, General Industry," (29 CFR 1910), Occupational Safety and Health Administration, OSHA 2206, (Revised January 1976).
6. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Safety, 3rd Edition, 1979.
7. "Handbook for Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268. March 1979.
8. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600/4-79-020. U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268. March 1979.
9. "EPA Method Validation Study 24, Method 602 (Purgeable Aromatics)," Report for EPA Contract 68-03-2856 (In preparation).

Table 1. *Chromatographic Conditions and Method Detection Limits*

Parameter	Retention Time (min.) Column 1	Retention Time (min.) Column 2	Method Detection Limit µg/L
Benzene	3.33	2.75	0.2
Toluene	5.75	4.25	0.2
Ethylbenzene	8.25	6.25	0.2
Chlorobenzene	9.17	8.02	0.2
1,4-Dichlorobenzene	16.8	16.2	0.3
1,3-Dichlorobenzene	18.2	15.0	0.4
1,2-Dichlorobenzene	25.9	19.4	0.4

Column 1 conditions: Supelcoport 100/120 mesh coated with 5% SP-1200 and 1.75% Bentone-34 packed in a 6 ft. × 0.085 in ID stainless steel column with helium carrier gas at 36 cc/min flow rate. Column temperature held at 50°C for 2 min. then programmed at 6°C/min to 90°C for a final hold.

Column 2 conditions: Chromosorb W-AW 60/80 mesh coated with 5% 1,2,3-Tris(2-cyanoethyoxy)propane packed in a 6 ft. × 0.085 in ID stainless steel column with helium carrier gas at 30 cc/min flow rate. Column temperature held at 40°C for 2 min then programmed at 2°C/min to 100°C for a final hold.

Table 2. *Single Operator Accuracy and Precision*

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
Benzene	91	10.0	0.5-9.7	21	3
Chlorobenzene	97	9.4	0.5-100	21	3
1,2-Dichlorobenzene	104	27.7	0.5-10.0	21	3
1,3-Dichlorobenzene	97	20.0	0.5-4.8	21	3
1,4-Dichlorobenzene	120	20.4	0.5-10.0	21	3
Ethylbenzene	98	12.4	0.5-9.9	21	3
Toluene	77	12.1	0.5-100	21	3

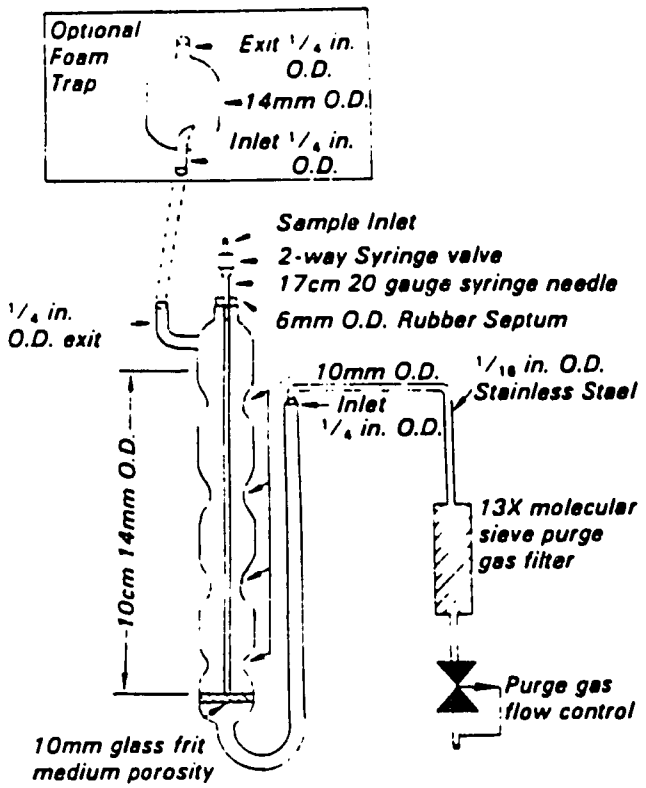


Figure 1. *Purging device*

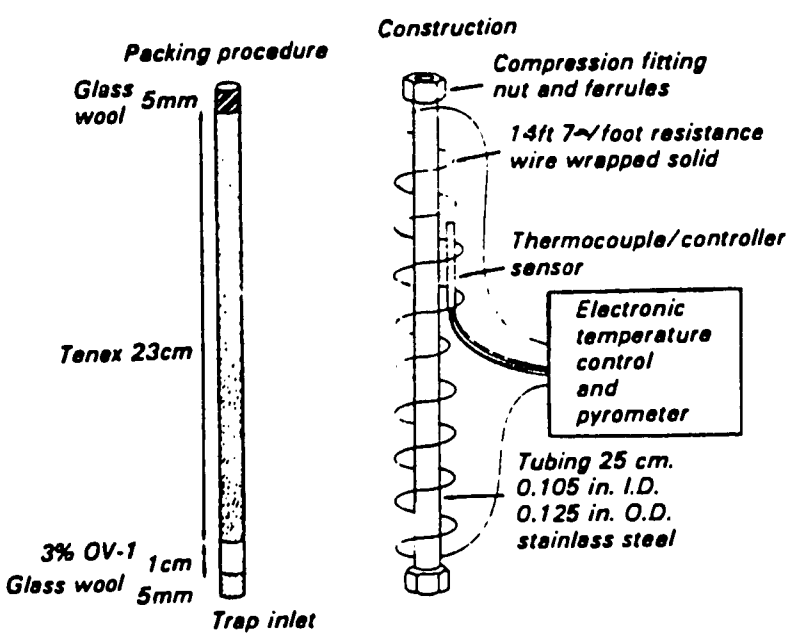


Figure 2. *Trap packings and construction to include desorb capability*

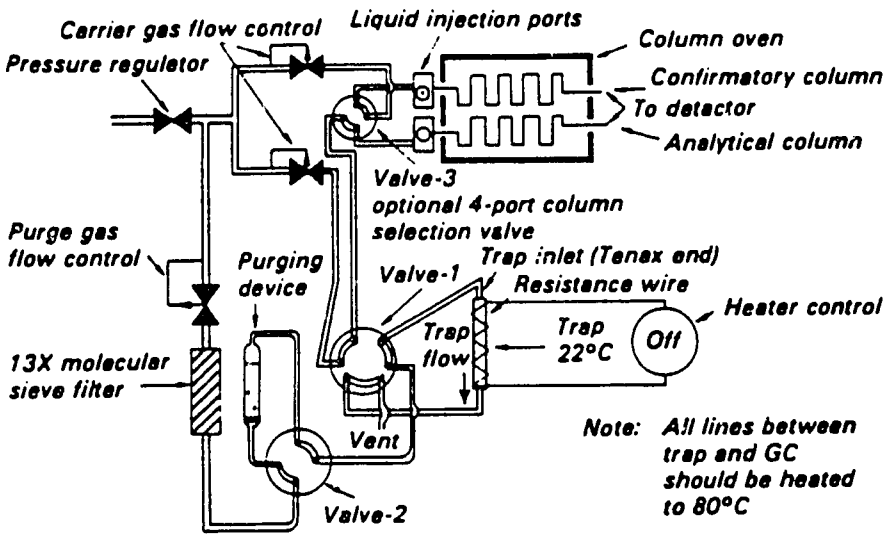


Figure 3. *Purge-trap system (Purge-sorb Mode)*

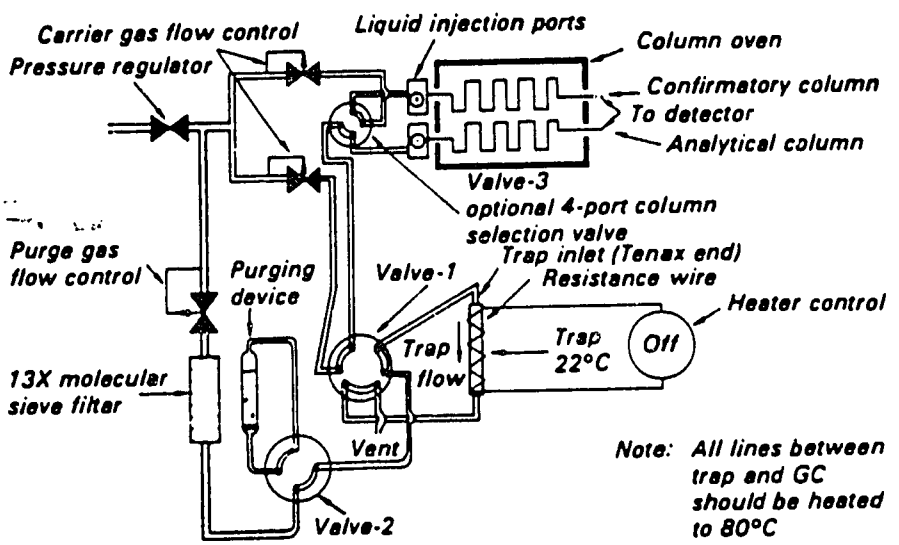


Figure 4. *Purge-trap system (Trap-dry Mode).*

262

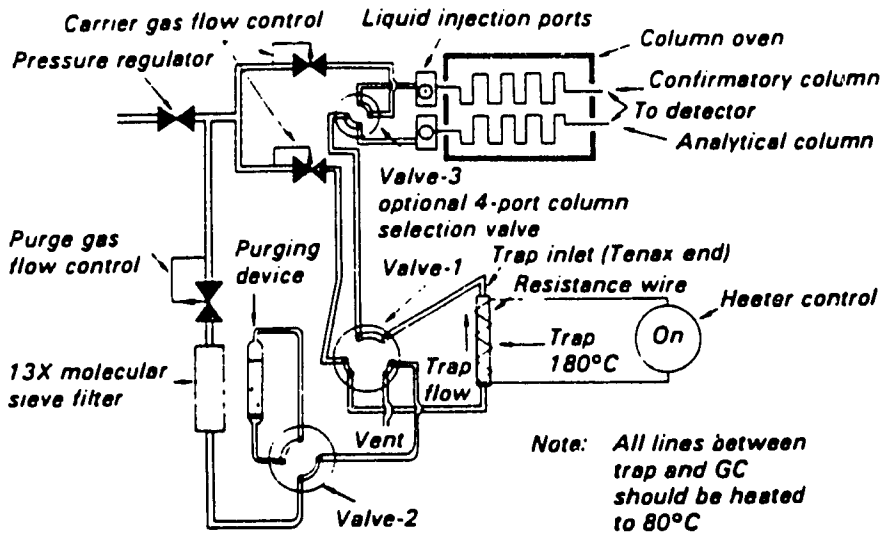


Figure 5. *Purge-trap system (Desorb Mode).*

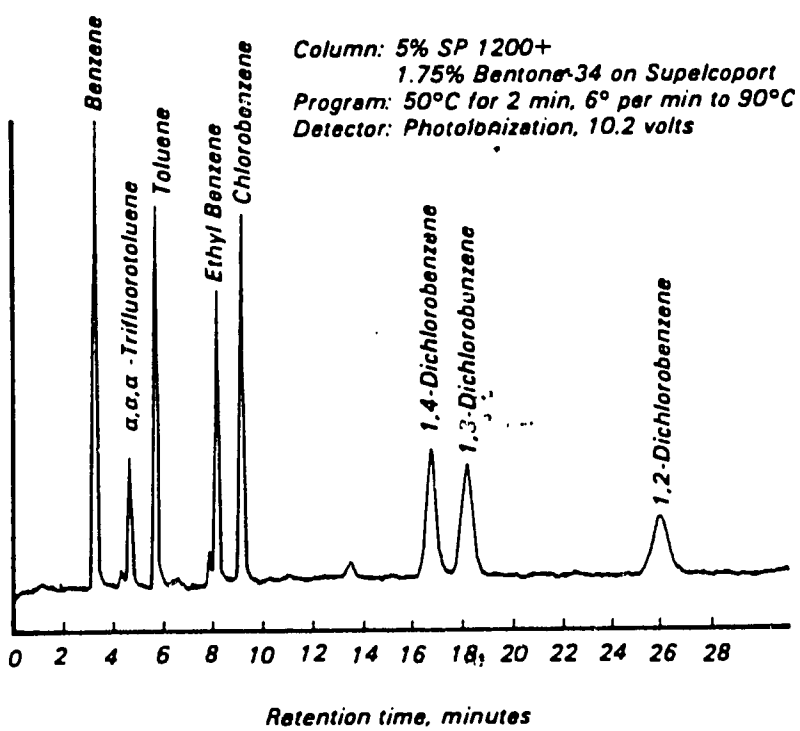


Figure 6. *Gas chromatogram of purgeable aromatics.*

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Acrolein and Acrylonitrile—Method 603

1. Scope and Application

1.1 This method covers the determination of acrolein and acrylonitrile. The following parameters may be determined by this method:

Parameter	STORET No.	CAS No.
Acrolein	34210	107-02-8
Acrylonitrile	34215	107-13-1

1.2 This is a purge and trap gas chromatographic method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for either or both of the compounds above, compound identifications should be supported by at least one additional qualitative technique. This method describes analytical conditions for a second gas chromatographic column that can be used to confirm measurements made with the primary column. Method 624 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results for the parameters listed above, if used with the purge and trap conditions described in this method.

1.3 The method detection limit (MDL, defined in Section 12.1)[1] for each parameter is listed in Table 1. The MDL for a specific wastewater may differ from those listed, depending upon the nature of interferences in the sample matrix.

1.4 Any modification of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval of alternate test procedures under 40 CFR 136.4 and 136.5

1.5 This method is restricted to use by or under the supervision of analysts experienced in the operation of a purge and trap system and a gas chromatograph (GC) and in the interpretation of GC chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 An inert gas is bubbled through a 5-mL water sample contained in a heated purging chamber. Acrolein and acrylonitrile are transferred from the aqueous phase to the vapor phase. The vapor is swept through a sorbent trap where the analytes are trapped. After the purge is completed, the trap is heated and backflushed with the inert gas to desorb the compounds onto a gas chromatographic column. The gas chromatograph is temperature

264

programmed to separate the analytes which are then detected with a flame ionization detector[2,3].

2.2 The method provides an optional gas chromatographic column that may be helpful in resolving the compounds of interest from the interferences that may occur.

3. Interferences

3.1 Impurities in the purge gas and organic compounds out-gassing from the plumbing ahead of the trap account for the majority of contamination problems. The analytical system must be demonstrated to be free from contamination under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5. The use of non-TFE plastic tubing, non-TFE thread sealants, or flow controllers with rubber components in the purging device should be avoided.

3.2 Samples can be contaminated by diffusion of volatile organics through the septum seal into the sample during shipment and storage. A field reagent blank prepared from reagent water and carried through the sampling and handling protocol can serve as a check on such contamination.

3.3 Contamination by carry-over can occur whenever high level and low level samples are sequentially analyzed. To reduce carry-over, the purging device and sample syringe must be rinsed out between samples with reagent water. Whenever an unusually concentrated sample is encountered, it should be followed by an analysis of reagent water to check for cross contamination. For samples containing large amounts of water-soluble materials, suspended solids, high boiling compounds or high analyte levels, it may be necessary to wash out the purging device with a detergent solution, rinse it with distilled water, and then dry it in a 105 °C oven between analyses. The trap and other parts of the system are also subject to contamination, therefore, frequent bakeout and purging of the entire system may be required.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[4-6] for the information of the analyst.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete sampling.

5.1.1 Vial—25-mL capacity or larger, equipped with a screw cap with a hole in the center (Pierce #13075 or equivalent). Detergent wash, rinse with tap and distilled water and dry at 105 °C before use.

5.1.2 Septum—Teflon-faced silicone (Pierce #12722 or equivalent). Detergent wash, rinse with tap and distilled water, and dry at 105 °C for one hour before use.

5.2 Purge and trap device—The purge and trap device consists of three separate pieces of equipment: the sample purger, the trap, and the desorber. Several complete devices are now commercially available.

5.2.1 The sample purger must be designed to accept 5-mL samples with a water column at least 3 cm deep. The gaseous head space between the water column and the trap must have a total volume of less than 15 mL. The purge gas must pass through the water column as finely divided bubbles with a diameter of less than 3 mm at the origin. The purge gas must be introduced no more than 5 mm from the base of the water column. The purge device must be capable of being heated to 85 °C within 3.0 minutes after transfer of the sample to the purge device and being held at 85 ± 2 °C during purge cycle. The entire water column in the purge device must be heated. Design of this modification to the standard purge device is optional, however, use of a water bath is suggested.

5.2.1.1 Heating mantle—To be used to heat water bath.

5.2.1.2 Temperature controller—equipped with thermocouple/sensor to accurately control water bath temperature to ± 2 °C. The sample purger illustrated in Figure 1 meets these design criteria.

5.2.2 The trap must be at least 25 cm long and have an inside diameter of at least 0.105 inch. The trap must be packed to contain 1.0 cm of methyl silicone coated packing (Section 6.5.2) and 23 cm of 2,6-diphenylene oxide polymer (Section 6.5.1). The minimum specifications for the trap are illustrated in Figure 2.

5.2.3 The desorber must be capable of rapidly heating the trap to 100 °C. The polymer section of the trap should not be heated higher than 180 °C. The desorber, illustrated in Figure 2, meets these design criteria.

5.2.4 The purge and trap device may be assembled as a separate unit as illustrated in Figure 3 or be coupled to a gas chromatograph.

5.3 pH paper—Narrow pH range, about 3.5 to 5.5 (Fisher Scientific Short Range Alkacid No. 2, #14-837-2 or equivalent).

5.4 Gas chromatograph—An analytical system complete with a temperature programmable gas chromatograph suitable for on-column injection and all required accessories including syringes, analytical columns, gases, detector, and strip-chart recorder. A data system is recommended for measuring peak areas.

5.4.1 Column 1—6 ft long × 0.1 in ID stainless steel or glass, packed with Durapak-Carbowax 400/Porasil-C (100/120 mesh) or equivalent. This column was used to develop the method performance statements given in Section 12. Guidelines for the use of alternate column packings are provided in Section 10.1.

5.4.2 Column 2—6 ft long × 0.1 in ID stainless steel or glass, packed with Chromosorb 101 (60/80 mesh) or equivalent.

5.4.3 Detector—Flame ionization. This type of detector has proven effective in the analysis of wastewaters for the parameters listed in the scope, and was used to develop the method performance statements in Section 12. Guidelines for the use of alternate detectors are provided in Section 10.1.

5.5 Syringes—5-mL, glass hypodermic with Luerlok tip (two each).

5.6 Micro syringes—25 µL.

5.7 Syringe valve, 2-way with Luer ends (three each).

5.8 Bottle—15-mL screw-cap with Teflon cap liner.

5.9 Balance—Analytical, capable of accurately weighing 0.0001 g.

6. Reagents

6.1 Reagent water—Reagent water is defined as a water in which an interferent is not observed at the MDL of the parameters of interest.

6.1.1 Reagent water can be generated by passing tap water through a carbon filter bed containing about one pound of activated carbon (Filtrasorb-300 or equivalent, Calgon Corp).

6.1.2 A water purification system (Millipore Super-Q or equivalent) may be used to generate reagent water.

6.1.3 Reagent water may also be prepared by boiling water for 15 minutes. Subsequently, while maintaining the temperature at 90 °C, bubble a contaminant-free inert gas through the water for one hour. While still hot, transfer the water to a narrow mouth screw cap bottle and seal with a Teflon lined septum and cap.

6.2 Sodium thiosulfate—(ACS) Granular.

6.3 Sodium hydroxide solution (10 N)—Dissolve 40 g NaOH in reagent water and dilute to 100 mL.

6.4 Hydrochloric acid solution (1 + 1)—Slowly add 50 mL concentrated HCl to 50 mL reagent water.

6.5 Trap Materials

6.5.1 2,6-Diphenylene oxide polymer, Tenax (60/80 mesh) chromatographic grade.

6.5.2 Methyl Silicone packing—3% OV-1 on Chromosorb-W (60/80 mesh) or equivalent.

6.6 Stock standard solutions—Stock standard solutions may be prepared from pure standard materials or purchased as certified solutions. Prepare stock standard solutions in reagent water using assayed liquids. Since acrolein and acrylonitrile are lachrymators, primary dilutions of these compounds should be prepared in a hood. A NIOSH/MESA approved toxic gas respirator should be used when the analyst handles high concentrations of such materials.

6.6.1 Place about 9.8 mL of reagent water into a 10-mL ground glass stoppered volumetric flask. For acrolein standards the reagent water must be adjusted to pH 4 to 5. Weigh the flask to the nearest 0.1 mg.

6.6.2 Using a 100-µL syringe, immediately add two or more drops of assayed reference material to the flask, then reweigh. Be sure that the drops fall directly into the water without contacting the neck of the flask.

6.6.3 Reweigh, dilute to volume, stopper, then mix by inverting the flask several times. Calculate the concentration in micrograms per microliter from the net gain in weight. When compound purity is assayed to be 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards may be used at any concentration if they are certified by the manufacturer or by an independent source.

6.6.4 Transfer the stock standard solution into a Teflon-sealed screw-cap bottle. Store at 4 °C and protect from light.

6.6.5 Prepare fresh standards weekly.

6.7 Secondary dilution standards—Using stock standard solutions, prepare secondary dilution standards in reagent water that contain the compounds of interest, either singly or mixed together. The secondary dilution standards should be prepared at concentrations such that the aqueous calibration standards prepared in Sections 7.3.1 or 7.4.1 will bracket the working range of the analytical system. Secondary dilution standards should be prepared weekly and stored at 4 °C. They should be checked frequently for signs of degradation or evaporation. Quality control check standards that can be used to determine the accuracy of calibration standards will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, in Cincinnati, Ohio.

7. Calibration

7.1 Assemble a purge and trap device that meets the specifications in Section 5.2. Condition the trap overnight at 180 °C by backflushing with an inert gas flow of at least 20 mL/min. Prior to use, daily condition traps 10 minutes while backflushing at 180 °C.

7.2 Connect the purge and trap device to a gas chromatograph. The gas chromatograph must be operated using temperature and flow rate parameters equivalent to those in Table 1. Calibrate the purge and trap-gas chromatographic system using either the external standard technique (Section 7.3) or the internal standard technique (Section 7.4).

7.3 External standard calibration procedure:

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter by carefully adding 20.0 µL of one or more secondary dilution standards to 100, 500, or 1000 mL of reagent water. A 25-µL syringe should be used for this operation. One of the external standards should be at a concentration near, but above, the method detection limit and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector. These standards must be prepared fresh daily.

7.3.2 Analyze each calibration standard according to Section 10, and tabulate peak height or area responses versus the concentration of the standard. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to concentration (calibration factor) is a constant over the working range (<10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.3.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ± 10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that compound.

7.4 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples.

7.4.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest as described in Section 7.3.1.

7.4.2 Prepare a spiking solution containing each of the internal standards using the procedures described in Sections 6.6 and 6.7. It is recommended that the secondary

266

dilution standard be prepared at a concentration of 15 µg/mL of each internal standard compound. The addition of 10 µL of this standard to 5.0 mL of sample or calibration standard would be equivalent to 30 µg/L.

7.4.3 Analyze each calibration standard according to Section 10 adding 10 L of internal standard spiking solution directly to the syringe (Section 10.4). Tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1.

$$\text{Eq. 1 } RF = (A_s C_{is})/(A_{is} C_s)$$

where:

A_s = Response for the parameter to be measured.

A_{is} = Response for the internal standard.

C_{is} = Concentration of the internal standard.

C_s = Concentration of the parameter to be measured.

If the RF value over the working range is a constant (<10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.4.4 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in reagent water 500 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a syringe, add 10 µL of the check sample concentrate to each of a minimum of four 5-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 2, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > 2p$ or $|X - R| > 2p$, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance:

Upper Control Limit (UCL) = R + 3s
Lower Control Limit (LCL) = R − 3s

where R and s are calculated as in Sections 8.2.3.

The UCL and LCL can be used to construct control charts[7] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[7].

8.4 The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 11.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Each day, the analyst must demonstrate through the analysis of reagent water, that interferences from the analytical system are under control.

8.6 It is recommended that the laboratory adopt additional quality

267

assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as gas chromatography with a dissimilar column, specific element detector, or mass spectrometer must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 The samples must be iced or refrigerated from the time of collection until extraction. If the sample contains free or combined chlorine, add sodium thiosulfate preservative (10 mg/40 mL is sufficient for up to 5 ppm Cl_2) to the empty sample bottles just prior to shipping to the sampling site. EPA methods 330.4 and 330.5 may be used for measurement of chlorine residual[8]. Field test kits are available for this purpose.

9.2 If acrolein is to be analyzed, collect about 500 mL sample in a clean glass container. Adjust the pH of the sample to 4 to 5 using acid or base, measuring with narrow range pH paper. Samples for acrolein analysis receiving no pH adjustment must be analyze within three days of sampling.

9.3. Fill a sample bottle just to overflowing in such a manner that no air bubbles pass through the sample as the bottle is being filled. Seal the bottle so that no air bubbles are entrapped in it. If preservative has been added, shake vigorously for one minute. Maintain the hermetic seal on the sample bottle until time of analysis.

9.4 All samples must be analyzed within 14 days of collection[3].

10. Sample Extraction and Gas Chromatography

10.1 Table 1 summarizes the recommended operating conditions for the gas chromatograph. Included in this Table are estimated retention times and MDL that can be achieved by this method. An example of the separations achieved by Column 1 is shown in Figure 4. Other packed columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met.

10.2 Calibrate the system daily as described in Section 7.

10.3 Adjust the purge gas (nitrogen or helium) flow rate to 20 mL/min. Attach the trap inlet to the purging device, and set the device to purge. Open the syringe valve located on the purging device sample introduction needle.

10.4 Remove the plunger from a 5-mL syringe and attach a closed syringe valve. Open the sample bottle (or standard) and carefully pour the sample into the syringe barrel to just short of overflowing. Replace the syringe plunger and compress the sample. Open the syringe valve and vent any residual air while adjusting the sample volume to 5.0 mL. Since this process of taking an aliquot destroys the validity of the sample for future analysis, the analyst should fill a second syringe at this time to protect against possible loss of data. Add 10.0 µL of the internal standard spiking solution (Section 7.4.2), if applicable, through the valve bore then close the valve.

10.5 Attach the syringe-syringe valve assembly to the syringe valve on the purging device. Open the syringe valves and inject the sample into the purging chamber.

10.6 Close both valves and purge the sample for 15.0 ± 0.1 minutes while heating at 85 ± 2 °C.

10.7 After the 15-minute purge time, attach the trap to the chromatograph, and adjust the device to the desorb mode. Begin the temperature program for the gas chromatograph. Introduce the trapped materials to the GC column by rapidly heating the trap to 100 ± 10 °C while backflushing the trap with an inert gas between 20 and 60 mL/min for two minutes.

10.8 While the trap is being desorbed into the gas chromatograph, empty the purging chamber using the sample introduction syringe. Wash the chamber with two 5-mL flushes of reagent water.

10.9 After desorbing the sample for 2.0 minutes recondition the trap by returning the purge and trap device to the purge mode. Wait 15 seconds then close the syringe valve on the purging device to begin gas flow through the trap. The trap temperature should be maintained at 100 °C. After approximately seven minutes turn off the trap heater and open the syringe valve to stop the gas flow through the trap. When cool the trap is ready for the next sample.

10.10 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size; however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

11. Calculations

11.1 Determine the concentration of individual compounds in the sample.

11.1.1 If the external sample calibration procedure is used, calculate the concentration of material from the peak response using the calibration curve or calibration factor determined in Section 7.3.2.

11.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.4.2 and the equation 2:

Eq. 2

Concentration $\mu g/L = (A_s C_{is})/(A_{is})(RF)$

where:

A_s = Response for the parameter to be measured.

A_{is} = Response for the internal standard.

C_{is} = Concentration of the internal standard.

11.2 Report results in micrograms per liter. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

11.3 For samples processed as part of a set where the spiked sample recovery falls outside of the control limits in 8.3, data for the affected parameters must be labeled as suspect.

12. Method Performance

12.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[1]. The MDL concentrations listed in Table 1 were obtained using reagent water[9]. Similar results were achieved using representative wastewaters.

12.2 This method is recommended for the concentration range from the MDL up to 1000 × MDL. Direct aqueous injection techniques should be used to measure concentration levels above 1000 × MDL.

268

12.3 In a single laboratory (EMSL-CI), using spiked wastewater, the average recoveries presented in Table 2 were obtained[3]. Seven replicate spiked samples were analyzed for each parameter. The relative standard deviation of the measurement is also included in Table 2.

12.4 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

Table 1. Chromatographic Conditions and Method Detection Limits

Parameter	Retention Time (min.) Column 1	Retention Time (min.) Column 2	Method Detection Limit µg/L
Acrolein	9.2	8.2	0.6
Acrylonitrile	13.5	9.8	0.5

Method detection limit based upon recovery of 5.0 µg/L dose into tap water.

Column 1 conditions: Durapak Carbowax 400/Porasil C, (100/120 mesh) packed in a 6 ft × 0.1 in. ID stainless steel or glass column with helium carrier gas at 30 mL/min flow rate. Column temperature held at 45 °C for 2 min, then programmed at 8 °C/min to 85 °C and held for 12 min. The column temperature should then be raised to 120 °C for 7 minutes to bake out water. Failure to dry the column may lead to irreproducable retention times[9].

Column 2 conditions: Chromosorb 101, (60/80 mesh) packed in a 6 ft x 0.1 in ID stainless steel, glass column with helium carrier gas at 40 mL/min flow rate. Column temperature held at 80°C for 4 min. then programmed at 15°C/min to 120°C and held 12 min.

Table 2. Single Operator Accuracy and Precision

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
Acrolein	96	11.6	20	7	1
Acrylonitrile	107	5.6	20	7	1

References

1. See Appendix A.
2. Bellar, T.A., and Lichtenberg, J.J. *Journal American Water Works Association, 66*, 739, (1974).
3. Kerns, E.H., et al, "Determination of Acrolein and Acrylonitrile in Water by Heated Purge and Trap Technique," 1980, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.
4. "Carcinogens—Working with Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health. Publication No. 77-206, Aug. 1977.
5. "OSHA Safety and Health Standards, General Industry," (29 CFR 1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
6. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.
7. "Handbook for Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory—Cincinnati, Ohio 45268, March 1979.
8. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600/4-79-020, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory—Cincinnati, Ohio 45268. March 1979.
9. "EPA Method Validation Study 25, Method 603 (Acrolein and Acrylonitrile)," Report for EPA Contract 68-03-2856 (In preparation).

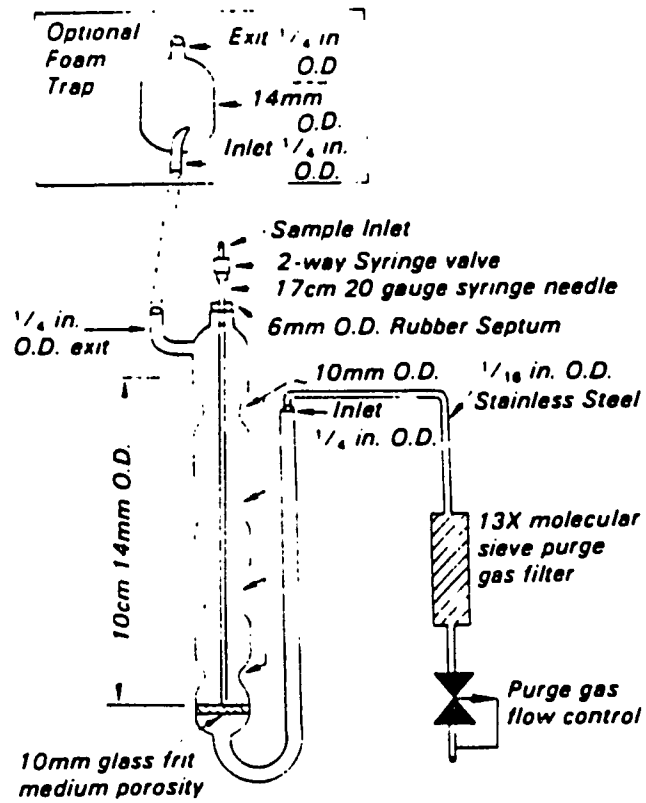


Figure 1. *Purging device*

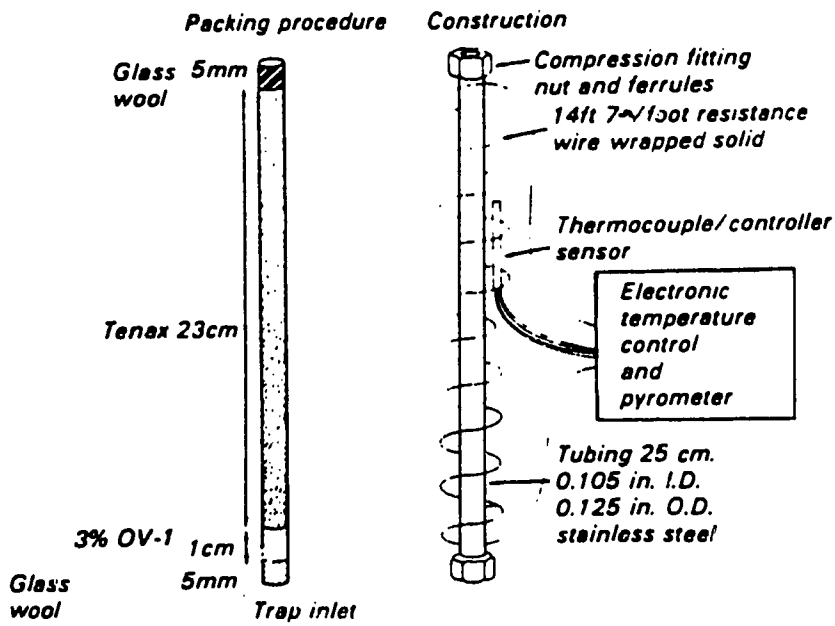
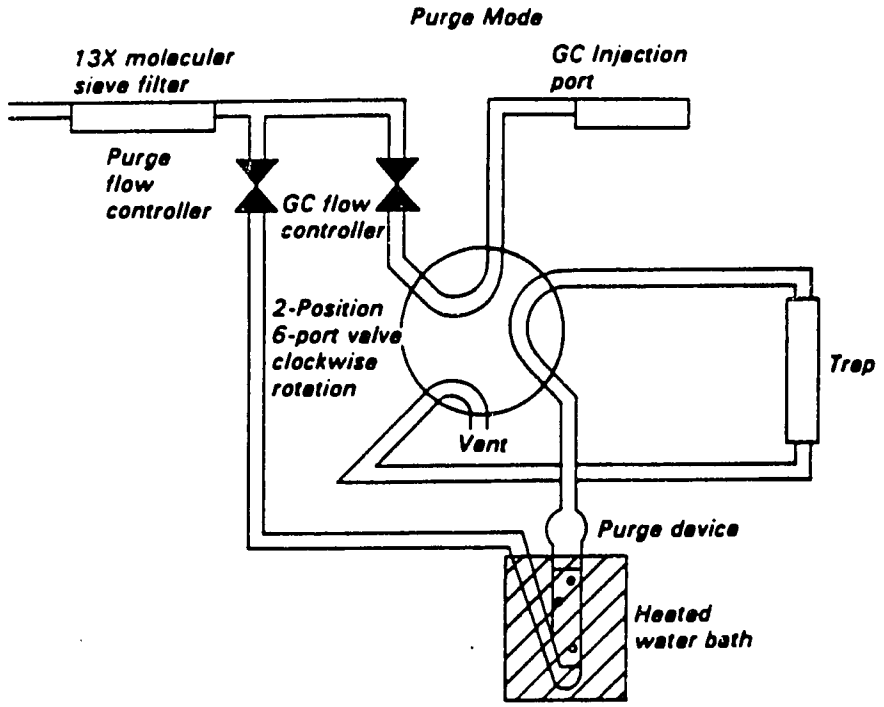


Figure 2. *Trap packings and construction to include desorb capability*

270



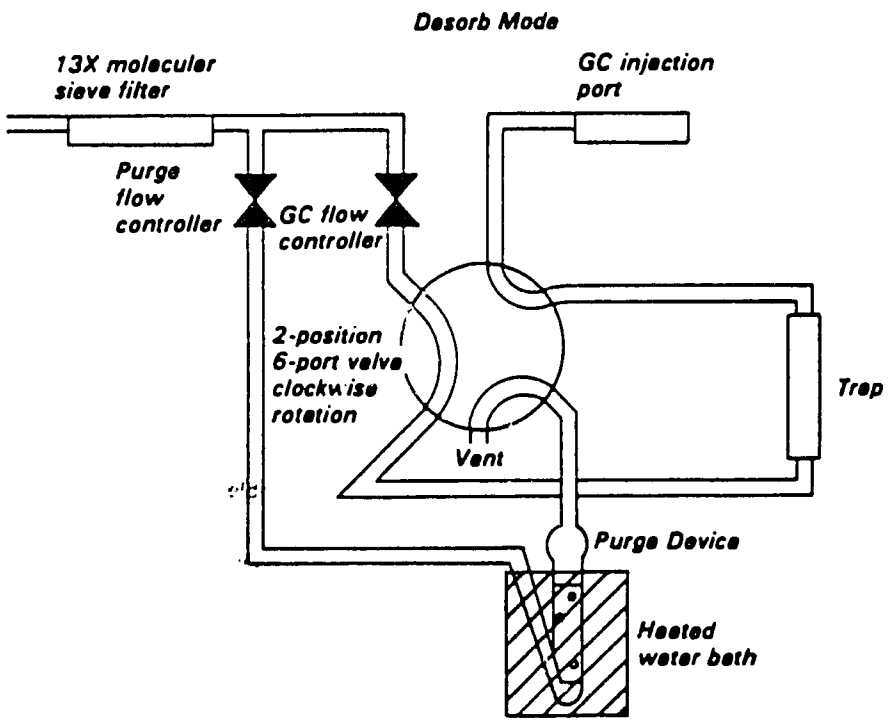


Figure 3. *Schematic of heated purge and trap device*

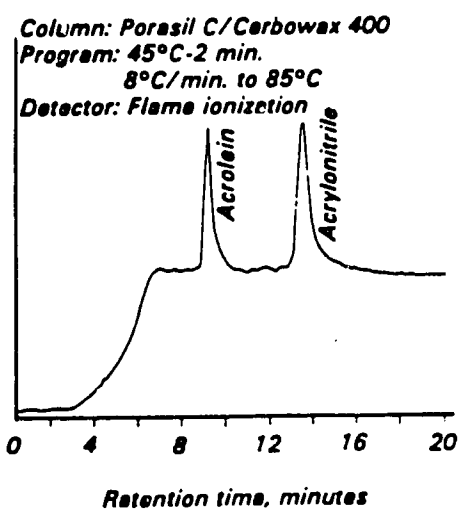


Figure 4. *Gas chromatogram of acrolein and acrylonitrile*

271

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Phenols — Method 604

1. Scope and Application

1.1 This method covers the determination of phenol and certain substituted phenols. The following parameters may be determined by this method:

Parameter	STORET No.	CAS No.
4-Chloro-3-methylphenol	34452	59-50-7
2-Chlorophenol	34586	95-57-8
2,4-Dichlorophenol	34601	120-83-2
2,4-Dimethylphenol	34606	105-67-9
2,4-Dinitrophenol	34616	51-28-5
2-Methyl-4,6-dinitrophenol	34657	534-52-1
2-Nitrophenol	34591	88-75-5
4-Nitrophenol	34646	100-02-7
Pentachlorophenol	39032	87-86-5
Phenol	34694	108-95-2
2,4,6-Trichlorophenol	34621	88-06-2

1.2 This is a gas chromatographic (GC) method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for any or all of the compounds above, compound identifications should be supported by at least one additional qualitative technique. This method describes analytical conditions for derivatization, cleanup and electron capture gas chromatography that can be used to confirm measurements made by flame ionization. Method 625 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results for all of the parameters listed above, using the extract produced by this method.

1.3 The method detection limit (MDL, defined in Section 14.1)[11] for each parameter is listed in Table 1. The MDL for a specific wastewater may differ from those listed, depending upon the nature of interferences in the sample matrix. The MDL listed in Table 1 for each parameter was achieved with a flame ionization detector. Comparable results were achieved when the derivatization cleanup and the electron capture detector were employed (See Table 2).

1.4 Any modification of this method, beyond that expressly permitted, shall be considered a ma, odification subject to application and approval of alternate test procedures under 40 CRF 136.4 and 136.5.

1.5 This method is restricted to use by or under the supervision of analysts experienced in the use of gas chromatography and in the interpretation of gas chromatograms. Each analyst must demonstrate the

272

ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 A 1-liter sample of wastewater is acidified and extracted with methylene chloride using separatory funnel techniques. The extract is dried and concentrated to a volume of 10 mL or less. During the concentration step, the solvent is exchanged to 2-propanol. Flame ionization gas chromatographic conditions are described which allow for the measurement of the compounds in the extract.[2]

2.2 A preliminary sample wash under basic conditions can be employed for samples having high general organic and organic base interferences.

2.3 The method also provides for the preparation of pentafluorobenzyl bromide (PFBB) derivatives for electron capture gas chromatography as an additional cleanup procedure to aid in the elimination of interferences.[2,3]

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in gas chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned.[4] Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and distilled water. It should then be drained dry, and heated in a muffle furnace at 400°C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are co-extracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedure in Section 12 can be used to overcome many of these interferences, but unique samples may require additional cleanup approaches to achieve the method detection limits listed in Tables 1 and 2.

3.3 The basic sample wash (Section 10.2) may cause significantly reduced recovery of phenol and 2,4-dimethylphenol. The analyst must recognize that results obtained under these conditions are minimum concentrations.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[5-7] for the information of the analyst.

4.2 Special care should be taken in handling pentafluorobenzyl bromide, which is a lachrymator, and 18 crown 6 ether, which is highly toxic.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle - Amber glass, one-liter or one-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container and capliner must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional) - The sampler must incorporate glass containers for the collection of a minimum of 250 mL of sample. Sample containers must be kept refrigerated at 4°C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing must be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.2 Glassware (All specifications are suggested. Catalog numbers are included for illustration only.)

5.2.1 Separatory funnel - 2000-mL, with Teflon stopcock.

5.2.2. Drying column - Chromatographic column 400-mm long x 19-mm ID with coarse frit.

5.2.3. Chromatographic column - 100-mm long x 10-mm ID, with Teflon stopcock.

5.2.4. Concentrator tube, Kuderna-Danish - 10-mL, graduated (Kontes K-570050-1025 or equivalent). Calibration must be checked at the volumes employed in the test. Ground glass stopper is used to prevent evaporation of extracts.

5.2.5 Evaporative flask, Kuderna-Danish - 500-mL (Kontes K-570001-0500 or equivalent). Attach to concentrator tube with springs.

5.2.6 Snyder column, Kuderna-Danish - three-ball macro (Kontes K-503000-0121 or equivalent).

5.2.7 Snyder column, Kuderna-Danish - two-ball micro (Kontes K-569001-0219 or equivalent).

5.3 Vials - Amber glass, 10- to 15-mL capacity, with Teflon-lined screwcap.

5.4 Reaction flask - Pyrex glass, 15- to 25-mL round bottom flask with standard tapered joint, fitted with a water cooled condenser and U-shaped drying tube containing granular calcium chloride.

5.5 Boiling chips - Approximately 10/40 mesh. Heat to 400°C for 30 minutes or Soxhlet extract with methylene chloride.

5.6 Water bath - Heated, with concentric ring cover, capable of

22

temperature control (± 2 C) The bath should be used in a hood.

5.7 Balance - Analytical, capable of accurately weighing 0 0001 g

5.8 Gas chromatograph - An analytical system complete with a temperature programmable gas chromatograph suitable for on-column injection and all required accessories including syringes, analytical columns, gases, detector, and strip-chart recorder A data system is recommended for measuring peak areas.

5.8.1 Column for underivatized phenols - 1 8 m long x 2 mm ID glass, packed with 1% SP-1240 DA on Supelcoport (80/100 mesh) or equivalent This column was used to develop the method performance statements in Section 14 Guidelines for the use of alternate column packings are provided in Section 11 1

5.8.2 Column for derivatized phenols - 1.8 m long x 2 mm ID glass column packed with 5% OV-17 on Chromosorb W-AW-DMCS (80/100 mesh). The carrier gas is 5% methane/95% Argon at a flow rate of 30 mL/min. The column temperature is 200° C. This column has proven effective in the analysis of wastewaters for derivatization products of the parameters listed in the scope (Section 1 1), and was used to develop the method performance statements in Section 14. Guidelines for the use of alternate columns are provided in Section 11 1

5.8.3 Detectors - flame ionization and electron capture. The flame ionization is used when determining the parent phenols. The electron capture is used when determining the derivatized phenols. Guidelines for use of alternate detectors are provided in Section 11 1

6. Reagents

6.1 Reagent water - Reagent water is defined as a water in which an interferent is not observed at the MDL of each parameter of interest.

6.2 Sodium hydroxide solution (10 N) - (ACS) Dissolve 40g NaOH in reagent water and dilute to 100 mL.

6.3 Sodium hydroxide solution (1 N) - (ACS) Dissolve 4g NaOH in reagent water and dilute to 100 mL.

6.4 Sodium sulfate - (ACS) Granular, anhydrous. Purify by heating at 400°C for four hours in a shallow tray

6.5 Sodium thiosulfate (ACS) Granular

6.6 Sulfuric acid solution (1 + 1) - (ACS) Slowly, add 50 mL H_2SO_4 (sp gr 1.84) to 50 mL of reagent water

6.7 Sulfuric acid (1 N) - (ACS) Slowly, add 29 mL H_2SO_4 (ACS, sp gr. 1 84) to reagent water and dilute to one liter

6.8 Potassium carbonate - (ACS) powdered.

6.9 Pentafluorobenzyl bromide (a-Bromopentafluorotoluene) - 97% minimum purity. NOTE. This chemical is a lachrymator (See Section 4 2.)

6.10 18-crown-6 ether (1,4,7,10,13,16 - Hexaoxacyclooctadecane) - 98% minimum purity NOTE. This chemical is highly toxic.

6.11 Derivatization reagent - Add one mL pentafluorobenzyl bromide and one gram 18 crown 6 ether to a 50-mL volumetric flask and dilute to volume with 2-propanol. Prepare fresh weekly. This operation should be carried out in a hood. Store 4°C and protect from light.

6.12 Acetone, hexane, methanol, methylene chloride, 2-propanol, hexane, toluene - Pesticide quality or equivalent.

6.13 Silica gel - Davison chemical, grade 923 (100/200 mesh) or equivalent. Activate at 130°C overnight and store in a desiccator

6.14 Stock standard solutions (1.00 μg/μL) - Stock standard solutions may be prepared from pure standard materials or purchased as certified solutions.

6.14.1 Prepare stock standard solutions by accurately weighing about 0.0100 grams of pure material. Dissolve the material in pesticide quality 2-propanol and dilute to volume in a 10-mL volumetric flask. Larger volumes may be prepared at the convenience of the analyst. If compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.14.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at 4°C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them Quality control check standards that can be used to determine the accuracy of calibration standards, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio, 45268

6.14.3 Stock standard solutions must be replaced after six months, or sooner if comparison with check standards indicates a problem.

7. Calibration

7.1 To calibrate the FIDGC for the analysis of underivatized phenols, establish gas chromatographic operating parameters equivalent to those indicated in Table 1 The gas chromatographic system can be calibrated using the external standard technique (Section 7 2) or the internal standard technique (Section 7 3)

7.2 External standard calibration procedure for FIDGC

7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with 2-propanol. One of the external standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector

7.2.2 Using injections of 2 to 5 μL of each calibration standard, tabulate peak height or area responses against the mass injected. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over the working range (< 10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.2.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard Alternatively, a new calibration curve or calibration factor must be prepared for that compound

274

7.3 Internal standard calibration procedure for FIDGC To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences Because of these limitations, no internal standard can be suggested that is applicable to all samples

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask To each calibration standard, add a known constant amount of one or more internal standards, and dilute to volume with 2-propanol. One of the standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector

7.3.2 Using injections of 2 to 5 µL of each calibration standard, tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1.

$$\text{Eq. 1} \quad RF = (A_s C_{is})/(A_{is} C_s)$$

where:

A_s = Response for the parameter to be measured

A_{is} = Response for the internal standard.

C_{is} = Concentration of the internal standard, (µg/L).

C_s = Concentration of the parameter to be measured, (µg/L).

If the RF value over the working range is a constant (< 10% RSD), the RF can be assumed to be nonvariant and the averge RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.3.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound.

7.4 To calibrate the ECGC for the analysis of phenol derivatives, establish gas chromatographic operating parameters equivalent to those indicated in Table 2

7.4.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with 2-propanol One of the external standards should represent a concentration near but above the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector

7.4.2 Each time samples are to be derivatived, simultaneously treat a one-mL aliquot of each calibration standard as described in Section 12.

7.4.3 After derivatization, inject 2 to 5 µL of each column eluate collected and tabulate peak height or area responses against the calculated equivalent mass of underivatized phenol injected. The results can be used to prepare a calibration curve for each compound.

7.5 Before using any cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2

8.1.3 The laboratory must spike and analyse a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8 4

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in 2-propanol 1000 times more concentrated that the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268

8.2.2 Using a pipet, add 1 00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 3, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If s > 2p or |X-R| > 2p, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

275

8.3.1 Calculate upper and lower control limits for method performance

Upper Control Limit (UCL) R 3 s

Lower Control Limit (LCL) R 3 s

where R and s are calculated as in Section 8.2.3. The UCL and LCL can be used to construct control charts[8] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± S. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[8].

8.4 The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 13.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate through the analysis of a one-liter aliquot of reagent water, that all glassware and reagents interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as gas chromatography with a dissimilar column, specific element detector, or mass spectrometer must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices[9] should be followed, except that the bottle must not be prewashed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon tubing and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4°C from the time of collection until extraction. Fill the sample bottle and at time of collection if residual chlorine is present, add 80 mg of sodium thiosulfate and mix well. U.S. Environmental methods 330.4 and 330.5 may be used for measurement of residual chlorine[10]. Field test kits are available for this purpose.

9.3 All samples must be extracted within 7 days and completely analyzed within 40 days of extraction[2].

10. Sample Extraction

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a two-liter separatory funnel.

10.2 For samples high inorganic content, the analyst may solvent wash the sample at a basic pH as prescribed in Section 10.2.1 and 10.2.2 to remove potential method interferences. Prolonged or exhaustive contact with solvent during the wash may result in low recovery of some of the phenols, notably phenol and 2,4-dimethyl phenol. For relatively clean samples, the wash should be omitted and the extraction, beginning with Section 10.3, should be followed.

10.2.1 Adjust the pH of the sample to 12.0 or greater with 10 N sodium hydroxide.

10.2.2 Add 60 mL of methylene chloride to the sample by shaking the funnel for one minute with periodic venting to release vapor pressure. Discard the solvent layer. The wash can be repeated up to two additional times if significant color is being removed.

10.3 Adjust the sample to a pH of 1 to 2 with sulfuric acid (1 + 1).

10.4 Add 60 mL of methylene chloride to the sample bottle, seal, and shake 30 seconds to rinse the inner walls. Transfer the solvent to the separatory funnel and shake for two minutes. Allow the solvent to separate from the sample and collect the methylene chloride in a 250-mL Erlenmeyer flask. If the emulsion interface between layers is more than one-third the size of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, or centrifugation.

10.5 Add a second 60-mL volume of methylene chloride to the sample bottle and complete the extraction procedure a second time, combining the extracts in the Erlenmeyer flask. Perform a third extraction in the same manner.

10.6 Assemble a Kuderna-Danish (K-D) concentrator by attaching a 10-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used in place of the KD if the requirements of Section 8.2 are met.

10.7 Pour the combined extract through a drying column containing three to four inches of anhydrous sodium sulfate, and collect in the K-D concentrator. Rinse the Erlenmeyer flask and column with 20 to 30 mL methylene chloride to complete the quantitative transfer.

10.8 Add one to two clean boiling chips to the flask and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL methylene chloride to the top. Place the K-D apparatus on a hot water bath (60 to 65°C) so that the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of the flask is bathed in vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will

276

not flood When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus and allow it to drain for at least 10 minutes while cooling

10.9 Increase the temperature of the hot water bath to 95 to 100°C Remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of 2-propanol A 5-mL syringe is recommended for this operation Attach a micro-Snyder column to the concentrator tube and prewet the column by adding about 0 5 mL of 2-propanol to the top Place the micro-K-D apparatus on the water bath so that the concentrator tube is partially immersed in the hot water Adjust the vertical position of the apparatus and the water temperature as required to complete concentration in 5 to 10 minutes At the proper rate of distillation, the balls of the column will actively chatter but the chambers will not flood When the apparent volume of the liquid reached 2.5 mL, remove the K-D apparatus and allow it to drain for at least 10 minutes while cooling Add an additional 2 mL of 2-propanol through the top of the micro-Snyder column and resume concentrating as before When the apparent volume of liquid reaches 0 5 mL, remove the K-D apparatus and allow it to drain for at least 10 minutes while cooling. Remove the micro-Snyder column and rinse its lower joint into the concentrator tube with a minimum amount of 2-propanol Adjust the extract volume to 1 0 mL. Stopper the concentrator tube and store in refrigerator at 4°C, if further processing will not be performed immediately If the sample extract requires no further cleanup, proceed with flame ionization gas chromatographic analysis (Section 11). If the sample requires further cleanup, proceed to Section 12 If the extracts will be stored longer than two days, they should be transferred to Teflon-sealed screw-cap vials.

10.10 Determine the original sample volume by refilling the sample bottle to the mark and transferring the liquid to a 1000-mL graduated cylinder Record the sample volume to the nearest 5 mL.

11. Gas Chromatography - Flame Ionization Detector

11.1 Table 1 summarizes the recommended gas chromatographic column and operating conditions. This Table includes retention times and MDL obtained under these conditions. An example of the parameter separation achieved by this column is shown in Figure 1 Other packed columns, chromatographic conditions, or detectors may be used if the requirements of Section 8 2 are met. Capillary (open-tubular) columns may also be used if the relative standard deviations of responses for replicate injections are demonstrated to be less than 6% and the requirements of Section 8.2 are met.

11.2 Calibrate the system daily as described in Section 7.1

11.3 If the internal standard approach is used, the standard must be added to the sample extract and mixed thoroughly immediately before injection into the instrument.

11.4 Inject 2 to 5 µL of the sample extract using the solvent-flush technique[111] Smaller (1.0 µL) volumes may be injected if automatic injectors are employed. Record the volume injected to the nearest 0.05 µL and the resulting responses in peak area or peak height units. If the response for the peak exceeds the working range of the system, dilute the extract and reanalyze.

11.5 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound may be used to calculate a suggested window size; however, the experience of the analyst should weigh heavily in the interpretation of chromatograms

11.6 If the measurement of the peak response is prevented by the presence of interferences, an alternate gas chromatographic procedure is required. Section 12 describes a derivatization and column chromatographic procedure which has been tested and found to be a practical means of analyzing phenols in complex extracts

12. Derivatization and Electron Capture Gas Chromatography

12.1 Pipet a 1.0-mL aliquot of the 2-propanol solution of standard or sample extract into a glass reaction vial. Add 1.0-mL of derivatizing reagent (Section 6.11). This is a sufficient amount of reagent to derivatize a solution whose total phenolic content does not exceed 0.3 mg/mL.

12.2 Add about 3 mg of potassium carbonate to the solution and shake gently.

12.3 Cap the mixture and heat it for four hours at 80°C in a hot water bath

12.4 Remove the solution from the hot water bath and allow it to cool.

12.5 Add 10 mL of hexane to the reaction flask and shake vigorously for one minute. Add 3.0 mL of distilled, deionized water to the reaction flask and shake for two minutes. Decant a portion of the organic layer into a concentrator tube and cap with a glass stopper.

12.6 Pack a 10-mm ID chromatographic column with 4.0 grams of activated silica gel. After settling the silica gel by tapping the column, add about two grams of anhydrous sodium sulfate to the top.

12.7 Pre-elute the column with 6 mL of hexane. Discard the eluate and just prior to exposure of the sulfate layer to air, pipet onto the column 2.0 mL of the hexane solution (Section 12.5) that contains the derivatized sample or standard Elute the column with 10 0 mL of hexane (Fraction 1) and discard this fraction Elute the column, in order, with 10 0 mL of 15% toluene in hexane (Fraction 2), 10.0 mL of 40% toluene in hexane (Fraction 3); 10.0 mL 75% toluene in hexane (Fraction 4), and 10.0 mL 15% 2-propanol in toluene (Fraction 5). All elution mixtures are prepared on a volume:volume basis. Elution patterns for the phenolic derivatives are shown in Table 2. Fractions may be combined as desired, depending upon the specific phenols of interest or level of interferences.

12.8 Analyze the fractions by electron capture gas chromatography Table 2 summarizes the recommended gas chromatographic column and operating conditions. This Table includes retention times and MDL obtained under these conditions. An example of the parameter separation achieved by this column is shown in Figure 2.

12.9 Calibrate the system daily with a minimum of three aliquots of calibration standards, containing each of the phenols of interest that are derivatized according to the procedure (See Section 7 4).

12.10 Inject 2 to 5 µL of the column fractions using the solvent-flush technique. Smaller (1.0 µL) volumes can be injected if automatic devices are employed. Record the volume injected to the nearest 0 05 µL, and the resulting peak size, in area units or height. If the peak response exceeds the

linear range of the system, dilute the extract and reanalyze

13. Calculations

13.1 Calculate the concentration of individual compounds in the sample determined by the flame ionization procedure (without derivatization) as indicated below

13.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the peak response using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated from equation 2.

Eq. 2. Concentration, $\mu g/L = \frac{(A)(V_t)}{(V_i)(V_s)}$

where

A = Amount of material injected, in nanograms

V_i = Volume of extract injected (μL)

V_t = Volume of total extract (μL)

V_s = Volume of water extracted (mL).

13.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and equation 3

Eq. 3. Concentration, $\mu g/L = \frac{(A_s)(I_s)}{(A_{is})(RF)(V_o)}$

where

A_s = Response for the parameter to be measured

A_{is} = Response for the internal standard

I_s = Amount of internal standard added to each extract (μg)

V_o = Volume of water extracted, in liters

13.2 Calculate the concentration of individual compounds in the sample when determined by the derivatization and electron capture procedure according to Equation 4:

Eq. 4 Concentration, $\mu g/L = \frac{(A)(B)(V_t)(10)}{(V_i)(V_s)(C)(D)}$

where:

A = Mass of underivatized phenol represented by area of peak in sample chromatogram, determined from calibration curve in Section 7.4.3, in nanograms.

B = Total volume of 2-propanol extract after concentration in mL

V_i = Volume of eluate injected ($\mu g/L$).

V_t = Total volume of column eluate ($\mu g/L$)

V_s = Volume of water extracted (mL)

C = Volume of hexane sample solution added to cleanup column, in mL

D = Volume of 2-propanol extract used for derivatization in mL.

13.3 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.4 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits in 8.4, data for the affected parameters must be labeled as suspect.

14. Method Performance

14.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[11]. The MDL concentrations listed in Tables 1 and 2 were obtained using reagent water[12]. Similar results were achieved using representative wastewaters.

14.2 In a single laboratory (I.T. Enviroscience, Inc.), using spiked reagent water and wastewater samples, the average recoveries presented in Table 3 were obtained using a flame ionization detector[12]. The standard deviation of the percent recovery is also included in Table 3.

14.3 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. See Appendix A.
2. "Determination of Phenols in Industrial and Municipal Wastewaters." Report for EPA Contract 68-03-2625. In preparation.
3. Kawahara, F.K., *Analytical Chemistry, 40*, 1009 (1968).
4. ASTM Annual Book of Standard, Part 31, D3694, "Standards Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 679, 1980
5. "Carcinogens - Working With Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977
6. "OSHA Safety and Health Standards, General Industry," (29 CFR 1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
7. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979
8. "Handbook for Analytical Quality Control in Wastewater and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268, March 1979
9. ASTM Annual Book of Standards, Part 31, D3370, "Standard Practice for Sampling Water," American Society for Testing and Materials, Philadelphia, PA, p. 76, 1980
10. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600/4-79-020, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268, March 1979
11. Burke, J.A., "Gas Chromatography for Pesticide Residue Analysis; Some Practical Aspects," *Journal of the Association of Official Analytical Chemists, 48*, 1037 (1965)
12. "Development of Detection Limits, EPA Method 604, Phenols." Special letter report for EPA contract 68-03-2625 Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268

278

Table 1. Chromatographic Conditions and Method Detection Limit

Parameter	Retention Time (min.)	Method Detection Limit (µg/L)
2-Chlorophenol	1.70	0.31
2-Nitrophenol	2.00	0.45
Phenol	3.01	0.14
2,4-Dimethylphenol	4.03	0.32
2,4-Dichlorophenol	4.30	0.39
2,4,6-Trichlorophenol	6.05	0.64
4-Chloro-3-methylphenol	7.50	0.36
2,4-Dinitrophenol	10.00	13.0
2-Methyl-4,6-dinitrophenol	10.24	16.0
Pentachlorophenol	12.42	7.4
4-Nitrophenol	24.25	2.8

Column conditions. Supelcoport (80/100 mesh) coated with 1% SP-1240 DA in 1.8 m long x 2 mm ID glass column with nitrogen carrier gas at a flow rate of 30 mL/min flow rate. Column temperature was 80°C at injection, programmed immediately at 8°C/min to 150°C final temperature. Method detection limits were determined with a flame ionization detector.

Table 2. Silica Gel Fractionation and Electron Capture Gas Chromatography of PFBB Derivatives

Parent Compound	Recovery (%) by Fraction[a]					Electron Capture	
	1	2	3	4	5	Retention Time (min.)	Method Detection Limit (µg/L)
2-Chlorophenol		·	90	1	·	3.3	0.58
2-Nitrophenol	·	·	·	9	90	9.1	0.77
Phenol		·	90	10	·	1.8	2.2
2,4-Dimethylphenol	·	·	95	7		2.9	0.63
2,4-Dichlorophenol	·	·	95	1	·	5.8	0.68
2,4,6-Trichlorophenol		50	50	·	·	7.0	0.58
4-Chloro-2-methylphenol	·	·	84	14	·	4.8	1.8
Pentachlorophenol	·	75	20	·		28.8	0.59
4-Nitrophenol		·	·	1	90	14.0	0.70

[a]Eluting solvent compositions as given in Section 12.7.
Column conditions. Chromosorb W-AW-DMCS (80/100 mesh) coated with 5% OV-17 packed in a 1.8 m long x 2.0 mm ID glass column with 5% methane/95% argon carrier gas at a flow rate of 30 mL/min. Column temperature isothermal at 200°C.

Table 3. Single Operator Accuracy and Precision

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
4-Chloro-3-methylphenol	82	15.0	0.70 - 3.5	21	3
2-Chlorophenol	67	14.8	0.74 - 3.7	21	3
2,4-Dichlorophenol	74	11.4	1.03 - 5.2	21	3
2,4-Dimethylphenol	51	14.0	0.82 - 4.1	21	3
2,4-Dinitrophenol	74	16.5	28.7	14	2
2-Methyl-4,6-dinitrophenol	86	12.4	34.6	21	3
2-Nitrophenol	67	12.9	0.80 - 4.0	21	3
4-Nitrophenol	45	7.9	15.9	21	3
Pentachlorophenol	79	8.8	21.0	21	3
Phenol	41	8.4	0.76 - 3.8	21	3
2,4,6-Trichlorophenol	71	14.5	1.20 - 6.0	21	3

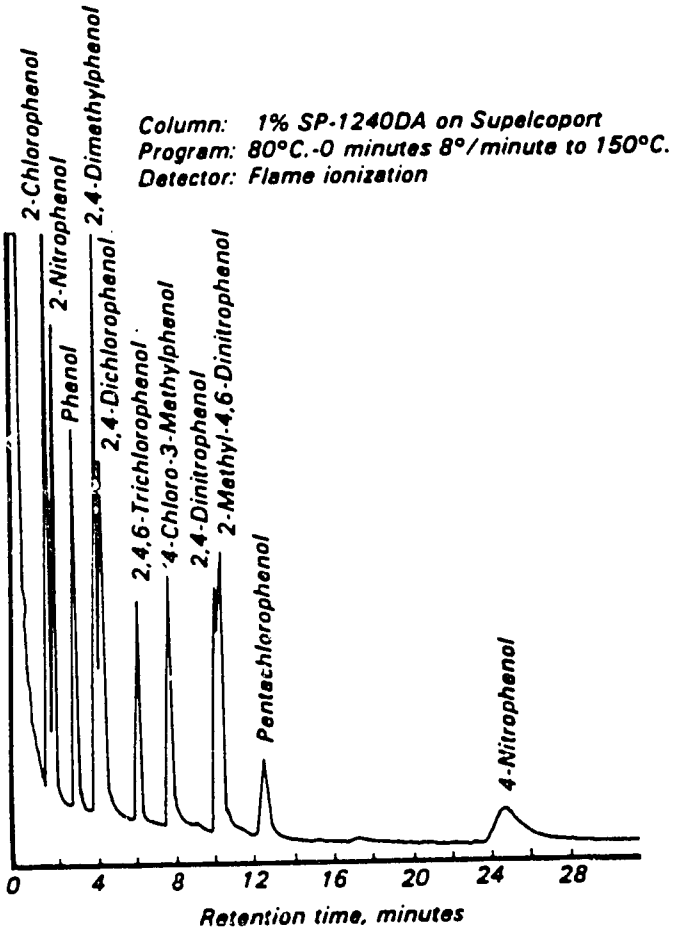


Figure 1. *Gas chromatogram of phenols*

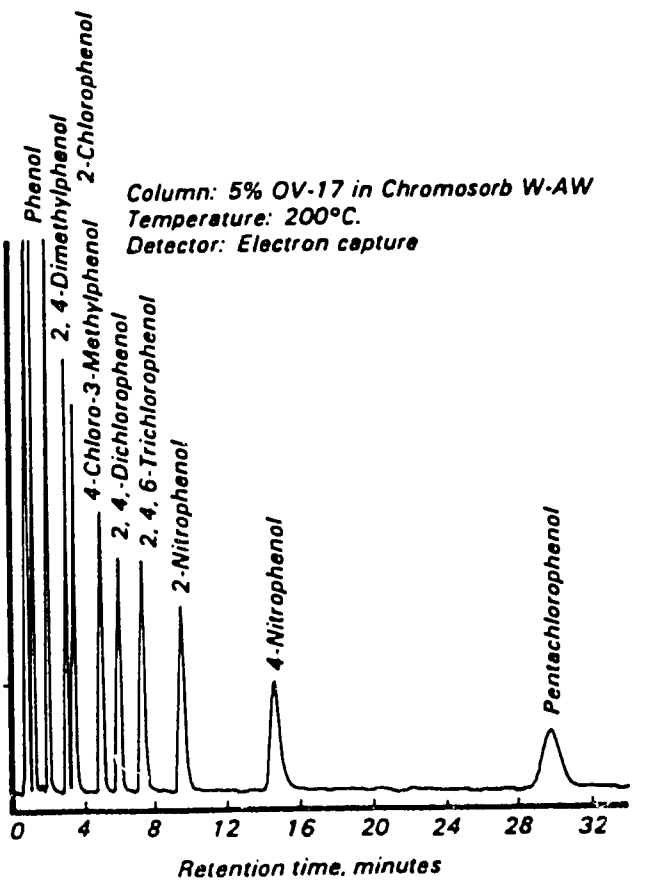


Figure 2. *Gas chromatogram of PFB derivatives of phenols.*

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Benzidines — Method 605

1. Scope and Application

1.1 This method covers the determination of certain benzidines. The following parameters can be determined by this method:

Parameter	STORET No	CAS No
Benzidine	39120	92-87-5
3,3'-Dichlorobenzidine	34631	91-94-1

1.2 This is a high performance liquid chromatography (HPLC) method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for the compounds above, identifications should be supported by, at least, one additional qualitative technique. This method decribes electrochemical conditions at a second potential which can be used to confirm measurements made with this method. Method 625 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results of the parameters listed above, using the extract produced by this method.

1.3 The method detection limit (MDL defined in Section 14)[1] for each parameter is listed in Table 1. The MDL for a specific wastewater may differ depending upon the nature of the interferences in the sample matrix.

1.4 Any modification of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval of alternate test procedures under 40 CFR 136.4 and 136.5.

1.5 This method is restricted to use by or under the supervision of analysts experienced in the use of HPLC instrumentation and in the interpretation of liquid chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 A measured volume of sample, approximately one-liter, is extracted with chloroform using liquid-liquid extractions in a separatory funnel. The chloroform extract is back-extracted with acid, neutralized, and extracted with chloroform. The chloroform is exchanged to methanol and concentrated using a rotary evaporator and nitrogen blowdown. It is then brought to a volume of 5 mL with an acetate buffer. HPLC conditions are described which permit the separation and measurement of the benzidine compounds using an electrochemical detector[2].

2.2 The acid back extraction step acts as a general purpose cleanup to aid in the elimination of interferences.

28

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned[3]. Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and reagent water. It should then be drained dry, and heated in a muffle furnace at 400°C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedures that are inherent in the extraction step are used to overcome many of these interferences, however unique samples may require additional cleanup approaches to achieve the MDL listed in Table 1.

3.3 Some dye plant effluents contain large amounts of components with retention times close to benzidine. In these cases, it has been found useful to reduce the electrode potential in order to eliminate interferences yet still detect benzidine. (See Section 12.7.)

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined, however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and are identified[4-6] for the benefit of the analyst.

4.2 Benzidine, 3,3'-dichlorobenzidine and chloroform, used for extraction of samples have been tentatively classified as known or suspected, human or mammalian carcinogens.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle - Amber glass, one-liter or one-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container must be washed, rinsed with acetone, or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional) - Must incorporate glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4°C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.2 Glassware (All specifications are suggested.)

5.2.1 Separatory funnels - 2000, 1000, and 250-mL, with Teflon stopcock.

5.2.2 Vials - Amber glass, 10- to 15-mL capacity, with Teflon-lined screw cap.

5.2.3 Rotary evaporator.

5.2.4 Flasks, round bottom, 100-mL, with 24/40 joints.

5.2.5 Centrifuge tubes - conical, screw-capped, graduated, equipped with Teflon lined caps.

5.2.6 Pipettes - Pasteur, with bulbs.

5.3 Balance - Analytical, capable of accurately weighing 0.0001 g.

5.4 HPLC - An analytical system complete with column supplies, compatible recorder, high pressure syringes and the following components.

5.4.1 Solvent delivery system - with pulse damper, Altex 110A, or equivalent.

5.4.2 Injection valve (Optional) - Waters U6K or equivalent.

5.4.3 Electrochemical detector - Bioanalytical Systems LC-2A with glassy carbon electrode, or equivalent. This detector has proven effective in the analysis of wastewaters for the parameters listed in the scope, and was used to develop the method performance statements in Section 14. Guidelines for the use of alternate detectors are provided in Section 12.1.

5.4.4 Electrode polishing kit - Princeton Applied Research Model 9320 or equivalent.

5.4.5 Column - Lichrosorb RP-2, 5 micron particle diameter, in a 25 cm x 4.6 mm ID stainless steel column. This column was used to develop the method performance statements in Section 14. Guidelines for the use of alternate column packings are provided in Section 12.1.

6. Reagents

6.1 Reagent water - Reagent water is defined as a water in which an interferent is not observed at the MDL of each parameter of interest.

6.2 Sodium hydroxide solution (5 N) - (ACS) Dissolve 20g NaOH in reagent water and dilute to 100 mL.

6.3 Sodium hydroxide (1 M)-(ACS) - Dissolve 40 g NaOH in reagent water and dilute to one liter.

6.4 Sodium thiosulfate - (ACS) Granular.

6.5 Sodium tribasic phosphate (0.4 M) - (ACS) Dissolve 160 g of trisodium phosphate decahydrate in reagent water and dilute to one liter.

6.6 Sulfuric acid solution (1+1) - (ACS) Slowly, add 50 mL H_2SO_4 (sp. gr. 1.84) to 50 mL of reagent water.

282

6.7 Sulfuric acid (1 M) - (ACS) Slowly add 58 mL H_2SO_4 (sp gr 1 84) to reagent water and dilute to one liter

6.8 Acetate buffer (0.1 M, pH-4 7) Dissolve 5 8 mL glacial acetic acid (ACS) and 13 6 g of sodium acetate trihydrate (ACS) in reagent water which has been purified by filtration through a RO-4 Millipore System or equivalent and dilute to one liter

6.9 Acetonitrile, chloroform (preserved with 1% ethanol), methanol - Pesticide quality or equivalent

6.10 Mobile phase - Place equal volumes of filtered acetonitrile (Millipore type FH filter or equivalent) and filtered acetate buffer (millipore type GS filter or equivalent) in a narrow-mouth glass container and mix thoroughly. Prepare fresh weekly. Degas daily by sonicating under vacuum, or by heating and stirring, or by purging with helium.

6.11 Stock standard solutions (1.00 μg/μL) - Stock standard solutions may be prepared from pure standard materials or purchased as certified solutions.

6.11.1 Prepare stock standard solutions by accurately weighing about 0 0100 g of pure material. Dissolve the material in pesticide quality methanol and dilute to volume in a 10-mL volumetric flask. Larger volumes can be used at the convenience of the analyst. If compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.11.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at 4°C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check samples that can be used to determine the accuracy of calibration standards will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

6.11.3 Stock standard solutions must be replaced after six months, or sooner if comparison with quality control check samples indicate a problem

7. Calibration

7.1 Establish chromatographic operating parameters equivalent to those given in Table 1. The HPLC system can be calibrated using the external standard technique (Section 7.2) or the internal standard technique (Section 7.3).

7.2 External standard calibration procedure:

7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with mobile phase One of the external standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.2.2 Using syringe injections of 5 to 25 μL or a constant volume injection loop, inject each calibration standard, and tabulate peak height or area responses against the mass injected. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over the working range (< 10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.2.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that compound. If serious loss of sensitivity occurs, polish the electrode and recalibrate.

7.3 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask To each calibration standard, add a known constant amount of one or more internal standards, and dilute to volume with mobile phase. One of the standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.3.2 Using 5 to 25 μL aliquots or a constant volume injection loop, inject each calibration standard, and tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1

Eq. 1 $RF = (A_s C_{is})/(A_{is} C_s)$

where:

A_s = Response for the parameter to be measured

A_{is} = Response for the internal standard.

C_{is} = Concentration of the internal standard, (μg/L).

C_s = Concentration of the parameter to be measured, (μg/L).

If the RF value over the working range is a constant (< 10% RSD), the RF can be assumed to be nonvariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF

7.3.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound. If serious loss of response occurs, polish the electrode and recalibrate.

7.4 Before using any alternate cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The

282

minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in methanol 1000 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 2, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > 2p$ or $|X-R| > 2p$, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance:

Upper Control Limit (UCL) = $R + 3s$

Lower Control Limit (LCL) = $R - 3s$

where R and s are calculated as in Section 8.2.3. The UCL and LCL can be used to construct control charts[7] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as $R \pm s$. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[7].

8.4 The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 13.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate through the analysis of a one-liter aliquot of reagent water, that all glassware and reagents interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as HPLC with a dissimilar column, gas chromatography, or mass spectrometry must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices[8] should be followed, except that the bottle must not be prewashed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be free as possible of Tygon and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4°C and stored in the dark from the time of collection until extraction. Both benzidine and 3,3'-dichlorobenzidine are easily oxidized. Fill the sample bottle and, at time of collection, if residual chlorine is present, add 80 mg of sodium thiosulfate per liter of sample, and mix thoroughly. U.S. Environmental Protection Agency methods 330.4 and 330.5 may be used for measurement

281

of residual chlorine[10]. Field test kits are available for this purpose. After mixing, adjust the pH of the sample to a range of 2 to 7 with sulfuric acid.

9.3 If 1,2-diphenyl hydrazine is likely to be present, adjust the pH of the sample to 4.0 ± 0.2 to prevent rearrangement to benzidine.

9.4 All samples must be extracted within seven days. Extracts may be held up to seven days before analysis, if stored under an inert (oxidant free) atmosphere[2]. The extract should be protected from light.

10. Sample Extraction

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a two-liter separatory funnel. Check the pH of the sample with wide-range pH paper and adjust to within the range of 6.5 to 7.5 with sodium hydroxide or sulfuric acid solutions.

10.2 Add 100 mL chloroform to the sample bottle, seal, and shake 30 seconds to rinse the inner walls. (Caution: Handle chloroform in a well ventilated area.) Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods. Collect the chloroform extract in a 250-mL separatory funnel.

10.3 Add a 50-mL volume of chloroform to the sample bottle and repeat the extraction procedure a second time, combining the extracts in the separatory funnel. Perform a third extraction in the same manner.

10.4 Separate and discard any aqueous layer remaining in the 250-mL separatory funnel after combining the organic extracts. Add 25 mL of 1 M sulfuric acid and extract the sample by shaking the funnel for two minutes. Transfer the aqueous layer to a 250-mL beaker. Extract with two additional 25-mL portions of 1 M sulfuric acid and combine the acid extracts in the beaker.

10.5 Place a stirbar in the 250-mL beaker and stir the acid extract while carefully adding 5 mL of 0.4 M sodium tribasic phosphate. While monitoring with a pH meter, neutralize the extract to a pH between 6 and 7 by dropwise addition of 5 N NaOH while stirring the solution vigorously. Approximately 25 to 30 mL of 5 N NaOH will be required and it should be added over at least a two-minute period. Do not allow the sample pH to exceed 8.

10.6 Transfer the neutralized extract into a 250-mL separatory funnel. Add 30 mL of chloroform and shake the funnel for two minutes. Allow the phases to separate, and transfer the organic layer to a second 250-mL separatory funnel.

10.7 Extract the aqueous layer with two additional 20-mL aliquots of chloroform as before. Combine the extracts in the 250-mL separatory funnel.

10.8 Add 20 mL of reagent water to the combined organic layers and shake for 30 seconds.

10.9 Transfer the organic extract into a 100-mL round bottom flask. Add 20 mL of methanol and concentrate to 5 mL with a rotary evaporator at reduced pressure and 35°C. An aspirator is recommended for use as the source of vacuum. Chill the receiver with ice. This operation requires approximately 10 minutes. Other concentration techniques may be employed, if the requirements of Section 8.2 are met.

10.10 Using a 9-inch Pasteur pipette, transfer the extract to a 15 mL conical screw-capped centrifuge tube. Rinse the flask, including the entire side wall, with 2-mL portions of methanol and combine with the original extract.

10.11 Carefully concentrate the extract to 0.5 mL using a gentle stream of nitrogen while heating in a 30°C water bath. Dilute to 2 mL with methanol, reconcentrate to 1 mL, and dilute to 5 mL with acetate buffer. Mix extract thoroughly. Cap the centrifuge tube and store refrigerated and protected from light if further processing will not be performed immediately.

10.12 Determine the original sample volume by refilling the sample bottle to the mark and transferring the liquid to a 1000-mL graduated cylinder. Record the sample volume to the nearest 5 mL.

11. Cleanup and Separation

11.1 Additional cleanup procedures may not be necessary for a relatively clean sample matrix. The single operator precision and accuracy data in Section 14 were gathered using only those cleanup procedures that are inherent in the extraction procedures of this method. If particular circumstances demand the use of an alternative cleanup procedure, the analyst must determine the elution profile and demonstrate that the recovery of each compound of interest is no less than 85%.

12. Liquid Chromatography

12.1 Table 1 summarizes the recommended operating conditions for the HPLC. This Table includes retention times, capacity factors, and MDL that were obtained under these conditions. An example of the parameter separation achieved by the HPLC column is shown in Figure 1. Other HPLC columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met. When the HPLC is idle, it is advisable to maintain a 0.1 mL/min flow through the column to prolong column life.

12.2 Calibrate the system daily as described in Section 7.

12.3 If the internal standard approach is being used, the standard must be added to the sample extract, and mixed thoroughly immediately, before injection into the instrument.

12.4 Inject 5 to 25 µL of the sample extract. If constant volume injection loops are not used, record the volume injected to the nearest 0.05 µL, and the resulting peak size in area or peak height units.

12.5 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size, however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

12.6 If the response for the peak exceeds the working range of the system, dilute the extract with mobile phase and reanalyze.

12.7 If the measurement of the peak response for benzidine is prevented by the presence of interferences, reduce the electrode potential to +0.6 volts and reanalyze. If the benzidine peak is still obscured by interferences, further cleanup is required.

13. Calculations

13.1 Determine the concentration of individual compounds in the sample.

13.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the peak response using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated from the equation 2.

$$\text{Eq. 2. Concentration, } \mu g/L \quad \frac{(A)(V_t)}{(V_i)(V_s)}$$

where

- A = Amount of material injected, in nanograms
- V_i = Volume of extract injected (μL)
- V_t = Volume of total extract (μL)
- V_s = Volume of water extracted (mL)

13.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and equation 3.

$$\text{Eq. 3. Concentration, } \mu g/L \quad \frac{(A_s)(I_s)}{(A_{is})(RF)(V_o)}$$

where

- A_s = Response for the parameter to be measured
- A_{is} = Response for the internal standard
- I_s = Amount of internal standard added to each extract (μg)
- V_o = Volume of water extracted, in liters

13.2 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.3 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits in Section 8.4, data for the affected parameters must be labeled as suspect.

14. Method Performance

14.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[11]. The MDL concentrations listed in Table 1 were obtained using reagent water[10]. Similar results were achieved using representative wastewaters.

14.2 This method has been tested for linearity of analyte recovery from reagent water and has been demonstrated to be applicable over the concentration range from 7 x MDL to 3000 x MDL[10].

14.3 In a single laboratory (Battelle, Columbus Laboratories), using spiked wastewater samples, the average recoveries presented in Table 2 were obtained[2]. Each spiked sample was analyzed in triplicate on two separate days. The standard deviation of the percent recovery is also included in Table 2.

14.4 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. See Appendix A.
2. "Determination of Benzidines in Industrial and Municipal Wastewaters," Report for EPA Contract 68-03-2624 (In preparation).
3. ASTM Annual Book of Standards, Part 31, D3694, "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 679, 1980.
4. "Carcinogens - Working With Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.
5. "OSHA Safety and Health Standards, General Industry," (29 CFR 1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
6. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.
7. "Handbook for Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268, March 1979.
8. ASTM Annual Book of Standards, Part 31, D3370, "Standard Practice for Sampling Water," American Society for Testing and Materials, Philadelphia, PA, p. 76, 1980.
9. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600/4-79-020, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268, March 1979.
10. "Determination of Method Detection Limit and Analytical Curve for EPA Method 605 - Benzidines," Special letter report for EPA Contract 68-03-2624, Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268.

236

Table 1 *Chromatographic Conditions and Method Detection Limits*

Parameter	Retention Time (min)	Column Capacity Factor	Method Detection Limit (µg/L)
Benzidine	6.1	1.44	0.08
3,3'-dichlorobenzidine	12.1	3.84	0.13

HPLC Column conditions. Lichrosorb RP-2, 5 micron particle size, in a 25 cm x 4.6 mm ID stainless steel column. Mobile Phase 0.8 mL/min of 50% acetonitrile 50% 0.1M pH 4.7 acetate buffer. The method detection limit was determined using an electrochemical detector operated at + 0.8 volts.

Table 2. *Single Operator Accuracy and Precision*

Parameter	Average Percent Recovery	Standard Deviation %	Spike range (µg/L)	Number of Analyses	Matrix Types
Benzidine	65	11.4	1.0-50	30	5
3,3'-Dichlorobenzidine	64	9.6	1.0-50	30	5

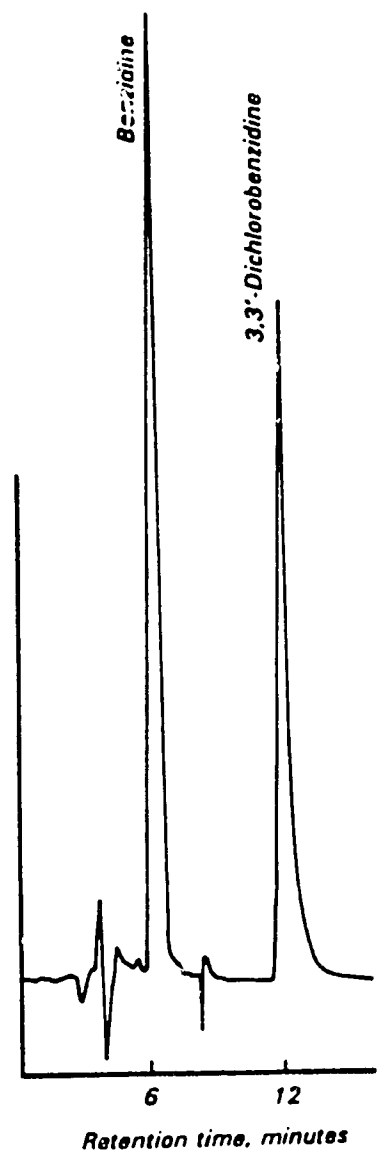


Figure 1. *Liquid chromatogram of benzidines*

287

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Phthalate Esters — Method 606

1. Scope and Application

1.1 This method covers the determination of certain phthalate esters The following parameters can be determined by this method:

Parameter	STORET No.	CAS No.
Bis(2-ethylhexyl) phthalate	39100	117-81-7
Butyl benzyl phthalate	34292	85-68-7
Di-n-butyl phthalate	39110	84-74-2
Diethyl phthalate	34336	84-66-2
Dimethyl phthalate	34341	131-11-3
Di-n-octyl phthalate	34596	117-84-0

1.2 This is a gas chromatographic (GC) method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136 1 When this method is used to analyze unfamiliar samples for any or all of the compounds above, compound identifications should be supported by at least one additional qualitative technique This method describes analytical conditions for a second gas chromatographic column that can be used to confirm measurements made with the primary column Method 625 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results for all of the parameters listed above, using the extract produced by this method

1.3 The method detection limit (MDL, defined in Section 14)[11] for each parameter is listed in Table 1 The MDL for a specific wastewater may differ from those listed, depending upon the nature of interferences in the sample matrix.

1.4 The sample extraction and concentration steps in this method are essentially the same as in methods 608, 609, 611 and 612 Thus, a single sample may be extracted to measure the parameters included in the scope of each of these methods When cleanup is required, the concentration levels must be high enough to permit selecting aliquots, as necessary to apply appropriate cleanup procedures The analyst is allowed the latitude, under Gas Chromatography (Section 12), to select chromatographic conditions appropriate for the simultaneous measurement of combinations of these parameters

1.5 Any modification of this method, beyond those expressly permitted, shall be considered a major modification subject to application and approval of alternate test procedures under 40 CRF 136 4 and 136 5

1.6 This method is restricted to use by or under the supervision of analysts experienced in the use of gas chromatography and in the interpretation of gas chromatograms Each analyst must demonstrate the

288

ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 A measured volume of sample, approximately 1-liter, is solvent extracted with methylene chloride using a separatory funnel. The methylene chloride extract is dried and exchanged to hexane during concentration to a volume of 10 mL or less. Gas chromatographic conditions are described which permit the separation and measurement of the compounds in the extract by electron capture gas chromatography[2].

2.2 Analysis for phthalates is especially complicated by their ubiquitous occurrence in the environment. This method provides Florisil and alumina column cleanup procedures to aid in the elimination of interferences that may be encountered.

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in gas chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned[3]. Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and distilled. It should then be drained dry, and heated in a muffle furnace at 400°C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Phthalate esters are contaminants in many products commonly found in the laboratory. It is particularly important to avoid the use of plastics because phthalates are commonly used as plasticizers and are easily extracted from plastic materials. Serious phthalate contamination can result at any time, if consistent quality control is not practiced. Great care must be experienced to prevent such contamination. Exhaustive cleanup of reagents and glassware may be required to eliminate background phthalate contamination[4,5].

3.3 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedures in Section 11 can be used to overcome many of these interferences, but unique samples may require additional cleanup approaches to achieve the MDL listed in Table 1.

4. Safety

The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[6-8] for the information of the analyst.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle - Amber glass, one-liter or one-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container and cap liner must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional) - Must incorporate glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4°C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.2 Glassware (All specifications are suggested. Catalog numbers are included for illustration only.)

5.2.1 Separatory funnel - 2000-mL, with Teflon stopcock.

5.2.2 Drying column - Chromatographic column 400 mm long x 19 mm ID with coarse frit.

5.2.3 Chromatographic column - 300 mm long x 10 mm ID with coarse fritted disc at bottom and Teflon stopcock (Kontes K-420540-0213 or equivalent).

5.2.4 Concentrator tube, Kuderna-Danish - 10-mL, graduated (Kontes K-570050-1025 or equivalent). Calibration must be checked at the volumes employed in the test. Ground glass stopper is used to prevent evaporation of extracts.

5.2.5 Evaporative flask, Kuderna-Danish - 500-mL (Kontes K-570001-0500 or equivalent). Attach to concentrator tube with springs.

5.2.6 Snyder column, Kuderna-Danish - three-ball macro (Kontes K-503000-0121 or equivalent).

5.2.7 Snyder column, Kuderna-Danish - two-ball micro (Kontes K-569001-0219 or equivalent).

5.2.8 Vials - Amber glass, 10- to 15-mL capacity, with Teflon-lined screw-cap.

5.3 Boiling chips - approximately 10/40 mesh. Heat to 400°C for 30 minutes or Soxhlet extract with methylene chloride.

5.4 Water bath - Heated, with concentric ring cover, capable of temperature control (± 2°C). The bath should be used in a hood.

5.5 Balance - Analytical, capable of accurately weighing 0.0001 g.

5.6 Gas chromatograph - Analytical system complete with gas

chromatograph suitable for on-column injection and all required accessories including syringes analytical columns, gases, detector, and strip-chart recorder A data system is recommended for measuring peak areas

5.6.1 Column 1 - 180 cm long x 4 mm ID glass, packed with 1.5% SP-2250 1.95% SP-2401 on Supelcoport (100 120 mesh) or equivalent This column was used to develop the method performance statements in Section 14 Guidelines for the use of alternate column packings are provided in Section 12.1

5.6.2 Column 2 - 180 cm long x 4 mm ID glass, packed with 3% OV-1 on Supelcoport (100 120 mesh) or equivalent

5.6.3 Detector - Electron capture This detector has proven effective in the analysis of wastewaters for the parameters listed in the scope and was used to develop the method performance statements in Section 14 Guidelines for the use of alternate detectors are provided in Section 12.1

6. Reagents

6.1 Reagent water - Reagent water is defined as a water in which an interferent is not observed at the MDL of each parameter of interest

6.2 Acetone, hexane, isooctane, methylene chloride, methanol - Pesticide quality or equivalent.

6.3 Ethyl ether - Nanograde, redistilled in glass if necessary.

6.3.1 Must be free of peroxides as indicated by EM Quant test strips. (Available from Scientific Products Co., Cat No P1126-8 and others.)

6.3.2 Procedures recommended for removal of peroxides are provided with the test strips After cleanup, 20 mL ethyl alcohol preservative must be added to each liter of ether

6.4 Sodium sulfate - (ACS) Granular, anhydrous. Several levels of purification may be required in order to reduce background phthalate levels to an acceptable level: 1) Heat four hours at 400°C in a shallow tray, 2) Heat 16 hours at 450-500°C in a shallow tray, 3) Soxhlet extract with methylene chloride for 48 hours.

6.5 Florisil - PR grade (60/100 mesh) Purchase activated at 1250°F and store in dark in glass container with ground glass stopper or foil-lined screw cap To prepare for use, place 100g of Florisil into a 500-mL beaker and heat for approximately 16 hours at 400 C After heating transfer to a 500-mL reagent bottle Tightly seal and cool to room temperature When cool add 3 mL of reagent water Mix thoroughly by shaking or rolling for 10 minutes and let it stand for at least two hours Keep the bottle sealed tightly

6.6 Alumina - Neutral activity Super I, W200 series, (ICN Life Sciences Group, No 404583). To prepare for use, place 100 g of alumina into a 500-mL beaker and heat for approximately 16 hours at 400°C. After heating transfer to a 500-mL reagent bottle. Tightly seal and cool to room temperature. When cool add 3 mL of reagent water Mix thoroughly by shaking or rolling for 10 minutes and let it stand for at least two hours. Keep the bottle sealed tightly

6.7 Stock standard solutions (1.00 µg/µL) - Stock standard solutions can be prepared from pure standard materials or purchased as certified solutions

6.7.1 Prepare stock standard solutions by accurately weighing about 0.0100 grams of pure material. Dissolve the material in pesticide quality isooctane, dilute to volume in a 10-mL volumetric flask. Larger volumes can be used at the convenience of the analyst. If compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.7.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at 4°C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check standards that can be used to determine the accuracy of calibration standards will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio, 45268

6.7.3 Stock standard solutions must be replaced after six months, or sooner if comparison with check standards indicate a problem.

7. Calibration

7.1 Establish gas chromatographic operating parameters equivalent to those indicated in Table 1 The gas chromatographic system can be calibrated using the external standard technique (Section 7.2) or the internal standard technique (Section 7.3)

7.2 External standard calibration procedure

7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with isooctane One of the external standards should be at a concentration near, but above, MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector

7.2.2 Using injections of 2 to 5 µL of each calibration standard, tabulate peak height or area responses against the mass injected The results can be used to prepare a calibration curve for each parameter Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over the working range (< 10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve

7.2.3. The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard Alternatively, a new calibration curve or calibration factor must be prepared for that parameter

7.3 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples.

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask. To each calibration standard, add a known constant amount of one or more internal standards, and dilute to

volume with isooctane. One of the standards should be at a concentration near, but above the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.3.2 Using injections of 2 to 5 µL of each calibration standard, tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1.

$$\text{Eq. 1} \quad RF = (A_s C_{is}) / (A_{is} C_s)$$

where

A_s = Response for the parameter to be measured.
A_{is} = Response for the internal standard.
C_{is} = Concentration of the internal standard, (µg/L).
C_s = Concentration of the parameter to be measured, (µg/L).

If the RF value over the working range is a constant (<10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.3.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound.

7.4 Before using any cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in acetone 1000 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 2, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > 2p$ or $|X-R| > 2p$, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance.

Upper Control Limit (UCL) = R + 3 s
Lower Control Limit (LCL) = R - 3 s

where R and s are calculated as in Section 8.2.3. The UCL and LCL can be used to construct control charts[9] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[9].

8.4 The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 13.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate through the analysis of a one-liter aliquot of reagent water, that all

glassware and reagents interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as gas chromatography with a dissimilar column, specific element detector, or mass spectrometer must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices [10] should be followed, except that the bottle must not be prerinsed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4°C from the time of collection until extraction.

9.3 All samples must be extracted within 7 days and completely analyzed within 40 days of extraction.[2]

10. Sample Extraction

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a two-liter separatory funnel.

10.2 Add 60 mL methylene chloride to the sample bottle, seal, and shake 30 seconds to rinse the inner walls. Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation or other physical methods. Collect the methylene chloride extract in a 250-mL Erlenmeyer flask.

10.3 Add a second 60-mL volume of methylene chloride to the sample bottle and repeat the extraction procedure a second time, combining the extracts in the Erlenmeyer flask. Perform a third extraction in the same manner.

10.4 Assemble a Kuderna-Danish (K-D) concentrator by attaching a 10-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used in place of the K-D if the requirements of Section 8.2 are met.

10.5 Pour the combined extract through a drying column containing about 10 cm of anhydrous sodium sulfate, and collect the extract in the K-D concentrator. Rinse the Erlenmeyer flask and column with 20 to 30 mL of methylene chloride to complete the quantitative transfer.

10.6 Add one or two clean boiling chips to the evaporative flask and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL methylene chloride to the top. Place the K-D apparatus on a hot water bath (60 to 65°C) so that the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood with condensed solvent. When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus and allow it to drain and cool for a least 10 minutes.

10.7 Increase the temperature of the hot water bath to about 80°C. Momentarily remove the Snyder column, add 50 mL of hexane and a new boiling chip and reattach the Snyder column. Pour about 1 mL of hexane into the top of the Snyder column and concentrate the solvent extract as before. Elapsed time of concentration should be 5 to 10 minutes. When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus and allow it to drain and cool for at least 10 minutes.

10.8 Remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of hexane and adjust the volume to 10 mL. A 5-mL syringe is recommended for this operation. Stopper the concentrator tube and store refrigerated if further processing will not be performed immediately. If the extracts will be stored longer than two days, they should be transferred to Teflon-sealed screw-cap bottles. If the sample extract requires no further cleanup, proceed with gas chromatographic analysis. If the sample requires cleanup, proceed to Section 11.

10.9 Determine the original sample volume by refilling the sample bottle to the mark and transferring the water to a 1000-mL graduated cylinder. Record the sample volume to the nearest 5 mL.

11. Cleanup and Separation

11.1 Cleanup procedures may not be necessary for a relatively clean sample matrix. The cleanup procedures recommended in this method have been used for the analysis of various clean waters and industrial effluents. If particular circumstances demand the use of an alternative cleanup procedure, the analyst must determine the elution profile and demonstrate that the recovery of each compound of interest is no less than 85%.

11.2 If the entire extract is to be cleaned up by one of the following two procedures, it must be concentrated to about 2 mL. To the concentrator tube in Section 10.8, add a clean boiling chip and attach a two-ball micro-Snyder column. Prewet the column by adding about 0.5 mL hexane to the top. Place the K-D apparatus on a hot water bath (80°C) so that the concentrator tube is partially immersed in hot water. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 5 to 10 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood. When the apparent volume of liquid reaches about 0.5 mL, remove the K-D apparatus and allow it to drain and

296

cool for at least 10 minutes. Remove the micro-Snyder column and rinse its lower joint into the concentrator tube with 0.2 mL of hexane. Proceed with one of the following cleanup procedures. Proper use of either procedure should yield acceptable results.

11.3 Florisil column cleanup for phthalate esters.

11.3.1 Place 10g of Florisil into a 10 mm ID chromatography column and tap the column to settle the Florisil. Add 1 cm of anhydrous sodium sulfate to the top of the Florisil.

11.3.3 Preelute the column with 40 mL of hexane. Discard this eluate and just prior to exposure of the sodium sulfate layer to the air, transfer the 2 mL sample extract onto the column, using an additional 2 mL of hexane to complete the transfer.

11.3.4 Just prior to exposure of the sodium sulfate layer to the air, add 40 mL hexane and continue the elution of the column. Discard this hexane eluate.

11.3.5 Next elute the phthalate esters with 100 mL of 20% ethyl ether/80% hexane (V/V) into a 500-mL K-D flask equipped with a 10-mL concentrator tube. Elute the column at a rate of about 2 mL/min for all fractions. Concentrate the collected fraction by standard K-D technique. No solvent exchange is necessary. After concentration and cooling, adjust the volume of the cleaned up extract to 10 mL in the concentrator tube and analyze by gas chromatography.

11.4 Alumina column cleanup for phthalate esters.

11.4.1 Place 10g of alumina into a 10 mm ID chromatography column and tap the column to settle the alumina. Add 1 cm of anhydrous sodium sulfate to the top of the alumina.

11.4.3 Preelute the column with 40 mL of hexane. Discard this eluate and just prior to exposure of the sodium sulfate layer to the air, transfer the 2 mL sample extract onto the column, using an additional 2 mL of hexane to complete the transfer.

11.4.4 Just prior to exposure of the sodium sulfate layer to the air add 35 mL of hexane and continue the elution of the column. Discard this hexane eluate.

11.4.5 Next elute the column with 140 mL of 20% ethyl ether/80% hexane (V/V) into a 500 mL K-D flask equipped with a 10-mL concentrator tube. Elute the column at a rate of about 2 mL/min for all fractions. Concentrate the collected fraction by standard K-D technique. No solvent exchange is necessary. After concentration and cooling, adjust the volume of the cleaned up extract to 10 mL in the concentrator tube and analyze by gas chromatography.

12. Gas Chromatography

12.1 Table 1 summarizes the recommended operating conditions for the gas chromatograph. Included in this table are estimated retention times and MDL that can be achieved by this method. Examples of the separations achieved by column 1 are shown in Figures 1 and 2. Other packed columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met. Capillary (open-tubular) columns may also be used if the relative standard deviations of responses for replicate injections are demonstrated to be less than 6% and the requirements of Section 8.2 are met.

12.2 Calibrate the system daily as described in Section 7.

12.3 If the internal standard approach is being used, the analyst must not add the internal standard to sample extracts until immediately before injection into the instrument. Mix thoroughly.

12.4 Inject 2 to 5 µL of the sample extract using the solvent-flush technique[11]. Smaller (1.0 µL) volumes can be injected if automatic devices are employed. Record the volume injected to the nearest 0.05 µL, and the resulting peak size in area or peak height units.

12.5 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size, however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

12.6 If the response for the peak exceeds the working range of the system, dilute the extract and reanalyze.

12.7 If the measurement of the peak response is prevented by the presence of interferences, further cleanup is required.

13. Calculations

13.1 Determine the concentration of individual compounds in the sample.

13.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the peak response using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated from equation 2.

Eq. 2. Concentration, µg/L $\frac{(A)(V_t)}{(V_i)(V_s)}$

where:

- A = Amount of material injected, in nanograms
- V_i = Volume of extract injected (µL).
- V_t = Volume of total extract (µL)
- V_s = Volume of water extracted (mL)

13.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and equation 3.

Eq. 3. Concentration, µg/L $\frac{(A_s)(I_s)}{(A_{is})(RF)(V_o)}$

where:

- A_s = Response for the parameter to be measured.
- A_{is} = Response for the internal standard.
- I_s = Amount of internal standard added to each extract (µg)
- V_o = Volume of water extracted, in liters

13.2 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.3 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits in Section 8.3, data for the affected parameters must be labeled as suspect.

14. Method Performance

14.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[11]. The MDL concentrations listed in Table 1 were obtained using reagent water[12]. Similar results were achieved using representative wastewaters.

14.2 This method has been tested for linearity of recovery from spiked reagent water and has been demonstrated to be applicable over

293

the concentration range from 5 X MDL to 1000 X MDL with the following exceptions dimethyl and diethyl phthalate recoveries at 1000 X MDL were low (70%), bis-2-ethylhexyl and di-n-octyl phthalate recoveries at 5 X MDL were low (60%)[12]

14.3 In a single laboratory (Southwest Research Institute), using spiked wastewater samples, the average recoveries presented in Table 2 were obtained Each spiked sample was analyzed in triplicate on two separate days The standard deviation of the percent recovery is also included in Table 2[12]

14.4 The U S Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. See Appendix A.

2 "Determination of Phthalates in Industrial and Municipal Wastewaters " Report for EPA Contract 68-03-2606 (in preparation).

3. ASTM Annual Book of Standards, Part 31, D3694, "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 679, 1980.

4 Giam, C.S., Chan, H.S. and Neff, G.S., "Sensitive Method for Determination of Phthalate Ester Plasticizers in Open-Ocean Biota Samples," *Analytical Chemistry, 47*, 2225, (1975).

5. Giam, C.S., Chan, H.S., "Control of Blanks in the Analysis of Phthalates in Air and Ocean Biota Samples," National Bureau of Standards (U.S.), Special Publication 442, pp. 701-708, 1976.

6. "Carcinogens - Working with Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.

7. "OSHA Safety and Health Standards, General Industry," (29 CFR 1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).

8 "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979

9 "Handbook for Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268, March 1979.

10. ASTM Annual Book of Standards, Part 31, D3370, "Standard Practice for Sampling Water," American Society for Testing and Materials, Philadelphia, PA, p. 76, 1980.

11. Burke, J.A., "Gas Chromatography For Pesticide Residue Analysis; Some Practical Aspects," *Journal of the Association of Official Analytical Chemists, 48*, 1037 (1965).

12. "Method Detection Limit and Analytical Curve Studies, EPA Methods 606, 607, and 608." Special letter report for EPA Contract 68-03-2605. Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268

Table 1. *Chromatographic Conditions and Method Detection Limits*

Parameter	Retention Time (min.) Column 1	Column 2	Method Detection Limit (µg/L)
Dimethyl phthalate	2.03	0.95	0.29
Diethyl phthalate	2.82	1.27	0.49
Di-n-butyl phthalate	8.65	3.50	0.36
Butyl benzyl phthalate	6.94*	5.11*	0.34
Bis(2-ethylhexyl) phthalate	8.92*	10.5 *	2.0
Di-n-octyl phthalate	16.2 *	18.0 *	3.0

Column 1 conditions: Supelcoport (100/120 mesh) coated with 1.5% SP-2250/1.95% SP-2401 packed in a 1.8 m long x 4 mm ID glass column with 5% methane/95% argon carrier gas at a flow rate of 60 mL/min. Column temperature, isothermal at 180°C, except where otherwise indicated.

Column 2 conditions: Supelcoport (100/120 mesh) coated with 3% OV-1 in a 1.8 m long x 4 mm ID glass column with 5% methane/95% argon carrier gas at 60 mL/min flow rate. Column temperature isothermal 200°C except where otherwise indicated.

**Retention time based upon isothermal oven temperature of 220°C.*

Table 2. *Single Operator Accuracy and Precision*

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
Bis(2-ethylhexyl) phthalate	85	4.2	24-1000	24	4
Butyl benzyl phthalate	82	6.5	3-100	24	4
Di-n-butyl phthalate	80	6.2	20-1500	24	4
Diethyl phthalate	94	1.3	15-50	18	3
Dimethyl phthalate	94	3.4	15-50	18	3
Di-n-octyl phthalate	86	4.9	40-150	24	4

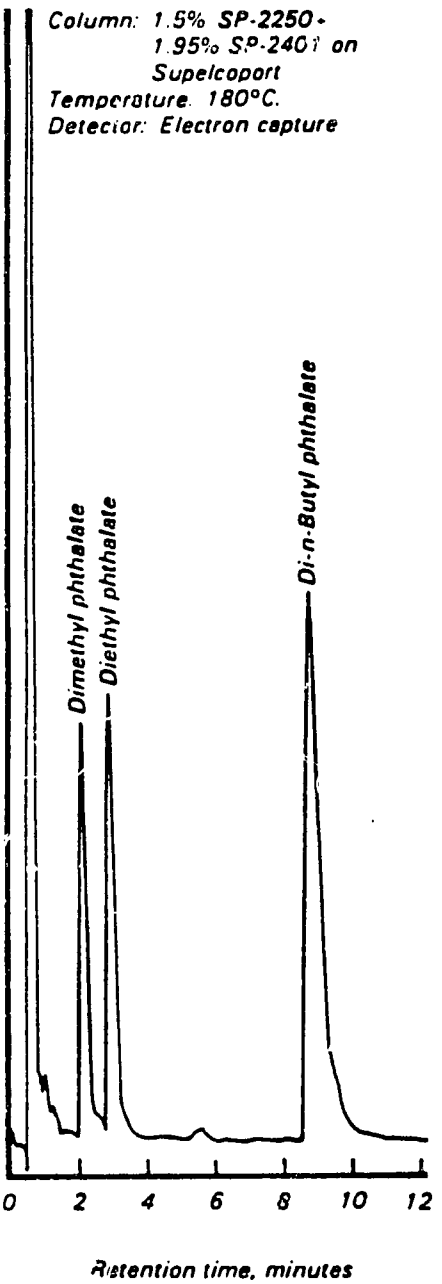


Figure 1. *Gas chromatogram of phthalates.*

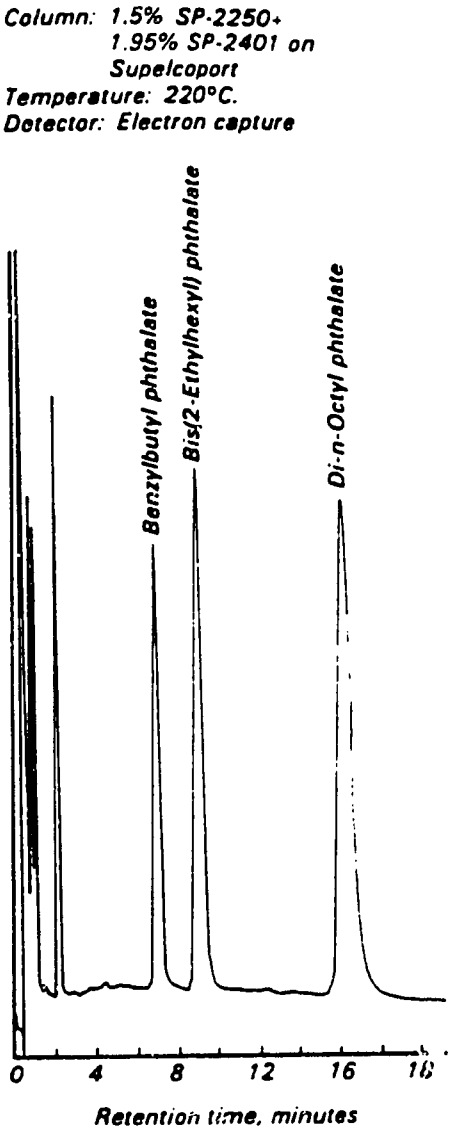


Figure 2. *Gas chromatogram of phthalates.*

795

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

Test Method

Nitrosamines — Method 607

1. Scope and Application

1.1 This method covers the determination of certain nitrosamines. The following parameters can be determined by this method:

Parameter	STORET No.	CAS No.
N-Nitrosodimethylamine	34438	62-75-9
N-Nitrosodiphenylamine	34433	86-30-6
N-Nitrosodi-n-propylamine	34428	621-64-7

1.2 This is a gas chromatographic (GC) method applicable to the determination of the parameters listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for any or all of the compounds above, compound identifications should be supported by at least one additional qualitative technique. This method describes analytical conditions for a second gas chromatographic column that can be used to confirm measurements made with the primary column. Method 625 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results for N-nitrosodi-n-propylamine. In order to confirm the presence of N-nitrosodiphyenylamine, the cleanup procedure specified in Section 11.3 or 11.4 must be used. In order to confirm the presence of N-nitrosodimethylamine by GC/MS, chromatographic column 1 of this method must be substituted for the column recommended in method 625. Confirmation of these parameters using GC-high resolution mass spectrometry or a Thermal Energy Analyzer is also recommended practice[1,2].

1.3 The method detection limit (MDL defined in Section 14.1)[3] for each parameter is listed in Table 1. The MDL for a specific wastewater may differ from those listed, depending upon the nature of interferences in the sample matrix.

1.4 Any modification of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval of alternate test procedures under 40 CFR 136.4 and 136.5.

1.5 This method is restricted to use by or under the supervision of analysts experienced in the use of gas chromatography and in the interpretation of gas chromatograms. Each analyst must demonstrate the ability to genarate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 A measured volume of sample, approximately one liter, is solvent extracted with methylene chloride using a separatory funnel. The methylene chloride extract is washed with dilute HCl to remove free amines, dried and concentrated to a volume of

296

10 mL or less. Gas chromatographic conditions are described which permit the separation and measurement of the compounds in the extract after it has been exchanged to methanol.[4]

2.2 The method provides Florisil and alumina column cleanup procedures to separate diphenylamine from the nitrosamines and to aid in the elimination of interferences that may be encountered.

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in gas chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned[5]. Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and distilled water. It should then be drained dry, and heated in a muffle furnace at 400 °C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedures (Section 11) can be used to overcome many of these interferences, but unique samples may require additional cleanup approaches to achieve the MDL listed in Table 1.

3.3 N-Nitrosodiphenylamine is reported[6-9] to undergo transnitrosation reactions. Care must be exercised in the heating or concentrating of solutions containing this compound in the presence of reactive amines.

3.4 The sensitive and selective Thermal Energy Analyzer and the reductive Hall detector may be used in place of the nitrogen-phosphorus detector when interferences are encountered. The Thermal Energy Analyzer offers the highest selectivity of the non-mass spectrometric detectors.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined, however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[10-12] for the information of the analyst.

4.2 These nitrosamines are known carcinogens[13-17], therefore, utmost care must be exercised in the handling of these materials. Nitrosamine reference standards and standard solutions should be handled and prepared in a ventilated glove box within a properly ventilated room.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle—Amber glass, one-liter or one-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional)—Must incorporate glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4 °C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.2 Glassware (All specifications are suggested. Catalog numbers are included for illustration only).

5.2.1 Separatory funnel—2000-mL and 250-mL, with Teflon stopcock.

5.2.2 Drying column—Chromatographic column approximately 400 mm long × 19 mm ID with coarse frit.

5.2.3 Concentrator tube, Kuderna-Danish—10-mL, graduated (Kontes K-570050-1025 or equivalent). Calibration must be checked at the volumes employed in the test. Ground glass stopper is used to prevent evaporation of extracts.

5.2.4 Evaporative flask, Kuderna-Danish—500-mL (Kontes K-570001-0500 or equivalent). Attach to concentrator tube with springs.

5.2.5 Snyder column, Kuderna-Danish—three-ball macro (Kontes K-503000-0121 or equivalent).

5.2.6 Snyder column, Kuderna-Danish—two-ball micro (Kontes K-569001-0219 or equivalent).

5.2.7 Vials—Amber glass, 10- to 15-mL capacity, with Teflon-lined screw cap.

5.2.8 Chromatographic column—Pyrex, approximately 400 mm long × 22 mm ID, with coarse fritted disc at bottom and Teflon stopcock (Kontes K-420540-0234 or equivalent), for use in Florisil column cleanup procedure.

5.2.9 Chromatographic column—Pyrex, approximately 300 mm long × 10 mm ID, with coarse fritted disc at bottom and Teflon stopcock (Kontes K-420540-0213 or equivalent), for use in alumina column cleanup procedure.

5.3 Boiling chips—approximately 10/40 mesh. Heat to 400 °C for 30 minutes or Soxhlet extract with methylene chloride.

5.4 Water bath—Heated, with concentric ring cover, capable of temperature control (± 2 °C). The bath should be used in a hood.

5.5 Balance—Analytical, capable of accurately weighing 0.0001 g.

5.6 Gas chromatograph—An analytical system complete with gas chromatograph suitable for on-column injection and all required accessories including syringes, analytical columns, gases, detector, and strip-chart recorder. A data system is recommended for measuring peak areas.

5.6.1 Column 1—1.8 m long × 4 mm ID Pyrex glass, packed with Chromosorb W AW (80/100 mesh) coated with 10% Carbowax 20 M/2% KOH or equivalent. This column was used to develop the method performance statements in Section 14. Guidelines for the use of alternate column packings are provided in Section 12.2.

5.6.2 Column 2—1.8 m long × 4 mm ID, Pyrex glass packed with Supelcoport (100/120 mesh) coated with 10% SP-2250.

5.6.3 Detector—Nitrogen-Phosphorus, reductive Hall or Thermal Energy Analyzer[1,2]. These detectors have proven effective in the analysis of wastewaters for the parameters listed in the scope. A nitrogen-phosphorus detector was used to develop the method performance statements in Section 14. Guidelines for the use of alternate detectors are provided in Section 12.2.

6. Reagents

6.1 Reagent water—Reagent water is defined as a water in which an interferent is not observed at the MDL of each parameter of interest.

6.2 Sodium hydroxide solution (10 N)—(ACS) Dissolve 40g NaOH in reagent water and dilute to 100 mL.

6.3 Sodium thiosulfate—(ACS) Granular.

6.4 Sulfuric acid solution (1 + 1)—(ACS) Slowly, add 50 mL H_2SO_4 (sp. gr. 1.84) to 50 mL of reagent water.

6.5 Sodium sulfate—(ACS) Granular, anhydrous. Purify by heating at 400 °C for 4 hours in a shallow tray.

6.6 Hydrochloric acid (1 + 9)—(ACS) Add one volume of conc. HCl to nine volumes of reagent water.

6.7 Acetone, methanol, methylene chloride and pentane—Pesticide quality or equivalent.

6.8 Ethyl ether—Nanograde, redistilled in glass if necessary.

6.8.1 Must be free of peroxides as indicated by EM Quant test strips. (Test strips are available from Scientific Products Co., Catalog No. P1126-8 and others.)

6.8.2 Procedures recommended for removal of peroxides are provided with the test strips. After cleanup, 20 mL ethyl alcohol preservative must be added to each liter of ether.

6.9 Florisil—PR grade (60/100 mesh); purchase activated at 1250 °F and store in dark in glass containers with glass stoppers or foil-lined screw caps. Before use, activate each batch at least 16 hours at 130 °C in a foil covered glass container.

6.10 Alumina—Basic activity Super I, W 200 series (ICN Life Sciences Group, No. 404571). Place 100 g of alumina, as it comes from the manufacturer, into a 500-mL reagent bottle and add 2 mL of reagent water. Mix the alumina preparation thoroughly by shaking or rolling for 10 minutes and let it stand for at least two hours. The preparation should be homogenous before use. Keep the bottle sealed tightly to ensure proper activity.

6.11 Stock standard solutions (1.00 μg/μL)—Stock standard solutions can be prepared from pure standard materials or purchased as certified solutions.

6.11.1 Prepare stock standard solutions by accurately weighing about 0.0100 grams of pure material. Dissolve the material in pesticide quality methanol, dilute to volume in a 10-mL volumetric flask. Larger volumes can be used at the convenience of the analyst. If compound purity is assayed to be 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.11.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at 4 °C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check standards that can be used to determine the accuracy of calibration standards will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

6.11.3 Stock standard solutions must be replaced after six months, or sooner if comparison with check standards indicate a problem.

7. Calibration

7.1 Establish gas chromatographic operating parameters equivalent to those indicated in Table 1. The gas chromatographic system can be calibrated using the external standard technique (Section 7.2) or the internal standard technique (Section 7.3).

7.2 External standard calibration procedure:

7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with methanol. One of the external standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.2.2 Using injections of 2 to 5 μL of each calibration standard, tabulate peak height or area responses against the mass injected. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over the working range (<10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.2.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that compound.

7.3 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples.

7.3.1 Prepare calibration standards at a minimum of three concentration

298

levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask. To each calibration standard, add a known constant amount of one or more internal standards, and dilute to volume with methanol. One of the standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.3.2 Using injections of 2 to 5 µL of each calibration standard, tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1.

Eq. 1. $RF = (A_s C_{is})/(A_{is} C_s)$

where:

A_s = Response for the parameter to be measured.

A_{is} = Response for the internal standard.

C_{is} = Concentration of the internal standard, (µg/L).

C_s = Concentration of the parameter to be measured, (µg/L).

If the RF value over the working range is a constant (<10% RSD), the RF can be assumed to be nonvariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.3.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound.

7.4 Before using any cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in methanol 1000 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 2, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > 2p$ or $|X - R| > 2p$, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance:

Upper Control Limit (UCL) = R + 3s
Lower Control Limit (LCL) = R − 3s

where R and s are calculated as in Section 8.2.3. The UCL and LCL can be used to construct control charts[18] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[18].

8.4. The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 13.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

291

8.5 Before processing any samples, the analyst should demonstrate through the analysis of a one-liter aliquot of reagent water, that all glassware and reagent interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as gas chromatography with a dissimilar column, specific element detector, or mass spectrometer must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices[19] should be followed, except that the bottle must not be prerinsed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4 °C from the time of collection until extraction. Fill the sample bottle and at time of collection, if residual chlorine is present, add 80 mg of sodium thiosulfate per liter of sample. U.S. Environmental Protection Agency methods 330.4 and 330.5 may be used for measurement of residual chlorine[20]. Field test kits are available for this purpose. If diphenylnitrosamine is to be determined, adjust the sample pH to 7 to 10 with sodium hydroxide or sulfuric acid.

9.3 All samples must be extracted within 7 days and completely analyzed within 40 days of extraction[4].

9.4 Nitrosamines are known to be light sensitive[7]. Samples should be stored in amber or foil-wrapped bottles in order to minimize photolytic decomposition.

10. Sample Extraction

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a two-liter separatory funnel. Check the pH of the sample with wide-range pH paper and adjust to within the range of 5 to 9 with sodium hydroxide or sulfuric acid.

10.2 Add 60 mL methylene chloride to the sample bottle, seal, and shake 30 seconds to rinse the inner surface. Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods. Collect the methylene chloride extract in a 250-mL Erlenmeyer flask.

10.3 Add a second 60-mL volume of methylene chloride to the sample bottle and repeat the extraction procedure a second time, combining the extracts in the Erlenmeyer flask. Perform a third extraction in the same manner.

10.4 Assemble a Kuderna-Danish (K-D) concentrator by attaching a 10-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used in place of the K-D if the requirements of Section 8.2 are met.

10.5 Add 10 mL of HCl (1 + 1) solution to the combined extracts and shake for two minutes. Allow the layers to separate. Transfer the combined extract to a drying column containing 10 cm of anhydrous sodium sulfate and collect it in the K-D concentrator. Rinse the Erlenmeyer flask and column with 20 to 30 mL of methylene chloride to complete the quantitative transfer.

10.6 Add one or two clean boiling chips to the evaporative flask and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL methylene chloride to the top. Place the K-D apparatus on a hot water bath (60 to 65 °C) so that the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood with condensed solvent. When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus and allow it to drain and cool for at least 10 minutes. Remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of methylene chloride. A 5-mL syringe is recommended for this operation. Stopper the concentrator tube and store refrigerated if further processing will not be performed immediately. If the extracts will be stored longer than two days, they should be transferred to Teflon-sealed screw-cap bottles.

10.7 If N-nitrosodiphenylamine is to be measured by gas chromatography, the analyst must first use a cleanup column to eliminate diphenylamine interference (Section 11). If N-nitrosodiphenylamine is of no interest, the analyst may proceed directly with gas chromatographic analysis (Section 12).

10.8 Determine the original sample volume by refilling the sample bottle to the mark with water and measure it in a 1000-mL graduated cylinder. Record the sample volume to the nearest 5 mL.

11. Cleanup and Separation

11.1 Cleanup procedures may not be necessary for a relatively clean sample matrix. The cleanup procedures recommended in this method have been used for the analysis of various clean waters and industrial effluents. If particular circumstances demand the use of an alternative cleanup procedure, the analyst must determine the elution profile and demonstrate that the recovery of each compound of interest is no less than 85%. Diphenylamine, if present in the original sample extract must be separate from the nitrosamines if N-nitrosodiphenylamine is to be determined by this method.

11.2 If the entire extract is to be cleaned up by one of the following procedures, it must be concentrated to 2.0 mL. To the concentrator tube in Section 10.6, add a clean boiling chip and attach a two-ball micro-Snyder column. Prewet the column by adding about 0.5 mL of methylene chloride to the top. Place the K-D apparatus on a hot water bath (60 to 65 °C) so that the concentrator tube is partially immersed in the hot water. Adjust the vertical position of the apparatus and the water temperature as required to

300

complete the concentration in 5 to 10 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood. When the apparent volume of liquid reaches about 0.5 mL, remove the K-D apparatus from the hot water bath and allow it to drain for at least 10 minutes while cooling. Remove the micro-Snyder column and rinse its lower joint into the concentrator tube with 0.2 mL of methylene chloride. Adjust the final volume to 2.0 mL and proceed with one of the following cleanup procedures.

11.3 Florisil column cleanup for nitrosamines:

11.3.1 Place 22 g of activated Florisil in a 22 mm ID chromatographic column. After settling the Florisil by tapping the column, add about a 5-mm layer of anhydrous granular sodium sulfate to the top.

11.3.2 Preelute the column, after colling, with 40 mL of ethyl ether/pentane (15 + 85)(V/V). Discard the eluate and just prior to exposure of the sodium sulfate layer to air, quantitatively transfer a 2.0 mL aliquot of the sample extract into the column by decantation using an additional 2 mL of pentane to complete the transfer.

11.3.3 Perform the first elution with 90 mL of ethyl ether/pentane (15 + 85)(V/V) and discard the eluate. This fraction will contain the diphenylamine, if it is present in the extract.

11.3.4 Perform the second elution with 100 mL of acetone/ethyl ether (5 + 95)(V/V) and collect the eluate in a 500-mL K-D flask equipped with a 10-mL concentrator tube. This fraction will contain all of the nitrosamines listed in the scope of the method.

11.3.5 Add 15 mL of methanol to the collected eluate and concentrate as in Section 10.6 at 70 to 75 °C, using pentane for the diluting and rinsing solvent.

11.3.6 Analyze by gas chromatography.

11.4 Alumina column cleanup for nitrosamines:

11.4.1 Place 12 g of the alumina preparation into a 10 mm ID chromatographic column and tap the column to settle the alumina. Add 1 to 2 cm of anhydrous sodium sulfate to the top of the alumina.

11.4.2 Preelute the column with 10 mL of ethyl ether/pentane (3 + 7)(V/V). Discard the eluate (about 2 mL) and, just prior to exposure of the sodium sulfate layer to air, transfer a 2.0 mL aliquot of the sample extract onto the column by decantation using an additional 2 mL of pentane to complete the transfer.

11.4.3 Just prior to exposure of the sodium sulfate layer to the air, add 70 mL of ethyl ether/pentane (3 + 7)(V/V). Discard the first 10 mL of eluate. Collect the remainder of the eluate in a 500-mL K-D flask equipped with a 10-mL concentrator tube. This fraction contains N-nitrosodiphenylamine and probably a small amount of N-nitrosodi-n-propylamine.

11.4.4 Next elute the column with 60 mL of ethyl ether/pentane (1 + 1) (V/V), collecting the eluate in a second K-D flask equipped with a 10-mL concentrator tube. Add 15 mL of methyl alcohol to the K-D flask. This fraction will contain N-nitrosodimethylamine, most of the N-nitrosodi-n-propylamine and any diphenylamine that is present.

11.4.5 Concentrate both fractions as in Section 10.6 using pentane as the diluting and rinsing solvent.

11.4.6 Analyze the fractions by gas chromatography.

12. Gas Chromatography

12.1 N-nitrosodiphenylamine completely reacts to form diphenylamine at the normal operating temperatures of a GC injection port (200 to 250 °C). Thus, N-nitrosodiphenylamine is chromatographed and detected as diphenylamine. Accurate determination depends on removal of diphenylamine that may be present in the original extract prior to GC (See Section 11).

12.2 Table 1 summarizes the recommended operating conditions for the gas chromatograph. This Table includes retention times and MDL that were obtained under these conditions. Examples of the parameter separations achieved by these columns are shown in Figures 1 and 2. Other packed columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met. Capillary (open-tubular) columns may also be used if the relative standard deviations of responses for replicate injections are demonstrated to be less than 6% and the requirements of Section 8.2 are met.

12.3 Calibrate the system daily as described in Section 7.

12.4 If the extract has not been submitted to one of the cleanup procedures in Section 11, it is necessary to exchange the solvent from methylene chloride to methyl alcohol before the thermionic detector can be used. To a 1-10 mL volume of methylene chloride extract in a concentrator tube, add 2 mL methyl alcohol, and a clean boiling chip. Attach a two-ball micro-Snyder column. Prewet the column by adding about 0.5 mL methylene chloride through the top. Place the K-D apparatus on a boiling water bath so that the concentrator tube is partially immersed in the hot water. Adjust the vertical position and insulate the apparatus as necessary to complete the concentration in 5 to 10 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood. When the apparent volume of liquid reaches about 0.5 mL, remove the K-D and allow it to drain for at least 10 minutes while cooling. Remove the micro-Snyder column and rinse its lower joint into the concentrator tube with 0.2 mL of methyl alcohol. Adjust the final volume to 2.0 mL.

12.5 If the internal standard approach is being used, add the internal standard to sample the extract and mix thoroughly, immediately before injection into the instrument.

12.6 Inject 2 to 5 µL of the sample extract using the solvent-flush technique[21]. Smaller (1.0 µL) volumes can be injected if automatic devices are employed. Record the volume injected to the nearest 0.05 µL, and the resulting peak size in area or peak height units.

12.7 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size; however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

12.8 If the response exceeds the working range of the system, dilute the extract and reanalyze.

12.9 If the measurement of the peak response is prevented by the presence of interferences, further cleanup is required.

13. Calculations

13.1 Determine the concentration of individual compounds in the sample.

13.1.1 If the external standard calibration procedure is used, calculate

301

the amount of material injected from the peak response using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated from equation 2:

$$\text{Eq. 2. Concentration, } \mu g/L = \frac{(A)(V_t)}{(V_i)(V_s)}$$

where:

A = Amount of material injected, in nanograms.
V_i = Volume of extract injected (µL).
V_t = Volume of total extract (µL).
V_s = Volume of water extracted (mL).

13.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and equation 3.

Eq. 3

$$\text{Concentration, } \mu g/L = \frac{(A_s)(I_s)}{(A_{is})(RF)(V_o)}$$

where:

A_s = Response for the parameter to be measured.
A_{is} = Response for the internal standard.
I_s = Amount of internal standard added to each extract (µg).
V_o = Volume of water extracted, in liters.

13.2 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.3 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits in Section 8.3, data for the affected parameters must be labeled as suspect.

14. Method Performance

14.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[3]. The MDL concentrations listed in Table 1 were obtained using reagent water[22]. Similar results were achieved using representative wastewaters.

14.2 This method has been tested for linearity of analyte recovery from reagent water and has been demonstrated to be applicable over the concentration range from 4 × MDL to 1000 × MDL[22].

14.3 In a single laboratory (Southwest Research Institute), using spiked wastewater samples, the average recoveries presented in Table 2 were obtained[4]. Each spiked sample was analyzed in triplicate on two separate days. The standard deviation of the percent recovery is also included in Table 2.

14.4 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. Fine, D.H., Lieb, D. and Rufeh, R., "Principle of Operation of the Thermal Energy Analyzer for the Trace Analysis of Volatile and Non-volatile N-nitroso Compounds." *Journal of Chromatography, 107,* 351, (1975).
2. Fine, D.H. Hoffman, F., Rounbehler, D.P. and Belcher, N.M., "Analysis of N-nitroso Compounds by Combined High Performance Liquid Chromatography and Thermal Energy Analysis," Walker, E.A., Bogovski, P. and Griciute, L., editors, N-Nitroso Compounds—Analysis and Formation Lyon, International Agency for Research on Cancer (IARC Scientific Publications No. 14), pp. 43-50, (1976)
3. See Appendix A.
4. "Determination of Nitrosamines in Industrial and Municipal Wastewaters." Report for EPA Contract 68-03-2606 (In preparation).
5. ASTM Annual Book of Standards, Part 31, D 3694. "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 76, 1980.
6. Buglass, A.J., Challis, B.C. and Osborn, M.R. "Transnitrosation and Decomposition of Nitrosamines." Bogovski, P. and Walker, E.A., editors, N-Nitroso Compounds in the Environment, Lyon, International Agency for Research on Cancer (IARC Scientific Publication No. 9), pp. 94-100, (1974).
7. Burgess, E.M. and Lavanish, J.M., "Photochemical Decomposition of N-nitrosamines," *Tetrahedon Letters,* 1221, (1964).
8. Druckrey, H., Preussmann, R., Ivankovic, S. and Schmahl, D., "Organotrope Carcionogene Wirkungen bei 65 Verschiedenen N-Nitroso-Verbindungen an BD-Ratten." *Z. Krebsforsch., 69,* 103 (1967).
9. Fiddler, W. "The Occurrence and Determination of N-nitroso Compounds," *Toxicol. Appl. Pharmacol., 31,* 352, (1975).
10. "Carcinogens—Working With Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.
11. "OSHA Safety and Health Standards, General Industry," (29CFR1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
12. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.
13. Lijinsky, W., "How Nitrosamines Cause Cancer." *New Scientist, 73,* 216, (1977)
14. Mirvish, S.S. "N-Nitroso compounds: Their Chemical and in vivo Formation and Possible Importance as Environmental Carcinogens." *J. Toxicol. Environ. Health, 3,* 1267 (1977).
15. "Reconnaissance of Environmental Levels of Nitrosamines in the Central United States." National Enforcement Investigations Center, Environmental Protection Agency, Report No. EPA-330/1-77-001, (1977)
16. "Atmospheric Nitrosamine Assessment Report." Office of Air Quality Planning and Standards, Environmental Protection Agency, Research Triangle Park, North Carolina, (1976).
17. "Scientific and Technical Assessment Report on Nitrosamines." Office of Research and Development, Environmental Protection Agency, Report No. EPA-560/6-7-001, (1976).
18. "Handbook of Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.
19. ASTM Annual Book of Standards, Part 31, D 3370, "Standard Practice for Sampling Water;" American Society for Testing and Materials, Philadelphia, PA, p. 679, 1980.
20. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600-4/79-020, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.
21. Burke, J.A., "Gas Chromatography for Pesticide Residue Analysis: Some Practical Aspects," *Journal of the Association of Official Analytical Chemists, 48,* 1037 (1965).

302

22. "Method Detection Limit and Analytical Curve Studies EPA Methods 606, 607, 608." special letter report for EPA Contract 68-03-2606, Environmental Monitoring and Support Laboratory – Cincinnati, Ohio 45268.

Table 1. Chromatographic Conditions and Method Detection Limits

Parameter	Retention Time (min.) Column 1	Retention Time (min.) Column 2	Method Detection Limit µg/L
N-Nitrosodimethylamine	4.1	0.88	0.15
N-Nitrosodi-n-propylamine	12.1	4.2	0.46
N-Nitrosodiphenylamine[a]	12.8[b]	6.4[c]	0.81

Column 1 conditions: Chromosorb W AW (80/100 mesh) coated with 10% Carbowax 20 M/2% KOH packed in a 1.8 m long × 4 mm ID glass column with helium carrier gas at a flow rate of 40 mL/min. column temperature Isothermal, at 110°C, except as otherwise indicated.

Column 2 conditions: Supelcoport (100/120 mesh) coated with 10% SP-2250 packed in a 1.8 m long × 4 mm ID glass column with helium carrier gas at a flow rate of 40 mL/min. column temperature, Isothermal at 120°C, except as otherwise indicated.

[a]*Measured as diphenylamine*

[b]*Determined isothermally at 220°C.*

[c]*Determined isothermally at 210°C.*

Table 2. Single Operator Accuracy and Precision

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
N-Nitrosodimethylamine	32	3.7	0.8	29	5
N-Nitrosodiphenylamine	79	7.1	1.2	29	5
N-Nitrosodi-n-propylamine	61	4.2	9.0	29	5

Column: 10% Carbowax 20M + 2% KOH on Chromosorb W-AW
Temperature: 110°
Detector: Phosphorus/Nitrogen

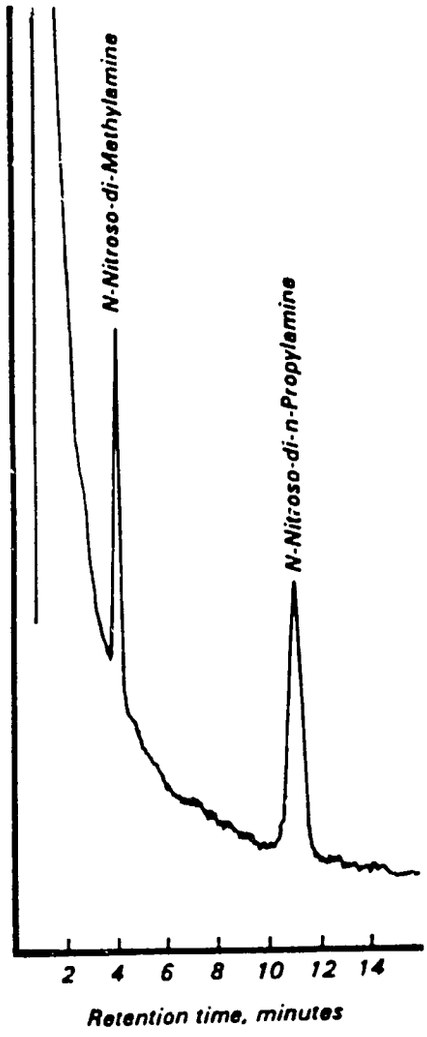


Figure 1. *Gas chromatogram of nitrosamines.*

Column: 10% Carbowax 20M + 2% KOH on Chromosorb W-AW
Temperature: 220°C.
Detector: Phosphorus/Nitrogen

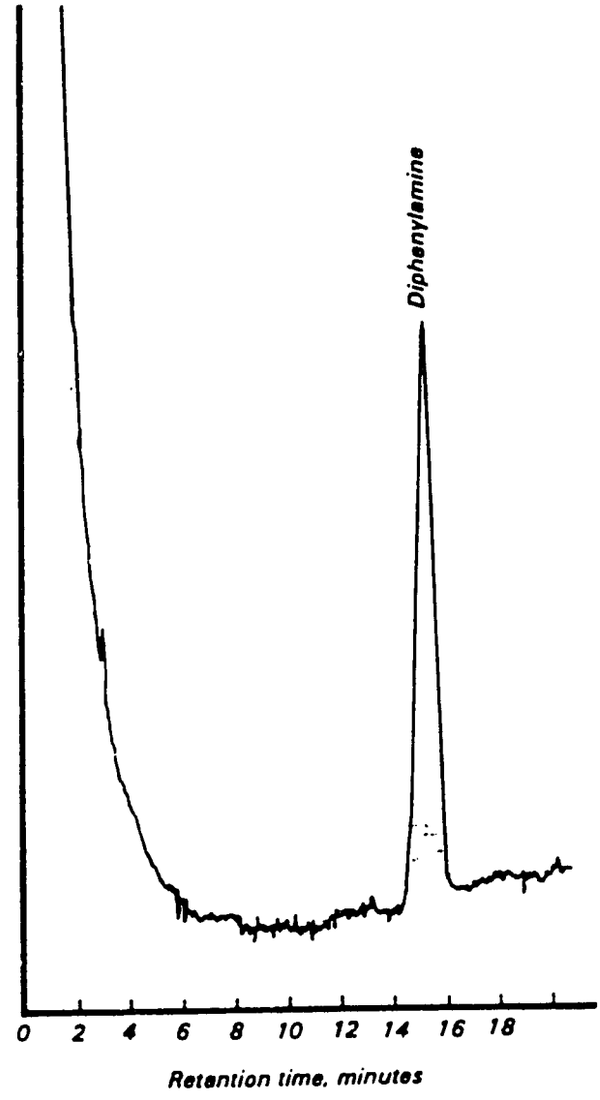


Figure 2. *Gas chromatogram of N-nitrosodiphenylamine as diphenylamine.*

304

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Organochlorine Pesticides and PCBs — Method 608

1. Scope and Application

1.1 This method covers the determination of certain organochlorine pesticides and PCBs. The following parameters can be determined by this method:

Parameter	STORET No.	CAS No.
Aldrin	39330	309-00-2
α-BHC	39337	319-84-6
β-BHC	39338	319-85-7
δ-BHC	34259	319-86-8
γ-BHC	39340	58-89-9
Chlordane	39350	57-74-9
4,4'-DDD	39310	72-54-8
4,4'-DDE	39320	72-55-9
4,4'-DDT	39300	50-29-3
Dieldrin	39380	60-57-1
Endosulfan I	34361	959-98-8
Endosulfan II	34356	33212-65-9
Endosulfan sulfate	34351	1031-07-8
Endrin	39390	72-20-8
Endrin aldehyde	34366	7421-93-4
Heptachlor	39410	76-44-8
Heptachlor epoxide	39420	1024-57-3
Toxaphene	39400	8001-35-2
PCB-1016	34671	12674-11-2
PCB-1221	39488	11104-28-2
PCB-1232	39492	11141-16-5
PCB-1242	39496	53469-21-9
PCB-1248	39500	12672-29-6
PCB-1254	39504	11097-69-1
PCB-1260	39508	11096-82-5

1.2 This is a gas chromatographic (GC) method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for any or all of the compounds above, compound identifications should be supported by at least one additional qualitative technique. This method describes analytical conditions for a second gas chromatographic column that can be used to confirm measurements made with the primary column. Method 625 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and

305

quantitative confirmation of results for all of the parameters listed above, using the extract produced by this method.

1.3 The method detection limit (MDL, defined in Section 14.1)[1] for each parameter is listed in Table 1. The MDL for a specific wastewater may differ from those listed, depending upon the nature of interferences in the sample matrix

1.4 The sample extraction and concentration steps in this method are essentially the same as in methods 606, 609, 611 and 612. Thus, a single sample may be extracted to measure the parameters included in the scope of each of these methods. When cleanup is required, the concentration levels must be high enough to permit selection of aliquots as necessary to apply appropriate cleanup procedures. The analyst is allowed the latitude to select gas chromatographic conditions appropriate for the simultaneous measurement of combinations of these parameters.

1.5 Any modification of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval of alternate test procedures under 40 CFR 136.4 and 136.5.

1.6 This method is restricted to use by or under the supervision of analysts experienced in the use of gas chromatography and in the interpretation of gas chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 A measured volume of sample, approximately one-liter, is solvent extracted with methylene chloride using a separatory funnel. The methylene chloride extract is dried and exchanged to hexane, during concentration to a final volume of 10 mL or less. Gas chromatographic conditions are described which permit the separation and measurement of the parameters in the extract by electron capture GC[2].

2.2 The method provides a Florisil column procedure and elemental sulfur removal procedure to aid in the elimination of interferences that may be encountered.

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in gas chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned[3]. Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and distilled water. It should then be drained dry and heated in a muffle furnace at 400 °C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Thorough rinsing with such solvents usually elminates PCB interference. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Interferences by phthalate esters can pose a major problem in pesticide analysis when using the elution capture detector. These compounds generally appear in the chromatogram as large eluting peaks, especially in the 15 and 50% fractions from Florisil. Common flexible plastics contain varying amounts of phthalates. These phthalates are easily extracted or leached from such materials during laboratory operations. Cross contamination of clean glassware routinely occurs when plastics are handled during extraction steps, especially when solvent wetted surfaces are handled. Interferences from phthalates can best be minimized by avoiding the use of plastics in the laboratory. Exhaustive cleanup of reagents and glassware may be required to eliminate background phthalate contamination[4,5]. The interferences from phthalate esters can be avoided by using a microcoulometric or electrolytic conductivity detector.

3.3 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedures in Section 11 can be used to overcome many of these interferences, but unique samples may require additional cleanup approaches to achieve the MDL listed in Table 1.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[6-8] for the information of the analyst.

4.2 The following parameters covered by this method have been tentatively classified as known or suspected, human or mammalian carcinogens: 4,4'-DDT, 4,4'-DDD, the BHCs, and the PCBs. Primary standards of these toxic compounds should be prepared in a hood.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle—Amber glass, one-liter or one-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional)—Must incorporate glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4 °C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible

silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.2 Glassware (All specifications are suggested. Catalog numbers are included for illustration only).

5.2.1 Separatory funnel—2000-mL, with Teflon stopcock.

5.2.2 Drying column—Chromatographic column approximately 400 mm long × 19 mm ID, with coarse frit.

5.2.3 Chromatographic column—Pyrex, 400 mm long × 22 mm ID, with coarse fritted plate and Teflon stopcock (Kontes K-42054 or equivalent).

5.2.4 Concentrator tube, Kuderna-Danish—10-mL, graduated (Kontes K-570050-1025 or equivalent). Calibration must be checked at the volumes employed in the test. Ground glass stopper is used to prevent evaporation of extracts.

5.2.5 Evaporative flask, Kuderna-Danish—500-mL (Kontes K-570001-0500 or equivalent). Attach to concentrator tube with springs.

5.2.6 Snyder column, Kuderna-Danish—three-ball macro (Kontes K-503000-0121 or equivalent).

5.2.7 Vials—Amber glass, 10- to 15-mL capacity, with Teflon-lined screw cap.

5.3 Boiling chips—approximately 10/40 mesh. Heat to 400 °C for 30 minutes or Soxhlet extract with methylene chloride.

5.4 Water bath—Heated, with concentric ring cover, capable of temperature control (± 2 °C). The bath should be used in a hood.

5.5 Balance—Analytical, capable of accurately weighing 0.0001 g.

5.6 Gas chromatograph—An analytical system complete with gas chromatograph suitable for on-column injection and all required accessories including syringes, analytical columns, gases, detector, and strip-chart recorder. A data system is recommended for measuring peak areas.

5.6.1 Column 1—1.8 m long × 4 mm ID glass, packed with 1.5% SP-2250/1.95% SP-2401 on Supelcoport (100/120 mesh) or equivalent. Column 1 was used to develop the method performance statements in Section 14. Guidelines for the use of alternate column packings are provided in Section 12.1.

5.6.2 Column 2—1.8 m long × 4 mm ID glass, packed with 3% OV-1 on Supelcoport (100/120 mesh) or equivalent.

5.6.3 Detector—Electron capture. This detector has proven effective in the analysis of wastewaters for the parameters listed in the scope, and was used to develop the method performance statements in Section 14. Guidelines for the use of alternate detectors are provided in Section 12.1.

6. Reagents

6.1 Reagent water—Reagent water is defined as a water in which an interferent is not observed at the MDL of each parameter of interest.

6.2 Sodium hydroxide solution (10 N)—(ACS). Dissolve 40g NaOH in reagent water and dilute to 100 mL.

6.3 Sodium thiosulfate—(ACS). Granular.

6.4 Sulfuric acid solution (1 + 1)—(ACS). Slowly, add 50 mL H_2SO_4 (sp. gr. 1.84) to 50 mL of reagent water.

6.5 Acetone, hexane, isooctane (2,2,4-trimethylpentane), methylene chloride—Pesticide quality or equivalent.

6.6 Ethyl ether—Pesticide quality or equivalent, redistilled in glass if necessary.

6.6.1 Must be free of peroxides as indicated by EM Laboratories Quant test strips (Available from Scientific Products Co., Cat. No. P1126-8, and others suppliers.)

6.6.2 Procedures recommended for removal of peroxides are provided with the test strips. After cleanup, 20 mL ethyl alcohol preservative must be added to each liter of ether.

6.7 Sodium sulfate—(ACS) Granular, anhydrous. Purify by heating at 400 °C for 4 hours in a shallow tray.

6.8 Florisil—PR grade (60/100 mesh); purchase activated at 1250 °F and store in dark in glass containers with glass stoppers or foil-lined screw caps. Before use, activate each batch at least 16 hours at 130 °C in a foil covered glass container.

6.9 Mercury—Triple distilled.

6.10 Copper powder—Activated.

6.11 Stock standard solutions (1.00 μg/μL)—Stock standard solutions can be prepared from pure standard materials or purchased as certified solutions.

6.11.1 Prepare stock standard solutions by accurately weighing about 0.0100 grams of pure material. Dissolve the material in isooctane, dilute to volume in a 10-mL volumetric flask. Larger volumes can be used at the convenience of the analyst. If compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.11.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at 4 °C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check standards that can be used to determine the accuracy of calibration standards will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

6.11.3 Stock standard solutions must be replaced after six months, or sooner if comparison with check standards indicate a problem.

7. Calibration

7.1 Establish gas chromatographic operating parameters which produce retention times equivalent to those indicated in Table 1. The gas chromatographic system may be calibrated using the external standard technique (Section 7.2) or the internal standard technique (Section 7.3).

7.2 External standard calibration procedure:

7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with isooctane. One of the external standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

307

7.2.2 Using injections of 2 to 5 µL of each calibration standard, tabulate peak height or area responses against the mass injected. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over the working range (<10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.2.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that compound.

7.3 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples.

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask. To each calibration standard, add a known constant amount of one or more internal standards, and dilute to volume with isooctane. One of the standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.3.2 Using injections of 2 to 5 µL of each calibration standard, tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1.

Eq. 1. $RF = (A_s C_{is})/(A_{is} C_s)$

where:

A_s = Response for the parameter to be measured.

A_{is} = Response for the internal standard.

C_{is} = Concentration of the internal standard, (µg/L).

C_s = Concentration of the parameter to be measured, (µg/L).

If the RF value over the working range is a constant (<10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.3.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound.

7.4 The cleanup procedure in Section 11 utilizes Florisil chromatography. Florisil from different batches or sources may vary in absorptive capacity. To standardize the amount of Florisil which is used, the use of lauric acid value[9] is suggested. The referenced procedure determines the adsorption from hexane solution of lauric acid (mg) per gram Florisil. The amount of Florisil to be used for each column is calculated by dividing this factor into 110 and multiplying by 20 g.

7.5 Before using any cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in acetone 1000 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 2, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > 2p$ or $|X - R| > 2p$, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define

308

the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance:

Upper Control Limit (UCL) = R + 3s
Lower Control Limit (LCL) = R − 3s

where R and s are calculated as in Section 8.2.3. The UCL and LCL can be used to construct control charts[10] that are useful in observing trends in performance. The control limits above be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[10].

8.4. he laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 13.5. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate through the analysis of a one-liter aliquot of reagent water, that all glassware and reagent interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as gas chromatography with a dissimilar column, specific element detector, or mass spectrometer must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices[11] should be followed, except that the bottle must not be prewashed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon tubing and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4 °C from the time of collection until extraction. If the samples will not be extracted within 72 hours of collection, the sample should be adjusted to a pH range of 5.0 to 9.0 with sodium hydroxide or sulfuric acid. Record the volume of acid or base used. If aldrin is to be determined, add sodium thiosulfate when residual chlorine is present. U.S. Environmental Protection Agency methods 330.4 and 330.5 may be used to measure chlorine residual[12]. Field test kits are available for this purpose.

9.3 All samples must be extracted within 7 days and completely analyzed within 40 days of extraction[2].

10. Sample Extraction

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a two-liter separatory funnel.

10.2 Add 60 mL methylene chloride to the sample bottle, seal, and shake 30 seconds to rinse the inner surface. Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods. Collect the methylene chloride extract in a 250-mL Erlenmeyer flask.

10.3 Add a second 60-mL volume of methylene chloride to the sample bottle and repeat the extraction procedure a second time, combining the extracts in the Erlenmeyer flask. Perform a third extraction in the same manner.

10.4 Assemble a Kuderna-Danish (K-D) concentrator by attaching a 10-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used in place of the Kuderna Danish if the requirements of Section 8.2 are met.

10.5 Pour the combined extract through a drying column containing about 10 cm of anhydrous sodium sulfate, and collect the extract in the K-D concentrator. Rinse the Erlenmeyer flask and column with 20 to 30 mL of methylene chloride to complete the quantitative transfer.

10.6 Add one or two clean boiling chips to the evaporative flask and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL methylene chloride to the top. Place the K-D apparatus on a hot water bath (60 to 65 °C) so that the concentrator tube is partially immersed in the hot water and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood with condensed solvent. When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus and allow it to drain and cool for at least 10 minutes.

10.7 Increase the temperature of the hot water bath to about 80 °C. Momentarily remove the Snyder column, add 50 mL of hexane and a new boiling chip and reattach the Snyder column. Prewet the column by adding about 1 mL of hexane to the top. Concentrate the solvent extract as before. The elapsed time of concentration should be 5 to 10 minutes. When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus and allow it to drain and cool at least 10 minutes.

309

10.8 Remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of hexane. A 5-mL syringe is recommended for this operation. Stopper the concentrator tube and store refrigerated if further processing will not be performed immediately. If the extracts will be stored longer than two days, they should be transferred to Teflon-sealed screw-cap bottles. If the sample extract requires no further cleanup, proceed with gas chromatographic analysis. If the sample requires cleanup proceed to Section 11.

10.9 Determine the original sample volume by refilling the sample bottle to the mark and transferring the liquid to a 1000-mL graduated cylinder. Record the sample volume to the nearest 5 mL.

11. Cleanup and Separation

11.1 Cleanup procedures may not be necessary for a relatively clean sample matrix. The cleanup procedures recommended in this method have been used for the analysis of various clean waters and industrial effluents. If particular circumstances demand the use of an alternative cleanup procedure, the analyst must determine the elution profile and demonstrate that the recovery of each compound of interest is no less than 85%. The Florisil column allows for a select fractionation of the compounds and will eliminate polar materials. Elemental sulfur interferes with the electron capture gas chromatography of certain pesticides, but can be removed by the techniques described below.

11.2 Florisil column cleanup:

11.2.1 Add a weight of Florisil (nominally 21 g) predetermined by calibration (Section 7.4 and 7.5), to a chromatographic column. Settle the Florisil by tapping the column. Add sodium sulfate to the top of the Florisil to form a layer 1 to 2 cm deep. Add 60 mL of hexane to wet and rinse the sodium sulfate and Florisil. Just prior to exposure of the sodium sulfate to air, stop the elution of the hexane by closing the stopcock on the chromatography column. Discard the eluate.

11.2.2 Adjust the sample extract volume to 10 mL with hexane and transfer it from the K-D concentrator tube to the Florisil column. Rinse the tube twice with 1 to 2 mL hexane, adding each rinse to the column.

11.2.3 Place a 500-mL K-D flask and clean concentrator tube under the chromatography column. Drain the column into the flask until the sodium sulfate layer is nearly exposed. Elute the column with 200 mL of 6% ethyl ether in hexane (V/V) (Fraction 1) using a drip rate of about 5 mL/min. Remove the K-D flask and set aside for later concentration. Elute the column again, using 200 mL of 15% ethyl ether in hexane (V/V)(Fraction 2), into a second K-D flask. Perform the third elution using 200 mL of 50% ethyl ether in hexane (V/V)(Fraction 3). The elution patterns for the pesticides an PCB's are shown in Table 2.

11.2.4 Concentrate the eluates by standard K-D techniques (Section 10.6), substituting hexane for the glassware rinses and using the water bath at about 85 °C. Adjust final volume to 10 mL with hexane. Analyze by gas chromatography.

11.3 Elemental sulfur will usually elute entirely in Fraction 1 of the Florisil column cleanup. To remove sulfur interference from this fraction or the original extract, pipet 1.00 mL of the concentrated extract into a clean concentrator tube or Teflon-sealed vial. Add one to three drops of mercury and seal[13]. Agitate the contents of the vial for 15 to 30 seconds. Prolonged shaking (two hours) may be required. If so, this may be accomplished with a reciprocal shaker. Alternatively, activated copper powder may be used for sulfur removal[14]. Analyze by gas chromatography.

12. Gas Chromatography

12.1 Table 1 summarizes the recommended operating conditions for the gas chromatograph. This table includes retention times and MDL that were obtained under these conditions. Examples of the parameter separations achieved by column 1 are shown in Figures 1 to 10. Other packed columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met. Capillary (open-tubular) columns may also be used if the relative standard deviations of responses for replicate injections are demonstrated to be less than 6% and the requirements of Section 8.2 are met.

12.2 Calibrate the system daily as described in Section 7.

12.3 If the internal standard approach is being used, the internal standard must be added to the sample extract and mixed thoroughly immediately, before injection into the instrument.

12.4 Inject 2 to 5 µL of the sample extract using the solvent-flush technique[15]. Smaller (1.0 µL) volumes can be injected if automatic devices are employed. Record the volume injected to the nearest 0.05 µL, the total extract volume, and the resulting peak size in area or peak height units.

12.5 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size; however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

12.6 If the response for the peak exceeds the working range of the system, dilute the extract and reanalyze.

12.7 If the measurement of the peak response is prevented by the presence of interferences, further cleanup is required.

13. Calculations

13.1 Determine the concentration of individual compounds in the sample.

13.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the peak response using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated from equation 2:

Eq. 2. $$\text{Concentration, } \mu g/L = \frac{(A)(V_t)}{(V_i)(V_s)}$$

where:

A = Amount of material injected, in nanograms.
V_i = Volume of extract injected (µL).
V_t = Volume of total extract (µL).
V_s = Volume of water extracted (mL).

13.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and equation 3.

Eq. 3 $$\text{Concentration, } \mu g/L = \frac{(A_s)(I_s)}{(A_{is})(RF)(V_o)}$$

where:

A_s = Response for the parameter to be measured.
A_{is} = Response for the internal standard.
I_s = Amount of internal standard added to each extract (µg).
V_o = Volume of water extracted, in liters.

310

13.2 When it is apparent that two or more PCB (Aroclor) mixtures are present, the Webb and McCall procedure[16] may be used to identify and quantify the Aroclors.

13.3 For multicomponent mixtures (chlordane, toxaphene and PCBs) match retention times of peaks in the standards with peaks in the sample. Quantitate every identifiable peak unless interference with individual peaks persist after cleanup. Add peak height or peak area of each identified peak in the chromatogram. Calculate as total response in the sample versus total response in the standard.

13.4 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.5 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits in Section 8.3, data for the affected parameters must be labeled as suspect.

14. Method Performance

14.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[1]. The MDL concentrations listed in Table 1 were obtained using reagent water[17]. Similar results were achieved using representative wastewaters.

14.2 This method has been tested for linearity of spike recovery from reagent water and has been demonstrated to be applicable over the concentration range from 4 × MDL up to 1000 × MDL with the following exceptions: Chlordane recovery at 4 × MDL was low (60%); Toxaphene recovery was demonstrated linear over the range of 10 × MDL to 1000 × MDL[17].

14.3 In a single laboratory (Southwest Research Institute), using spiked wastewater samples, the average recoveries presented in Table 3 were obtained[4]. Each spiked sample was analyzed in triplicate on two separate days. The standard deviation of the percent recovery is also included in Table 3.

14.4 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1 See Appendix A

2. "Determination of Pesticides and PCBs in Industrial and Municipal Wastewaters." Report for EPA Contract 68-03-2606. In preparation.

3. ASTM Annual Book of Standards, Part 31, D3694, "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 679, 1980.

4. Giam, D.S., Chan, H.S. and Nef, G.S., "Sensitive Method for Determination of Phthalate Ester Plasticizers in Open-Ocean Biota Samples," *Analytical Chemistry, 47*, 2225, (1975).

5. Giam, C.S., Chan, H.S., "Control of Blanks in the Analysis of Phthalates in Air and Ocean Biota Samples," U.S. National Bureau of Standards, Special Publication 442, pp. 701-708, 1976.

6. "Carcinogens—Working With Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.

7. "OSHA Safety and Health Standards, General Industry," (29 CFR 1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).

8. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.

9. Mills, P.A., "Variation of Florisil Activity: Simple Method for Measuring Absorbent Capacity and Its Use in Standardizing Florisil Columns," *Journal of the Association of Official Analytical Chemists, 51*, 29 (1968).

10. "Handbook for Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.

11. ASTM Annual Book of Standards, Part 31, D3370, "Standard Practice for Sampling Water," American Society for Testing and Materials, Philadelphia, PA, p. 76, 1980.

12. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600-4/79-020, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.

13. Goerlitz, D.F. and Law, L.M., *Bulletin for Environmental Contamination and Toxicology, 6* 9 (1971).

14. "Manual of Analytical Methods for the Analysis of Pesticides in Human Environmental Samples," U.S. Environmental Protection Agency, Health Effects Research Laboratory, Research Triangle Park, N.C., EPA Report 600/8-80-038, Section 11,B, p.6.

15. Burke, J.A., "Gas Chromatography for Pesticide Residue Analysis; Some Practical Aspects," *Journal of the Association of Official Analytical Chemists, 48*, 1037 (1965).

16. Webb, R.G., and McCall, A.C., "Quantitative PCB Standards for Electron Capture Gas Chromatography," *Journal of Chromatographic Science, 11*, 366 (1973).

17. "Method Detection Limit and Analytical Curve Studies, EPA Methods 606, 607, and 608," Special letter report for EPA Contract 68-03-2606. Environmental Monitoring and Support Laboratory--Cincinnati, Ohio 45268.

Table 1. *Chromatographic Conditions and Method Detection Limits*

Parameter	Retention Time (min.) Column 1	Retention Time (min.) Column 2	Method Detection Limit µg/L
α-BHC	1.35	1.82	0.003
γ-BHC	.70	2.13	0.004
β-BHC	1.90	1.97	0.006
Heptachlor	2.00	3.35	0.003
δ-BHC	2.15	2.20	0.009
Aldrin	2.40	4.10	0.004
Hepachlor epoxide	3.50	5.00	0.083
Endosulfan I	4.50	6.20	0.014
4,4'-DDE	5.13	7.15	0.004
Dieldrin	5.45	7.23	0.002
Endrin	6.55	8.10	0.006
4,4'-DDD	7.83	9.08	0.011
Endosulfan II	8.00	8.28	0.004
4,4'-DDT	9.40	11.75	0.012
Endrin aldehyde	11.82	9.30	0.023
Endosulfan sulfate	14.22	10.70	0.066
Chlordane	mr	mr	0.014
Toxaphene	mr	mr	0.24
PCB-1016	mr	mr	nd
PCB-1221	mr	mr	nd
PCB-1232	mr	mr	nd
PCB-1242	mr	mr	0.065
PCB-1248	mr	mr	nd
PCB-1254	mr	mr	nd
PCB-1260	mr	mr	nd

Column 1 conditions: Supelcoport (100/120 mesh) coated with 1.5% SP-2250/1.95% SP-2401 packed in a 1.8 m long × 4 mm ID glass column with 5% Methane/95% Argon carrier gas at a flow rate of 60 mL/min. Column temperature isothermal at 200°C, except for PCB-1016 through PCB-1248, which should be measured at 160°C.

Column 2 conditions: Supelcoport (100/120 mesh) coated with 3% OV-1 in a 1.8 m long × 4 mm ID glass column with 5% Methane/95% Argon carrier gas at a flow rate of 60 mL/min. Column temperature, isothermal at 200°C, for the pesticides; 140°C for PCB-1221 and 1232; 170°C for PCB-1016 and 1242 to 1268.

mr — Multiple peak response. See Figures 2 thru 10.

nd — Not determined.

Table 2. *Distribution of Chlorinated Pesticides and PCBs into Florisil Column Fractions[2]*

Parameter	Percent Recovery by Fraction: Fraction 1	Fraction 2	Fraction 3
Aldrin	100		
α-BHC	100		
β-BHC	97		
δ-BHC	98		
γ-BHC	100		
Chlordane	100		
4,4'-DDD	99		
4,4'-DDE	98		
4,4'-DDT	100		
Dieldrin	0	100	
Endosulfan I	37	64	
Endosulfan	0	7	91
Endosulfan sulfate	0	0	106
Endrin	4	96	
Endrin aldehyde	0	68	26
Heptachlor	100		
Heptachlor epoxide	100		
Toxaphene	96		
PCB-1016	97		
PCB-1221	97		
PCB-1232	95		
PCB-1242	97		
PCB-1248	103		
PCB-1254	90		
PCB-1260	95		

Eluant composition by fraction:
Fraction 1 — 6% ethyl ether in hexane
Fraction 2 — 15% ethyl ether in hexane
Fraction 3 — 50% ethyl ether in hexane

Table 3. *Single Operator Accuracy and Precision*

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
Aldrin	89	2.5	2.0	15	3
α-BHC	89	2.0	1.0	15	3
β-BHC	88	1.3	2.0	15	3
δ-BHC	86	3.4	2.0	15	3
γ-BHC	97	3.3	1.0	15	3
Chlorane	93	4.1	20	21	4
4-4'-DDD	92	1.9	6.0	15	3
4,4'-DDE	89	2.2	3.0	15	3
4,4'-DDT	92	3.2	8.0	15	3
Dieldrin	95	2.8	3.0	15	2
Endosulfan I	96	2.9	3.0	12	2
Endosulfan II	97	2.4	5.0	14	3
Endosulfan sulfate	99	4.1	15	15	3
Endrin	95	2.1	5.0	12	2
Endrin aldehyde	87	2.1	12	11	2
Heptachlor	88	3.3	1.0	12	2
Heptachlor epoxide	93	1.4	2.0	15	3
Toxaphene	95	3.8	200	18	3
PCB-1016	94	1.8	25	12	2
PCB-1221	96	4.2	55-110	12	2
PCB-1232	88	2.4	110	12	2
PCB-1242	92	2.0	28-56	12	2
PCB-1248	90	1.6	40	12	2
PCB-1254	92	3.3	40	18	3
PCB-1260	91	5.5	80	18	3

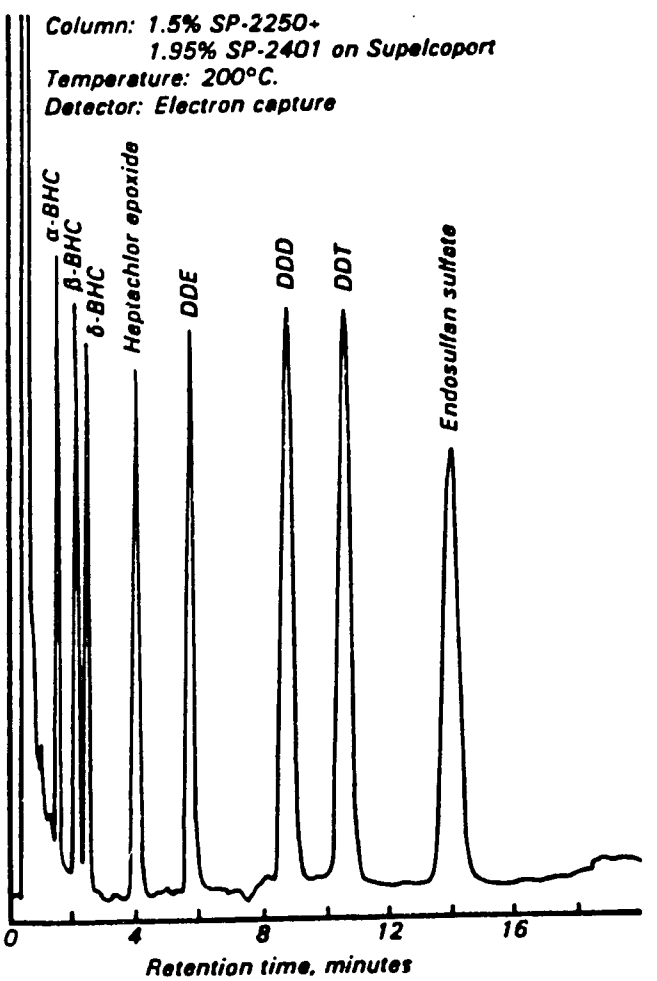


Figure 1. *Gas chromatogram of pesticides.*

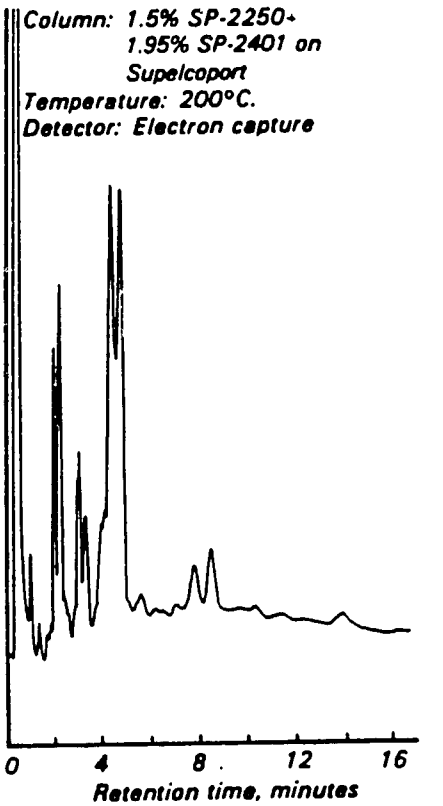


Figure 2. *Gas chromatogram of chlordane.*

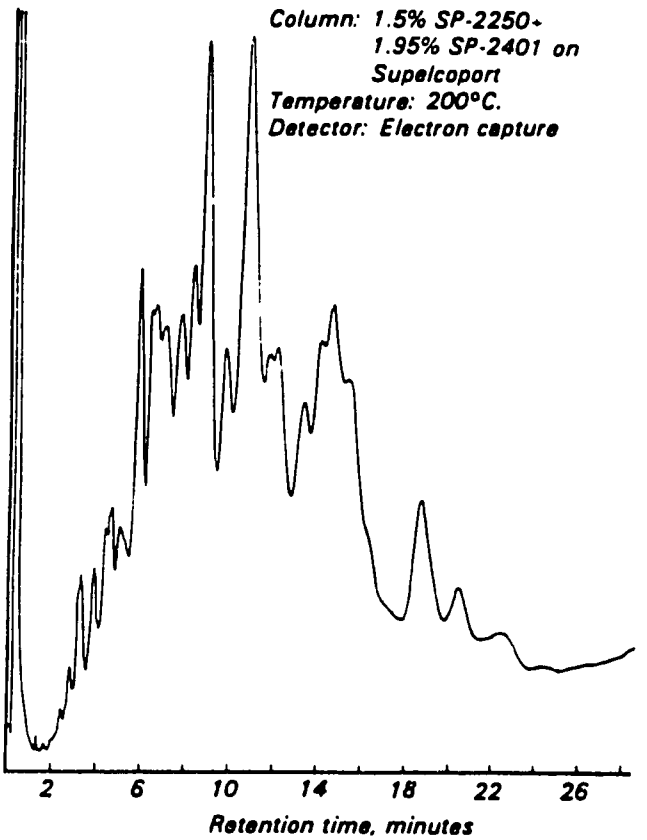


Figure 3. *Gas chromatogram of toxaphene.*

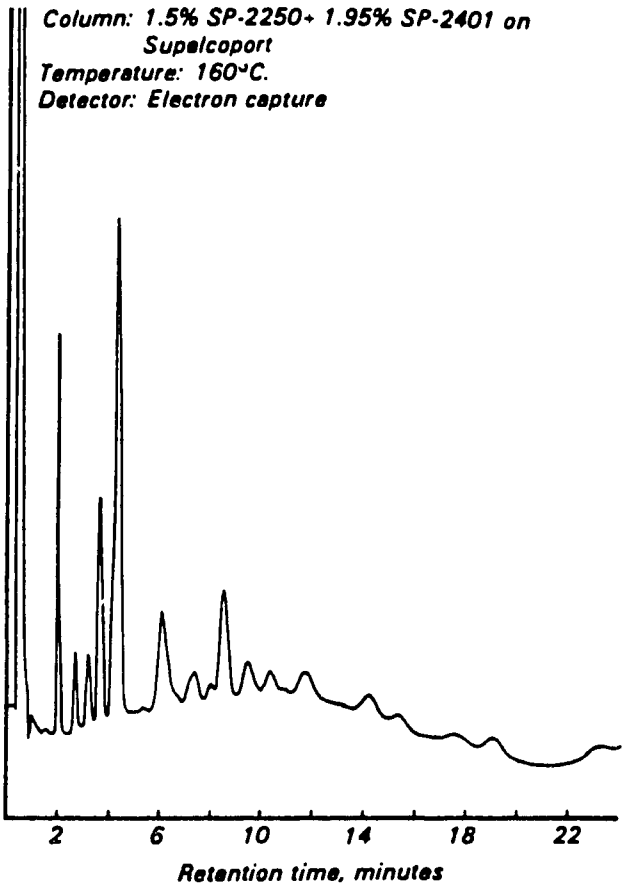


Figure 5. *Gas chromatogram of PCB-1221.*

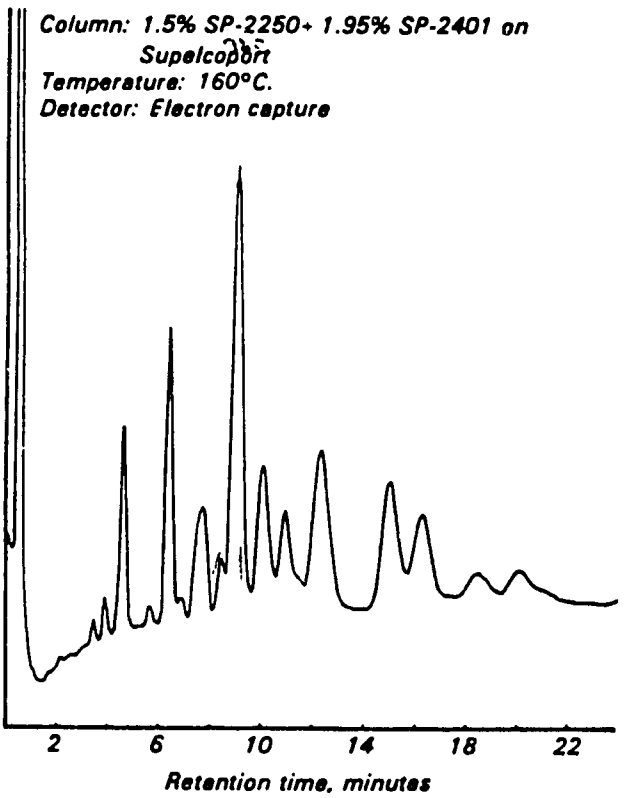


Figure 4. *Gas chromatogram of PCB-1016.*

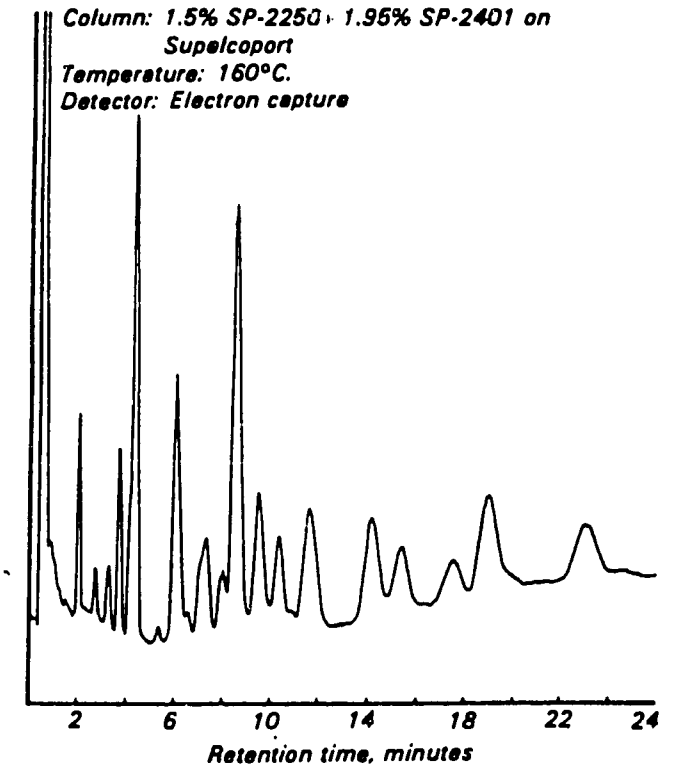


Figure 6. *Gas chromatogram of PCB-1232.*

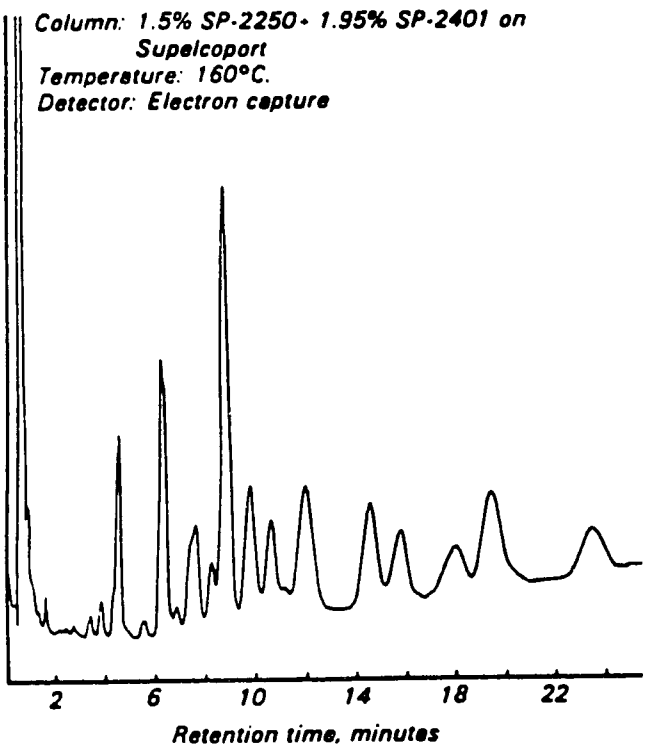


Figure 7. *Gas chromatogram of PCB-1242.*

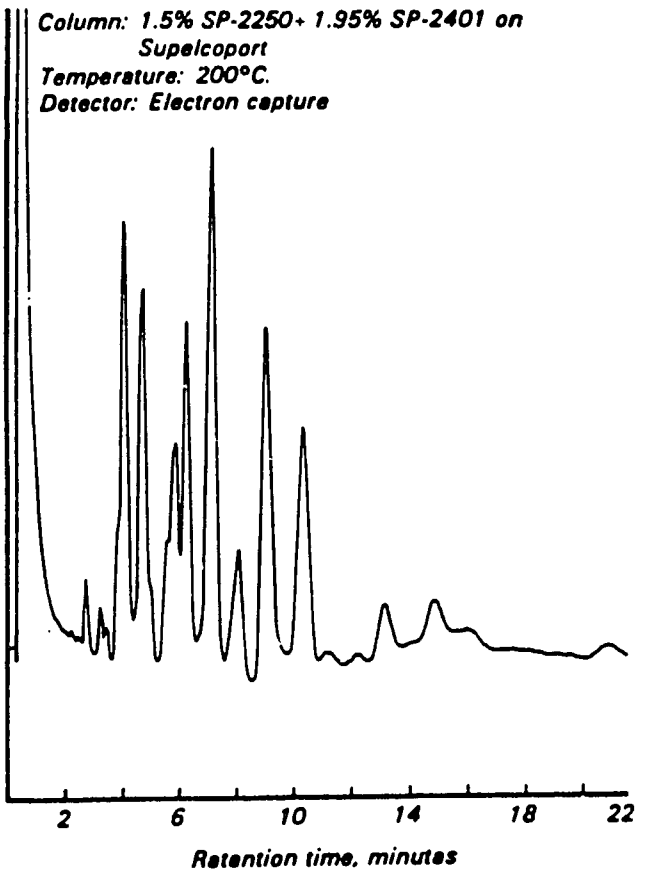


Figure 9. *Gas chromatogram of PCB-1254.*

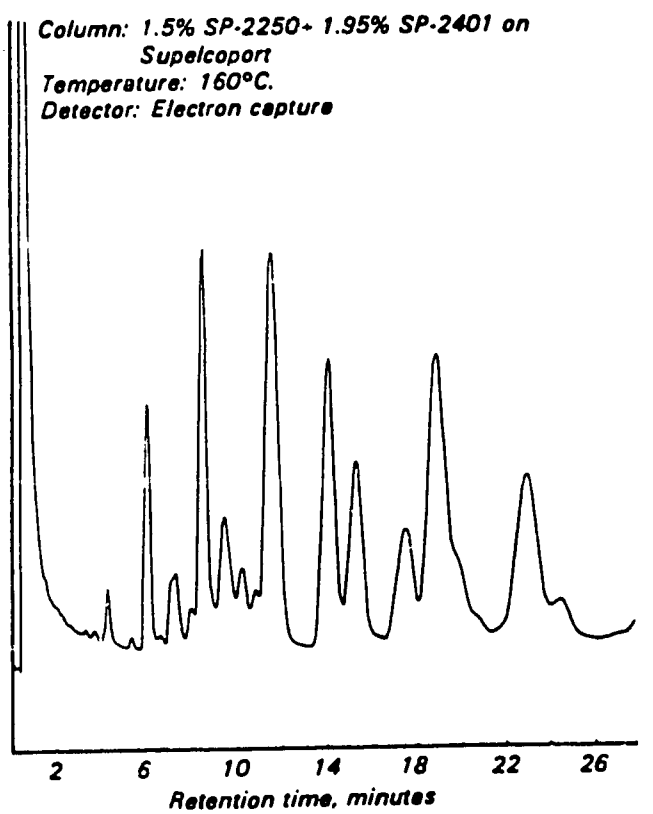


Figure 8. *Gas chromatogram of PCB-1248.*

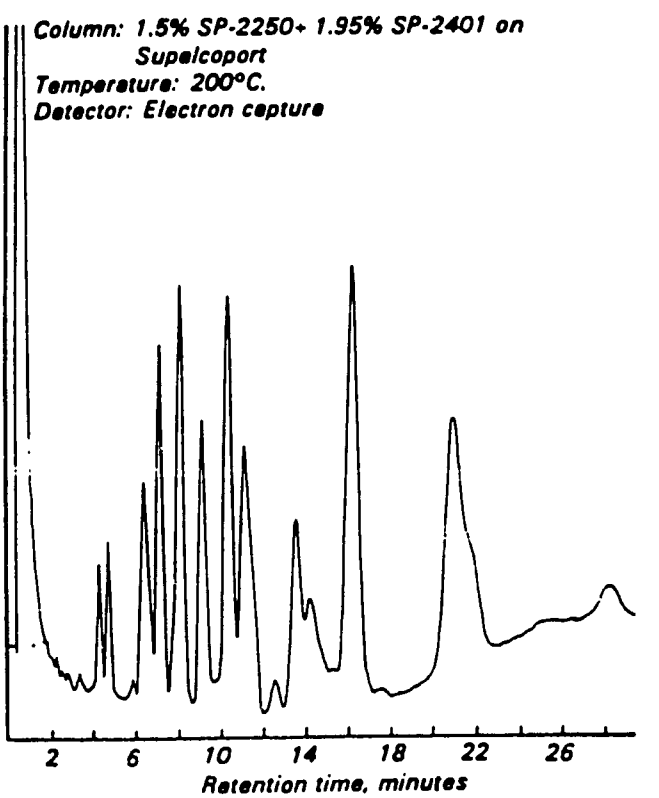


Figure 10. *Gas chromatogram of PCB-1260.*

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

Test Method

Nitroaromatics and Isophorone — Method 609

1. Scope and Application

1.1 This method covers the determination of certain nitroaromatics and isophorone. The following parameters may be determined by this method:

Parameter	STORET No.	CAS No.
2,4-Dinitrotoluene	34611	121-14-2
2,6-Dinitrotoluene	34626	606-20-2
Isophorone	34408	78-59-1
Nitrobenzene	34447	98-95-3

1.2 This is a gas chromatographic (GC) method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for any or all of the compounds above, compound identifications should be supported by at least one additional qualitative technique. This method describes analytical conditions for a second gas chromatographic column that can be used to confirm measurements made with the primary column. Method 625 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results for all of the parameters listed above, using the extract produced by this method.

1.3 The method detection limit (MDL defined in Section 14.1)[1] for each parameter is listed in Table 1. The MDL for a specific wastewater may differ from those listed, depending upon the nature of interferences in the sample matrix.

1.4 The sample extraction and concentration steps in this method are essentially the same as in method 606, 608, 611 and 612. Thus, a single sample may be extracted to measure all of the parameters included in the scope of each of these methods. When cleanup is required the concentration levels must be high enough to permit selection of aliquots of the extract, as necessary, to apply appropriate cleanup procedures. The analyst is allowed the latitude, under Gas Chromatography (Section 12), to select chromatographic conditions appropriate for the simultaneous measurement of combinations of these parameters.

1.5 Any modifications of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval of alternate test procedures under 40 CFR 136.4 and 136.5.

1.6 This method is restricted to use by or under the supervision of analysts experienced in the use of gas chroma-

tography and in the interpretation of gas chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 6.2.

2. Summary of Method

2.1 A measured volume of sample, approximately one-liter, is extracted with methylene chloride using separatory funnel techniques. The extract is dried and exchanged to hexane during concentration to 1.0 mL by evaporation. Isophorone and nitrobenzene are measured by flame ionization gas chromatography (FIDGC). The dinitrotoluenes are measured by electron capture GC (ECGC)[2].

2.2 The method provides a Florisil chromatographic cleanup procedure to aid in the elimination of interferences that may be encountered.

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in gas chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned[3]. Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and distilled water. It should then be drained dry, and heated in a muffle furnace at 400 °C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Thorough rinsing with such solvents usually eliminates PCB interferences. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedures in Section 11 can be used to overcome many of these interferences, but unique samples may require additional cleanup approaches to achieve the MDL listed in Table 1.

4. Safety

The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[4-6] for the information of the analyst.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle—Amber glass, one-liter or one-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional)—Must incorporate glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4 °C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.2 Glassware (All specifications are suggested. Catalog numbers are included for illustration only).

5.2.1 Separatory funnel—2000-mL, with Teflon stopcock.

5.2.2 Drying column—Chromatographic column approximately 400 mm long × 19 mm ID with coarse frit.

5.2.3 Concentrator tube, Kuderna-Danish—10-mL, graduated (Kontes K-570050-1025 or equivalent). Calibration must be checked at the volumes employed in the test. Ground glass stopper is used to prevent evaporation of extracts.

5.2.4 Evaporative flask, Kuderna-Danish—500-mL (Kontes K-570001-0500 or equivalent). Attach to concentrator tube with springs.

5.2.5 Snyder column, Kuderna-Danish—Three-ball macro (Kontes K-503000-0121 or equivalent).

5.2.6 Snyder column, Kuderna-Danish—Two-ball micro (Kontes K-569001-0219 or equivalent).

5.2.7 Vials—Amber glass, 10- to 15-mL capacity, with Teflon-lined screw-cap.

5.2.8 Chromatographic column—100 mm long × 10 mm ID, Teflon stopcock.

5.3 Boiling chips—approximately 10/40 mesh. Heat to 400 °C for 30 minutes or Soxhlet extract with methylene chloride.

5.4 Water bath—Heated, with concentric ring cover, capable of temperature control (± 2 °C). The bath should be used in a hood.

5.5 Balance—Analytical, capable of accurately weighing 0.0001 g.

5.6 Gas chromatograph—Analytical system complete with gas chromatograph suitable for on-column injection and all required accessories including syringes, analytical columns, gases, detector, and strip-chart recorder. A data system is recommended for measuring peak areas.

5.6.1 Column 1—1.2 m (4 ft.) long × 2 mm or 4 mm ID, Pyrex glass, packed with Gas-Chrom Q (80/100 mesh) coated with 1.95% OF-1/1.5% OV-17. This column was used to develop the method performance statements in Section 14. Guidelines for the use of alternate column packings are provided in Section 12.1.

5.6.2 Column 2—3.0 m (10 ft.) long × 2 or 4 mm ID, Pyrex glass, packed

317

with Gas-Chrom Q (80/100 mesh) coated with 3% OV-101.

5.6.3 Detector—Flame ionization. This detector has proven effective in the analysis of wastewaters for isophorone and nitrobenzene, and was used to develop the method performance statements in Section 14. Guidelines for the use of alternate detectors are provided in Section 12.1.

5.6.4 Detector—Electron capture. This detector has proven effective in the analysis of wastewaters for the dinitrotoluenes, and was used to develop the method performance statements in Section 14. Guidelines for the use of alternate detectors are provided in Section 12.1.

6. Reagents

6.1 Reagent water—Reagent water is defined as a water in which an interferent is not observed at the MDL of each parameter of interest.

6.2 Sodium hydroxide solution (10 N)—(ACS) Dissolve 40g NaOH in reagent water and dilute to 100 mL.

6.3 Sulfuric acid solution (1 + 1)—(ACS) Slowly, add 50 mL H_2SO_4 (sp. gr. 1.84) to 50 mL of reagent water.

6.4 Acetone, hexane, methanol, methylene chloride,—Pesticide quality or equivalent.

6.5 Sodium sulfate—(ACS) Granular, anhydrous. Purify by heating at 400 °C for 4 hours in a shallow tray.

6.6 Florisil—PR grade (60/100 mesh), purchase activated at 1250 °F and store in dark in glass containers with glass stoppers or foil-lined screw caps. Before use, activate each batch overnight at 200 °C in glass containers loosely covered with foil.

6.7 Stock standard solutions (1.00 μg/μL)—Stock standard solutions can be prepared from pure standard materials or purchased as certified solutions.

6.7.1 Prepare stock standard solutions by accurately weighing about 0.0100 g of pure material. Dissolve the material in pesticide quality hexane, dilute to volume in a 10-mL volumetric flask. Larger volumes can be used at the convenience of the analyst. If compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.7.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at 4 °C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check standards that can be used to determine the accuracy of calibration standards, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Systems Laboratory, Cincinnati, Ohio 45268.

6.7.3 Stock standard solutions must be replaced after six months, or sooner if comparison with check standards indicate a problem.

7. Calibration

7.1 Establish gas chromatographic operating conditions to produce resolution of the parameters equivalent to those indicated in Table 1. The gas chromatographic system may be calibrated using the external standard technique (Section 7.2) or the internal standard technique (Section 7.3).

7.2 External standard calibration procedure:

7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with hexane. One of the external standards should be at a concentration near, but above, the MDL limit and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.2.2 Using injections of 2 to 5 μL of each calibration standard, tabulate peak height or area responses against the mass injected. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over the working range (<10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.2.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ± 10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that compound.

7.3 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples.

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask. To each calibration standard, add a known constant amount of one or more internal standards, and dilute to volume with hexane. One of the standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.3.2 Using injections of 2 to 5 μL of each calibration standard, tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1.

Eq. 1. $RF = (A_s C_{is})/(A_{is} C_s)$

where:

A_s = Response for the parameter to be measured.

A_{is} = Response for the internal standard.

C_{is} = Concentration of the internal standard (μg/L).

C_s = Concentration of the parameter to be measured (μg/L).

If the RF value over the working range is a constant (<10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.3.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ± 10%, the test must be repeated using a fresh calibration standard.

Alternatively, a new calibration curve must be prepared for that compound.

7.4 Before using any cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in acetone 1000 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 2, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > 2p$ or $|X - R| > 2p$, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before an samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance:

Upper Control Limit (UCL) = R + 3s
Lower Control Limit (LCL) = R − 3s

where R and s are calculated as in Section 8.2.3.

The UCL and LCL can be used to construct control charts[7] that are useful in observing trends in performance. The control limits above be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[7].

8.4. The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 13.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate through the analysis of a one-liter aliquot of reagent water, that all glassware and reagent interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as gas chromatography with a dissimilar column, specific element detector, or mass spectrometer must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices[8] should be followed, except that the bottle must not be prerinsed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon tubing and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4 °C from the time of collection until extraction.

319

9.3 All samples must be extracted within 7 days and completely analyzed within 40 days of extraction[2].

10. Sample Extraction

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a two-liter separatory funnel. Check the pH of the sample with wide-range pH paper and adjust to within the range of 5 to 9 with diluted sodium hydroxide or sulfuric acid.

10.2 Add 60 mL methylene chloride to the sample bottle, seal, and shake for 30 seconds to rinse the inner surface. Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods. Collect the methylene chloride extract in a 250-mL Erlenmeyer flask.

10.3 Add a second 60-mL volume of methylene chloride to the sample bottle and repeat the extraction procedure a second time, combining the extracts in the Erlenmeyer flask. Perform a third extraction in the same manner.

10.4 Assemble a Kuderna-Danish (K-D) concentrator by attaching a 10-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used in place of the K-D if the requirements of Section 8.2 are met.

10.5 Pour the combined extract through a drying column containing about 10 cm of anhydrous sodium sulfate, and collect the extract in the K-D concentrator. Rinse the Erlenmeyer flask and column with 20 to 30 mL of methylene chloride to complete the quantitative transfer.

10.6 Sections 10.7 and 10.8 describe a procedure for exchanging the methylene chloride solvent to hexane while concentrating the extract volume to 1.0 mL. When it is not necessary to achieve the MDL in Table 2, the solvent exchange may be made by the addition of 50 mL of hexane and concentration to 10 mL as described in method 606, Section 10.7.

10.7 Add one or two clean boiling chips to the evaporative flask and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL methylene chloride to the top. Place the K-D apparatus on a hot water bath (60 to 65 °C) so that the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood with condensed solvent. When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus and allow it to drain and cool for at least 10 minutes. Remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of methylene chloride. A 5-mL syringe is recommended for this operation.

10.8 Add 1 to 2 mL of hexane to the concentrator tube, and a clean boiling chip. Attach a two-ball micro-Snyder column. Prewet the micro-Snyder column by adding about 0.5 mL of hexane to the top. Place this micro K-D apparatus on a water bath (60 to 65 °C) so that the concentrator tube is partially immersed in the hot water. Adjust the vertical position of the apparatus and water temperature as required to complete the concentration in 5 to 10 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood. When the apparent volume of liquid reaches 0.5 mL, remove the K-D apparatus and allow it to drain for at least 10 minutes while cooling. Remove the micro-Snyder column and rinse its lower joint into the concentrator tube with a small volume of hexane. Adjust the final volume to 1.0 mL and stopper the concentrator tube and store refrigerated if further processing will not be performed immediately. Unless the sample is known to require cleanup, proceed with gas chromatographic analysis.

10.9 Determine the original sample volume by refilling the sample bottle to the mark with water and measuring the volume in a 1000-mL graduated cylinder. Record the sample volume to the nearest 5 mL.

11. Cleanup and Separation

11.1 Cleanup procedures may not be necessary for a relatively clean sample matrix. The cleanup procedure recommended in this method has been used for the analysis of various clean waters and industrial effluents. If particular circumstances demand the use of an alternative cleanup procedure, the analyst must demonstrate that the recovery of each compound of interest is no less than 85%.

11.2 Prepare a slurry of 10g of activated Florisil in methylene chloride in hexane (1 + 9)(V/V). Use it to pack a 10-mm ID chromatography column, gently tapping the column to settle the Florisil. Add 1 cm of anhydrous sodium sulfate to the top of the Florisil.

11.2.1 Just prior to exposure of the sodium sulfate layer to the air transfer the 1 mL of sample extract onto the column using an additional 2 mL of hexane to complete the transfer.

11.2.2 Just prior to exposure of the sodium sulfate layer to the air, add 30 mL of methylene chloride in hexane (1 + 9)(V/V) and continue the elution of the column. Elution of the column should be at a rate of about 2 mL skill per min. Discard the eluate from this fraction.

11.2.3 Next elute the column with 30 mL of acetone/methylene chloride (1 + 9)(V/V) into a 500-mL K-D flask equipped with a 10-mL concentrator tube. Concentrate the collected fraction by the K-D technique prescribed in Sections 10.6, 10.7, and 10.8 including the solvent exchange in 1 mL of hexane. This fraction should contain the nitroaromatics and isophorone.

11.2.4 Analyze by gas chromatography.

12. Gas Chromatography

12.1 Isophorone and nitrobenzene are analyzed by injection of a portion of the extract into an FIDGC. The dinitrotoluenes are analyzed by a separate injection into an ECGC. Table 1 summarizes some recommended gas chromatographic column materials and operating conditions for the instruments. This Table includes retention times and MDL obtained under these conditions. Examples of the parameter separations achieved by Column 1 are shown in Figures 1 and 2. Other packed columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met. Capillary (open-tubular) columns may also be used if the relative standard deviations of responses for replicate injections are demonstrated to be less than 6% and the requirements of Section 8.2 are met.

329

12.2 Calibrate the system daily as described in Section 7.

12.3 If the internal standard approach is being used, the analyst must not add the internal standard to sample the extract until immediately before injection into the instrument. Mix thoroughly.

12.4 Inject 2 to 5 µL of the sample extract using the solvent-flush technique[9]. Smaller (1.0 µL) volumes can be injected if automatic devices are employed. Record the volume injected to the nearest 0.05 µL, and the resulting peak size in area or peak height units.

12.5 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size; however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

12.6 If the response exceeds the working range of the system, dilute the extract and reanalyze.

12.7 If the measurement of the peak response is prevented by the presence of interferences, further cleanup is required.

13. Calculations

13.1 Determine the concentration of individual compounds in the sample.

13.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the peak response using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated from equation 2:

$$\text{Eq. 2. Concentration, } \mu g/L = \frac{(A)(V_t)}{(V_i)(V_s)}$$

where:

- A = Amount of material injected, in nanograms.
- V_i = Volume of extract injected (µL).
- V_t = Volume of total extract (µL).
- V_s = Volume of water extracted (mL).

calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and equation 3.

Eq. 3

$$\text{Concentration, } \mu g/L = \frac{(A_s)(I_s)}{(A_{is})(RF)(V_o)}$$

where:

- A_s = Response for the parameter to be measured.
- A_{is} = Response for the internal standard.
- I_s = Amount of internal standard added to each extract (µg).
- V_o = Volume of water extracted, in liters.

13.2 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.3 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits in Section 8.3, data for the affected parameters must be labeled as suspect.

14. Method Performance

14.1 Method detection limits—The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[1]. The MDL concentrations listed in Table 1 were obtained using reagent water[10]. Similar results were achieved using representative wastewaters.

14.2 This method has been tested for linearity of recovery from reagent water and has been demonstrated to be applicable over the concentration range from 7 × MDL to 1000 × MDL[10].

14.3 In a single laboratory (Battelle, Columbus Laboratories), using spiked wastewater samples, the average recoveries presented in Table 2 were obtained[2]. Each spiked sample was analyzed in triplicate on two separate days. The standard deviation of the percent recovery is also included in Table 2.

14.4 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. See Appendix A.
2. "Determination of Nitroaromatics and Isophorone in Industrial and Municipal Wastewaters." Report for EPA Contract 68-03-2624 (In preparation).
3. ASTM Annual Book of Standards, Part 31, D 3694. "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 679, 1980.
4. "Carcinogens—Working With Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.
5. "OSHA Safety and Health Standards, General Industry," (29CFR1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
6. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.
7. "Handbook of Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.
8. ASTM Annual Book of Standards, Part 31, D 3370, "Standard Practice for Sampling Water," American Society for Testing and Materials, Philadelphia, PA, p. 76, 1980.
9. Burke, J.A., "Gas Chromatography for Pesticide Residue Analysis; Some Practical Aspects," *Journal of the Association of Official Analytical Chemists, 48*, 1037 (1965).
10. "Determination of Method Detection Limit and Analytical Curve for EPA Methods 609—Nitroaromatics and Isophorone," special letter report for EPA Contract 68-03-2624. Environmental Monitoring and Support Laboratory—Cincinnati, Ohio 45268.

Table 1. Chromatographic Conditions and Method Detection Limits

Parameter	Retention Time (min.) Column 1	Retention Time (min.) Column 2	Method Detection Limit (µg/L) EC	Method Detection Limit (µg/L) FID
Nitrobenzene	3.31	4.31	13.7	3.6
2,6-Dinitrotoluene	3.52	4.75	0.01	—
Isophorone	4.49	5.72	15.7	5.7
2,4-Dinitrotoluene	5.35	6.54	0.02	—

Column 1 conditions: Gas-Chrom Q (80/100 mesh) coated with 1.95% QF-1/1.5% OV-17 packed in a pyrex glass column 1.2 m (4 ft) long × 2 mm or 4 mm ID. Nitrogen carrier gas at a flow rate of 44 mL/min was used when determining isophorone and nitrobenzene by FID. The Column temperature was isothermal at 85 °C. Methane (10%)/Argon (90%) carrier gas at flow rate of 44 mL/min was used when determining the dinitrotoluenes by ECGC. The column temperature was isothermal at 145 °C.

Column 2 conditions: Gas-Chrom Q (80/100 mesh) coated with 3% OV-1 packed in a pyrex glass column 3.0 m (10 ft) long × 2 mm or 4 mm ID. Nitrogen carrier gas at a flow rate of 44 mL/min was used when determining isophorone and nitrobenzene by FID. The column temperature was isothermal at 100 °C. Methane (10%)/Argon (90%) carrier gas flow rate of 44 mL/min was used when determining the dinitroltoluenes by ECGC. The column temperature was isothermal, 150 °C.

A 2 mm ID column was used with the FIDGC and a 4 mm ID column was used with the ECGC.

Table 2. Single Operator Accuracy and Precision

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
2,4-Dinitrotoluene	63	3.1	5-100	21	4
2,6-Dinitrotoluene	66	3.2	5-50	24	4
Isophorone	73	4.6	50-60	21	4
Nitrobenzene	71	5.9	90-100	24	4

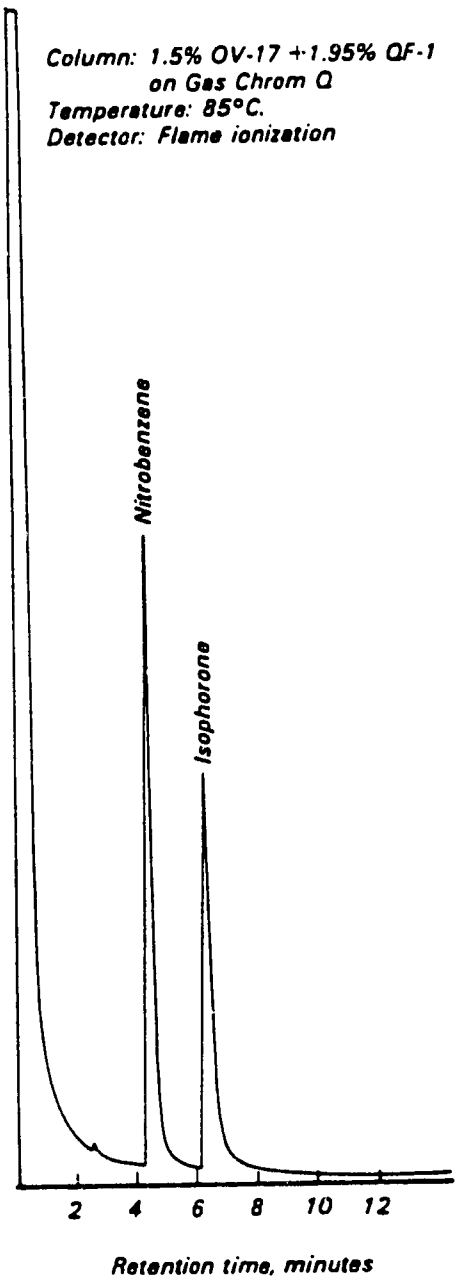


Figure 1. *Gas chromatogram of nitrobenzene and isophorone.*

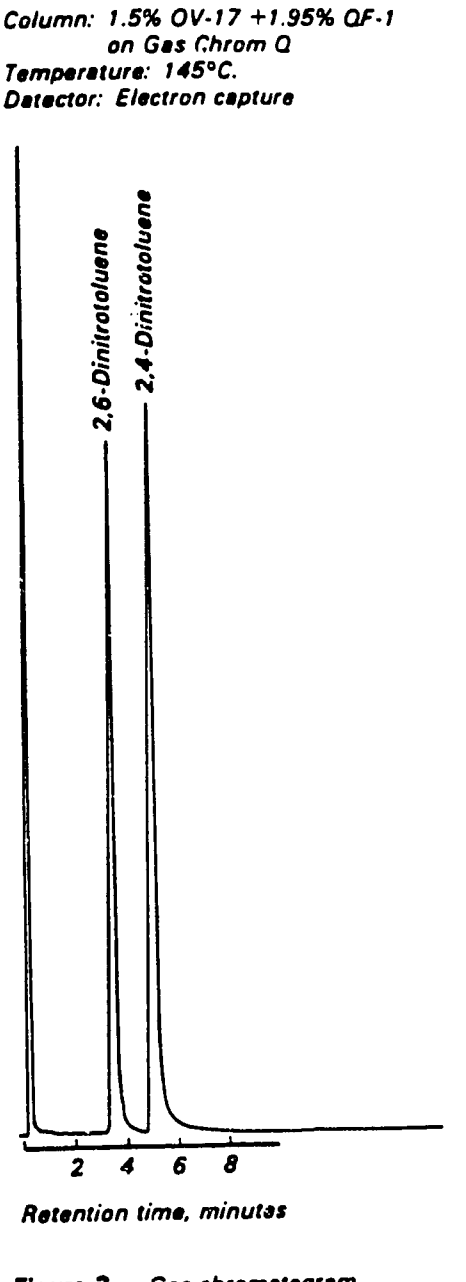


Figure 2. *Gas chromatogram of dinitrotoluenes.*

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Polynuclear Aromatic Hydrocarbons — Method 610

1. Scope and Application

1.1 This method covers the determination of certain polynuclear aromatic hydrocarbons (PAH). The following parameters may be determined by this method:

Parameter	STORET No.	CAS No.
Acenaphthene	34205	83-32-9
Acenaphthylene	34200	208-96-8
Anthracene	34220	120-12-7
Benzo (a) anthracene	34526	56-55-3
Benzo (a) pyrene	34247	50-32-8
Benzo (b) fluoranthene	34230	205-99-2
Benzo (ghi) perylene	34521	191-24-2
Benzo (k) fluoranthene	34242	207-08-9
Chrysene	34320	218-01-9
Dibenzo (a, h) anthracene	34556	53-70-3
Fluoranthene	34376	206-44-0
Fluorene	34381	86-73-7
Indeno (1, 2, 3-cd) pyrene	34403	193-39-5
Naphthalene	34696	91-20-3
Phenanthrene	34461	85-01-8
Pyrene	34469	129-00-0

1.2 This is a chromatographic method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for any or all of the compounds above, compound identifications should be supported by at least one additional qualitative technique. Method 625 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results for many of the parameters listed in Section 1.1, using the extract produced by this method.

1.3 This method provides for both high performance liquid chromatographic (HPLC) and gas chromatographic (GC) approaches to the determination of PAHs. The gas chromatographic procedure does not adequately resolve the following four pairs of compounds: anthracene and phenanthrene; chrysene and benzo (a) anthracene; benzo (b) fluoranthene and benzo (k) fluoranthene; and dibenzo (a, h) anthracene and indeno (1, 2, 3-cd) pyrene. Unless the purpose for the analysis can be served by reporting the sum of an unresolved pair, the liquid chromatographic approach must be used for these compounds. The liquid

chromatographic method does resolve all 16 of the PAHs listed.

1.4 The method detection limit (MDL, defined in Section 15)[11] for each parameter is listed in Table 1. The MDL for a specific wastewater may differ depending upon the nature of interferences in the sample matrix.

1.5 The sample extraction and concentration steps in this method are essentially the same as in methods 606, 608, 609, 611 and 612. Therefore, a single sample may be extracted to measure the parameters included in the scope of each of these methods, provided the concentration is high enough to permit selecting aliquots of the extract for cleanup, when required. Selection of the aliquots must be made prior to the solvent exchange steps of this method. The analyst is allowed the latitude, under Gas Chromatography (Section 13), to select chromatographic conditions appropriate for the simultaneous measurement of combinations of these parameters.

1.6 Any modification of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval of alternate test procedures under 40 CFR 136.4 and 136.5.

1.7 This method is restricted to use by or under the supervision of analysts experienced in the use of HPLC and GC and in the interpretation of liquid and gas chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 A measured volume of sample, approximately one-liter, is solvent extracted with methylene chloride using a separatory funnel. The methylene chloride extract is dried and concentrated to a volume of 10 mL or less. The solvent is exchanged to cyclohexane prior to cleanup. Following cleanup, when using HPLC for determination of the PAHs, the solvent is exchanged to acetonitrile. Ultraviolet (UV) and fluorescence detectors are used with HPLC. When cleanup is not required and when flame ionization detector GC is used for determination, the methylene chloride extract may be analyzed directly. When cleanup is required, the cyclohexane exchange is made. Instrumental conditions are described which permit the separation and measurement of the PAH compounds[12].

2.2 A silica gel column cleanup procedure is provided to aid in the elimination of interferences that may be encountered.

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in the chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned[13]. Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and distilled water. It should then be drained dry, and heated in a muffle furnace at 400°C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedure in Section 11 can be used to overcome many of these interferences, but unique samples may require additional cleanup approaches to achieve the MDL listed in Table 1.

3.3 The extent of interferences that may be encountered using liquid chromatographic techniques has not been fully assessed. Although the HPLC conditions described allow for a unique resolution of the specific PAH compounds covered by this method, other PAH compounds may interfere.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[14-6] for the information of the analyst.

4.2 The following paramenters covered by this method have been tentatively classified as known or suspected, human or mammalian carcinogens: benzo (a) anthracene, benzo (a) pyrene and dibenzo (a, h) anthracene.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling:

5.1.1 Grab sample bottle - Amber glass, one-liter or one-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional) - Must incorporate glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4°C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

325

5.2 Glassware (All specifications are suggested. Catalog numbers are included for illustration only.)

5.2.1 Separatory funnel - 2000-mL, with Teflon stopcock.

5.2.2 Drying column - Chromatographic column 400 mm long x 19 mm ID with coarse frit.

5.2.3 Concentrator tube, Kuderna-Danish - 10-mL, graduated (Kontes K-570050-1025 or equivalent). Calibration must be checked at the volumes employed in the test. Ground glass stopper is used to prevent evaporation of extracts.

5.2.4 Evaporative flask, Kuderna-Danish - 500-mL (Kontes K-570001-0500 or equivalent). Attach to concentrator tube with springs.

5.2.5 Snyder column, Kuderna-Danish - three-ball macro (Kontes K-503000-0121 or equivalent).

5.2.6 Snyder column, Kuderna-Danish - two-ball micro (Kontes K-569001-0219 or equivalent).

5.2.7 Vials - Amber glass, 10- to 15- mL capacity, with Teflon-lined screwcap.

5.2.8 Chromatographic column - 250 mm long x 10 mm ID with coarse fritted disc at bottom and Teflon stopcock.

5.3 Boiling chips - approximately 10/40 mesh. Heat to 400°C for 30 minutes or Soxhlet extract with methylene chloride.

5.4 Water bath - Heated, with concentric ring cover, capable of temperature control (±2°C). The bath should be used in a hood.

5.5 Balance — Analytical, capable of accurately weighing 0.0001g.

5.6 High performance liquid chromatographic apparatus (modular):

5.6.1 Gradient pumping system, constant flow.

5.6.2 Reverse phase column, 5 micron HC-ODS Sil-X, 250 mm x 2.6 mm ID (Perkin-Elmer No. 089-0716 or equivalent).

5.6.3 Fluorescence detector, for excitation at 280 nm and emission greater than 389 nm cutoff (Corning 3-75 or equivalent). Fluorometers should have dispersive optics for excitation and can utilize either filter or dispersive optics at the emission detector.

5.6.4 UV detector, 254 nm, coupled to fluorescence detector.

5.6.5 Strip-chart recorder compatible with detectors. Use of a data system for measuring peak areas and retention times is recommended.

5.7 Gas chromatograph - An analytical system complete with temperature programmable gas chromatograph suitable for on-column injection or splitless injection and all required accessories including syringes, analytical columns, gases, detector, and strip-chart recorder. A data system is recommended for measuring peak areas.

5.7.1 Column - 1.8 m long x 2 mm ID pyrex glass packed with 3% OV-17 on Chromosorb W-AW-DCMS (100/120 mesh) or equivalent. This column was used to develop the retention time data in Table 2. Guidelines for the use of alternate column packings are provided in Section 13.

5.7.2 Detector - Flame ionization. This detector has proven effective in the analysis of wastewaters for the compounds listed in the scope excluding the four pairs of unresolved compounds listed in Section 1.3. Guidelines for the use of alternate detectors are provided in Section 12.2.

6. Reagents

6.1 Reagent water - Reagent water is defined as a water in which an interferent is not observed at the MDL of each parameter of interest.

6.2 Sodium thiosulfate - (ACS) Granular.

6.3 Cyclohexane, methanol, acetone, methylene chloride, and pentane - Pesticide quality or equivalent.

6.4 Acetonitrile, high purity HPLC quality, distilled in glass.

6.5 Sodium sulfate - (ACS) Granular, anhydrous. Purify by heating at 400°C for four hours in a shallow tray.

6.6 Silica gel - Grade 923 (100/200 mesh) dessicant (Davison Chemical or equivalent). Before use, activate for at least 16 hours at 130°C in a shallow glass tray, loosely covered with foil.

6.7 Stock standard solutions (1.00 µg/µL) - Stock standard solutions can be prepared from pure standard materials or purchased as certified solutions.

6.7.1 Prepare stock standard solutions by accurately weighing about 0.0100 g of pure material. Dissolve the material in HPLC quality acetonitrile, dilute to volume in a 10-mL volumetric flask. Larger volumes can be used at the convenience of the analyst. If compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.7.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at 4°C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check standards that can be used to determine the accuracy of calibration standards will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

6.7.3 Stock standard solutions must be replaced after six months, or sooner if comparison with check standards indicate a problem.

7. Calibration

7.1 Establish liquid or gas chromatographic operating parameters to produce resolution of the parameters equivalent to that indicated in Tables 1 or 2. The chromatographic system can be calibrated using the external standard technique (Section 7.2) or the internal standard technique (Section 7.3).

7.2 External standard calibration procedure:

7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with acetonitrile. One of the external standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.2.2 Analyze each calibration standard (5 to 25 µL for HPLC and 2 to 5 µL for GC), and tabulate peak height or area responses against the mass injected. The results may be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over the working range (< 10% relative

326

standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.2.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that compound.

7.3 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples.

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask. To each calibration standard, add a known constant amount of one or more internal standards, and dilute to volume with acetonitrile. One of the standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.3.2 Analyze each calibration standard (5 to 25 µL for HPLC and 2 to 5 µL for GC) and tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using Equation 1.

$$\text{Eq. 1} \quad RF = (A_s C_{is})/(A_{is} C_s)$$

where:

A_s = Response for the parameter to be measured.
A_{is} = Response for the internal standard.
C_{is} = Concentration of the internal standard, (µg/L).
C_s = Concentration of the parameter to be measured, (µg/L).

If the RF value over the working range is a constant (< 10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.3.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than 10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound.

7.4 Before using any cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in acetronitrile 1000 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 3, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > 2p$ or $|X-R| > 2p$, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance:

Upper Control Limit (UCL) = R + 3 s
Lower Control Limit (LCL) = R − 3 s

where R and s are calculated as in Section 8.2.3.

The UCL and LCL can be used to construct control charts[7] that are useful in observing trends in perfor-

327

mance The control limits above must be replaced by method performance criteria as they become available from the U S Environmental Protection Agency

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[7]

8.4 The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 14.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate through the analysis of a one-liter aliquot of reagent water, that all glassware and reagents interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend on the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as chromatography with a dissimilar column or detector must be used. This may include the use of a mass spectrometer Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers Conventional sampling practices[8] should be followed, except that the bottle must not be prewashed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon tubing and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4° C from the time of collection until extraction. PAHs are known to be light sensitive, therefore, samples, extracts and standards should be stored in amber or foil wrapped bottles in order to minimize photolytic decomposition. Fill the sample bottle and, if residual chlorine is present, add 80 mg of sodium thiosulfate per liter of sample. U.S. Environmental Protection Agency methods 330.4 and 330.5 may be used for measurement of residual chlorine[9] Field test kits are available for this purpose.

9.3 All samples must be extracted within 7 days, and analysis completed within 40 days of extraction[2]

10. Sample Extraction

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume Pour the entire sample into a two-liter separatory funnel.

10.2 Add 60 mL methylene chloride to the sample bottle, seal, and shake 30 seconds to rinse the inner surface. Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of ten minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods Collect the methylene chloride extract in a 250-mL Erlenmeyer flask

10.3 Add a second 60-mL volume of methylene chloride to the sample bottle, rinse and repeat the extraction procedure a second time, combining the extracts in the Erlenmeyer flask Perform a third extraction in the same manner

10.4 Assemble a Kuderna-Danish (K-D) concentrator by attaching a 10-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used in place of the K-D if the requirements of Section 8.2 are met

10.5 Pour the combined extract through a drying column containing about 10 cm of anhydrous sodium sulfate, and collect the extract in the K-D concentrator Rinse the Erlenmeyer flask and column with 20 to 30 mL of methylene chloride to complete the quantitative transfer.

10.6 Add one or two clean boiling chips to the evaporative flask and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL methylene chloride to the top. Place the K-D apparatus on a hot water bath (60 to 65°C) so that the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood with condensed solvent When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus and allow it to drain and cool for at least 10 minutes. Remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of methylene chloride. A 5-mL syringe is recommemded for this operation. Stopper the concentrator tube and store refrigerated if further processing will not be performed immediately. If the extracts will be stored longer than two days, they should be transferred to Teflon-sealed screw-cap bottles and protected from light.

10.7 Determine the original sample volume by refilling the sample bottle to the mark and transferring the water to a 1000-mL graduated cylinder Record the sample volume to the nearest 5 mL.

328

11. Cleanup and Separation

11.1 Cleanup procedures may not be necessary for a relatively clean sample matrix. The cleanup procedures recommended in this method have been used for the analysis of various clean waters and industrial effluents. If particular circumstances demand the use of an alternative cleanup procedure, the analyst must determine the elution profile and demonstrate that the recovery of each compound of interest is no less than 85%.

11.2 Before the silica gel cleanup technique can be utilized, the extract solvent must be exchanged to cyclohexane. Add a 1- to 10- mL aliquot of sample extract (in methylene chloride) and a boiling chip to a clean K-D concentrator tube. Add 4 mL cyclohexane and attach a micro-Snyder column. Prewet the micro-Snyder column by adding 0.5 mL methylene chloride to the top. Place the micro-K-D apparatus on a boiling (100°C) water bath so that the concentrator tube is partially immersed in the hot water. Adjust the vertical position of the apparatus and the water temperature as required to complete concentration in 5 to 10 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood. When the apparent volume of the liquid reaches 0.5 mL, remove the K-D apparatus and allow it to drain for at least 10 minutes while cooling. Remove the micro-Snyder column and rinse its lower joint into the concentrator tube with a minimum of cyclohexane. Adjust the extract volume to about 2 mL.

11.3 Silica gel column cleanup for PAHs.

11.3.1 Prepare a slurry of 10g activated silica gel in methylene chloride and place this in a 10 mm ID chromatography column. Gently tap the column to settle the silica gel and elute the methylene chloride. Add 1 to 2 cm of anhydrous sodium sulfate to the top of the silica gel.

11.3.2 Preelute the column with 40 mL of pentane. Discard the eluate and just prior to exposure of the sodium sulfate layer to the air, transfer the 2 mL of cyclohexane sample extract onto the column, using an additional 2 mL of cyclohexane to complete the transfer.

11.3.3 Just prior to exposure of the sodium sulfate layer to the air, add 25 mL pentane and continue elution of the column. Discard the pentane eluate.

11.3.4 Elute the column with 25 mL of methylene chloride/pentane (4 + 6) (V/V) and collect the eluate in a 500-mL K-D flask equipped with a 10-mL concentrator tube. Elution of the column should be at a rate of about 2 mL/min.

11.3.5 Concentrate the collected fraction to less than 10 mL by K-D techniques as in Section 10.6, using pentane to rinse the walls of the glassware. Proceed with HPLC or GC analysis.

12. High Performance Liquid Chromatography (HPLC)

12.1 To the extract in the concentrator tube, add 4 mL of acetonitrile and a new boiling chip, then attach a micro-Snyder column. Increase the temperature of the hot water bath to 95 to 100°C. Concentrate the solvent as in Section 10. After cooling, remove the micro-Snyder column and rinse its lower joint into the concentrator tube with about 0.2 mL acetonitrile. Adjust the extract volume to 1.0 mL.

12.2 Table 1 summarizes the recommended HPLC column materials and operating conditions for the instrument. This table includes retention times, capacity factors, and MDL that were obtained under these conditions. The UV detector is recommended for the determination of naphthalene, acenaphthylene, acenapthene, and fluorene, and the fluorescence detector is recommended for the remaining PAHs. Examples of the parameter separations achieved by this HPLC column are shown in Figures 1 and 2. Other HPLC columns, chromatrograpic conditions or detectors may be used if the requirements of Section 8.2 are met.

12.3 Calibrate the system daily as described in Section 7.

12.4 If the internal standard approach is being used, the internal standard must be added to sample extract and mixed thoroughly, immediately, before injection into the instrument.

12.5 Inject 5 to 25 µL of the sample extract using a high pressure syringe or a constant volume sample injection loop. Record the volume injected to the nearest 0.1 µL, and the resulting peak size in height or area units. Re-equilibrate the liquid chromatographic column at the initial gradient conditions for at least 10 minutes between injections.

12.6 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size, however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

12.7 If the peak height or area exceeds the linear range of the system, dilute the extract with acetonitrile and reanalyze.

12.8 If the peak area measurement is prevented by the presence of interferences, further cleanup is required.

13. Gas Chromatography

13.1 The packed column GC procedure will not resolve certain isomeric pairs as indicated in Section 1.3 and Table 2. The liquid chromatographic procedure (Section 12) must be used for these materials. Capillary (open-tubular) columns may be used if the relative standard deviations of responses for replicate injections are demonstrated to be less than 6% and the requirements of Section 8.2 are met.

13.2 To achieve maximum sensitivity with this method, the extract must be concentrated to 1.0 mL. Add a clean boiling chip to the methylene chloride extract in the concentrator tube. Attach a two-ball micro-Snyder column. Prewet the micro-Snyder column by adding about 0.5 mL of methylene chloride to the top. Place the micro K-D apparatus on a hot water bath (60 to 65°C) so that the concentrator tube is partially immersed in the hot water. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 5 to 10 minutes. At the proper rate of distillation the balls will actively chatter but the chambers will not flood. When the apparent volume of liquid reaches 0.5 mL, remove the K-D apparatus. Drain and cool for at least 10 minutes. Remove the micro-Snyder column and rinse its lower joint into the concentrator tube with a small volume of methylene chloride. Adjust the final volume to 1.0 mL and stopper the concentrator tube.

13.3 Table 2 describes the recommended GC column and operating conditions for the instrument. This table includes

retention times that were obtained under these conditions. An example of the parameter separations achieved by this column is shown in Figure 3. Other packed columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met. Capillary (open-tubular) columns may also be used if the relative standard deviations of responses for replicate injections are demonstrated to be less than 6% and the requirements of Section 8.2 are met.

13.4 Calibrate the GC system daily as described in Section 7.

13.5 If the internal standard approach is being used, add the internal standard to sample extract and mix thoroughly, immediately, before injection into the instrument.

13.6 Inject 2 to 5 µL of the sample extract using the solvent-flush technique[10]. Smaller (1.0 µL) volumes may be injected if automatic devices are employed. Record the volume injected to the nearest 0.05 µL, and the resulting peak size in area or peak height units.

13.7 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size; however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

13.8 If the response for the peak exceeds the working range of the system, dilute the extract and reanalyze.

13.9 If the measurement of the peak response is prevented by the presence of interferences, further cleanup is required.

14. Calculations

14.1 Determine the concentration of individual parameters in the sample.

14.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the peak response using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated from Equation 2:

Eq. 2. Concentration, µg/L $= \frac{(A)(V_t)}{(V_i)(V_s)}$

where:

- A = Amount of material injected, in nanograms
- V_i = Volume of extract injected (µL).
- V_t = Volume of total extract (µL).
- V_s = Volume of water extracted (mL).

14.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and Equation 3.

Eq. 3. Concentration, µg/L $= \frac{(A_s)(I_s)}{(A_{is})(RF)(V_o)}$

where:

- A_s = Response for the parameter to be measured.
- A_{is} = Response for the internal standard.
- I_s = Amount of internal standard added to each extract (µg).
- V_o = Volume of water extracted, in liters.

14.2 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

14.3 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits established in Section 8.4, data for the affected parameters must be labeled as suspect.

15. Method Performance

15.1 Method detection limits - The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[11]. The MDL concentrations listed in Table 1 were obtained using reagent water[11]. Similar results were achieved using representative wastewaters. MDL for the GC approach were not determined.

15.2 This method has been tested for linearity of recovery from spiked reagent water and has been demonstrated to be applicable over the concentration range from 8 x MDL to 800 x MDL[11], with the following exception: benzo(ghi)perylene recovery at 80 x and 800 x MDL were low (35% and 45% respectively).

15.3 In a single laboratory (Battelle Columbus Laboratories), using spiked wastewater samples, the average recoveries presented in Table 3 were obtained[12]. Each spiked sample was analyzed in triplicate on two separate days. The standard deviation of the precent recovery is also included in Table 3.

15.4 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. See Appendix A.
2. Determination of Polynuclear Aromatic Hydrocarbons in Industrial and Municipal Wastewaters," Report for EPA Contract 68-03-2624 (In preparation).
3. ASTM Annual Book of Standards, Part 31, D 3694, "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 679, 1980.
4. "Carcinogens - Working With Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.
5. "OSHA Safety and Health Standards, General Industry," (29CFR-1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
6. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.
7. "Handbook of Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.
8. ASTM Annual Book of Standards, Part 31, D 3370, "Standard Practice for Sampling Water," American Society for Testing and Materials, Philadelphia, PA, p. 76, 1980

330

9 "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric: DPD) for Chlorine, Total Residual." Methods for Chemical Analysis of Water and Wastes. EPA 600-4 79-020. U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268. March 1979.

10 Burke, J. A., "Gas Chromatography for Pesticide Residue Analysis; Some Practical Aspects." *Journal of the Association of Official Analytical Chemists*, *48*, 1037 (1965).

11 Cole, T., Riggins, R., and Glaser, J., "Evaluation of Method Detection Limits and Analytical Curve for EPA Method 610 - PNAs." International Symposium on Polynuclear Aromatic Hydrocarbons, 5th. Battelle Columbus Laboratory, Columbus, Ohio (1980).

Table 1. High Performance Liquid Chromatography Conditions and Method Detection Limits

Parameter	Retention Time (min)	Capacity Factor (k')	Method Detection Limit (µg/L)[a]
Naphthalene	16.6	12.2	1.8
Acenaphthylene	18.5	13.7	2.3
Acenaphthene	20.5	15.2	1.8
Fluorene	21.2	15.8	0.21
Phenanthrene	22.1	16.6	0.64
Anthracene	23.4	17.6	0.66
Fluoranthene	24.5	18.5	0.21
Pyrene	25.4	19.1	0.27
Benzo(a)anthracene	28.5	21.6	0.013
Chrysene	29.3	22.2	0.15
Benzo(b)fluoranthene	31.6	24.0	0.018
Benzo(k)flouranthene	32.9	25.1	0.017
Benzo(a)pyrene	33.9	25.9	0.023
Dibenzo(a,h)anthracene	35.7	27.4	0.030
Benzo(ghi)perylene	36.3	27.8	0.076
Indeno(1,2,3-cd)pyrene	37.4	28.7	0.043

HPLC conditions: Reverse phase HC-ODS Sil-X 2.6 mm x 250 mm Perkin-Elmer column, isocratic elution for 5 min using acetonitrile/water (4 : 6), then linear gradient elution to 100% acetonitrile over 25 minutes; flow rate is 0.5 mL/min. If columns having other internal diameters are used, the flow rate should be adjusted to maintain a linear velocity of 2 mm/sec.

[a]The method detection limit for naphthalene, acenaphthylene, acenaphthene, and fluorene were determined using a UV detector. All others were determined using a fluorescence detector.

Table 2. Gas Chromatographic Operating Conditions and Retention Times

Parameter	Retention Time (min)
Naphthalene	4.5
Acenaphthylene	10.4
Acenaphthene	10.8
Fluorene	12.6
Phenanthrene	15.9
Anthracene	15.9
Fluoranthene	19.8
Pyrene	20.6
Benzo(a)anthracene	20.6
Chrysene	24.7
Benzo(b)fluoranthene	28.0
Benzo(k)fluoranthene	28.0
Benzo(a)pyrene	29.4
Dibenzo(a,h)anthracene	36.2
Indeno(1,2,3-cd)pyrene	36.2
Benzo(ghi)perylene	38.6

GC conditions: Chromosorb W-AW-DCMS (100/120 mesh) coated with 3% OV-17, packed in a 1.8 m long x 2 mm ID glass column, with nitrogen carrier gas at a flow rate of 40 mL/min. Column temperature was held at 100°C for 4 min, then programmed at 8°/minute to a final hold at 280°C.

Table 3 *Single Operator Accuracy and Precision*

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
Acenaphthene	88	5.7	11.6-25	24	4
Acenaphthylene	93	6.4	250-450	24	4
Anthracene	93	6.3	7.9-11.3	24	4
Benzo(a)anthracene	89	6.9	0.64-0.66	24	4
Benzo(a)pyrene	94	7.4	0.21-0.30	24	4
Benzo(b)fluoranthene	97	12.9	0.24-0.30	24	4
Benzo(ghi)perylene	86	7.3	0.42-3.4	24	4
Benzo(k)fluoranthene	94	9.5	0.14-6.2	24	4
Chrysene	88	9.0	2.0-6.8	24	4
Dibenzo(a,h)anthracene	87	5.8	0.4-1.7	24	4
Fluoranthene	116	9.7	0.3-2.2	24	4
Fluorene	90	7.9	6.1-23	24	4
Indeno(1,2,3-cd)pyrene	94	6.4	0.96-1.4	24	4
Naphthalene	78	8.3	20-70	24	4
Phenanthrene	98	8.4	3.8-5.0	24	4
Pyrene	96	8.5	2.3-6.9	24	4

Column: HC-ODS SIL-X
Mobile phase: 40% to 100% Acetonitrile in water
Detector: Ultra violet at 254nm

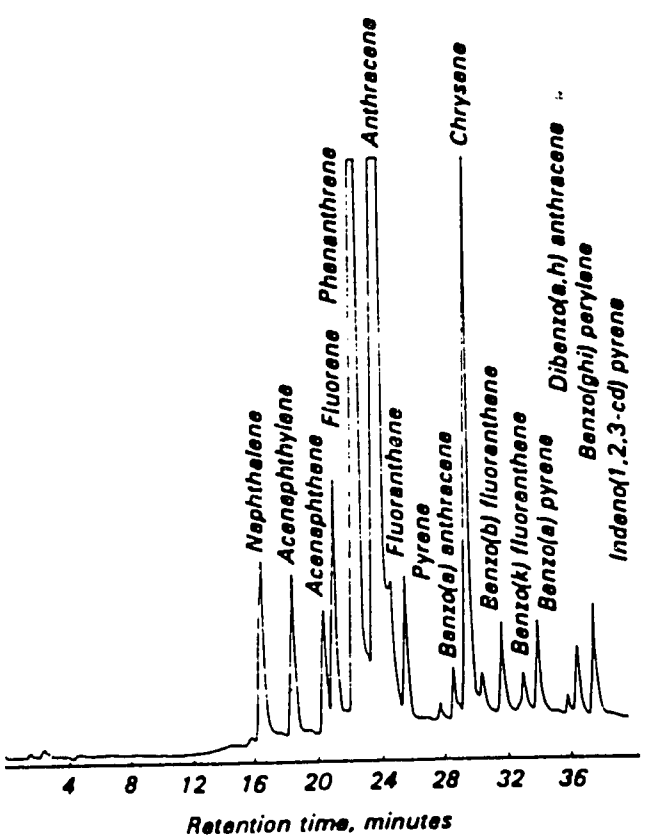


Figure 1. *Liquid chromatogram of polynuclear aromatic hydrocarbons.*

332

Column: HC-ODS SIL-X
Mobile phase: 40% to 100% Acetonitrile in water
Detector: Fluorescence

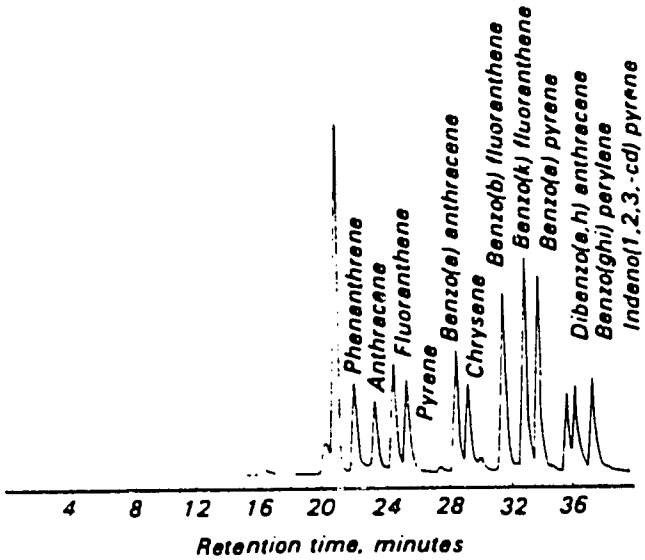


Figure 2. *Liquid chromatogram of polynuclear aromatic hydrocarbons.*

Column: 3% OV-17 on Chromosorb W-AW-DCMS
Program: 100°C. 4 min.,8° per min. to 280°C.
Detector: Flame ionization

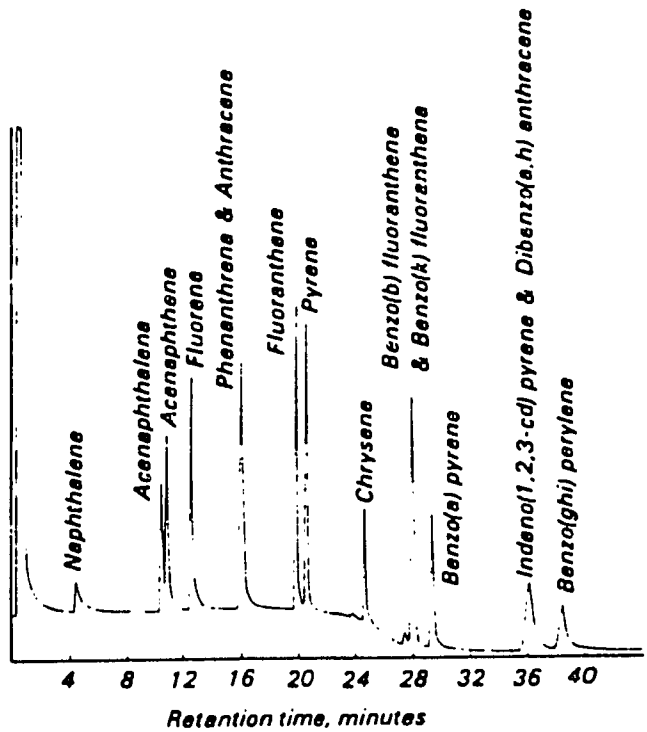


Figure 3. *Gas chromatogram of polynuclear aromatic hydrocarbons.*

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Haloethers — Method 611

1. Scope and Application

1.1 This method covers the determination of certain haloethers. The following parameters can be determined by this method:

Parameter	STORET No.	CAS No.
Bis(2-chloroethyl) ether	34273	111-44-4
Bis(2-chloroethoxy) methane	34278	111-91-1
Bis(2-chloroisopropyl) ether	34283	108-60-1
4-Bromophenyl phenyl ether	34636	101-55-3
4-Chlorophenyl phenyl ether	34641	7005-72-3

1.2 This is a gas chromatographic (GC) method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for any or all of the compounds above, compound identifications should be supported by at least one additional qualitative technique. This method describes analytical conditions for a second GC column that can be used to confirm measurements made with the primary column. Method 625 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results for all of the parameters listed above, using the extract from this method.

1.3 The method detection limit (MDL, defined in Section 14.1)[11] for each parameter is listed in Table 1. The MDL for a specific wastewater may differ from that listed, depending upon the nature of interferences in the sample matrix.

1.4 The sample extraction and concentration steps in this method are essentially the same as in methods 606, 608, 609, and 612. Thus, a single sample may be extracted to measure the parameters included in the scope of each of these methods. When cleanup is required, the concentration levels must be high enough to permit selecting aliquots, as necessary, to apply appropriate cleanup procedures. The analyst is allowed the latitude, under Gas Chromatography (Section 12), to select chromatographic conditions appropriate for the simultaneous measurement of combinations of these parameters.

1.5 Any modification of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval of alternate test procedures under 40 CFR 136.4 and 136.5.

1.6 This method is restricted to use by or under the supervision of analysts experienced in the use of

334

gas chromatography and in the interpretation of gas chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 A measured volume of sample, approximately one-liter, is solvent extracted with methylene chloride using a separatory funnel. The methylene chloride extract is dried and exchanged to hexane during concentration to a volume of 10 mL or less. GC conditions are described which permit the separation and measurement of the compounds in the extract using a halide specific detector.[2]

2.2 The method provides a Florisil column cleanup procedure to aid in the elimination of interferences that may be encountered.

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in gas chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned.[3] Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and reagent water. It should then be drained dry, and heated in a muffle furnace at 400°C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedures in Section 11 can be used to overcome many of these interferences, but unique samples may require additional cleanup approaches to achieve the MDL listed in Table 1.

3.3 Dichlorobenzenes are known to coelute with haloethers under some gas chromatographic conditions. If these materials are present together in a sample, it may be necessary to analyze the extract with two different column packings to completely resolve all of the compounds.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[4-6] for the information of the analyst.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle - Amber glass, one-liter or one-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional) - Must incorporate glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4°C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.2 Glassware (All specifications are suggested. Catalog numbers are included for illustration only.)

5.2.1 Separatory funnel - 2000-mL, with Teflon stopcock.

5.2.2 Drying column - Chromatographic column 400 mm long x 19 mm ID, with coarse frit.

5.2.3 Chromatographic column - 400-mm long x 19 mm ID glass with coarse fritted plate on bottom and Teflon stopcock (Kontes K-420540-0224 or equivalent).

5.2.4 Concentrator tube, Kuderna-Danish - 10-mL, graduated (Kontes K-570050-1025 or equivalent). Calibration must be checked at the volumes employed in the test. Ground glass stopper is used to prevent evaporation of extracts.

5.2.5 Evaporative flask, Kuderna-Danish - 500-mL (Kontes K-570001-0500 or equivalent). Attach to concentrator tube with springs.

5.2.6 Snyder column, Kuderna-Danish - Three-ball macro (Kontes K-503000-0121 or equivalent).

5.2.7 Vials - Amber glass, 10- to 15- mL capacity, with Teflon-lined screwcap.

5.3 Boiling chips - Approximately 10/40 mesh. Heat to 400°C for 30 minutes or Soxhlet extract with methylene chloride.

5.4 Water bath - Heated, with concentric ring cover, capable of temperature control (±2°C). The bath should be used in a hood.

5.5 Balance - Analytical, capable of accurately weighing 0.0001 g.

5.6 Gas chromatograph - An analytical system complete with temperature programmable gas chromatograph suitable for on-column injection and all required accessories including syringes, analytical columns, gases, detector, and strip-chart recorder. A data system is recommended for measuring peak areas.

5.6.1 Column 1 - 1.8 m long x 2 mm ID pyrex glass, packed with Supelcoport, (100/120 mesh) coated

335

with 3% SP-1000 or equivalent. This column was used to develop the method performance statements in Section 14. Guidelines for the use of alternate column packings are provided in Section 12.1.

5.6.2 Column 2 - 1.8 m long x 2 mm ID pyrex glass, packed with Tenax-GC (60/80 mesh) or equivalent.

5.6.3 Detector - Halide specific electrolytic conductivity or microcoulometric. These detectors have proven effective in the analysis of wastewaters for the parameters listed in the scope of this method. The Hall conductivity detector was used to develop the method performance statements in Section 14. Guidelines for the use of alternate detectors are provided in Section 12.1. Although less selective, an electron capture detector is an acceptable alternative.

6. Reagents

6.1 Reagent water - Reagent water is defined as a water in which an interferent is not observed at the MDL of each parameter of interest.

6.2 Sodium thiosulfate - (ACS) Granular.

6.3 Acetone, methanol, methylene chloride, hexane, and petroleum ether (boiling range 30 to 60°C) - Pesticide quality or equivalent.

6.4 Sodium sulfate - (ACS) Granular, anhydrous. Purify by heating at 400°C for four hours in a shallow tray.

6.5 Florisil - PR Grade (60/100 mesh); purchase activated at 1250°F and store in the dark in glass container with glass stoppers or foil-lined screw caps. Before use, activate each batch overnight at 130°C in a foil-covered glass container.

6.6 Ethyl ether - Nanograde, redistilled in glass, if necessary.

6.6.1 Must be free of peroxides as indicated by EM Laboratories Quant test strips. (Available from Scientific Products Co., Cat. No. P1126-8, and other suppliers.)

6.6.2 Procedures recommended for removal of peroxides are provided with the test strips. After cleanup 20 mL ethyl alcohol preservative must be added to each liter of ether.

6.7 Stock standard solutions (1.00 µg/µL) - Stock standard solutions can be prepared from pure standard materials or purchased as certified solutions.

6.7.1 Prepare stock standard solutions by accurately weighing about 0.0100 g of pure material. Dissolve the material in pesticide quality acetone and dilute to volume in a 10-mL volumetric flask. Larger volumes can be used at the convenience of the analyst. If compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.7.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at 4°C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check standards that can be used to determine the accuracy of calibration standards will be available for the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

6.7.3 Stock standard solutions must be replaced after six months, or sooner if comparison with check standards indicate a problem.

7. Calibration

7.1 Establish gas chromatographic operating parameters to produce retention times equivalent to those listed in Table 1. The GC chromatographic system may be calibrated using the external standard technique (Section 7.2) or the internal standard technique (Section 7.3).

7.2 External standard calibration procedure.

7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with hexane. One of the external standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.2.2 Using injections of 2 to 5 µL of each calibration standard, tabulate peak height or area responses against the mass injected. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over the working range (± 10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.2.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ± 10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that compound.

7.3 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples.

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask. To each calibration standard, add a known constant amount of one or more internal standards, and dilute to volume with hexane. One of the standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.

7.3.2 Using injections of 2 to 5 µL of each calibration standard, tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1.

$$\text{Eq. 1} \quad RF = \frac{(A_s C_{is})}{(A_{is} C_s)}$$

where:

- A_s Response for the parameter to be measured.
- A_{is} Response for the internal standard.
- C_{is} Concentration of the internal standard, (µg/L).
- C_s Concentration of the parameter to be measured, (µg/L).

336

If the RF value over the working range is a constant (< 10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_x/A_{is} vs. RF.

7.3.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound.

7.4 Before using any cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

7.5 The cleanup procedure in Section 11 utilizes Florisil column chromatography. Florisil from different batches or sources may vary in adsorption capacity. To standardize the amount of Florisil which is used, the use of lauric acid value[7] is suggested. The referenced procedure determines the adsorption from hexane solution of lauric acid (mg) per gram Florisil. The amount of Florisil to be used for each column is calculated by dividing 110 by this ratio and multiplying by 20 g.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in acetone 1000 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 2, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > 2p$ or $|X-R| > 2p$, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance:

Upper Control Limit (UCL) = R + 3 s
Lower Control Limit (LCL) = R − 3 s

where R and s are calculated as in Section 8.2.3. The UCL and LCL can be used to construct control charts[8] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[8].

8.4 The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 13.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate through the analysis of a one-liter aliquot of reagent water, that all glassware and reagent interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality

337

assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as GC with a dissimilar column, specific element detector, or mass spectrometer must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices[9] should be followed, except that the bottle must not be prewashed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be a free as possible of Tygon and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4°C from the time of collection until extraction. Fill the sample bottles and, if residual chlorine is present, add 80 mg of sodium thiosulfate per each liter of water. U.S. Environmental Protection Agency methods 330.4 and 330.5 may be used to measure the residual chlorine[10]. Field test kits are available for this purpose.

9.3 All samples must be extracted within 7 days and completely analyzed within 40 days of extraction[2].

10. Sample Extraction

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a two-liter separatory funnel.

10.2 Add 60 mL methylene chloride to the sample bottle, seal, and shake 30 seconds to rinse the inner walls. Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods. Collect the methylene chloride extract in a 250-mL Erlenmeyer flask.

10.3 Add a second 60-mL volume of methylene chloride to the sample bottle and repeat the extraction procedure a second time, combining the extracts in the Erlenmeyer flask. Perform a third extraction in the same manner.

10.4 Assemble a Kuderna-Danish (K-D) concentrator by attaching a 10-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used in place of the K-D if the requirements of Section 8.2 are met.

10.5 Pour the combined extract through a drying column containing about 10 cm of anhydrous sodium sulfate, and collect the extract in the K-D concentrator. Rinse the Erlenmeyer flask and column with 20 to 30 mL of methylene chloride to complete the quantitative transfer.

10.6 Add one or two clean boiling chips to the evaporative flask and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL methylene chloride to the top. Place the K-D apparatus on a hot water bath (60° to 65°C) so that the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood with condensed solvent. When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus and allow it to drain and cool for at least 10 minutes.

NOTE: Some of the haloethers are very volatile and significant losses will occur in concentration steps if care is not exercised. It is important to maintain a constant gentle evaporation rate and not to allow the liquid volume to fall below 1 to 2 mL before removing the K-D from the hot water bath.

10.7 Momentarily remove the Snyder column, add 50 mL of hexane and a new boiling chip and replace the column. Raise the temperature of the water bath to 85 to 90°C. Concentrate the extract as in Section 10.6 except use hexane to prewet the column. When the apparent volume of liquid reaches 1 to 2 mL, remove the K-D and allow it to drain and cool at least 10 minutes. Remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of hexane. A 5-mL syringe is recommended for this operation. Stopper the concentrator tube and store refrigerated if further processing will not be performed immediately. If the extracts will be stored longer than two days, they should be transferred to Teflon-sealed screw-cap bottles.

10.8 Determine the original sample volume by refilling the sample bottle to the mark and transferring the water to a 1000-mL graduated cylinder. Record the sample volume to the nearest 5 mL.

11. Cleanup and Separation

11.1 Cleanup procedures may not be necessary for a relatively clean sample matrix. The cleanup procedure recommended in this method has been used for the analysis of various clean waters and industrial effluents. If particular circumstances demand the use of an alternative cleanup procedure, the analyst must determine the elution profile and demonstrate that the recovery of each compound of interest is no less than 85%.

11.2 Florisil column cleanup for haloethers:

11.2.1 Adjust the sample extract volume to 10 mL.

11.2.2 Place a charge (nominally 20 g, actual amount determined as in Section 7.5) of activated Florisil in a 19-mm ID chromatographic column. After settling the Florisil by tapping the column, add about one-half inch layer of anhydrous granular sodium sulfate to the top. Allow the Florisil to cool.

11.2.3 Pre-elute the column with 50 to 60 mL of petroleum ether. Discard the eluate and just prior to exposure of the sulfate layer to air, quantitatively transfer the sample extract into the column by decantation and subsequent petroleum ether washings. Discard the eluate. Just prior to exposure of the sodium sulfate layer to the air, begin eluting the column with 300 mL of ethyl ether/petroleum ether (6 : 94) (V/V). Adjust the elution rate to approx-

338

imately 5 mL min and collect the eluate in a 500 mL K-D flask equipped with a 10 mL concentrator tube. This fraction should contain all of the haloethers.

11.2.4 Concentrate the fraction by K-D as in Section 10.6 except prewet the Snyder column with hexane. When the apparatus is cool, remove the column and rinse the flask and its lower joint into the concentrator tube with hexane. Adjust the volume to 10 mL. Analyze by GC (Section 12.)

12. Gas Chromatography

12.1 Table 1 summarizes the recommended operating conditions for the gas chromatograph. This table includes retention times and MDL that were obtained under these conditions. Examples of the parameter separations achieved by these columns are shown in Figures 1 and 2. Other packed columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met. Capillary (open-tubular) columns may also be used if the relative standard deviations of responses for replicate injections are demonstrated to be less than 6% and the requirements of Section 8.2 are met.

12.2 Calibrate the system daily as described in Section 7.

12.3 If the internal standard approach is being used, the analyst must not add the internal standard to sample extracts until immediately before injection into the instrument. Mix thoroughly.

12.4 Inject 2 to 5 µL of the sample extract using the solvent-flush technique.[11] Smaller (1.0 µL) volumes can be injected if automatic devices are employed. Record the extract volume to the nearest 0.1 mL and the volume injected to the nearest 0.05 µL, and the resulting peak size in area or peak height units.

12.5 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size, however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

12.6 If the response for the peak exceeds the working range of the system, dilute the extract and reanalyze.

12.7 If the measurement of the peak response is prevented by the presence of interferences, further cleanup is required.

13. Calculations

13.1 Determine the concentration of individual compounds in the sample.

13.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the peak response using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated from equation 2.

Eq. 2 Concentration, µg/L $\frac{(A)(V_t)}{(V_i)(V_s)}$

where:

- A — Amount of material injected, in nanograms
- V_i — Volume of extract injected (µL).
- V_t — Volume of total extract (µL)
- V_s — Volume of water extracted (mL)

13.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and equation 3.

Eq. 3 Concentration, µg/L $\frac{(A_s)(I_s)}{(A_{is})(RF)(V_o)}$

where:

- A_s — Response for the parameter to be measured.
- A_{is} — Response for the internal standard
- I_s — Amount of internal standard added to each extract (µg).
- V_o — Volume of water extracted, in liters.

13.2 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.3 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits in Section 8.4, data for the affected parameters must be labeled as suspect.

14. Method Performance

14.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero.[11] The MDL concentrations listed in Table 1 were obtained using reagent water.[12] Similar results were achieved using representative wastewaters.

14.2 This method has been tested for linearity of recovery from spiked reagent water and has been demonstrated to be applicable for the concentration range from 4X MDL to 1000 x MDL.[12]

14.3 In a single laboratory (Monsanto Research Center), using spiked wastewater samples, the average recoveries presented in Table 2 were obtained.[2] Each spiked sample was analyzed in triplicate on three separate occasions. The standard deviation of the percent recovery is also included in Table 2.

14.4 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. See Appendix A.
2. "Determination of Haloethers in Industrial and Municipal Wastewaters." Report for EPA Contract 68-03-2633 (In preparation).
3. ASTM Annual Book of Standards, Part 31, D 3694 "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 679, 1980.
4. "Carcinogens - Working With Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.
5. "OSHA Safety and Health Standards, General Industry," (29CFR1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
6. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.

7. Mills, P.A., "Variation of Florisil Activity: Simple Method for Measuring Absorbent Capacity and Its Use in Standardizing Florisil Columns," *Journal of the Association of Official Analytical Chemists, 51*, 29 (1968).

8. "Handbook of Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.

9. ASTM Annual Book of Standards, Part 31, D 3370, "Standard Practice for Sampling Water," American Society for Testing and Materials, Philadelphia, PA, p. 76, 1980.

10. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600-4/79-020, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.

11. Burke, J. A., "Gas Chromatography for Pesticide Residue Analysis; Some Practical Aspects," *Journal of the Association of Official Analytical Chemists, 48*, 1037 (1965).

12. "EPA Method Validation Study 21 Method 611 (Haloethers)," Report for EPA Contract 68-03-2633 (In Preparation).

Table 1. Chromatographic Conditions and Method Detection Limits

Parameter	Retention Time (min) Column 1	Retention Time (min) Column 2	Method Detection Limit (µg/L)
Bis(2-chloroisopropyl) ether	8.4	9.7	0.8
Bis(2-chloroethyl) ether	9.3	9.1	0.3
Bis(2-chloroethoxy) methane	13.1	10.0	0.5
4-Chlorophenyl phenyl ether	19.4	15.0	3.9
4-Bromophenyl phenyl ether	21.2	16.2	2.3

Column 1 conditions: Supelcoport (100/120 mesh) coated with 3% SP-1000 packed in 1.8 m long x 2 mm ID glass column with helium carrier gas at a flow rate of 40 mL/min. Column temperature: 60°C for 2 min after injection then program at 8°C/min to 230°C and hold for 4 min. Under these conditions the retention time for Aldrin is 22.6 min.

Column 2 conditions: Tenax-GC (60/80 mesh) packed in a 1.8 m long x 2mm ID glass column with helium carrier gas at 40 mL/min flow rate. Column temperature: 150°C for 4 min after injection then program at 16°C/min to 310°C. Under these conditions the retention time for Aldrin is 18.4 min.

Table 2. Single Operator Accuracy and Precision

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
Bis(2-chloroethyl)ether	59	4.5	97	27	3
Bis(2-chloroethoxy)methane	62	5.3	138	27	3
Bis(2-chloroisopropyl)ether	67	4.0	54	27	3
4-Bromophenyl phenyl ether	78	3.5	14	27	3
4-Chlorophenyl phenyl ether	73	4.5	30	27	3

340

Column: 3% SP-1000 on Supelcoport
Program. 60°C.-2 minutes 8°/minute to 230°C.
Detector: Hall electrolytic conductivity

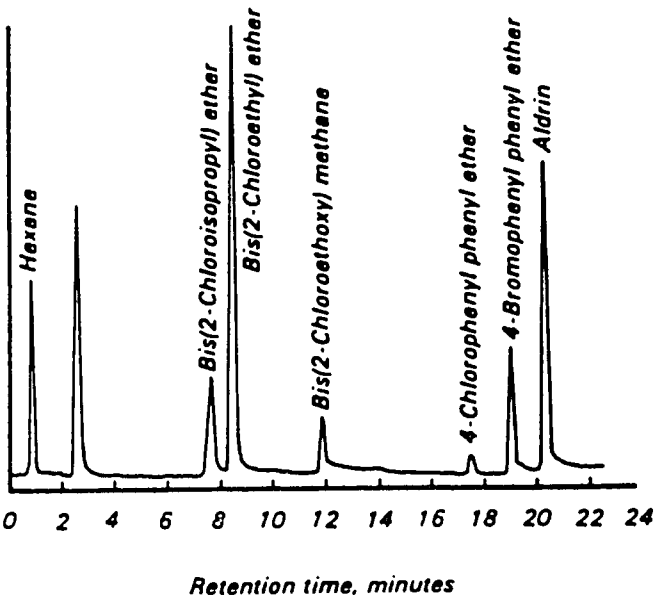


Figure 1. *Gas chromatogram of haloethers.*

Column: Tenax GC
Program: 150°C.-4 minutes 16°/minute to 310°C.
Detector: Hall electrolytic conductivity

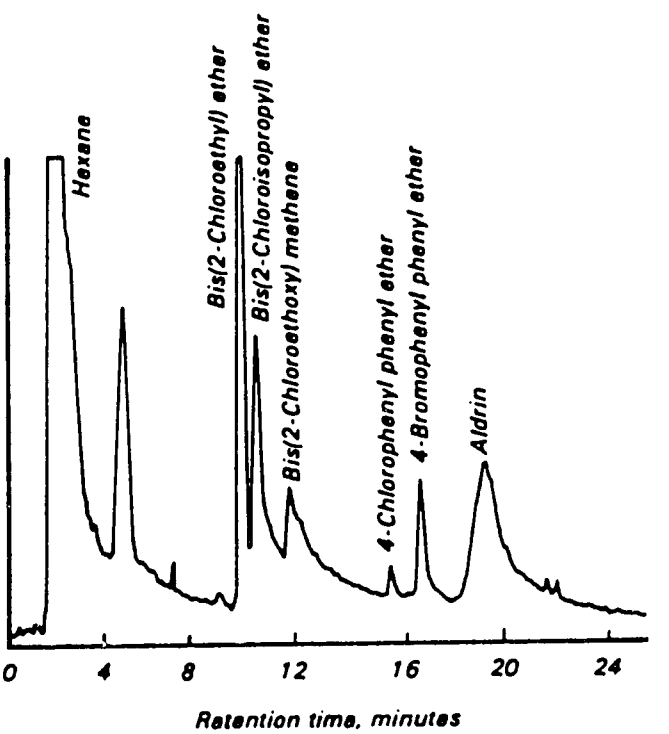


Figure 2. *Gas chromatogram of haloethers.*

341

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Chlorinated Hydrocarbons — Method 612

1. Scope and Application

1.1 This method covers the determination of certain chlorinated hydrocarbons. The following parameters can be determined by this method:

Parameter	STORET No.	CAS No.
2-Chloronaphthalene	34581	91-58-7
1,2-Dichlorobenzene	34536	95-50-1
1,3-Dichlorobenzene	34566	541-73-1
1,4-Dichlorobenzene	34571	106-46-7
Hexachlorobenzene	39700	118-74-1
Hexachlorobutadiene	34391	87-68-3
Hexachlorocyclopentadiene	34386	77-47-4
Hexachloroethane	34396	67-72-1
1,2,4-Trichlorobenzene	34551	120-82-1

1.2 This is a gas chromatographic (GC) method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1. When this method is used to analyze unfamiliar samples for any or all of the compounds above, compound identifications should be supported by at least one additional qualitative technique. Method 625 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results for all of the parameters listed above, using the extract produced by this method.

1.3 The method detection limit (MDL defined in Section 14.1)[11] for each parameter is listed in Table 1 The MDL for a specific wastewater may differ from that listed, depending upon the nature of interferences in the sample matrix

1.4 The sample extraction and concentration steps in this method are essentially the same as in methods 606, 608, 609, and 611 Thus, a single sample may be extracted to measure the parameters included in the scope of each of these methods. When cleanup is required, the concentration levels must be high enough to permit selecting aliquots, as necessary, to apply appropriate cleanup procedures. The analyst is allowed the latitude, under Gas Chromatography (Section 12), to select chromatographic conditions appropriate for the simultaneous measurement of combinations of these parameters, provided that the requirements of Section 8.2 are met.

1.5 Any modification of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval of alternate test procedures under 40 CFR 136 4 and 136 5

342

1.6 This method is restricted to use by or under the supervision of analysts experienced in the use of gas chromatography and in the interpretation of gas chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 A measured volume of sample, approximately one-liter, is solvent extracted with methylene chloride using a separatory funnel. The methylene chloride extract is dried and solvent exchanged to hexane during concentration to a volume of 10 mL or less. GC conditions are described which permit the separation and measurement of the compounds in the extract using an ECD[2].

2.2 The method provides a Florisil column cleanup procedure to aid in the elimination of interferences that may be encountered.

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in gas chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned[3]. Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and distilled water. It should then be drained dry, and heated in a muffle furnace at 400°C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedure in Section 11 can be used to overcome many of these interferences, but unique samples may require additional cleanup approaches to achieve the detection limits listed in Table 1.

4. Safety

The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[4-6] for the information of the analyst.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle - Amber glass, one-liter or one-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional) - Must incorporate glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4°C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.2 Glassware (All specifications are suggested. Catalog numbers are included for illustration only).

5.2.1 Separatory funnel - 2000-mL, with Teflon stopcock.

5.2.2 Drying column - Chromatographic column 400 mm long x 19 mm ID with coarse frit.

5.2.3 Concentrator tube, Kuderna-Danish - 10-mL, graduated (Kontes K-570050-1025 or equivalent). Calibration must be checked at the volumes employed in the test. Ground glass stopper is used to prevent evaporation of extracts.

5.2.4 Evaporative flask, Kuderna-Danish - 500-mL (Kontes K-570001-0500 or equivalent). Attach to concentrator tube with springs.

5.2.5 Snyder column, Kuderna-Danish - three-ball macro (Kontes K-503000-0121 or equivalent).

5.2.6 Snyder column, Kuderna-Danish - two-ball micro (Kontes K-569001-0219 or equivalent).

5.2.7 Vials - Amber glass, 10- to 15- mL capacity, with Teflon- lined screwcap.

5.2.8 Chromatography column - 300 mm long x 10 mm ID with coarse fritted disc at bottom and Teflon stopcock.

5.3 Boiling chips - approximately 10/40 mesh. Heat to 400°C for 30 minutes or Soxhlet extract with methylene chloride.

5.4 Water bath - Heated, with concentric ring cover, capable of temperature control (± 2°C). The bath should be used in a hood.

5.5 Balance - Analytical, capable of accurately weighing 0.0001 g.

5.6 Gas chromatograph - An analytical system complete with gas chromatograph suitable for on-column injection and all required accessories including syringes, analytical columns, gases, ECD, and strip-chart recorder. A data system is recommended for measuring peak areas.

5.6.1 Column - 1.8 m long x 2 mm ID pyrex glass, packed with 1.5% OV-1 + 2.4% OV-225 on Supelcoport (80/100 mesh) or equivalent. This column was used to develop the method performance statements in Section

343

14 Guidelines for the use of alternate column packings are provided in Section 12.1

5.6.2 Detector - Electron capture This detector has proven effective in the analysis of wastewaters for the parameters listed in the scope, and was used to develop the accuracy and precision statements in Section 14 Guidelines for the use of alternate detectors are provided in Section 12.1

6. Reagents

6.1 Reagent water - Reagent water is defined as a water in which an interferent is not observed at the MDL of each parameter of interest.

6.2 Acetone, methanol, methylene chloride, hexane, petroleum ether (Boiling range 30 to 60°C) - Pesticide quality or equivalent

6.3 Sodium sulfate - (ACS) Granular, anhydrous. Purify by heating at 400°C for four hours in a shallow tray

6.4 Florisil - PR grade (60/100 mesh); purchase activated at 1250°F and store in the dark in glass containers with glass stoppers or foil-lined screw caps Before use, activate each batch at 130°C in foil-covered glass containers.

6.5 Stock standard solutions (1.00 µg/µL) - Stock standard solutions can be prepared from pure standard materials or purchased as certified solutions.

6.5.1 Prepare stock standard solutions by accurately weighing about 0.0100 g of pure material. Dissolve the material in pesticide quality isooctane, dilute to volume in a 10-mL volumetric flask. Larger volumes can be used at the convenience of the analyst. If compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.5.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at 4°C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check standards that can be used to determine the accuracy of calibration standards will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio, 45268

6.5.3 Stock standard solutions must be replaced after six months, or sooner if comparison with check standards indicate a problem

7. Calibration

7.1 Establish GC operating conditions to produce resolution of the parameters equivalent to those indicated in Table 1. The GC system may be calibrated using external standard technique (Section 7.2) or the internal standard technique (Section 7.3)

7.2 External standard calibration procedure

7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with isooctane. One of the external standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector

7.2.2 Using injections of 2 to 5 µL of each calibration standard, tabulate peak height or area responses against the mass injected The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over the working range (≤ 10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.2.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that compound.

7.3 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask To each calibration standard, add a known constant amount of one or more internal standards, and dilute to volume with isooctane One of the standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector

7.3.2 Using injections of 2 to 5 µL of each calibration standard, tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1

$$\text{Eq 1} \quad RF = (A_s C_{is})/(A_{is} C_s)$$

where

- A_s Response for the parameter to be measured
- A_{is} Response for the internal standard
- C_{is} Concentration of the internal standard, (µg/L)
- C_s Concentration of the parameter to be measured, (µg/L)

If the RF value over the working range is a constant (≤ 10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is} vs RF

7.3.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards If the response for any parameter varies from the predicted response by more than ±10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound

7.4 Before using any cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents

344

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each compound to be measured. Using stock standards, prepare a quality control check sample concentrate in acetone 1000 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 2, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If s > 2p or |X-R| > 2p, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance.

Upper Control Limit (UCL) R + 3 s
Lower Control Limit (LCL) R - 3 s

where R and s are calculated as in Section 8.2.3.

The UCL and LCL can be used to construct control charts[7] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[7].

8.4 The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for a particular parameter does not fall within the control limits for method performance, the results reported for that parameter in all samples processed as part of the same set must be qualified as described in Section 13.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate through the analysis of one-liter aliquot of reagent water, that all glassware and reagents interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as gas chromatography with a dissimilar column, specific element detector, or mass spectrometer must be used. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices[8] should be followed, except that the bottle must not be prewashed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4°C from the time of collection until extraction.

345

9.3 All samples must be extracted within 7 days and completely analyzed within 40 days of extraction.[8]

10. Sample Extraction

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a two-liter separatory funnel.

10.2 Add 60 mL methylene chloride to the sample bottle, seal, and shake 30 seconds to rinse the inner surface. Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods. Collect the methylene chloride extract in a 250-mL Erlenmeyer flask.

10.3 Add a second 60-mL volume of methylene chloride to the sample bottle and repeat the extraction procedure a second time, combining the extracts in the Erlenmeyer flask. Perform a third extraction in the same manner.

10.4 Assemble a Kuderna-Danish (K-D) concentrator by attaching a 10-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used in place of the Kuderna-Danish if the requirements of Section 8.2 are met.

10.5 Pour the combined extract through a drying column containing about 10 cm of anhydrous sodium sulfate, and collect the extract in the K-D concentrator. Rinse the Erlenmeyer flask and column with 20 to 30 mL of methylene chloride to complete the quantitative transfer.

10.6 Add one or two clean boiling chips to the evaporative flask and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL methylene chloride to the top. Place the K-D apparatus on a hot water bath (60 to 65°C) so that the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood with condensed solvent. When the apparent volume of liquid reaches 1 to 2 mL, remove the K-D apparatus from the water bath and allow it to drain for at least 10 minutes while cooling.

NOTE: The dichlorobenzenes have a sufficiently high volatility that significant losses may occur in concentration steps if care is not exercised. It is important to maintain a constant gentle evaporation rate and not to allow the liquid volume to fall below 1 to 2 mL before removing the K-D from the hot water bath.

10.7 Momentarily remove the Snyder column, add 50 mL hexane and a new boiling chip and replace the column. Raise the temperature of the water bath to 85 to 90°C. Concentrate the extract as in Section 10.6, except using hexane to prewet the column. Remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of hexane. A 5-mL syringe is recommended for this operation. Stopper the concentrator tube and store refrigerated if further processing will not be performed immediately.

10.8 Determine the original sample volume by refilling the sample bottle to the mark and transferring the water to a 1000-mL graduated cylinder. Record the sample volume to the nearest 5 mL.

10.9 Unless the sample is known to require cleanup, proceed to analysis by gas chromatography.

11. Cleanup and Separation

11.1 Cleanup procedures may not be necessary for a relatively clean sample matrix. The cleanup procedures recommended in this method have been used for the analysis of various clean waters and industrial effluents. If particular circumstances demand the use of an alternative cleanup procedure, the analyst must determine the elution profile and demonstrate that the recovery of each compound of interest is no less than 85%.

11.2 Florisil column cleanup for chlorinated hydrocarbons.

11.2.1 Adjust the sample extract to 10 mL with hexane.

11.2.2 Place a 12-g charge of activated Florisil in a 10-mm ID chromatography column. After settling the Florisil by tapping the column, add a 1 to 2 cm layer of anhydrous granular sodium sulfate to the top. Allow to cool, then pre-elute the column, with 100 mL of petroleum ether. Discard the eluate and just prior to exposure of the sodium sulfate layer to air, quantitatively transfer the sample extract into the column by decantation and subsequent petroleum ether washings. Discard the eluate. Just prior to exposure of the sodium sulfate layer to the air, begin eluting the column with 200 mL petroleum ether and collect the eluate in a 500-mL K-D flask equipped with a 10-mL concentrator tube. This fraction should contain all of the chlorinated hydrocarbons.

11.2.3 Concentrate the fraction by K-D as in section 10.6 except prewet the column with hexane. When the apparatus is cool, remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of hexane. Analyze by GC.

12. Gas Chromatography

12.1 Table 1 summarizes the recommended operating conditions for the gas chromatograph. This table includes retention times and MDL that were obtained under these conditions. Examples of the parameter separations achieved by this column are shown in Figures 1 and 2. Other packed columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met. Capillary (open-tubular) columns may also be used if the relative standard deviations of responses for replicate injections are demonstrated to be less than 6% and the requirements of Section 8.2 are met.

12.2 Calibrate the system daily as described in Section 7.

12.3 If the internal standard approach is being used, the internal standard must be added to the sample extract and mixed thoroughly, immediately before injection into the instrument.

12.4 Inject 2 to 5 µL of the sample extract using the solvent-flush technique.[9] Smaller (1.0 µL) volumes can be injected if automatic devices are employed. Record the volume of the extract to the nearest 0.1 mL, the volume injected to the nearest 0.05 µL, and the resulting peak size in area or peak height units.

2,46

12.5 The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size, however the experience of the analyst should weigh heavily in the interpretation of chromatograms.

12.6 If the response for the peak exceeds the working range of the system, dilute the extract and reanalyze.

12.7 If the measurement of the peak response is prevented by the presence of interferences, further cleanup is required.

13. Calculations

13.1 Determine the concentration of individual compounds in the sample.

13.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the peak response using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated from Equation 2.

Eq. 2 Concentration, $\mu g/L$ $\frac{(A)(V_t)}{(V_i)(V_s)}$

where

- A Amount of material injected, in nanograms
- V_i Volume of extract injected (μL).
- V_t Volume of total extract (μL)
- V_s Volume of water extracted (mL).

13.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and Equation 3.

Eq. 3. Concentration, $\mu g/L$ $\frac{(A_s)(I_s)}{(A_{is})(RF)(V_o)}$

where:

- A_s Response for the parameter to be measured.
- A_{is} Response for the internal standard.
- I_s Amount of internal standard added to each extract (μg).
- V_o Volume of water extracted, in liters.

13.2 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.3 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits in section 8.4, data for the affected parameters must be labeled as suspect.

14. Method Performance

14.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero.[11] The MDL concentrations listed in Table 1 were obtained using reagent water.[10] Similar results were achieved using representative wastewaters.

14.2 This method has been tested for linearity of spike recovery from reagent water and has been demonstrated to be applicable over the concentration range from the 4 x MDL up to 1000 x MDL.[10]

14.3 In a single laboratory (EMSL-Cincinnati), using three wastewaters spiked at six concentration levels, the average recoveries presented in Table 2 were obtained. The standard deviation of the percent recovery is also included in Table 2.

14.4 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. See Appendix A.
2. "Determination of Chlorinated Hydrocarbons in Industrial and Municipal Wastewaters." Report for EPA Contract 68-03-2625 (In preparation).
3. ASTM Annual Book of Standards, Part 31, D 3694, "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 679, 1980.
4. "Carcinogens - Working With Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.
5. "OSHA Safety and Health Standards, General Industry," (29CFR1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
6. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.
7. "Handbook of Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.
8. ASTM Annual Book of Standards, Part 31, D 3370, "Standard Practice for Sampling Water," American Society for Testing and Materials, Philadelphia, PA, p. 76, 1980.
9. Burke, J. A. "Gas Chromatography for Pesticide Residue Analysis; Some Practical Aspects," *Journal of the Association of Official Analytical Chemists, 48*, 1037 (1965).
10. "Development of Detection Limits, EPA Method 612, Chlorinated Hydrocarbons," Special letter report for EPA Contract 68-03-2625, Environmental Monitoring and Support Laboratory - Cincinnati, Ohio 45268

347

Table 1 *Chromatographic Conditions and Method Detection Limits*

Parameter	Retention Time (min.)	Method Detection Limit (µg/L)
1,3-Dichlorobenzene	6.8	1.19
1,4-Dichlorobenzene	7.6	1.34
Hexachloroethane	8.3	0.03
1,2-Dichlorobenzene	9.3	1.14
Hexachlorobutadiene	20.0	0.34
1,2,4-Trichlorobenzene	22.3	0.05
2-Chloronaphthalene	3.6[a]	0.94
Hexachlorobenzene	10.1[a]	0.05

Column conditions: Supelcoport (80/100 mesh) coated with 1.5% OV-1/2.4% OV-225 packed in a 1.8 m x 2 mm ID glass column with 5% Methane/95% Argon carrier gas at a flow rate of 25 mL/min. Column temperature, isothermal at 75 C, except as otherwise indicated.

[a] - Column temperature 165 C.

Table 2. *Single Operator Accuracy and Precision*

Parameter	Average Percent Recovery	Standard Deviation %	Spike Range (µg/L)	Number of Analyses	Matrix Types
2-Chloronaphthalene	76	25	19.1-268	18	3
1,2-Dichlorobenzene	82	10	29.8-356	18	3
1,3-Dichlorobenzene	86	18	20.4-238	18	3
1,4-Dichlorobenzene	89	20	23.0-324	18	3
Hexachlorobenzene	95	12	1.29-14.9	18	3
Hexachlorobutadiene	96	10	3.12-36.8	18	3
Hexachloroethane	99	12	1.02-14.8	18	3
1,2,4-Trichlorobenzene	96	16	15.1-216	18	3

3,48

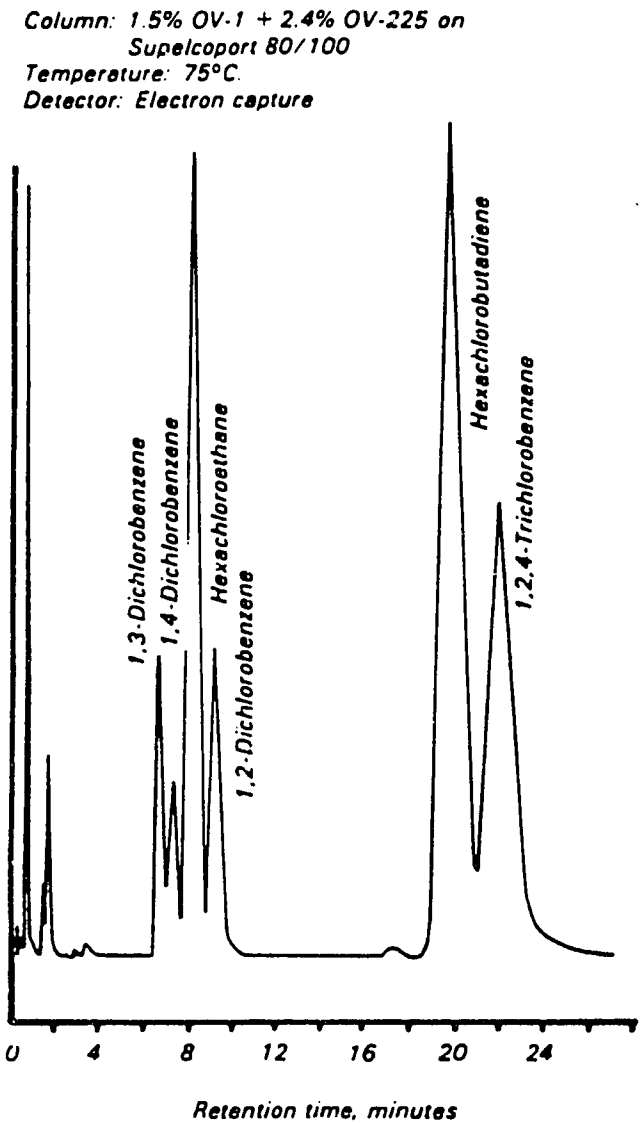


Figure 1. *Gas chromatogram of chlorinated hydrocarbons.*

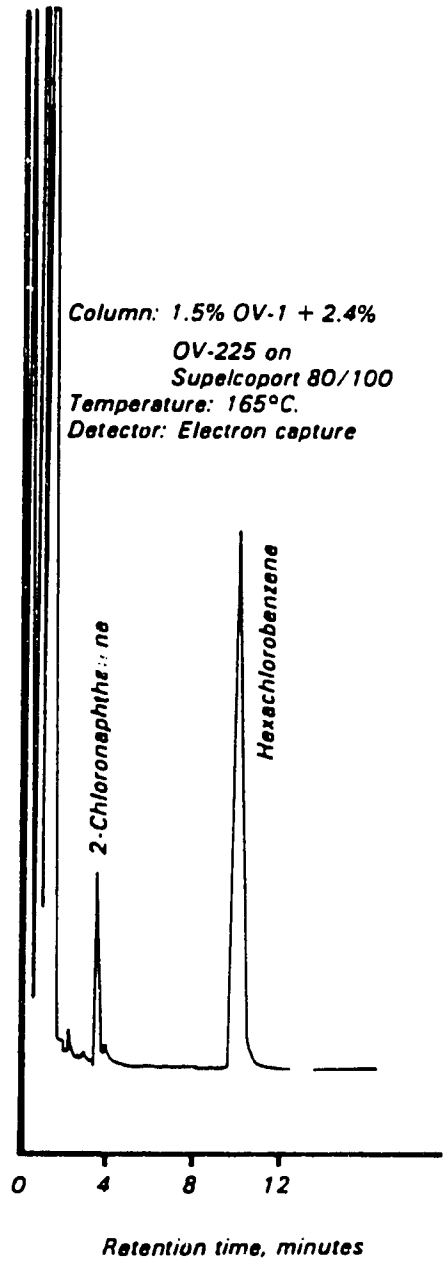


Figure 2. *Gas chromatogram of chlorinated hydrocarbons.*

349

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

2,3,7,8-Tetrachlorodibenzo-p-Dioxin — Method 613

1. Scope and Application

1.1 This method covers the determination of 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD). The following parameter may be determined by this method:

Parameter	STORET No.	CAS No.
2,3,7,8-TCDD	34675	1746-01-6

1.2 This is a gas chromatographic/mass spectrometer (GC/MS) method applicable to the determination of 2,3,7,8-TCDD in municipal and industrial discharges as provided under 40 CFR 136.1. Method 625 may be used to screen samples for 2,3,7,8-TCDD. When the screening test is positive, the final qualitative confirmation and quantification must be made using method 613.

1.3 The method detection limit (MDL, defined in Section 14.1)[1] for 2,3,7,8-TCDD is listed in Table 1. The MDL for a specific wastewater may be different depending upon the nature of interferences in the sample matrix.

1.4 Because of the extreme toxicity of this compound, the analyst must prevent exposure to himself, or to others, by materials known or believed to contain 2,3,7,8-TCDD. Section 4 of this method contains guidelines and protocols that serve as minimum safe-handling standards in a limited access laboratory.

1.5 Any modification of this method, beyond those expressly permitted, shall be considered as major modifications subject to application and approval of alternate test procedures under 40 CFR 136.4 and 136.5.

1.6 This method is restricted to use only by or under the supervision of analysts experienced in the use of gas chromatograph/mass spectrometers and skilled in the interpretation of mass spectra. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 A one-liter sample of wastewater is spiked with an internal standard of labeled 2,3,7,8-TCDD. The spiked sample is then extracted with methylene chloride using separatory funnel techniques. The extract is concentrated and exchanged to hexane while being concentrated to a volume of 1.0 mL or less. Capillary column GC/MS conditions are described which allow for the separation and measurement of 2,3,7,8-TCDD in the extract[2,3].

2.2 The method provides selected column chromatographic cleanup procedures to aid in the elimination of interferences that may be encountered.

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample

350

processing hardware that lead to discrete artifacts and/or elevated backgrounds at the ions monitored. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned.[4] Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and distilled water. Glassware should then be drained dry, and heated in a muffle furnace at 400 °C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store it inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are co-extracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. 2,3,7,8-TCDD is often associated with other interfering chlorinated compounds which are at concentrations several magnitudes higher than that of 2,3,7,8-TCDD. The cleanup procedures in Section 11 can be used to overcome many of these interferences, but unique samples may require additional cleanup approaches[1,5-7] to eliminate false positives and achieve the method detection limit listed in Table 1.

3.3 The primary column, SILAR-10C, resolves 2,3,7,8-TCDD from the other 21 isomers[3]. Positive results obtained using any other gas chromatographic column must be confirmed using this column.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are identified[8-10]. Benzene and 2,3,7,8-TCDD have been identified as suspected human or mammalian carcinogens.

4.2 Each laboratory must develop a strict safety program for handling of 2,3,7,8-TCDD. The following laboratory practices are recommended:

4.2.1 Contamination of the laboratory will be minimized by conducting all manipulations in a hood.

4.2.2 The effluents of sample splitters for the gas chromatograph and roughing pumps on the GC/MS should pass through either a column of activated charcoal or be bubbled through a trap containing oil or high-boiling alcohols.

4.2.3 Liquid waste should be dissolved in methanol or ethanol and irradiated with ultraviolet light with wavelength greater than 290 nm for several days. (Use F 40 BL lamps or equivalent.) Analyze liquid wastes and dispose of the solutions when 2,3,7,8-TCDD can no longer be detected.

4.3 Dow Chemical U.S.A. has issued the following precautions (revised 11/78) for safe handling of 2,3,7,8-TCDD in the laboratory:

4.3.1 The following statements on safe handling are as complete as possible on the basis of available toxicological information. The precautions for safe handling and use are necessarily general in nature since detailed, specific recommendations can be made only for the particular exposure and circumstances of each individual use. Inquiries about specific operations or uses may be addressed to the Dow Chemical Company. Assistance in evaluating the health hazards of particular plant conditions may be obtained from certain consulting laboratories and from State Departments of Health or of Labor, many of which have an industrial health service. 2,3,7,8-TCDD is extremely toxic to laboratory animals. However, it has been handled for years without injury in analytical and biological laboratories. Techniques used in handling radioactive and infectious materials are applicable to 2,3,7,8-TCDD.

4.3.1.1 Protective Equipment: Throw-away plastic gloves, apron or lab coat, safety glasses and lab hood adequate for radioactive work.

4.3.1.2 Training: Workers must be trained in the proper method of removing of contaminated gloves and clothing without contacting the exterior surfaces.

4.3.1.3 Personal Hygiene: Thorough washing of hands and forearms after each manipulation and before breaks (coffee, lunch, and shift).

4.3.1.4 Confinement: Isolated work area, posted with signs, segregated glassware and tools, plastic-backed absorbent paper on benchtops.

4.3.1.5 Waste: Good technique includes minimizing contaminated waste. Plastic bag liners should be used in waste cans. Janitors must be trained in safe handling of waste.

4.3.1.6 Disposal of Wastes: 2,3,7,8-TCDD decomposes above 800 °C. Low-level waste such as the absorbent paper, tissues, animal remains and plastic glvoes may be burned in a good incinerator. Gross quantities (milligrams) should be packaged securely and disposed through commercial or governmental channels which are capable of handling high-level radioactive wastes or extremely toxic wastes. Liquids should be allowed to evaporate in a good hood and in a disposable container. Residues may then be handled as above.

4.3.1.7 Decontamination: Personal—any mild soap with plenty of scrubbing action: Glassware, Tools, and Surfaces—Chlorothene NU Solvent (Trademark of the Dow Chemical Company) is the least toxic solvent shown to be effective. Satisfactory cleaning may be accomplished by rinsing with Chlorothene, then washing with any detergent and water. Dish water may be disposed to the sewer. It is prudent to minimize solvent wastes because they may require special disposal through commercial sources which are expensive.

4.3.1.8 Laundry: Clothing known to be contaminated should be disposed with the precautions described under "Disposal of Wastes." Lab coats or other clothing worn in 2,3,7,8-TCDD

work area may be laundered. Clothing should be collected in plastic bags. Persons who convey the bags and launder the clothing should be advised of the hazard and trained in proper handling. The clothing may be put into a washer without contact if the launderer knows the problem. The washer should be run through a cycle before being used again for other clothing.

4.3.1.9 Wipe Tests: A useful method of determining cleanliness of work surfaces and tool is to wipe the surface with a piece of filter paper. Extraction and analysis by gas chromatography can achieve a limit of sensitivity of 0.1 µg per wipe. Less than 1 µg 2,3,7,8-TCDD per sample indicates acceptable cleanliness; anything higher warrants further cleaning. More than 10 µg on a wipe sample indicates an acute hazard and requires prompt cleaning before further use of the equipment or work space and indicates further that unacceptable work practices have been employed in the past.

4.3.1.10 Inhalation: Any procedure that may produce airborne contamination must be done with good ventilation. Gross losses to a ventilation system must not be allowed. Handling of the dilute solutions normally used in analytical and animal work presents no inhalation hazards except in case of an accident.

4.3.1.11 Accidents: Remove contaminated clothing immediately, taking precautions not to contaminate skin or other articles. Wash exposed skin vigorously and repeatedly until medical attention is obtained.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle—Amber glass, one-liter or one-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional)—Must incorporate glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4 °C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.1.3 Clearly label all samples as "POISON" and ship according to U.S.D.O.T. requirements.

5.2 Glassware (All specifications are suggested. Catalog numbers are included for illustration only).

5.2.1 Separatory funnel—2000-mL and 125-mL, with Teflon stopcock.

5.2.2 Concentrator tube, Kuderna-Danish—10-mL, graduated (Kontes K-570050-1025 or equivalent). Calibration must be checked at the volumes employed in the test. Ground glass stopper is used to prevent evaporation of extracts.

5.2.3 Evaporative flask, Kuderna-Danish—500-mL (Kontes K-570001-0500 or equivalent). Attach to concentrator tube with springs.

5.2.4 Snyder column, Kuderna-Danish—three-ball macro (Kontes K-503000-0121 or equivalent).

5.2.5 Snyder column, Kuderna-Danish—two-ball micro (Kontes K-569001-0219 or equivalent).

5.2.6 Vials—Amber glass, 10- to 15-mL capacity, with Teflon-lined screw cap.

5.2.7 Chromatography column—300 mm long × 10 mm ID with coarse fritted disc at bottom and Teflon stopcock.

5.2.8 Chromatography column—400 mm long × 11 mm ID with coarse fritted disc at bottom and Teflon stopcock.

5.3 Boiling chips—approximately 10/40 mesh. Heat to 400 °C for 30 minutes or Soxhlet extract with methylene chloride.

5.4 Water bath—Heated, with concentric ring cover, capable of temperature control (± 2 °C). The bath should be used in a hood.

5.5 GC/MS system.

5.5.1 Gas chromatograph—An analytical system complete with all required accessories including syringes, analytical columns, and gases. The injection port must be designed for capillary columns. Either split, splitless, or on-column injection techniques may be employed, as long as the requirements of Section 7.1.1 are achieved.

5.5.2 Primary column—50 m long × 0.25 mm ID glass, coated with SILAR-10C (or equivalent). An equivalent column must resolve 2,3,7,8-TCDD from the other 21 TCDD isomers. Guidelines for the use of alternate columns are provided in Section 12.1.

5.5.3 Mass Spectrometer—Either low resolution mass spectrometers (LRMS) or high resolution mass spectrometers (HRMS) may be used. The mass spectrometer must be equipped with a 70 volt (nominal) ion source and be capable of acquiring ion abundance data in real time Selected Ion Monitoring (SIM) for groups of four or more ions.

5.5.4 GC/MS interface—Any gas chromatograph to mass spectrometer interface can be used that achieves the requirements of Section 7.1.1 constructed of glass or glass-lined materials are recommended. Glass surfaces can be deactivated by silanizing with dichlorodimethylsilane. To achieve maximum sensitivity, the exit end of the capillary column should be placed in the ion source. A short piece of fused silica capillary can be used as the interface to overcome problems associated with straightening the exit end of glass capillary columns.

5.5.5 The SIM data acquired during the chromatographic program is defined as the Selected Ion Current Profile (SICP). The SICP can be acquired under computer control or as real time analog output. If computer control is used, there must be software available to plot the SICP and report peak height or area data for any ion in the SICP between specified time or scan number limits.

5.6 Balance—Analytical, capable of accurately weighing 0.0001g.

6. Reagents

6.1 Reagent water—Reagent water is defined as a water in which an interferent is not observed at the MDL of 2,3,7,8-TCDD.

6.2 Sodium hydroxide solution—(ACS) Dissolve 400 g NaOH in reagent water and dilute to one liter. Wash the solution with methylene chloride and with hexane before use.

6.3 Sodium thiosulfate—(ACS) Granular.

6.4 Sulfuric acid (Conc.)—(ACS) sp. gr. 1.84.

352

6.5 Methylene chloride, hexane, benzene, tetradecane – Pesticide quality or equivalent.

6.6 Sodium sulfate – (ACS) Granular, anhydrous (purified by heating at 400 °C for four hours in a shallow tray).

6.7 Alumina – neutral, 80/200 mesh (Fisher Scientific Co., No. A-540 or equivalent). Before use, activate for 24 hours at 130 °C in a foil covered glass container.

6.8 Silica gel – high purity grade, 100/120 mesh. (Fisher Scientific Co., No. S-679 or equivalent).

6.9 Stock standard solutions (1.00 µg/µL) – Stock standard solutions can be prepared from pure standard materials or purchased as certified solutions. Acetone should be used as the solvent for spiking solutions; ortho-xylene is recommended for calibration standards for split injectors; and tetradecane is recommended for splitless or on-column injectors. Analyze stock internal standards to verify the absence of native 2,3,7,8-TCDD.

6.9.1 Prepare stock standard solutions of 2,3,7,8-TCDD (mol wt 320) and either $^{37}Cl_4$ 2,3,7,8-TCDD (mol wt 328) or $^{13}C_{12}$ 2,3,7,8-TCDD (mol wt 332) in an isolated area by accurately weighing about 0.0100 g of pure material. Dissolve the material in pesticide quality solvent, dilute to volume in a 10-mL volumetric flask. If compound purity is certified at 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.

6.9.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store in an isolated refrigerator protected from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards or spiking solutions from them.

6.9.3 Stock standard solutions must be replaced after six months or sooner if comparison with check standards indicates a problem.

6.10 Internal standard spiking solutions (25 ng/mL) – Using stock standard solution. Prepare a spiking solution in acetone of either $^{13}C_{12}$ or $^{37}Cl_4$ 2,3,7,8-TCDD at a concentration of 25 ng/mL. (See Section 10.2.1.)

7. Calibration

7.1 Establish gas chromatographic conditions for the GC/MS system equivalent to those indicated in Table 1 and SIM conditions for the mass spectrometer as described in Section 12.2. The GC/MS system must be calibrated using the internal standard technique. By injecting calibration standards, establish ion response factors for 2,3,7,8-TCDD vs. an internal standard (either $^{13}C_{12}$ or $^{37}Cl_4$ 2,3,7,8-TCDD).

7.1.1 Using stock standards, prepare GC/MS calibration standard solutions that will allow measurement of relative response factors of at least three concentration ratios of 2,3,7,8-TCDD to internal standard. Each solution must be prepared to contain the internal standard at a concentration of 25 ng/mL. If any interferences are contributed by the internal standard at m/e 320 and 322, its concentration may be reduced in calibration standards and in the internal spiking solution (Section 6.10). One of the calibration standard solutions should be prepared to contain 2,3,7,8-TCDD representing a concentration near, but above, the MDL. The other 2,3,7,8-TCDD concentrations should correspond to the expected range of concentrations found in real samples.

7.1.2 Using injections of 2 to 5 µL, tabulate peak height or area response against the concentration of 2,3,7,8-TCDD and internal standard in each calibration standard, and calculate response factors (RF) for 2,3,7,8-TCDD using equation 1.

$$\text{Eq. 1} \quad RF = (A_s C_{is})/(A_{is} C_s)$$

where:

A_s = SIM response for 2,3,7,8-TCDD m/e 320.

A_{is} = SIM response for internal standard, m/e 332 for $^{13}C_{12}$ 2,3,7,8-TCDD, m/e 328 for $^{37}Cl_4$ 2,3,7,8-TCDD.

C_{is} = Concentration of the internal standard, (µg/L).

C_s = Concentration of 2,3,7,8-TCDD (µg/L).

If the RF value over the working range is a constant (<10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.1.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more 2,3,7,8-TCDD calibration standards. If the response for 2,3,7,8-TCDD varies from the predicted response by more than ± 10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared.

7.2 Before using any cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in both mass spectrometry and gas chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike and analyze a minimum of 10% of all samples with native 2,3,7,8-TCDD to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Using stock standard solution, prepare a quality control check sample concentrate containing 2,3,7,8-TCDD at a concentration of 15 ng/mL in acetone.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for the results. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 2, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > 2p$ or $|X - R| > 2p$, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the result of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria and define the performance of the laboratory for each spike concentration and parameter being measured.

8.3.1 Calculate upper and lower control limits for method performance:

Upper Control Limit (UCL) = R + 3s
Lower Control Limit (LCL) = R − 3s

where R and s are calculated as in Section 8.2.3. The UCL and LCL can be used to construct control charts[11] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 The laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[10].

8.4 The laboratory is required to collect a portion of their samples in duplicate to monitor spike recoveries. The frequency of spiked sample analysis must be at least 10% of all samples or one sample per month, whichever is greater. One aliquot of the sample must be spiked and analyzed as described in Section 8.2. If the recovery for 2,3,7,8-TCDD does not fall within the control limits for method performance, the results reported for 2,3,7,8-TCDD in all samples processed as part of the same set must be qualified as described in Section 13.4. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate through the analysis of a one-liter aliquot of reagent water, that all glassware and reagent interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices[12] should be followed, except that the bottle must not be prewashed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4 °C and protected from light from the time of collection until extraction. If the sample contains residual chlorine, add 80 mg of sodium thiosulfate per each liter of sample. U.S. Environmental Protection Agency methods 330.4 and 330.5 may be used for measurement of residual chlorine[13]. Field test kits are available for this purpose.

9.3 Label all samples and containers POISON and ship according to applicable U.S. Department of Transportation regulations.

9.4 All samples must be extracted within 7 days and completely analyzed within 40 days of extraction[2].

10. Sample Extraction

CAUTION: When using this method to analyze for 2,3,7,8-TCDD, all of the following operations must be performed in a limited access laboratory with the analyst wearing full protective covering for all exposed skin surfaces. See Section 4.2.

10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a two-liter separatory funnel.

10.2 Add 1.00 mL of internal standard spiking solution to the sample in the separatory funnel. If the final extract will be concentrated to a fixed volume below 1.00 mL (Section 12.3), only that volume of spiking solution should be added to the sample so that the final extract will represent 25 ng/mL at the time of analysis.

10.3 Add 60 mL methylene chloride to the sample bottle, seal, and shake 30 seconds to rinse the inner surface. Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods. Collect the methylene chloride extract in a 250-mL Erlenmeyer flask.

10.4 Add a second 60-mL volume of methylene chloride to the sample bottle and repeat the extraction procedure a second time, combining the extracts in the Erlenmeyer flask. Perform a third extraction in the same manner.

10.5 Assemble a Kuderna-Danish (K-D) concentrator by attaching a

354

10-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used in place of the K-D if the requirements of Section 8.2 are met.

10.6 Pour the combined extract into the K-D concentrator. Rinse the Erlenmeyer flask and column with 20 to 30 mL of methylene chloride to complete the quantitative transfer.

10.7 Add one or two clean boiling chips to the evaporative flask and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL methylene chloride to the top. Place the K-D apparatus on a hot water bath (60 to 65 °C) so that the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood with condensed solvent. When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus from the water bath and allow it to drain and cool for at least 10 minutes.

10.8 Momentarily remove the Snyder column, add 50 mL hexane and a new boiling chip and replace the column. Raise the temperature of the water bath to 85 to 90 °C. Concentrate the extract as in Section 10.7, except using hexane to prewet the column. Remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of hexane. A 5-mL syringe is recommended for this operation. Set aside a K-D glassware for reuse in Section 10.14.

10.9 Pour the hexane from the concentrator tube into a 125-mL separatory funnel. Rinse the concentrator tube four times with 10-mL aliquots of hexane. Combine all rinses in the 125-mL separatory funnel.

10.10 Add 50 mL of 10N sodium hydroxide solution to the funnel and shake for 30 to 60 seconds. Discard the aqueous phase.

10.11 Perform a second wash of the organic layer with 50 mL of reagent water. Discard the aqueous phase.

10.12 Wash the hexane layer with at least two 50-mL aliquots of concentrated sulfuric acid. Continue washing the hexane layer with 50-mL aliquots of concentrated sulfuric acid until the acid layer remains colorless. Discard all acid fractions.

10.13 Wash the hexane layer with two 50-mL aliquots of reagent water. Discard the aqueous phases.

10.14 Transfer the hexane layer into a 125-mL Erlenmeyer flask containing 1 to 2 g anhydrous sodium sulfate. Swirl the flask for 30 seconds and decant the hexane into the reassembled K-D apparatus. Complete the quantitative transfer with two 10-mL hexane rinses of the Erlenmeyer flask.

10.15 Replace the one or two clean boiling chips and concentrate the extract to 6 to 10 mL as described in Section 10.8.

10.16 Add a clean boiling chip and attach a micro-Snyder column. Prewet the column by adding about 1 mL hexane to the top. Place the K-D apparatus on the 80 °C water bath so that the concentrator tube is partially immersed in the hot water. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 5 to 10 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood. When the apparent volume of liquid reaches about 0.5 mL, remove the K-D apparatus and allow it to drain for at least 10 minutes while cooling. Remove the micro-Snyder column and rinse its lower joint into the concentrator tube with 0.2 mL hexane. Adjust the extract volume to 1.0 mL with hexane. Stopper the concentrator tube and store refrigerated and protected from light if GC/MS analysis or cleanup will not be performed immediately.

10.17 Determine the original sample volume by refilling the sample bottle to the mark with water and measuring the volume in a 1000-mL graduated cylinder. Record the sample volume to the nearest 5 mL.

11. Cleanup and Separation

11.1 Cleanup procedures may not be necessary for a relatively clean sample matrix. The cleanup procedures recommended in this method have been used for the analysis of various clean waters and industrial affluents. The single operator precision and accuracy data in Table 2 was gathered using the recommended cleanup procedures. If particular circumstances demand the use of an alternative cleanup procedure, the analyst must determine the elution profile and demonstrate that the recovery of 2,3,7,8-TCDD is reproducible and equivalent to the recovery of the internal standard. Two cleanup column options are offered to the analyst in this section. The alumina column should be used first to overcome interferences. If background problems are still encountered, the silica gel column may be helpful. Other cleanup procedures have been described to overcome special interference problems[1,5-7].

11.2 Alumina column cleanup for 2,3,7,8-TCDD.

11.2.1 Fill a 300 mm long × 10 mm ID chromatography column with activated alumina to the 150 mm level, tapping the column gently to settle the alumina. Add 10 mm anhydrous sodium sulfate to the top of the alumina.

11.2.2 Preelute the column with 50 mL hexane. Adjust the elution rate to 1 mL/min. Discard the eluate and just prior to exposure of the sodium sulfate layer to the air, transfer the entire 1.0 mL sample extract onto the column, using two additional 2-mL portions of hexane to complete the transfer.

11.2.3 Just prior to exposure of the sodium sulfate layer to the air, add 50 mL 3% methylene chloride/97% hexane (V/V) and continue the elution of the column. Discard the eluate.

11.2.4 Next elute the column with 50 mL 20% methylene chloride/80% hexane (V/V) into a 500 mL K-D flask equipped with a 10-mL concentrator tube. Concentrate the collected fraction to 1.0 mL by standard K-D techniques and analyze by GC/MS.

11.3 Silica gel column cleanup for 2,3,7,8-TCDD.

11.3.1 Fill a 400 mm long × 11 mm ID chromatography column with silica gel to the 300 mm level, tapping the column gently to settle the silica gel. Add 10 mm anhydrous sodium sulfate to the top of the silica gel.

11.3.2 Preelute the column with 50 mL 20% benzene/80% hexane (V/V). Adjust the elution rate to 1 mL/min. Discard the eluate and just prior to exposure of the sodium sulfate layer to the air, transfer the entire 1.0 mL sample extract onto the column, using two 2 mL portions of 20% benzene/80% hexane to complete the transfer.

11.3.3 Just prior to exposure of the sodium sulfate layer to the air, add 40 mL 20% benzene/80% hexane to the column. Collect the eluate in a clean

500-mL K-D flask equipped with a 10-mL concentrator tube.

11.3.4 Evaporate the fraction to 1.0 mL by standard K-D techniques and analyze by GC/MS.

12. GC/MS Analysis

12.1 Table 1 summarizes the recommended gas chromatographic capillary column and operating conditions for the instrument. Included in this table are the estimated retention time and MDL that can be achieved by this method. Other capillary columns or chromatographic conditions may be used to screen samples if the requirements of Section 8.2 are met. Confirmation of 2,3,7,8-TCDD must be accomplished using a GC column that separates 2,3,7,8-TCDD from all other TCDD isomers.

12.2 Analyze standards and samples with the mass spectrometer operating in the SIM mode using a dwell time to give at least seven points per peak. For LRMS, use ions at m/e 320, 322, and 257 for 2,3,7,8-TCDD and either the ion at m/e 328 for ^{37}Cl 2,3,7,8-TCDD or m/e 332 for ^{13}C 2,3,7,8-TCDD. For HRMS, use ions at m/e 319.8965 and 321.8936 for 2,3,7,8-TCDD and either the ion at m/e 327.8847 for ^{37}Cl 2,3,7,8-TCDD or m/e 331.9367 for ^{13}C 2,3,7,8-TCDD.

12.3 If lower detection limits are required, the extract may be carefully evaporated to dryness under a gentle stream of nitrogen with the concentrator tube in a water bath at about 40 °C. Do this immediately before GC/MS analysis. Redissolve the extract in the desired final volume of orthoxylene or tetradecane. The method performance data reported in Section 14 was gathered using a final extract volume of 0.2 mL.

12.4 Calibrate the system daily as described in Section 7.1.3. The volume of calibration standard injected must be measured, or be the same as all sample injection volumes.

12.5 Inject a 2 to 5 µL aliquot of the sample extract.

12.6 The presence of 2,3,7,8-TCDD is qualitatively confirmed if all of the following criteria are achieved.

12.6.1 The gas chromatographic column must resolve 2,3,7,8-TCDD from the other 21 TCDD isomers.

12.6.2 The ions for native 2,3,7,8-TCDD (LRMS-m/e 320, 322, and 257 and HRMS-m/e 320 and 322) and labeled 2,3,7,8-TCDD (m/e 328 or 332) must exhibit a simultaneous maximum at a retention time that matches that of native 2,3,7,8-TCDD in the calibration standard, within the performance specifications of the analytical system.

12.6.3 The chlorine isotope ratio at m/e 320 and m/e 322 must agree to within ± 10% of that in the calibration standard.

12.6.4 The signal of all peaks must be greater than 2.5 times the noise level.

12.7 For quantitation, measure the response of the m/e 320 peak for 2,3,7,8-TCDD and the m/e 332 peak for $^{13}C_{12}$ 2,3,7,8-TCDD or the m/e 328 peak for $^{37}Cl_4$ 2,3,7,8-TCDD.

12.8 Co-eluting impurities are suspected if all criteria are achieved except those in Section 12.6.3. In this case, another SIM analysis using ions at m/e 257, 259, 320 and either m/e 328 or m/e 322 can be performed. The ions at m/e 257 and m/e 259 are indicative of the loss of one chlorine and one carbonyl group from 2,3,7,8-TCDD. If the ions m/e 257 and m/e 259 give a chlorine isotope ratio that agrees to within ± 10% of the same cluster in the calibration standards, than the presence of TCDD can be confirmed. Co-eluting DDD, DDE, and PCB residues can be confirmed, but will require another injection using the appropriate SIM ions or full repetitive mass scans. If the response for ^{37}Cl 2,3,7,8-TCDD at m/e 328 is too large, PCB contamination is suspected and can be confirmed by examining the response at both m/e 326 and m/e 328. The ^{37}Cl 2,3,7,8-TCDD internal standard gives negligible response at m/e 326. These pesticide residues can be removed using the alumina column cleanup.

12.9 If broad background interference restricts the sensitivity of the GC/MS analysis, the analyst should employ additional cleanup procedures and reanalyze by GC/MS.

12.10 In those circumstances where these procedures do not yield a definitive conclusion, then the use of high resolution mass spectrometry is suggested.[5]

13. Calculations

13.1 Calculate the concentration of 2,3,7,8-TCDD in the sample using the response factor (RF) determined in 7.1.2 and equation 2.

Eq. 2

$$\text{Concentration, } \mu g/l = \frac{(A_{is})(I_s)}{(A_{is})(RF)(V_o)}$$

where:

A_s = SIM response for 2,3,7,8-TCDD ion at m/e 320.

A_{is} = SIM response for the internal standard ion at m/e 328 or 332.

I_s = Amount of internal standard added to each extract (µg).

V_o = Volume of water extracted, in liters.

13.2 For each sample, calculate the percent recovery of the internal standard by comparing the area of the ion peak measured in the sample to the area of the same peak in the calibration standard. If the recovery is below 50%, the results of the sample analysis must be qualified as described in Section 13.4.

13.3 Report results in micrograms per liter. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.4 For samples processed as part of a set where the laboratory spiked sample recovery falls outside of the control limits in Section 8.3 or the internal standard recovery is below 50%, the data for 2,3,7,8-TCDD must be labeled as suspect.

14. Method Performance

14.1 Method detection limit—The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[1]. The MDL concentration listed in Table 1 was obtained using reagent water[14].

14.2 In a single laboratory (Monsanto Research Corporation), using spiked samples, the average recovery presented in Table 2 was obtained.[14] The average standard deviation of the percent recovery is also included in Table 2. The results for quality control checks for this study are presented in Table 3.

14.3 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1 See Appendix A.

356

2. "Determination of 2,3,7,8-TCDD in Industrial and Municipal Wastewaters." Report for EPA Control 68-03-2635 (In preparation).
3. Buser, H.R., and Rappe, C. "High Resolution Gas Chromatography of the 22 Tetrachlorodibenzo-p-dioxin Isomers," *Analytical Chemistry, 52,* 2257, (1980).
4. ASTM Annual Book of Standards, Part 31, D 3694. "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Test and Materials, Philadelphia, PA, p. 679, (1980).
5. Harless, R.L., E.O. Oswald, and M.K. Wilkinson, "Sample Preparation and Gas Chromatography/Mass Spectrometry Determination of 2,3,7,8-Tetrachlorodibenzo-p-dioxin," *Analytical Chemistry, 52,* 1239 (1980).
6. Lamparski, L.L. and T.J. Nestrick. "Determination of Tetra-, Hepta-, and Octachlorodibenzo-p-dioxin Isomers in Particulate Samples at Parts per Trillion Levels," *Analytical Chemistry, 52,* 2045 (1980).
7. Longhorst, M.L. and L.A. Shadoff, "Determination of Parts-per-Trillion Concentrations of Tetra-, Hexa-, and Octachlorodibenzo-p-dioxins in Human Milk," *Analytical Chemistry, 52,* 2037, (1980).
8. "Carcinogens—Working with Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.
9. "OSHA Safety and Health Standards, General Industry," (29CFR1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
10. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.
11. "Handbook of Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.
12. ASTM Annual Book of Standards, Part 31, D 3370, "Standard Practice for Sampling Water," American Society for Testing and Materials, Philadelphia, PA 1980.
13. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600/4-79-020, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.
14. "Determination of Method Detection Limits for EPA Method 613," Special letter report for EPA Contract 68-03-2863, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

Table 1. *Chromatographic Conditions and Method Detection Limit*

Parameter	*Retention Time (min.)*	*Detection Limit (g/L)*
2,3,7,8-TCDD	*34.5*	*0.002*

Column conditions: SILAR-10C coated on a 50 m long 0.25 mm ID glass column with helium carrier gas at 30 cm/sec linear velocity, splitless injector. Column temperature programmed: isothermal 100°C for 3 minutes, then programmed at 20°C/min to 180°C, and 2°C/min to 250°C.

Table 2. *Single Operator Accuracy and Precision*

Parameter	*Matrix*	*Spike µg/L*	*Average Percent Recovery*	*Standard Deviation %*
2,3,7,8-TCDD	*Reagent water*	*0.005*	*95.4*	*10.2*
2,3,7,8-TCDD	*Industrial waste*	*0.005*	*85.8*	*6.6*
2,3,7,8-TCDD	*Municipal waste*	*0.025*	*92.4*	*18.7*

Table 3. *Quality Control Results (14)*

Matrix	*320/322 Isotope Ratios*	*Internal Standard Recovery*
Reagent water	*0.79 ± 0.04*	*87%*
Industrial waste	*0.81 ± 0.01*	*104%*
Municipal waste	*0.83 ± 0.02*	*14%*

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Purgeables — Method 624

1. Scope and Application

1.1 This method covers the determination of a number of purgeable organics. The following parameters may be determined by this method:

Parameter	STORET No.	CAS No.
Benzene	34030	71-43-2
Bromodichloromethane	32101	75-27-4
Bromoform	32104	75-25-2
Bromomethane	34413	74-83-9
Carbon tetrachloride	32102	56-23-5
Chlorobenzene	34301	108-90-7
Chloroethane	34311	75-00-3
2-Chloroethylvinyl ether	34576	110-75-8
Chloroform	32106	67-66-3
Chloromethane	34418	74-87-3
Dibromochloromethane	32105	124-48-1
1,2-Dichlorobenzene	34536	95-50-1
1,3-Dichlorobenzene	34566	541-73-1
1,4-Dichlorobenzene	34571	106-46-7
1,1-Dichloroethane	34496	75-34-3
1,2-Dichloroethane	34531	107-06-2
1,1-Dichloroethene	34501	75-35-4
trans-1,2-Dichloroethene	34546	156-60-5
1,2-Dichloropropane	34541	78-87-5
cis-1,3-Dichloropropene	34704	10061-01-5
trans-1,3-Dichloropropene	34699	10061-02-6
Ethyl benzene	34371	100-41-4
Methylene chloride	34423	75-09-2
1,1,2,2-Tetrachloroethane	34516	79-34-5
Tetrachloroethene	34475	127-18-4
Toluene	34010	108-88-3
1,1,1-Trichloroethane	34506	71-55-6
1,1,2-Trichloroethane	34511	79-00-5
Trichloroethene	39180	79-01-6
Trichlorofluoromethane	34488	75-69-4
Vinyl chloride	39175	75-01-4

1.2 The method may be extended to screen samples for acrolein (STORET No. 34210, CAS No. 107-02-8) and acrylonitrile (STORET 34215, CAS No. 107-13-1), however, the preferred method for these two compounds is method 603.

1.3 This is a purge and trap gas chromatographic/mass spectrometer

358

(GC/MS) method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR 136.1.

1.4 The method detection limit (MDL, defined in Section 14.1)[1] for each parameter is listed in Table 1. The MDL for a specific wastewater differ from those listed, depending upon the nature of interferences in the sample matrix.

1.5 Until the U.S. Environmental Protection Agency establishes performance criteria based upon the results of interlaboratory testing, any alternative GC/MS method which meets the performance criteria described in Section 8.2 will be permitted. Performance must be verified for such modification by analyzing wastewater as described in Section 8.2.2. In addition, the laboratory must successfully participate in the applicable performance evaluation studies.

1.6 This method is restricted to use by or under the supervision of analysts experienced in the use of purge and trap systems and gas chromatograph/mass spectrometers and skilled in the interpretation of mass spectra. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 An inert gas is bubbled through a 5-mL sample contained in a specially-designed purging chamber at ambient temperature. The purgeables are efficiently transferred from the aqueous phase to the vapor phase. The vapor is swept through a sorbent column where the purgeables are trapped. After purging is completed, the sorbent column is heated and backflushed with the inert gas to desorb the purgeables onto a gas chromatographic column. The gas chromatograph is temperature programmed to separate the purgeables which are then detected with a mass spectrometer[2,3].

3. Interferences

3.1 Impurities in the purge gas, organic compounds out-gassing from the plumbing ahead of the trap and solvent vapors in the laboratory account for the majority of contamination problems. The analytical system must be demonstrated to be free from contamination under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5. The use of non-TFE plastic tubing, non-TFE thread sealants, or flow controllers with rubber components in the purging device should be avoided.

3.2 Samples can be contaminated by diffusion of volatile organics (particularly fluorocarbons and methylene chloride) through the septum seal into the sample during shipment and storage. A field reagent blank prepared from reagent water and carried through the sampling and handling protocol can serve as a check on such contamination.

3.3 Contamination by carry over can occur whenever high level and low level samples are sequentially analyzed. To reduce carry over, the purging device and sample syringe must be rinsed with reagent water between sample analyses. Whenever an unusually concentrated sample is encountered, it should be followed by an analysis of reagent water to check for cross contamination. For samples containing large amounts of water-soluble materials, suspended solids, high boiling compounds or high purgeable levels, it may be necessary to wash out the purging device with a detergent solution, rinse it with distilled water, and then dry it in a 105 °C oven between analyses. The trap and other parts of the system are also subject to contamination; therefore, frequent bakeout and purging of the entire system may be required.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified[5-7] for the information of the analyst.

4.2 The following parameters covered by this method have been tentatively classified as known or suspected, human or mammalian carcinogens: benzene, carbon tetrachloride, chloroform, 1,4-dichlorobenzene, and vinyl chloride. Primary standards of these toxic compounds should be prepared in a hood. A NIOSH/MESA approved toxic gas respirator should be worn when the analyst handles high concentrations of these toxic compounds.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete sampling.

5.1.1 Vial—25-mL capacity or larger, equipped with a screw cap with hole in center (Pierce #13075 or equivalent). Detergent wash, rinse with tap and distilled water, and dry at 105 °C before use.

5.1.2 Septum—Teflon-faced silicone (Pierce #12722 or equivalent). Detergent wash, rinse with tap and distilled water, and dry at 105 °C for one hour before use.

5.2 Purge and trap device—The purge and trap device consists of three separate pieces of equipment: the sample purger, trap, and the desorber. Several complete devices are now commercially available.

5.2.1 The sample purger must be designed to accept 5-mL samples with a water column at least 3 cm deep. The gaseous head space between the water column and the trap must have a total volume of less than 15-mL. The purge gas must pass through the water column as finely divided bubbles with a diameter of less than 3 mm at the origin. The purge gas must be introduced no more than 5 mm from the base of the water column. The sample purger, illustrated in Figure 1, meets these design criteria.

5.2.2 The trap must be at least 25 cm long and have an inside diameter of at least 0.105 inch. The trap must be packed to contain the following minimum lengths of adsorbents: 1.0 cm of methyl silicone coated packing (Section 6.3.2), 15 cm of 2,6-diphenylene oxide polymer (Section 6.3.1), and 8 cm of silica gel, (Section 6.3.3). The minimum specifications for the trap are illustrated in Figure 2.

5.2.3 The desorber should be capable of rapidly heating the trap to 180 °C. The polymer section of the trap should not be heated higher than 180 °C and the remaining sections should not exceed 220 °C. The desorber design, illustrated in Figure 2, meets these criteria.

5.2.4 The purge and trap device may be assembled as a separate unit or be coupled to a gas chromatograph as illustrated in Figures 3 and 4.

5.3 GC/MS system.

5.3.1 Gas chromatograph—An analytical system complete with a temperature programmable gas chromatograph suitable for on-column injection and all required accessories including syringes, analytical columns, and gases.

5.3.2 Column—6 ft long × 0.1 in ID stainless steel or glass, packed with 1% SP-1000 on Carbopack B (60/80 mesh) or equivalent. This column was used to develop the method performance statements in Section 14. Guidelines for the use of alternate column packings are provided in Section 11.1.

5.3.3 Mass spectrometer—Capable of scanning from 20 to 260 amu every seven seconds or less, utilizing 70 volts (nominal) electron energy in the electron impact ionization mode and producing a mass spectrum which meets all the criteria in Table 2 when 50 ng of 4-bromofluorobenzene (BFB) is injected through the gas chromatograph inlet.

5.3.4 GC/MS interface—Any gas chromatograph to mass spectrometer interface that gives acceptable calibration points at 50 ng or less per injection for each of the parameters of interest and achieves all acceptable performance criteria (see Section 10) may be used. Gas chromatograph to mass spectrometer interfaces constructed of all-glass or glass-lined materials are recommended. Glass can be deactivated by silanizing with dichloro-dimethylsilane.

5.3.5 Data system—A computer system must be interfaced to the mass spectrometer that allows the continuous acquisition and storage on machine readable media of all mass spectra obtained throughout the duration of the chromatographic program. The computer must have software that allows searching any GC/MS data file for ions of a specified mass and plotting such ion abundances versus time or scan number. This type of plot is defined as an Extracted Ion Current Profile (EICP). Software must also be available that allows integrating the abundance in any EICP between specified time or scan number limits.

5.4 Syringes—5-mL glass hypodermic with Luerlok tip (two each), if applicable to the purging device.

5.5 Micro syringes—25-mL, 0.006 inch ID needle.

5.6 Syringe valve—two-way, with Luer ends (three each), if applicable to the purging device.

5.7 Syringe—5-mL, gas-tight with shut-off valve.

5.8 Bottle—15-mL, screw-cap, with Teflon cap liner.

5.9 Balance—Analytical, capable of accurately weighing 0.0001 g.

6. Reagents

6.1 Reagent water—Reagent water is defined as a water in which an interferent is not observed at the MDL of the parameters of interest.

6.1.1 Reagent water may be generated by passing tap water through a carbon filter bed containing about 453 g of activated carbon (Calgon Corp., Filtrasorb-300 or equivalent).

6.1.2 A water purification system (Millipore Super-Q or equivalent) may be used to generate reagent water.

6.1.3 Reagent water may also be prepared by boiling water for 15 minutes. Subsequently, while maintaining the temperature at 90 °C, bubble a contaminant-free inert gas through the water for one hour. While still hot, transfer the water to a narrow-mouth screw-cap bottle and seal with a Teflon-lined septum and cap.

6.2 Sodium thiosulfate—(ACS) Granular.

6.3 Trap materials

6.3.1 2,6-Diphenylene oxide polymer—Tenax (60/80 mesh), chromatographic grade or equivalent.

6.3.2 Methyl silicone packing—3% OV-1 on Chromosorb-W (60/80 mesh) or equivalent.

6.3.3 Silica gel, Davison Chemical, (35/60 mesh), grade-15 or equivalent.

6.4 Methanol—Pesticide quality or equivalent.

6.5 Stock standard solutions—Stock standard solutions may be prepared from pure standard materials or purchased as certified solutions. Prepare stock standard solutions in methanol using assayed liquids or gases as appropriate. Because of the toxicity of some of the organohalides, primary dilutions of these materials should be prepared in a hood. A NIOSH/MESA approved toxic gas respirator should be used when the analyst handles high concentrations of such materials.

6.5.1 Place about 9.8 mL of methanol into a 10-mL ground glass stoppered volumetric flask. Allow the flask to stand, unstoppered, for about 10 minutes or until all alcohol wetted surfaces have dried. Weigh the flask to the nearest 0.1 mg.

6.5.2 Add the assayed reference material as described below:

6.5.2.1 Liquids—Using a 100-µL syringe, immediately add two or more drops of assayed reference material to the flask, then reweigh. The liquid must fall directly into the alcohol without contacting the neck of the flask.

6.5.2.2 Gases—To prepare standards for any of the four halocarbons that boil below 30 °C (bromomethane, chloroethane, chloromethane, and vinyl chloride), fill a 5-mL valved gas-tight syringe with the reference standard to the 5.0-mL mark. Lower the needle to 5 mm above the methanol meniscus. Slowly introduce the reference standard above the surface of the liquid. The heavy gas rapidly dissolves in the methanol.

6.5.3 Reweigh, dilute to volume, stopper, then mix by inverting the flask several times. Calculate the concentration in micrograms per microliter from the net gain in weight. When compound purity is assayed to be 96% or greater, the weight may be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards may be used at any concentration if they are certified by the manufacturer or by an independent source.

6.5.4 Transfer the stock standard solution into a Teflon-sealed screw-cap bottle. Store, with minimal headspace, at −10 ° to −20 °C and protect from light.

6.5.5 Prepare fresh standards weekly for the four gases and 2-chloroethyl-vinyl ether. All other standards must be replaced after one month, or sooner if comparison with check standards indicate a problem.

6.6 Secondary dilution standards—Using stock standard solutions, prepare secondary dilution standards in methanol that contain the compounds of interest, either singly or mixed together. The secondary dilution standards should be prepared at concentrations such that the aqueous calibration standards prepared in Section 7.3.1 or 7.4.1 will bracket the

36D

working range of the analytical system. Secondary dilution standards should be stored with minimal headspace and should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check standards that can used to determine the accuracy of calibration standards, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

6.7 Surrogate standard spiking solution—Select a minimum of three surrogate compounds from Table 3. Prepare stock standard solutions for each surrogate standard in methanol as described in Section 6.5. Prepare a surrogate standard spiking solution from these stock standards at a concentration of 150 µg/10 mL in water. Store the spiking solution at 4 °C in Teflon sealed glass containers with a minimum of headspace. The solutions should checked frequently for stability. They should be replaced after six months. The addition of 10 µL of this solution to 5 mL of sample or standard is equivalent to a concentration of 30 µg/L of each surrogate standard. Surrogate standard spiking solutions, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

6.8 BFB Standard—Prepare a 25 µg/µL solution of BFB in methanol.

7. Calibration

7.1 Assemble a purge and trap device that meets the specifications in Section 5.2. Condition the trap overnight at 180 °C by back flushing with an inert gas flow of at least 20 mL/min. Prior to use, daily condition traps 10 minutes while backflushing at 180 °C.

7.2 Connect the purge and trap device to a gas chromatograph. The gas chromatograph must be operated using temperature and flow rate parameters equivalent to those in Table 1. Calibrate the purge and trap-GC/MS system using either the external standard technique (Section 7.3) or the internal standard technique (Section 7.4).

7.3 External standard calibration procedure:

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter by carefully adding 20.0 µL of one or more secondary dilution standards to 50, 250, or 500 mL of reagent water. A 25-µL syringe with a 0.006 inch ID needle should be used for this operation. One of the external standards should be at a concentration near, but above, the MDL (See Table 1) and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the GC/MS system. Aqueous standards may be stored up to 24 hours, if held in sealed vials with zero headspace as described in Section 9.2. If not so stored, they must be discarded after one hour.

7.3.2 Analyze each calibration standard according to Section 11, and tabulate the area response of the primary characteristic ion (See Table 4) against the concentration in the standard. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to concentration (calibration factor) is a constant over the working range (<10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.

7.3.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ± 10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that parameter.

7.4 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples. Due to their generally unique retention times, bromochloromethane, 2-bromo-1-chloropropane, and 1,4-dichlorobutane have been used successfully as internal standards.

7.4.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest as described in Section 7.3.1.

7.4.2 Prepare a spiking solution containing each of the internal standards using the procedures described in Sections 6.5 and 6.6. It is recommended that the secondary dilution standard be prepared at a concentration of 15 µg/mL of each internal standard compound. The addition of 10 µL of this standard to 5.0 mL of sample or calibration standard would be equivalent to 30 µg/L.

7.4.3 Analyze each calibration standard, according to Section 11, adding 10 µL of internal standard spiking solution directly to the syringe (Section 11.4). Tabulate the area response of the characteristic ions against concentration for each compound and internal standard and calculate response factors (RF) for each compound using equation 1.

$$\text{Eq. 1} \quad RF = (A_s C_{is})/(A_{is} C_s)$$

where:

A_s = Area of the characteristic ion for the parameter to be measured.

A_{is} = Area of the characteristic ion for the internal standard.

C_{is} = Concentration of the internal standard.

C_s = Concentration of the parameter to be measured.

If the RF value over the working range is a constant (<10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve or response ratios, A_s/A_{is}, vs. RF.

7.4.4 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ± 10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared for that compound.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted to certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike all samples with surrogate standards to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each parameter to be measured. Using stock standards, prepare a quality control check sample concentrate in methanol 500 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a syringe, add 10 µL of the check sample concentrate and 10 µL of the surrogate standard dosing solution (Section 6.7) to each of a minimum of four 5-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 11.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for all parameters and surrogate standards. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 5, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If $s > p$ or $|X - R| > p$, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the results of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria for each of the surrogate standards.

8.3.1 Calculate upper and lower control limits for method performance for each surrogate standard, using the values for R and s calculated in Section 8.2.3:

Upper Control Limit (UCL) = R + 3s
Lower Control Limit (LCL) = R − 3s

The UCL and LCL can be used to construct control charts[8] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become available from the U.S. Environmental Protection Agency.

8.3.2 For each surrogate standard, the laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly[8].

8.4 The laboratory is required to spike all of their samples with the surrogate standard spiking solution to monitor spike recoveries. If the recovery for any surrogate standard does not fall within the control limits for method performance, the results reported for that sample must be qualified as described in Section 13.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Each day, the analyst must demonstrate, through the analysis of reagent water, that interferences from the analytical system are under control.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 All samples must be iced or refrigerated from the time of collection until extraction. If the sample contains residual chlorine, add sodium thiosulfate preservative (10 mg/40 mL is sufficient for up to 5 ppm Cl_2) to the empty sample bottles just prior to shipping to the sampling site. U.S. Environmental Protection Agency methods 330.4 and 330.5 may be used for measurement of residual chlorine[9]. Field test kits are available for this purpose.

9.2 Grab samples must be collected in glass containers having a total volume of at least 25 mL. Fill the sample bottle just to overflowing in such a manner that no air bubbles pass through the sample as the bottle is being filled. Seal the bottle so that no air bubbles are entrapped in it. If preservative has been added, shake vigorously for one minute. Maintain the hermetic seal on the sample bottle until time of analysis.

9.3 Experimental evidence indicates that some aromatic compounds, notably benzene, toluene, and ethyl benzene are susceptible to rapid biological degradation under certain environmental conditions[3]. Refrigeration alone may not be adequate to preserve these compounds in wastewaters for more than seven days. For this reason, a separate sample should be collected, acidified, and analyzed when these aromatics are to be determined. Collect about 500 mL of sample in a clean container. Adjust the pH of the sample to about 2 by adding HCl (1 + 1) while stirring. Check pH with narrow range (1.4 to 2.8) pH paper. Fill a sample container as described in Section 9.2. If chlorine residual is present, add sodium thiosulfate to another sample container and fill as in Section 9.2 and mix thoroughly.

9.4 All samples must be analyze within 14 days of collection.

10. Daily GC/MS Performance Tests

10.1 At the beginning of each day that analyses are to be performed, the

362

GC MS system must be checked to see if acceptable performance criteria are achieved for BFB[10]. The performance test must be passed before any samples, blanks, or standards are analyzed, unless the instrument has met the DFTPP test described in method 625 earlier in the day[11].

10.2 These performance tests require the following instrumental parameters.

Electron Energy:	70 Volts (nominal)
Mass Range:	20 to 260
Scan Time:	to give at least 5 scans per peak but not to exceed 7 seconds per scan.

10.3 At the beginning of each day, inject 2 µL of BFB solution directly on column. Alternately, add 2 µL of BFB solution to 5.0 mL of reagent water or standard solution and analyze according to Section 11. Obtain a background corrected mass spectrum of BFB and check that all the key ion criteria in Table 2 are achieved. If all the criteria are not achieved, the analyst must retune the mass spectrometer and repeat the test until all criteria are achieved.

11. Sample Extraction and Gas Chromatography

11.1 Table 1 summarizes the recommended operating conditions for the gas chromatograph. This table includes retention times and method detection limits that were achieved under these conditions. An example of the parameter separations achieved by Column 1 is shown in Figure 5. Other packed columns or chromatographic conditions may be used if the requirements of Section 8.2 are met.

11.2 After achieving the key ion abundance criteria in Section 10, calibrate the system daily as described in Section 7.

11.3 Adjust the purge gas (helium) flow rate to 40 ± 3 mL/min. Attach the trap inlet to the purging device, and set the device to purge. Open the syringe valve located on the purging device sample introduction needle.

11.4 Remove the plunger from a 5-mL syringe and attach a closed syringe valve. Open the sample or standard bottle which has been allowed to come to ambient temperature, and carefully pour the sample into the syringe barrel to just short of overflowing. Replace the syringe plunger and compress the sample. Open the syringe valve and vent any residual air while adjusting the sample volume to 5.0 mL. Since this process of taking an aliquot destroys the validity of the sample for future analysis, the analyst should fill a second syringe at this time to protect against possible loss of data. Add 10.0 µL of the surrogate spiking solution (Section 6.7) and, if applicable, 10.0 µL of the internal standard spiking solution (Section 7.4.2) through the valve bore, then close the valve. The surrogate and internal standards may be mixed and added as a single spiking solution.

11.5 Attach the syringe-syringe valve assembly to the syringe valve on the purging device. Open the syringe valves and inject the sample into the purging chamber.

11.6 Close both valves and purge the sample for 11.0 ± 0.1 minutes at ambient temperature.

11.7 At the conclusion of the purge time, attach the trap to the chromatograph, adjust the device to the desorb mode, and begin the gas chromatographic temperature program. Concurrently, introduce the trapped materials to the gas chromatographic column by rapidly heating the trap to 180 °C while backflushing the trap with an inert gas between 20 and 60 mL/min for four minutes. If this rapid heating requirement cannot be met, the gas chromatographic column must be used as a secondary trap by cooling it to 30 °C (or subambient, if problems persist) instead of the recommended initial temperature of 45 °C.

11.8 While the trap is being desorbed into the gas chromatograph, empty the purging chamber using the sample introduction syringe. Wash the chamber with two 5-mL flushes of reagent water.

11.9 After desorbing the sample for four minutes, recondition the trap by returning the purge and trap device to the purge mode. Wait 15 seconds then close the syringe valve on the purging device to begin gas flow through the trap. The trap temperature should be maintained at 180 °C. Trap temperatures up to 230 °C may be employed, however, the higher temperature will shorten the useful life of the trap. After approximately seven minutes turn off the trap heater and open the syringe valve to stop the gas flow through the trap. When cool, the trap is ready for the next sample.

11.10 If the response for any ion exceeds the working range of the system, dilute the sample aliquot in the second syringe with reagent water and reanalyze.

12. Qualitative Identification

12.1 Obtain EICPs for the primary ion (Table 4) and at least two secondary ions for each parameter of interest. The following criteria must be met to make a qualititative identification.

12.1.1 The characteristic ions of each parameter of interest must maximize in the same or within one scan of each other.

12.1.2 The retention time must fall within ± 30 seconds of the retention time of the authentic compound.

12.1.3 The relative peak heights of the three characteristic ions in the EICPs must fall within ± 20% of the relative intensities of these ions in a reference mass spectrum. The reference mass spectrum can be obtained from a standard analyzed in the GC/MS system or from a reference library.

12.2 Structural isomers that have very similar mass spectra and less than 30 seconds difference in retention time, can be explicitly identified only if the resolution between authentic isomers in a standard mix is acceptable. Acceptable resolution is achieved if the baseline to valley height between the isomers is less than 25% of the sum of the two peak heights. Otherwise, structural isomers are identified as isomeric pairs.

13. Calculations

13.1 When a parameter has been identified, the quantitation of that parameter should be based on the integrated abundance from the EICP of the first listed characteristic ion given in Table 4. If the sample produces an interference for the primary ion, use a secondary characteristic ion to quantitate. Quantitation may be performed using the external or internal standard techniques.

13.1.1 If the external standard calibration procedure is used, calculate the concentration of the parameter being measured from the area of the characteristic ion using the calibration curve or calibration factor in Section 7.3.2.

13.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.4.3 and equation 2.

Eq. 2.

Concentration µg/L = $(A_s C_{is})/(A_{is})(RF)$

where:

367

A_s = Area of the characteristic ion for the parameter or surrogate standard to be measured.

A_{is} = Area of the characteristic ion for the internal standard.

C_{is} = Concentration of the internal standard.

13.2 Report results in micrograms per liter. The results for cis- and trans-1,3 dichloropropene should be reported as total 1,3-dichloropropene (STORET No. 34561, CAS No. 542-75-6). When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

13.3 If any of the surrogate standard recoveries fall outside the control limits which were established as directed in Section 8.4, data for all parameters determined by this method in that sample must be labeled as suspect.

14. Method Performance

14.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero[1]. The MDL concentrations listed in Table 1 were obtained using reagent water[12]. Similar results were achieved using representative wastewaters.

14.2 The average recoveries and the average standard deviations of the percent recoveries, presented in Table 5, were the result of a study of the accuracy and precision of this method by several laboratories. The values listed represent the results from 2 to 4 laboratories[13].

14.3 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

References

1. See Appendix A.
2. Bellar, T.A., and J.J. Lichtenberg, *Journal American Water Works Association, 66*, p. 739, (1974).
3. Bellar, T.A., and J.J. Lichtenberg, "Semi-Automated Headspace Analysis of Drinking Waters and Industrial Waters for Purgeable Volatile Organic Compounds," Measurement of Organic Pollutants in Water and Wastewater, C.E. Van Hall, editor, American Society for Testing and Materials, Philadelphia, PA. Special Technical Publication 686, 1978.
4. "Sampling and Analysis Procedures for Screening of Industrial Effluents for Priority Pollutants." U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, OH 45268, March 1977, Revised April 1977. Effluent Guidelines Division, Washington, DC 10460.
5. "Carcinogens—Working with Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.
6. "OSHA Safety and Health Standards, General Industry," (29CFR1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).
7. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.
8. "Handbook of Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.
9. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600/4-79-020, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.
10. Budde, W.L. and Eichelberger, J.W., "Performance Tests for the Evaluation of Computerized Gas Chromatography/Mass Spectrometry Equipment and Laboratories," EPA-600/4-80-025, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, OH 45268, p. 16, April 1980.
11. Eichelberg, J.W. Harris, L.E., and Budde, W.L., "Reference Compound to Calibrate Ion Abundance Measurement in Gas Chromatography—Mass Spectrometry Systems.", *Analytical Chemistry, 47*, 995-1000 (1979).
12. "Method Detection Limit for Methods 624 and 625," Olynyk, P., Budde, W.L. Eichelberger, J.W., unpublished report, October 1980.
13. Kleopfer, R.D., "POTW Toxic Study, Analytical Quality Assurance Final Report," U.S. Environmental Protection Agency, Region VII, Kansas City, Kansas 66115, 1981.

36 41

Table 1. Chromatographic Conditions and Method Detection Limits

Parameter	Retention Time (min.) Column 1	Method Detection Limit (µg/L)
Chloromethane	2.3	nd
Bromomethane	3.1	nd
Vinyl chloride	3.8	nd
Chloroethane	4.6	nd
Methylene chloride	6.4	2.8
Trichlorofluoromethane	8.3	nd
1,1-Dichloroethene	9.0	2.8
1,1-Dichloroethane	10.1	4.7
trans-1,2-Dichloroethene	10.8	1.6
Chloroform	11.4	1.6
1,2-Dichloroethane	12.1	2.8
1,1,1-Trichloroethane	13.4	3.8
Carbon tetrachloride	13.7	2.8
Bromodichloromethane	14.3	2.2
1,2-Dichloropropane	15.7	6.0
trans-1,3-Dichloropropene	15.9	5.0
Trichloroethene	16.5	1.9
Benzene	17.0	4.4
Dibromochloromethane	17.1	3.1
1,1,2-Trichloroethane	17.2	5.0
cis-1,3-Dichloropropene	17.2	nd
2-Chloroethylvinyl ether	18.6	nd
Bromoform	19.8	4.7
1,1,2,2-Tetrachloroethane	22.1	6.9
Tetrachloroethene	22.2	4.1
Toluene	23.5	6.0
Chlorobenzene	24.6	6.0
Ethyl benzene	26.4	7.2
1,3-Dichlorobenzene	33.9	nd
1,2-Dichlorobenzene	35.0	nd
1,4-Dichlorobenzene	35.4	nd

nd = not determined

Column conditions: Carbopak B (60/80 mesh) coated with 1% SP-1000 packed in a 6 ft by 2 mm ID glass column with helium carrier gas at a flow rate of 30 mL/min. Column temperature is isothermal at 45°C for 3 min, then programmed at 8°C per minute to 220°C and held for 15 min.

Table 2. BFB Key Ion Abundance Criteria

Mass	Ion Abundance Criteria
50	15 to 40% of mass 95
75	30 to 60% of mass 95
95	Base Peak, 100% Relative Abundance
96	5 to 9% of mass 95
173	<2% of mass 174
174	>50% of mass 95
175	5 to 9% of mass 174
176	>95% but < 101% of mass 174
177	5 to 9% of mass 176

Table 3. Suggested Surrogate and Internal Standards

Compound	Retention Time (min.)[a]	Primary Ion	Secondary Ions
Surrogate Standards			
Benzene d-6	17.0	84	–
4-Bromofluorobenzene	28.3	95	174, 176
1,2-Dichloroethane d-4	12.1	102	–
1,4-Difluorobenzene	19.6	114	63, 88
Ethylbenzene d-5	26.4	111	–
Ethylbenzene d-10	26.4	98	–
Fluorobenzene	18.4	96	70
Pentafluorobenzene	23.5	168	–
Internal Standards			
Bromochloromethane	9.3	126	49, 130, 51
2-Bromo-1-chloropropane	19.2	77	79, 156
1,4-Dichlorobutane	25.8	55	90, 92

[a]For chromatographic conditions, see Table 1.

Table 4. Characteristic Ions for Purgeable Organics

Parameter	Primary Ion	Secondary Ions
Chloromethane	50	52
Bromomethane	94	96
Vinyl chloride	62	64
Chloroethane	64	66
Methylene chloride	84	49, 51, 86
Trichlorofluoromethane	101	103
1,1-Dichloroethene	96	61, 98
1,1-Dichloroethane	63	65, 83, 85, 98, 100
trans-1,2-Dichloroethene	96	61, 98
Chloroform	83	85
1,2-Dichloroethane	98	62, 64, 100
1,1,1-Trichloroethane	97	99, 117, 119
Carbon tetrachloride	117	119, 121
Bromodichloromethane	127	83, 85, 129
1,2-Dichloropropane	112	63, 65, 114
trans-1,3-Dichloropropene	75	77
Trichloroethene	130	95, 97, 132
Benzene	78	
Dibromochloromethane	127	129, 208, 206
1,1,2-Trichloroethane	97	83, 85, 99, 132, 134
cis-1,3-Dichrloropropene	75	77
2-Chloroethylvinyl ether	106	63, 65
Bromoform	173	171, 175, 250, 252, 254, 256
1,1,2,2-Tetrachloroethane	168	83, 85, 131, 133, 166
Tetrachloroethene	164	129, 131, 166
Toluene	92	91
Chlorobenzene	112	114
Ethyl benzene	106	91
1,3-Dichlorobenzene	146	148, 113
1,2-Dichlorobenzene	146	148, 113
1,4-Dichlorobenzene	146	148, 113

36.6

Table 5. *Accuracy and Precision for Purgeable Organics*

Parameter	Reagent Water Average Percent Recovery	Reagent Water Standard Deviation (%)	Wastewater Average Percent Recovery	Wastewater Standard Deviation (%)
Benzene	99	9	98	10
Bromodichloromethane	102	12	103	10
Bromoform	104	14	105	16
Bromomethane	100	20	88	23
Carbon tetrachloride	102	16	104	15
Chlorobenzene	100	7	102	9
Chloroethane	97	22	103	31
2-Chloroethylvinyl ether	101	13	95	17
Chloroform	101	10	101	12
Chloromethane	99	19	99	24
Dibromochloromethane	103	11	104	14
1,1-Dichloroethane	101	10	104	15
1,2-Dichloroethane	100	8	102	10
1,1-Dichloroethene	102	17	99	15
trans-1,2-Dichloroethene	99	12	101	10
1,2-Dichloropropane	102	8	103	12
cis-1,3-Dichloropropene	105	15	102	19
trans-1,3-Dichloropropene	104	11	100	18
Ethyl benzene	100	8	103	10
Methylene chloride	96	16	89	28
1,1,2,2-Tetrachloroethane	102	9	104	14
Tetrachloroethene	101	9	100	11
Toluene	101	9	98	14
1,1,1-Trichloroethane	101	11	102	16
1,1,2-Trichloroethane	101	10	104	15
Trichloroethene	101	9	100	12
Trichlorofluoromethane	103	11	107	19
Vinyl chloride	100	13	98	25

Samples were spiked between 10 and 1000 µg/L.

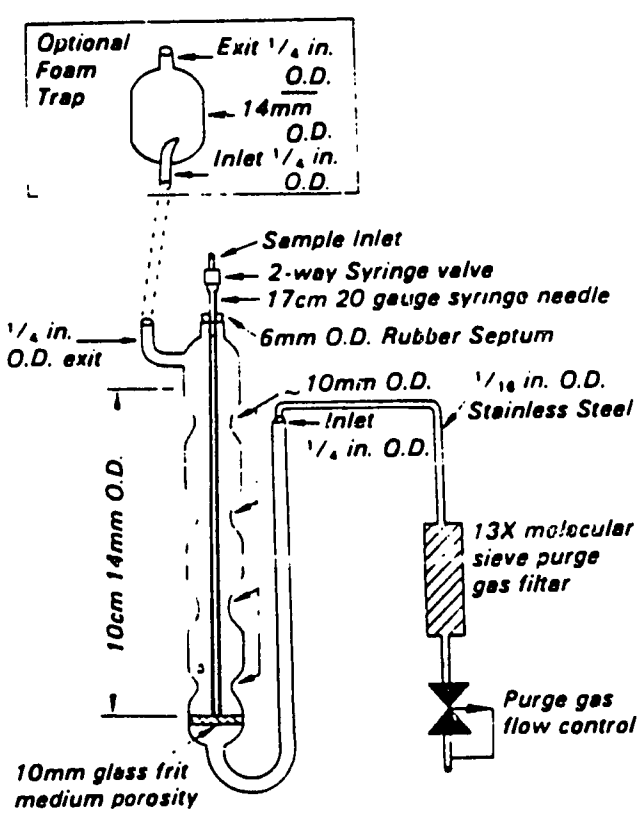


Figure 1. *Purging device*

367

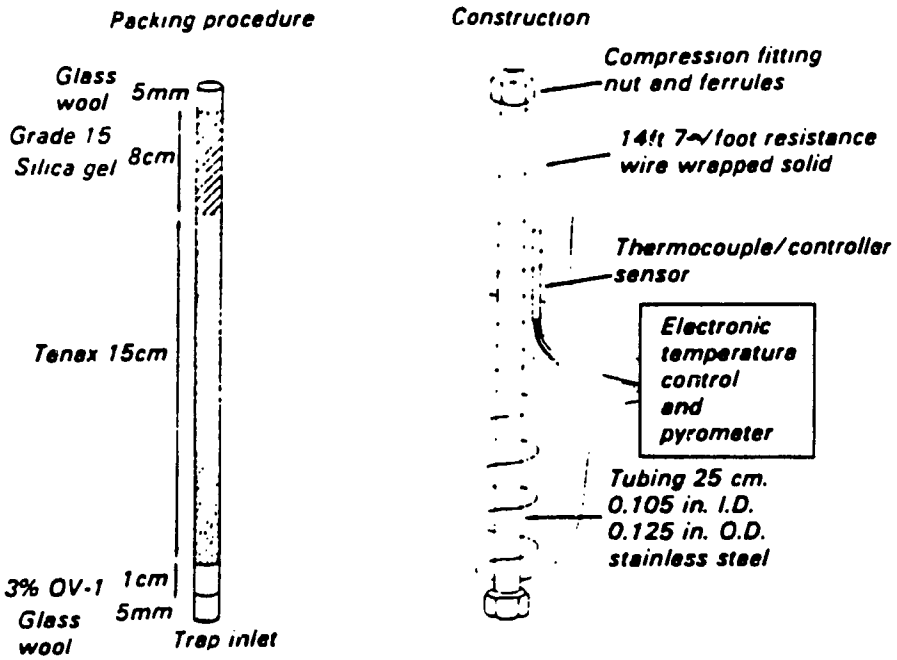


Figure 2. *Trap packings and construction to include desorb capability*

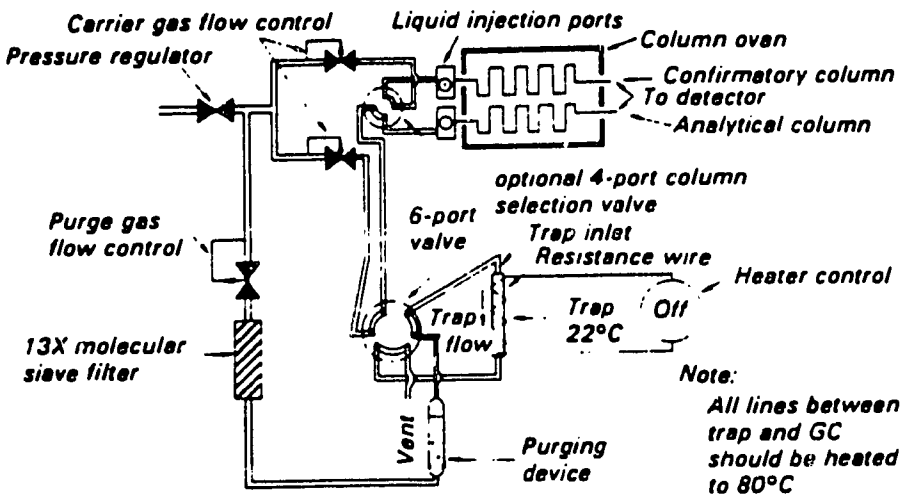


Figure 3. *Schematic of purge and trap device — purge mode*

369

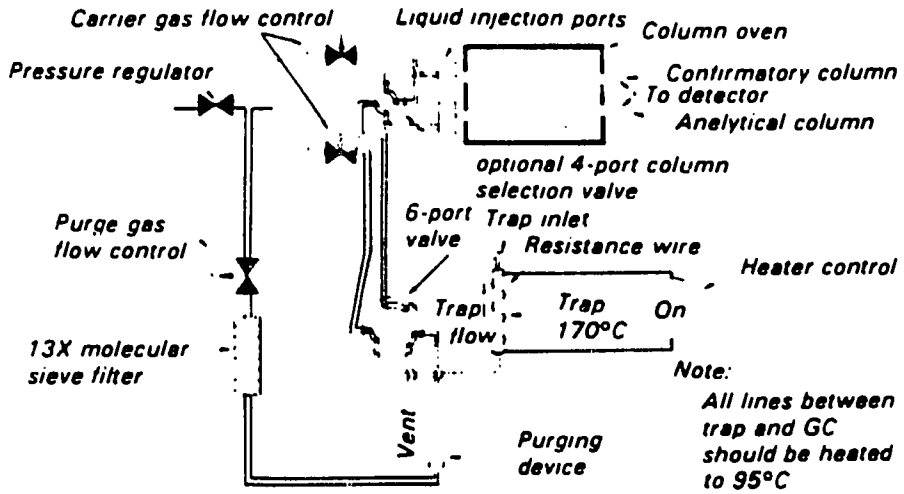


Figure 4. *Schematic of purge and trap device — desorb mode*

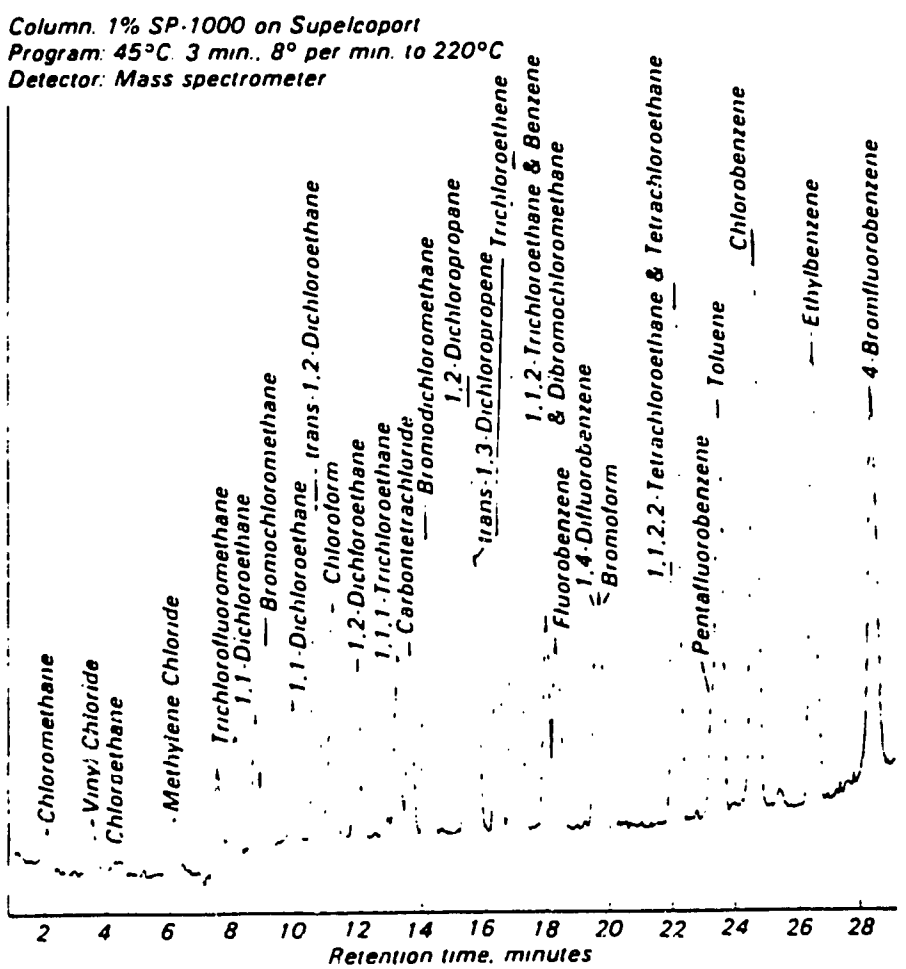


Figure 5. *Gas chromatogram of volatile organics by purge and trap*

369

United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA

Test Method

Base/Neutrals and Acids — Method 625

1. Scope and Application

1.1 This method covers the determination of a number of organic compounds that are partitioned into an organic solvent and are amenable to gas chromatography. The parameters listed in Tables 1 and 2 may be qualitatively and quantitatively determined using this method.

1.2 The method may be extended to include the parameters listed in Table 3. Benzidine can be subject to oxidative losses during solvent concentration. α-BHC, γ-BHC, endosulfan I and II, and endrin are subject to decomposition under the alkaline conditions of the extraction step. Hexachlorocyclopentadiene is subject to thermal decomposition in the inlet of the gas chromatograph, chemical reaction in acetone solution and photochemical decomposition. N-nitrosodimethylamine is difficult to separate from the solvent under the chromatographic conditions described. N-nitrosodiphenylamine decomposes in the gas chromatographic inlet and cannot be separated from diphenylamine. The preferred method for each of these paramaters is listed in Table 3.

1.3 This is a gas chromatography/mass spectrometry (GC/MS) method applicable to the determination of the compounds listed in Tables 1, 2, and 3 in municipal and industrial discharges as provided under 40 CFR 136.1. Until the U.S. Environmental Protection Agency establishes performance criteria based upon the results of interlaboratory testing, any alternative GC/MS method which meets the performance criteria described in Section 8.2 will be permitted. Performance must be verified for such modification by analyzing wastewater as described in Section 8.2.2. In addition, the laboratory must successfully participate in the applicable performance evaluation studies.

1.4 The method detection limit (MDL, defined in Section 16)[1] for each parameter is listed in Tables 4 and 5. The MDL for a specific wastewater differ from those listed, depending upon the nature of interferences in the sample matrix.

1.5 This method is restricted to use by or under the supervision of analysts experienced in the operation of gas chromatograph/mass spectrometers and skilled in the interpretation of mass spectra. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

2. Summary of Method

2.1 A measured volume of sample, approximately one-liter, is serially extracted with methylene chloride at a pH greater than 11 and again at pH less than 2 using a separatory funnel or a continuous extractor. The methylene chloride extract is dried and concentrated to a volume of 1 mL. Chromatographic conditions are described which permit the separation and measurement of the parameters in the extract. Qualitative identification is performed using the retention time and the relative abundance of three characteristic ions. Quantitative analysis is performed using either

370

external or internal standard techniques with a single characteristic ion.

3. Interferences

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in the total ion current profiles. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.5.

3.1.1 Glassware must be scrupulously cleaned[3]. Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water, and rinses with tap water and reagent water. It should then be drained dry, and heated in a muffle furnace at 400 °C for 15 to 30 minutes. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store it inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled.

3.3 The base-neutral extraction may cause significantly reduced recovery of phenol, 2-methylphenol, and 2,4-dimethylphenol. The analyst must recognize that results obtained under these conditions are minimum concentrations.

3.4 The packed gas chromatographic columns recommended for the basic fraction may not exhibit sufficient resolution for certain isomeric pairs. These include anthracene and phenanthrene; chrysene and benzo(a)anthracene; and benzo(b)fluoranthene and benzo(k)fluoranthene. The gas chromatograph retention time and mass spectra are not sufficiently different to make an unambiguous distinction between these compounds. Alternative techniques should be used to identify and quantify these specific compounds. See method 610.

3.5 In samples that contain an inordinate number of interferences, the use of chemical ionization (CI) mass spectrometry may make identification easier. Tables 6 and 7 give characteristic CI ions for most of the compounds covered by this method. The use of CI mass spectrometry to support electron ionization (EI) mass spectrometry is encouraged but not required.

4. Safety

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified for the information of the analyst[4-6].

4.2 The following parameters covered by this method have been tentatively classified as known or suspected, human or mammalian carcinogens; benzo(a)anthracene, benzidine, 3,3'-dichlorobenzidine, benzo(a)pyrene, α-BHC, β-BHC, δ-BHC, γ-BHC, dibenzo(a,h) anthracene, N-nitrosodimethylamine, 4,4'-DDT and polychlorinated biphenyls.

5. Apparatus and Materials

5.1 Sampling equipment, for discrete or composite sampling.

5.1.1 Grab sample bottle—Amber glass, one-liter or one-quart volume, fitted with screw caps lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The container must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.

5.1.2 Automatic sampler (optional)—Must incorporate glass sample containers for the collection of a minimum of 250 mL. Sample containers must be kept refrigerated at 4 °C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.

5.2 Glassware (All specifications are suggested. Catalog numbers are included for illustration only).

5.2.1 Separatory funnel—2000-mL, with Teflon stopcock.

5.2.2 Drying column—19 mm ID chromatographic column with coarse frit.

5.2.3 Concentrator tube, Kuderna-Danish—10-mL, graduated (Kontes K-570050-1025 or equivalent). Calibration must be checked at the volumes employed in the test. Ground glass stopper is used to prevent evaporation of extracts.

5.2.4 Evaporative flask, Kuderna-Danish—500-mL (Kontes K-570001-0500 or equivalent). Attach to concentrator tube with springs.

5.2.5 Snyder column, Kuderna-Danish—Three-ball macro (Kontes K-503000-0121 or equivalent).

5.2.6 Snyder column, Kuderna-Danish—Two-ball micro (Kontes K-569001-0219 or equivalent).

5.2.7 Vials—Amber glass, 10- to 15-mL capacity, with Teflon-lined screw cap.

5.2.8 Continuous liquid-liquid extractors—Equipped with Teflon or glass connecting joints and stoococks requiring no lubrication. (Hershberg-Wolf Extractor-Ace Glass Company, Vineland, N.J. P/N 6841-10 or equivalent.)

5.3 Boiling chips—approximately 10/40 mesh. Heat to 400 °C for 30 minutes or Soxhlet extract with methylene chloride.

5.4 Water bath—Heated, with concentric ring cover, capable of temperature control (± 2 °C). The bath should be used in a hood.

5.5 Balance—Analytical, capable of accurately weighing 0.0001 g.

5.6 GC/MS system.

5.6.1 Gas chromatograph—An analytical system complete with a temperature programmable gas chromatograph and all required accessories including syringes, analytical columns, and gases. The injection port must be designed for on-column injection when using packed columns and for splitless injection when using capillary columns.

5.6.2 Column for Base Neutrals—1.8 m long × 2 mm ID glass, packed with Supelcoport (100/120 mesh) coated with 3% SP-2250 or equivalent. This column was used to develop the accuracy and precision statements in Table 6 and the MDL data in Table 4. Guidelines for the use of alternate column packings are provided in Section 13.1.

5.6.3 Column for Acids—1.8 m long × 2 mm ID glass, packed with Supelcoport (100/120 mesh) coated with 1% SP-1240 DA or equivalent. This column was used to develop the accuracy and precision statements in Table 7, and the MDL data in Table 5. Guidelines for the use of alternate column packings are given in Section 13.1.

5.6.4 Mass Spectrometer—Capable of scanning from 35 to 450 amu every seven seconds or less utilizing a 70 volt (nominal) electron energy in the electron impact ionization mode and producing a mass spectrum which meets all the criteria in Table 9 when 50 ng of decafluorotriphenyl phosphine (DFTPP: bis(perfluorophenyl) phenyl phosphine) is injected through the gas chromatographic inlet. Any gas chromatograph to mass spectrometer interface that gives acceptable calibration points at 50 ng per injection for each compound of interest in Tables 1 through 3 and achieves all acceptable performance criteria (Section 12) may be used. Gas chromatograph to mass spectrometer interfaces constructed of all glass or glass lined materials are recommended. Glass can be deactivated by silanizing with dichlorodimethylsilane.

5.6.5 A computer system must be interfaced to the mass spectrometer that allows the continuous acquisition and storage on machine readable media of all mass spectra obtained throughout the duration of the chromatographic program. The computer must have software that allows searching any GC/MS data file for ions of a specific mass and plotting such ion abundances versus time or scan number. This type of plot is defined as an Extracted Ion Current Profile (EICP). Software must also be available that allows integrating the abundance in any EICP between specified time or scan number limits.

6. Reagents

6.1 Reagent water—Reagent water is defined as a water in which an interferent is not observed at the method detection limit of each parameter of interest.

6.2 Sodium hydroxide solution (10 N)—Dissolve 40g NaOH in reagent water and dilute to 100 mL.

6.3 Sodium thiosulfate—(ACS) Granular.

6.4 Sulfuric acid solution (1 + 1)—Slowly add 50 mL of H_2SO_4 (sp. gr. 1.84) to 50 mL of reagent water.

6.5 Acetone, methanol, methylene chloride—Pesticide quality or equivalent.

6.6 Sodium sulfate—(ACS) Granular, anhydrous. Purify by heating at 400 °C for four hours in a shallow tray.

6.7 Stock standard solutions (1.00 µg/µL)—Stock standard solutions can be prepared from pure standard materials or purchased as certified solutions.

6.7.1 Prepare stock standard solutions by accurately weighing about 0.0100 g of pure material. Dissolve the material in pesticide quality acetone or other suitable solvent and dilute to volume in a 10-mL volumetric flask. Larger volumes may be used at the convenience of the analyst. If compound purity is assayed at 96% or greater, the weight may be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards may be used at any concentration if they are certified by the manufacturer or by an independent source.

6.7.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store in at 4 °C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Quality control check samples, that can be used to determine the accuracy of calibration standards will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

6.7.3 Stock standard solutions must be replaced after six months or sooner if comparison with quality control check samples indicate a problem.

6.8 Surrogate standard spiking solutions—select a minimum of three surrogate compounds from Table 8. Prepare a surrogate standard spiking solution at a concentration of 100 µg/1.00 ML in acetone. Addition of 1.00 mL of this solution to 1000-mL of sample is equivalent to a concentration of 100 µg/L of each surrogate standard. Store the spiking solutions at 4 °C in Teflon-sealed containers. The solutions should be checked frequently for stability. These solutions must be replaced after six months, or sooner if comparison with quality control check samples indicate a problem. Surrogate standard spiking solutions, appropriate for use with this method will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

6.9 DFTPP standard—Prepare a 25 ng/µL solution of DFTPP in acetone.

7. Calibration

7.1 Establish gas chromatographic operating parameters equivalent to those indicated in Tables 4 or 5. The GC/MS system can be calibrated using the external standard technique (Section 7.2) or the internal standard technique (Section 7.3).

7.2 External standard calibration procedure:

7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with acetone. One of the external standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the GC/MS system.

7.2.2 Analyze 2 to 5 µL of each calibration standard and tabulate the area responses of the primary characteristic ion of each standard (Tables 4 and 5) against the mass injected. The results may be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over

the working range (<10% relative standard deviation, RSD), linearity through the origin may be assumed and the average ratio or calibration factor may be used in place of a calibration curve.

7.2.3 The working calibration curve or calibration factor must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ± 10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve or calibration factor must be prepared for that compound.

7.3 Internal standard calibration procedure. To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal standard is not affected by method or matrix interferences. Table 8 lists some recommended internal standards. Phenanthrene-d_{10} has been used for this purpose. Use the base peak ion as the primary ion for quantification of the standards. If interferences are noted, use the next two most intense ions as the secondary ions.

7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding appropriate volumes of one or more stock standards to a volumetric flask. To each calibration standard or standard mixture, add a known constant amount of one or more internal standards, and dilute to volume with acetone. One of the calibration standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the GC/MS system.

7.3.2 Analyze 2 to 5 µL of each calibration standard and tabulate the area of the primary characteristic ion (Tables 4 and 5) against concentration for each compound and internal standard, and calculate response factors (RF) for each compound using equation 1.

$$\text{Eq. 1} \quad RF = (A_s C_{is})/(A_{is} C_s)$$

where:

A_s = Area of the characteristic ion for the parameter to be measured.

A_{is} = Area of the characteristic ion for the internal standard.

C_{is} = Concentration of the internal standard, (µg/L).

C_s = Concentration of the parameter to be measured, (µg/L).

If the RF value over the working range is a constant (<10% RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. RF.

7.3.3 The working calibration curve or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than ± 10%, the test must be repeated using a fresh calibration standard. Alternatively, a new calibration curve must be prepared.

8. Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.1.3 The laboratory must spike all samples with surrogate standards to monitor continuing laboratory performance. This procedure is described in Section 8.4.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each parameter to be measured. Using stock standards, prepare a quality control check sample concentrate in acetone 1000 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate and 1.0 mL of the surrogate standard dosing solution (Section 6.8) to each of a minimum of four 1000-mL aliquots of reagent water. A representative wastewater may be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots according to the method beginning in Section 10.

8.2.3 Calculate the average percent recovery, (R), and the standard deviation of the percent recovery (s), for all parameters and surrogate standards. Wastewater background corrections must be made before R and s calculations are performed.

8.2.4 Using Table 6 or 7, note the average recovery (X) and standard deviation (p) expected for each method parameter. Compare these to the calculated values for R and s. If s > p or |X − R| > p, review potential problem areas and repeat the test.

8.2.5 The U.S. Environmental Protection Agency plans to establish performance criteria for R and s based upon the result of interlaboratory testing. When they become available, these criteria must be met before any samples may be analyzed.

8.3 The analyst must calculate method performance criteria for each of the surrogate standards.

8.3.1 Calculate upper and lower control limits for method performance for each surrogate standard, using the values for R and s calculated in Section 8.2.3:

Upper Control Limit (UCL) = R + 3s
Lower Control Limit (LCL) = R − 3s

The UCL and LCL can be used to construct control charts[7] that are useful in observing trends in performance. The control limits above must be replaced by method performance criteria as they become avail-

able from the U.S. Environmental Protection Agency

8.3.2 For each surrogate standard, the laboratory must develop and maintain separate accuracy statements of laboratory performance for wastewater samples. An accuracy statement for the method is defined as R ± s. The accuracy statement should be developed by the analysis of four aliquots of wastewater as described in Section 8.2.2, followed by the calculation of R and s. Alternately, the analyst may use four wastewater data points gathered through the requirement for continuing quality control in Section 8.4. The accuracy statements should be updated regularly.[7]

8.4 The laboratory is required to spike all samples with the surrogate standard spiking solution to monitor spike recoveries. If the recovery for any surrogate standard does not fall within the control limits for method performance, the results reported for that sample must be qualified as described in Section 15.3. The laboratory should monitor the frequency of data so qualified to ensure that it remains at or below 5%.

8.5 Before processing any samples, the analyst should demonstrate through the analysis of a one-liter aliquot of reagent water, that all glassware and reagent interferences are under control. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9. Sample Collection, Preservation, and Handling

9.1 Grab samples must be collected in glass containers. Conventional sampling practices[8] should be followed, except that the bottle must not be prewashed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon and other potential sources of contamination.

9.2 The samples must be iced or refrigerated at 4 °C from the time of collection until extraction. Fill the sample bottles and, if residual chlorine is present, add 80 mg of sodium thiosulfate per each liter of water. U.S. Environmental Protection Agency methods 330.4 and 330.5 may be used to measure the residual chlorine[9]. Field test kits are available for this purpose.

9.3 All samples must be extracted within 7 days and completely analyzed within 40 days of extraction.

10. Separatory Funnel Extraction

10.1 Samples are usually extracted using separatory funnel techniques. If emulsions will prevent achieving acceptable solvent recovery with separatory funnel extractions, continuous extraction (Section 11) may be used. The separatory funnel extraction scheme described below assumes a sample volume of one-liter. When sample volumes of two liters are to be extracted, use 250-, 100-, and 100-mL volumes of methylene chloride for the serial extraction of the base/neutrals and 200-, 100-, and 100-mL volumes of methylene chloride for the acids.

10.2 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a two-liter separatory funnel. Pipet 1.00 mL surrogate standard spiking solution into the separatory funnel and mix well. Check the pH of the sample with wide-range pH paper and adjust to pH > 11 with 10 N sodium hydroxide.

10.3 Add 60 mL methylene chloride to the sample bottle, seal, and shake for 30 seconds to rinse the inner surface. Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool, centrifugation, or other physical methods. Collect the methylene chloride extract in a 250-ml Erlenmeyer flask. If the emulsion cannot be broken (recovery of less than 80% of the methylene chloride, corrected for the water solubility of methylene chloride), transfer the sample, solvent, and emulsion into the extraction chamber of a continuous extractor and proceed as described in Section 11.3.

10.4 Add a second 60-mL volume of methylene chloride to the sample bottle and repeat the extraction procedure a second time, combining the extracts in the Erlenmeyer flask. Perform a third extraction in the same manner. Label the combined extract as base/neutral fraction.

10.5 Adjust the pH of the aqueous phase to less than 2 using sulfuric acid (1 + 1). Serially extract three times with 60-mL aliquots of methylene chloride. Collect and combine the extracts in a 250-mL Erlenmeyer flask and label the combined extract as the acid fraction.

10.6 For each fraction, assemble a Kuderna-Danish (K-D) concentrator by attaching a 10-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used in place of the K-D if the requirements of Section 8.2 are met.

10.7 For each fraction, pour the combined extract through a drying column containing about 10 cm of anhydrous sodium sulfate, and collect the extract in the K-D concentrator. Rinse the Erlenmeyer flask and column with 20 to 30 mL of methylene chloride to complete the quantitative transfer.

10.8 To the evaporative flask for each fraction, add one or two clean boiling chips and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL methylene chloride to the top of the column. Place the K-D apparatus on a hot water bath (60 ° to 65 °C) so that the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood with condensed solvent. When the apparent volume of liquid reaches 1 mL, remove the K-D apparatus from the water bath and

374

allow it to drain and cool for at least 10 minutes. Remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of methylene chloride. A 5-mL syringe is recommended for this operation.

10.9 Add another one or two clean boiling chips to the concentrator tube and attach a two-ball micro Snyder column. Prewet the Snyder column by adding about 0.5 mL of methylene chloride to the top of the column. Place the K-D apparatus on a hot water bath (60° to 65°C) so that the concentrator tube is partially immersed in the hot water. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 5 to 10 minutes. At the proper rate of distillation the balls of the column will actively chatter but the chambers will not flood with condensed solvent. When the apparent volume of liquid reaches about 0.5 mL, remove the K-D apparatus from the water bath and allow it to drain for at least 10 minutes while cooling. Remove the Snyder column and rinse its the flask and its lower joint into the concentrator tube with 0.2 mL of acetone or methylene chloride. Adjust the final volume to 1.0 mL with the solvent. Stopper the concentrator tube and store refrigerated if GC/MS analysis will not be performed immediately. If the extracts will be stored longer than two days, they should be transferred to Teflon-sealed screw-cap bottles and labeled base/neutral or acid fraction as appropriate.

10.10 Determine the original sample volume by refilling the sample bottle to the mark and transferring the water to a 1000-mL graduated cylinder. Record the sample volume to the nearest 5 mL.

11. Continuous Extraction

11.1 When experience with a sample from a given source indicates that a serious emulsion problem will result or an emulsion is encountered in Section 10.3, using a separatory funnel, a continuous extractor should be used.

11.2 Mark the water meniscus on the side of the sample bottle for later measurement of the sample volume. Check the pH of the sample with wide-range pH paper and adjust to pH 11 with 10 N sodium hydroxide. Transfer the sample to the continuous extractor and using a pipet, add 1.00 mL of surrogate standard spiking solution and mix well. Add 60 mL of methylene chloride to the sample bottle, seal and shake for 30 seconds to rinse the inner surface. Transfer the solvent to the extractor.

11.3 Repeat the sample bottle rinse with an additional 50- to 100-mL portion of methylene chloride and add the rinse to the extractor.

11.4 Add 200 to 500 mL of methylene chloride to the distilling flask, add sufficient reagent water to ensure proper operation, and extract for 24 hours. Allow to cool, then detach the boiling flask, and dry, concentrate and seal the extract as in Section 10.6 through 10.9. Hold the aqueous phase for acid extraction (See Section 11.5).

11.5 Charge a clean distilling flask with 500 mL of methylene chloride and attach it to the continuous extractor. Carefully, adjust the pH of the aqueous phase to less than 2 using sulfuric acid (1 + 1). Extract for 24 hours. Dry, concentrate and label and seal the extract as described in Sections 10.6 through 10.9.

12. Daily GC/MS Performance Tests

12.1 At the beginning of each day that analyses are to be performed, the GC/MS system must be checked to see that acceptable performance criteria are achieved for DFTPP. Each day that benzidine is to be determined, the tailing factor criterion described in Section 12.4 must be achieved. Each day the acids are to be determined, the tailing factor criterion in Section 12.5 must be achieved.

12.2 These DFTPP performance test require the following instrumental parameters.

Electron Energy	70 volts (nominal)
Mass Range	35 to 450 amu
Scan Time	to give at least 5 scans per peak but not to exceed 7 seconds per scan.

12.3 DFTPP performance test[10]—At the beginning of each day, inject 2µL (50 ng) of DFTPP standard solution. Obtain a background corrected mass spectra of DFTPP and check that all the key ion criteria in Table 9 are achieved. If all the criteria are not achieved, the analyst must retune the mass spectrometer and repeat the test until all criteria are achieved. The performance criteria must be achieved before any samples, blanks, or standards are analyzed. The tailing factor tests in Section 12.4 and 12.5 may be performed simultaneously with the test.

12.4 Column performance test for base/neutrals - At the beginning of each day that the base-neutral fraction is to be analyzed for benzidine, the benzidine tailing factor must be calculated. Inject 100 ng of benzidine either separately or as a part of a standard mixture that may contain DFTPP and calculate the tailing factor. The benzidine tailing factor must be less than 3.0. Calculation of the tailing factor is illustrated in Figure 13.[11] Replace the column packing if the tailing factor criterion cannot be achieved.

12.5 Column performance for acids—At the beginning of each day that the acids are to be determined, inject 50 ng of pentachlorophenol either separately or as a part of a standard mix that may contain DFTPP. The tailing factor for pentachlorophenol must be less than five. Calculation of the tailing factor is illustrated in Figure 13[11]. Replace the column packing if the tailing factor criterion cannot be achieved.

13. Gas Chromatography/ Mass Spectrometry

13.1 Table 4 summarizes the recommended gas chromatographic operating conditions for the base/neutral fraction. Table 5 summarizes the recommended gas chromatographic operating conditions for determination of the acid fraction. These tables include retention times and MDL that were achieved under these conditions. Examples of the parameter separations achieved by these columns are shown in Figures 1 through 12. Other packed columns or chromatographic conditions may be used if the requirements of Section 8.2 and Section 12 are met. Capillary (open-tubular) columns may also be used if the relative standard deviations of responses for replicate injections are demonstrated to be less than 6% and the requirements of Section 8.2 and Section 12 are met.

13.2 After the GC/MS performance requirements of Section 12, calibrate the system daily as described in Section 7.

13.3 If the internal standard approach is being used, the internal standard must be added to sample extract and mixed thoroughly, immediately, before injection into the instrument. This minimizes losses due to adsorption, chemical reaction or evaporation.

13.4 Inject 2 to 5 µL of the sample extract using the solvent-flush

375

technique[12]. Smaller (1.0 µL) volumes may be injected if automatic devices are employed. Record the volume injected to the nearest 0.05 µL.

13.5 If the response for any ion exceeds the working range of the GC/MS system, dilute the extract and reanalyze.

13.6 Perform all qualitative and quantitative measurements as described in Sections 14 and 15. When the extracts are not being used for analyses, store them at 4 °C protected from light in screw-cap vials equipped with unpierced Teflon-lined septa.

14. Qualitative Identification

14.1 Obtain an EICP for the primary ion and the two other ions listed in Tables 4 and 5. See Section 7.3 for ions to be used with internal and surrogate standards. The following criteria must be met to make a qualitative identification.

14.1.1 The characteristic ions for each compound of interest must maximize in the same or within one scan of each other.

14.1.2 The retention time must fall within ±30 seconds of the retention time of the authentic compound.

14.1.3 The relative peak heights of the three characteristic ions in the EICP's must fall within ±20% of the relative intensities of these ions in a reference mass spectrum. The reference mass spectrum can be obtained by a standard analyzed in the GC/MS system or from a reference library.

14.2 Structural isomers that have very similar mass spectra and less than 30 seconds difference in retention time, can be explicitly identified only if the resolution between authentic isomers in a standard mix is acceptable. Acceptable resolution is achieved if the baseline to valley height between the isomers is less than 25% of the sum of the two peak heights. Otherwise, structural isomers are identified as isomeric pairs.

15. Calculations

15.1 When a compound has been identified, the quantitation of that compound will be based on the integrated abundance from the EICP of the primary characteristic ion in Tables 4 and 5. Use the base peak ion for internal and surrogate standards. If the sample produces an interference for the first listed ion, use a secondary ion to quantitate. Quantitation will be performed using external or internal standard techniques.

15.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the area of the characteristic ion using the calibration curve or calibration factor in Section 7.2.2. The concentration in the sample can be calculated from equation 2:

Eq. 2. $$\text{Concentration, } \mu g/L = \frac{(A)(V_t)}{(V_i)(V_s)}$$

where:

A = Amount of material injected, in nanograms.
V_i = Volume of extract injected (µL).
V_t = Volume of total extract (µL).
V_s = Volume of water extracted (mL).

15.1.2 If the internal standard calibration procedure was used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and equation 3.

Eq. 3

$$\text{Concentration, } \mu g/L = \frac{(A_s)(I_s)}{(A_{is})(RF)(V_o)}$$

where:

A_s = Area of the characteristic ion for the parameter to be measured.
A_{is} = Area of the characteristic ion for the internal standard.
I_s = Amount of internal standard added to each extract (µg).
V_o = Volume of water extracted (liters).

15.2 Report results in micrograms per liter without correction for recovery data. When duplicate and spiked samples are analyzed, report all data obtained with the sample results.

15.3 If the surrogate standard recovery falls outside the control limits in Section 8.3, data for all parameters in that fraction of the sample must be labeled as suspect.

16. Method Performance

16.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with a 99% confidence that the value is above zero[11]. The MDL concentrations listed in Tables 4 and 5 were obtained using reagent water[13].

16.2 The average recoveries and the average standard deviations of the percent recoveries, presented in Table 5, were the result of a study of the accuracy and precision of this method by several laboratories. The values listed represent the results from two to four laboratories[14].

16.3 The U.S. Environmental Protection Agency is in the process of conducting an interlaboratory method study to fully define the performance of this method.

17. Screening Procedure for 2,3,7,8-TCDD

17.1 If the sample must be screened for the presence of 2,3,7,8-TCDD, it is recommended that the reference material not be handled in the laboratory unless extensive safety precautions are employed. It is sufficient to analyze the base/neutral extract by selected ion monitoring (SIM) GC/MS techniques, as follows:

17.1.1 Concentrate the base/neutral extract to a final volume of 0.2 mL.

17.1.2 Adjust the temperature of the base/neutral column (Section 5.6.2) to 220 °C.

17.1.3 Operate the mass spectrometer to acquire data in the SIM mode using the ions at m/e 257, 320 and 322 and a dwell time no greater than 333 milliseconds per ion.

17.1.4 Inject 5 to 7 µL of the base/neutral extract. Collect SIM data for a total of 10 minutes.

17.1.5 The possible presence of 2,3,7,8-TCDD is indicated if all three ions exhibit simultaneous peaks at any point in the selected ion current profiles.

17.1.6 For each occurrence where the possible presence of 2,3,7,8-TCDD is indicated, calculate and retain the relative abundances of each of the three ions.

17.2 False positives to this test may be caused by the presence of single or coeluting combinations of compounds whose mass spectra contain all of these ions.

17.3 Conclusive results of the presence and concentration level of 2,3,7,8-TCDD can be obtained only from a properly equipped laboratory through the use of method 613 of other approved alternate test procedures.

376

References

1 See Appendix A

2. "Sampling and Analysis Procedures for Screening of Industrial Effluents for Priority Pollutants." U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1977, Revised April 1977. Available from Effluent Guidelines Division, Washington, DC 20460.

3. ASTM Annual Book of Standards, Part 31, D 3694. "Standard Practice for Preparation of Sample Containers and for Preservation," American Society for Testing and Materials, Philadelphia, PA, p. 679, (1980).

4. "Carcinogens—Working with Carcinogens," Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health, Publication No. 77-206, Aug. 1977.

5. "OSHA Safety and Health Standards, General Industry," (29CFR1910), Occupational Safety and Health Administration, OSHA 2206, (Revised, January 1976).

6. "Safety in Academic Chemistry Laboratories," American Chemical Society Publication, Committee on Chemical Safety, 3rd Edition, 1979.

7. "Handbook of Analytical Quality Control in Water and Wastewater Laboratories," EPA-600/4-79-019, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268 March 1979.

8. ASTM Annual Book of Standards, Part 31, D 3370, "Standard Practice for Sampling Water," American Society for Testing and Materials, Philadelphia, PA, p. 76, 1980.

9. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine, Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA 600-4/79-02U, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.

10. Eichelberger, J.W., Harris, L.E., and Budde, W.L., "Reference Compound to Calibrate Ion Abundance Measurement in Gas Chromatography-Mass Spectrometry," *Analytical Chemistry*, 47, 995 (1975).

11. McNair, H.M. and Bonelli, E.J., "Basic Chromatography," Consolidated Printing, Berkeley, California, p. 52, 1969.

12. Burke, J.A., "Gas Chromatography for Pesticide Residue Analysis; Some Practical Aspects," *Journal of the Association of Official Analytical Chemists*, *48*, 1037 (1965).

13. "Method Detection Limit for Methods 624 and 625," Olynyk, P., Budde, W.L., Eichelberger, J.W., unpublished report October, 1980.

14. Kloepfer, R.D., "POTW Toxic Study, Analytical Quality Assurance Final Report," U.S. Environmental Protection Agency, Region VII, Kansas City, Kansas 66115, 1981.

Table 1. Base/Neutral Extractables

Parameter	STORET No.	CAS No.
Acenaphthene	34205	83-32-9
Acenaphthylene	34200	208-96-8
Anthracene	34220	120-12-7
Aldrin	39330	309-00-2
Benzo(a)anthracene	34526	56-55-3
Benzo(b)fluoranthene	34230	205-99-2
Benzo(k)fluoranthene	34242	207-08-9
Benzo(a)pyrene	34247	50-32-8
Benzo(ghi)perylene	34521	191-24-2
Benzyl butyl phthalate	34292	85-68-7
β-BHC	39338	319-85-7
δ-BHC	34259	319-86-8
Bis(2-chloroethyl)ether	34273	111-44-4
Bis(2-chloroethoxy)methane	34278	111-91-1
Bis(2-ethylhexyl)phthalate	39100	117-81-7
Bis(2-chloroisopropyl)ether	34283	108-60-1
4-Bromophenyl phenyl ether	34636	101-55-3
Chlordane	39350	57-74-9
2-Chloronaphthalene	34581	91-58-7
4-Chlorophenyl phenyl ether	34641	7005-72-3
Chrysene	34320	218-01-9
4,4'-DDD	39310	72-54-8
4,4'-DDE	39320	72-55-9
4,4'-DDT	39300	50-29-3
Dibenzo(a,h)anthracene	34556	53-70-3
Di-n-butylphthalate	39110	84-74-2
1,3-Dichlorobenzene	34566	541-73-1
1,2-Dichlorobenzene	34536	95-50-1
1,4-Dichlorobenzene	34571	106-46-7
3,3'-Dichlorobenzidine	34631	91-94-1
Dieldrin	39380	60-57-1
Diethyl phthalate	34336	84-66-2
Dimethyl phthalate	34341	131-11-3
2,4-Dinitrotoluene	34611	121-14-2
2,6-Dinitrotoluene	34626	606-20-2
Di-n-octylphthalate	34596	117-84-0
Endosulfan sulfate	34351	1031-07-8
Endrin aldehyde	34366	7421-93-4
Fluoranthene	34376	206-44-0
Fluorene	34381	86-73-7
Heptachlor	39410	76-44-8
Heptachlor epoxide	39420	1024-57-3
Hexachlorobenzene	39700	118-74-1
Hexachlorobutadiene	34391	87-68-3
Hexachloroethane	34396	67-72-1
Indeno(1,2,3-cd)pyrene	34403	193-39-5
Isophorone	34408	78-59-1
Naphthalene	34696	91-20-3
Nitrobenzene	34447	98-95-3
N-Nitrosodi-n-propylamine	34428	621-64-7
PCB-1016	34671	12674-11-2
PCB-1221	39488	11104-28-2
PCB-1232	39492	11141-16-5
PCB-1242	39496	53469-21-9
PCB-1248	39500	12672-29-6
PCB-1254	39504	11097-69-1
PCB-1260	39508	11096-82-5
Phenanthrene	34461	85-01-8
Pyrene	34469	129-00-0
Toxaphene	39400	8001-35-2
1,2,4-Trichlorobenzene	34551	120-82-1

Table 2. *Acid Extractables*

Parameter	STORET No.	CAS No.
4-Chloro-3-methylphenol	34452	59-50-7
2-Chlorophenol	34586	95-57-8
2,4-Dichlorophenol	34601	120-83-2
2,4-Dimethylphenol	34606	105-67-9
2,4-Dinitrophenol	34616	51-28-5
2-Methyl-4,6-dinitrophenol	34657	534-52-1
2-Nitrophenol	34591	88-75-5
4-Nitrophenol	34646	100-02-7
Pentachlorophenol	39032	87-86-5
Phenol	34694	108-95-2
2,4,6-Trichlorophenol	34621	88-06-2

Table 3. *Additional Extractable Parameters*[a]

Parameter	STORET No.	CAS No.	Method
Benzidine	39120	92-87-5	605
α-BHC	39337	319-84-6	608
γ-BHC	39340	58-89-8	608
Endosulfan I	34361	959-98-8	608
Endosulfan II	34356	33213-65-9	608
Endrin	39390	72-20-8	608
Hexachlorocyclopentadiene	34386	77-47-4	612
N-Nitrosodimethylamine	34438	62-75-9	605
N-Nitrosodiphenylamine	34433	86-30-6	605

[a]See Section 1.2 of method

Table 4. *Chromatographic Conditions, Method Detection Limits and Characteristic Ions for Base/Neutral Extractables*

Parameter	Retention Time (min.)	Method Detection Limit (µg/L)	Characteristic Ions: Electron Impact, Primary	Electron Impact, Secondary		Chemical Ionization (Methane)		
1,3-Dichlorobenzene	7.4	1.9	146	148	113	146	148	150
1,4-Dichlorobenzene	7.8	4.4	146	148	113	146	148	150
Hexachloroethane	8.4	1.6	117	201	199	199	201	203
Bis(2-chloroethyl)ether	8.4	5.7	93	63	95	63	107	109
1,2-Dichlorobenzene	8.4	1.9	146	148	113	146	148	150
Bis(2-chloroisopropyl)ether	9.3	5.7	45	77	79	77	135	137
N-Nitrosodi-n-propyl amine			130	42	101			
Nitrobenzene	11.1	1.9	77	123	65	124	152	164
Hexachlorobutadiene	11.4	0.9	225	223	227	223	225	227
1,2,4-Trichlorobenzene	11.6	1.9	180	182	145	181	183	209
Isophorone	11.9	2.2	82	95	138	139	167	178
Naphthalene	12.1	1.6	128	129	127	129	157	169
Bis(2-chloroethoxy)methane	12.2	5.3	93	95	123	65	107	137
Hexachlorocyclopentadiene*	13.9		237	235	272	235	237	239
2-Chloronaphthalene	15.9	1.9	162	164	127	163	191	203
Acenaphthylene	17.4	3.5	152	151	153	152	153	181
Acenaphthene	17.8	1.9	154	153	152	154	155	183
Dimethyl phthalate	18.3	1.6	163	194	164	151	163	164
2,6-Dinitrotoluene	18.7	1.9	165	89	121	183	211	223
Fluorene	19.5	1.9	166	165	167	166	167	195
4-Chlorophenyl phenyl ether	19.5	4.2	204	206	141			
2,4-Dinitrotoluene	19.8	5.7	165	63	182	183	211	223
Diethylphthalate	20.1	22	149	177	150	177	223	251
N-Nitrosodiphenylamine*	20.5	1.9	169	168	167	169	170	198
Hexachlorobenzene	21.0	1.9	284	142	249	284	286	288
α-BHC*	21.1		183	181	109			
4-Bromophenyl phenyl ether	21.2	1.9	248	250	141	249	251	277
γ-BHC*	22.4		183	181	109			
Phenanthrene	22.8	5.4	178	179	176	178	179	207
Anthracene	22.8	1.9	178	179	176	178	179	207
β-BHC	23.4	4.2	181	183	109			
Heptachlor	23.4	1.9	100	272	274			
δ-BHC	23.7	3.1	183	109	181			
Aldrin	24.0	1.9	66	263	220			

Table 4. *(Continued)*

Parameter	Retention Time (min.)	Method Detection Limit (µg/L)	Characteristic Ions: Electron Impact, Primary	Electron Impact, Secondary		Chemical Ionization (Methane)		
Dibutyl phthalate	24.7	2.5	149	150	104	149	205	279
Heptachlor epoxide	25.6	2.2	353	355	351			
Endosulfan I*	26.4	—	237	339	341			
Fluoranthene	26.5	2.2	202	101	100	203	231	243
Dieldrin	27.2	2.5	79	263	279			
4,4'-DDE	27.2	5.6	246	248	176			
Pyrene	27.3	1.9	202	101	100	203	231	243
Endrin*	27.9	—	81	263	82			
Endosulfan II*	28.6	—	237	339	341			
4,4'-DDD	28.6	2.8	235	237	165			
Benzidine*	28.8	44	184	92	185	185	213	225
4,4'-DDT	29.3	4.7	235	237	165			
Endosulfan sulfate	29.8	5.6	272	387	422			
Endrin aldehyde	—	—	67	345	250			
Butyl benzyl phthalate	29.9	2.5	149	91	206	149	299	327
Bis(2-ethylhexyl) phthalate	30.6	2.5	149	167	279	149		
Chrysene	31.5	2.5	228	226	229	228	229	257
Benzo(a)anthracene	31.5	7.8	228	229	226	228	229	257
3,3'-Dichlorobenzidine	32.2	16.5	252	254	126			
Di-n-octylphthalate	32.5	2.5	149					
Benzo(b)fluoranthene	34.9	4.8	252	253	125	252	253	281
Benzo(k)fluoranthene	34.9	2.5	252	253	125	252	253	281
Benzo(a)pyrene	36.4	2.5	252	253	125	252	253	281
Indeno(1,2,3-c,d)pyrene	42.7	3.7	276	138	277	276	277	305
Dibenzo(a,h)anthracene	43.2	2.5	278	139	279	278	279	307
Benzo(ghi)perylene	45.1	4.1	276	138	277	276	277	305
N-Nitrosodimethyl amine*	—	—	42	74	44			
Chlordane**	19 to 30	—	373	375	377			
Toxaphene**	25 to 34	—	159	231	233			
PCB 1016**	18 to 30	—	224	260	294			
PCB 1221**	15 to 30	30	190	224	260			
PCB 1232**	15 to 32	—	190	224	260			
PCB 1242**	15 to 32	—	224	260	294			
PCB 1248**	12 to 34	—	234	330	362			
PCB 1254**	22 to 34	36	294	330	362			
PCB 1260**	23 to 32	—	330	362	394			

*See Section 1.2.

**These compounds are mixtures of various isomers. (See Figures 2 to 12)

Gas Chromatographic conditions: Glass column 1.8 m long × 2 mm ID packed with Supelcoport (100/120 mesh) coated with 3% SP-2250. Carrier gas: helium at a flow rate of 30 mL per min.
Temperature: Isothermal at 50°C for 4 min., then 8° per min to 270°C. Hold at 270°C for 30 min.

Table 5. Chromatographic Conditions, Method Detection Limits and Characteristic Ions for Acid Extractables

Parameter	Retention Time (min.)	Method Detection Limit (µg/L)	Characteristic Ions: Electron Impact, Primary	Electron Impact, Secondary		Chemical Ionization (Methane)		
2-Chlorophenol	5.9	3.3	128	64	130	129	131	157
2-Nitrophenol	6.5	3.6	139	55	109	140	168	122
Phenol	8.0	1.5	94	65	66	95	123	135
2,4-Dimethylphenol	9.4	2.7	122	107	121	123	151	163
2,4-Dichlorophenol	9.8	2.7	162	164	98	163	165	167
2,4,6-Trichlorophenol	11.8	2.7	196	198	200	197	199	201
4-Chloro-3-methylphenol	13.2	3.0	142	107	144	143	171	183
2,4-Dinitrophenol	15.9	42	184	63	154	185	213	225
2-Methyl-4,6-dinitrophenol	16.2	24	198	182	77	199	227	239
Pentachlorophenol	17.5	3.6	266	264	268	267	265	269
4-Nitrophenol	20.3	2.4	65	139	109	140	168	122

Chromatographic conditions: 1.8 m long × 2 mm ID glass column packed with Supelcoport (100/120 mesh) coated with 1% SP-1240. Carrier gas: helium at a flow rate of 30 mL per min. Column temperature, isothermal at 70°C for 2 min, then 8° per min, to 200°.

Table 6. Accuracy and Precision for Base/Neutral Extractables

Parameter	Reagent Water		Wastewater	
	Average Percent Recovery	Standard Deviation (%)	Average Percent Recovery	Standard Deviation (%)
Acenaphthene	77	23	83	29
Acenaphthylene	78	22	82	23
Aldrin	72	6	—	—
Anthracene	84	14	76	22
Benzo(a)anthracene	83	19	75	28
Benzo(b)fluoranthene	96	68	41	21
Benzo(k)fluoranthene	96	68	47	27
Benzo(ghi)perylene	80	45	68	40
Benzo(a)pyrene	90	22	43	21
Benzidine	87	61	63	55
Butyl benzyl phthalate	47	32	74	43
β-BHC	69	25	—	—
δ-BHC	56	18	—	—
Bis(2-chloroethoxy)methane	84	33	82	74
Bis(2-chloroethyl)ether	56	36	72	37
Bis(2-chloroisopropyl)ether	71	33	71	39
Bis (2-ethylhexyl) phthalate	129	50	82	63
4-Bromophenyl phenyl ether	80	17	75	20
2-Chloronaphthalene	73	24	79	27
4-Chlorophenyl phenyl ether	45	11	—	—
Chrysene	83	19	75	28
4,4'-DDD	80	9	—	—
4,4'-DDE	69	20	—	—
4,4'-DDT	63	15	—	—
Dibenzo(a,h)anthracene	82	39	70	40
Di-n-butyl phthalate	70	25	93	51
1,2-Dichlorobenzene	59	27	62	28
1,3-Dichlorobenzene	55	28	54	24
1,4-Dichlorobenzene	61	31	63	35
3,3'-Dichlorobenzidine	184	174	143	145
Diethylphthalate	42	28	43	28
Dimethyl phthalate	25	33	35	36
2,4-Dinitrotoluene	83	32	79	34
2,6-Dinitrotoluene	79	18	79	25
Di-n-octylphthalate	97	37	89	62
Endosulfan sulfate	79	29	—	—
Fluoranthene	89	19	80	26
Fluorene	77	16	80	20
Heptachlor	69	6	—	—
Heptachlor epoxide	82	7	—	—
Hexachlorobenzene	79	20	71	22
Hexachlorobutadiene	46	25	48	28
Hexachlorocyclopentadiene	27	25	12	12
Hexachloroethane	46	21	52	26
Indeno (1,2,3-cd) pyrene	65	37	81	43
Isophorone	75	33	77	42
Naphthalene	6	32	75	35
Nitrobenzene	72	31	82	54
N-Nitrosodi-n-propylamine	68	39	76	45
N-Nitrosodiphenylamine	84	24	86	31
PCB-1221	77	11	—	—
PCB-1254	80	13	—	—
Phenanthrene	84	14	76	22
Pyrene	86	15	80	23
1,2,4-Trichlorobenzene	64	16	69	26

Spiked between 5 to 2400 μg/L.

Table 7. Accuracy and Precision for Acid Extractables

Parameter	Reagent Water Average Percent Recovery	Reagent Water Standard Deviation (%)	Wastewater Average Percent Recovery	Wastewater Standard Deviation (%)
4-Chloro-3-methylphenol	79	18	75	21
2-Chlorophenol	70	23	71	25
2,4-Dichlorophenol	74	24	80	21
2,4-Dimethylphenol	64	25	58	26
2,4-Dinitrophenol	78	21	108	56
2-Methyl-4,6-dinitrophenol	83	18	90	35
4-Nitrophenol	41	20	43	16
2-Nitrophenol	75	25	75	27
Pentachlorophenol	86	20	66	36
Phenol	36	14	36	21
2,4,6-Trichlorophenol	77	20	81	20

Spiked from 10 to 1500 µg/L.

Table 8. Suggested Internal and Surrogate Standards

Base/Neutral Fraction	Acid Fraction
Aniline-d_5	2-Fluorophenol
Anthracene-d_{10}	Pentafluorophenol
Benzo(a)anthracene-d_{12}	Phenol-d_5
4,4'-Dibromobiphenyl	2-Perfluoromethyl phenol
4,4'-Dibromooctafluorobiphenyl	
Decafluorobiphenyl	
2,2'-Difluorobiphenyl	
4-Fluoroaniline	
1-Fluoronaphthylene	
2-Fluoronaphthylene	
Naphthalene-d_8	
Nitrobenzene-d_5	
2,3,4,5,6-Pentafluorobiphenyl	
Phenanthrene-d_{10}	
Pyridine-d_5	

Table 9. DFTPP Key Ions and Ion Abundance Criteria

Mass	Ion Abundance Criteria
51	30-60% of mass 198
68	less than 2% of mass 69
70	less than 2% of mass 69
127	40-60% of mass 198
197	less than 1% of mass 198
198	base peak, 100% relative abundance
199	5-9% of mass 198
275	10-30% of mass 198
365	greater than 1% of mass 198
441	present but less than mass 443
442	greater than 40% of mass 198
443	17-23% of mass 442

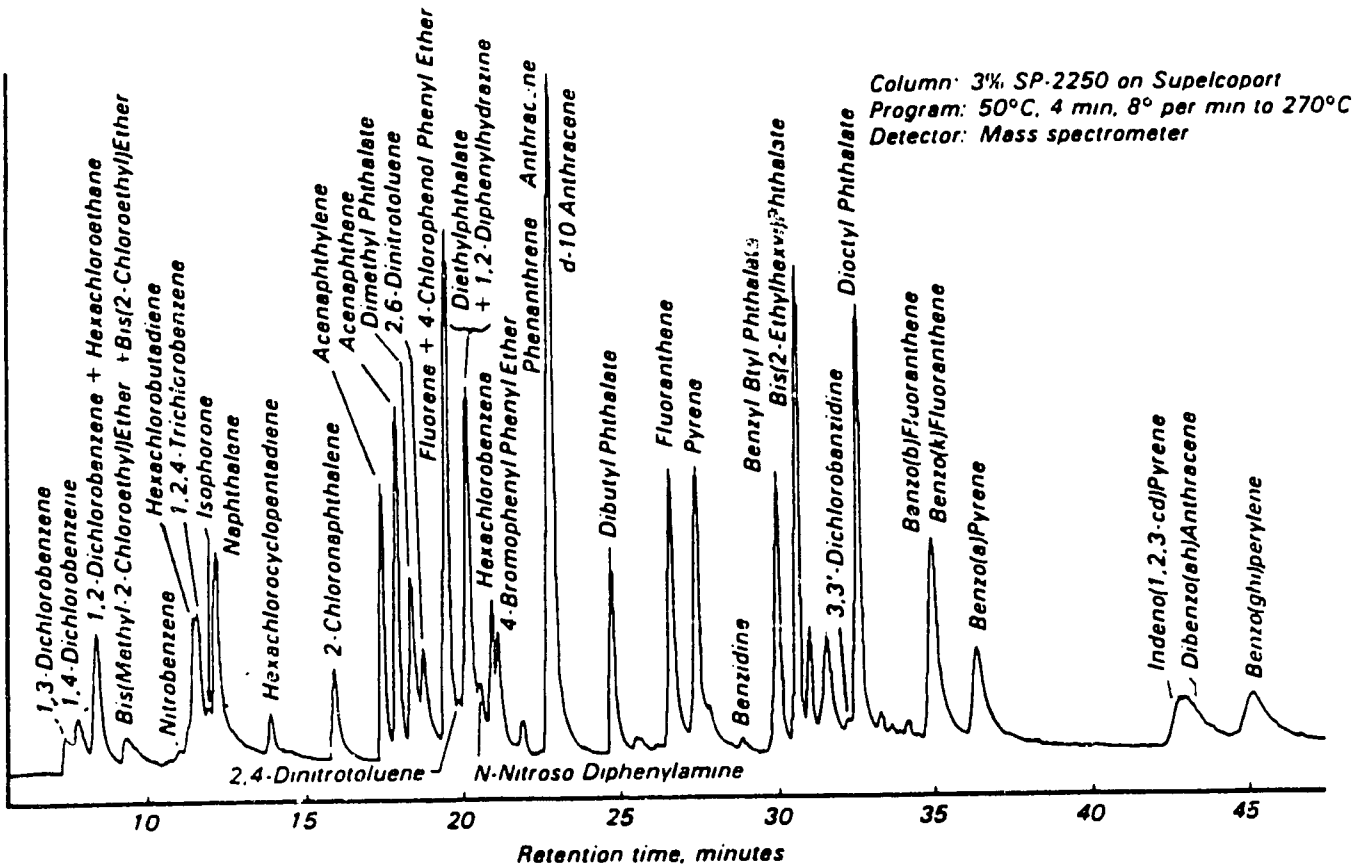


Figure 1. *Gas chromatogram of base/neutral fraction.*

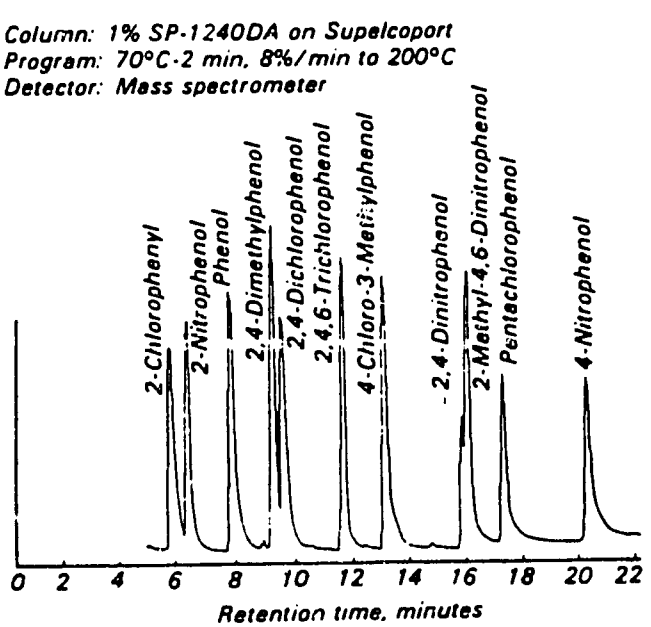


Figure 2. *Gas chromatogram of acid fraction.*

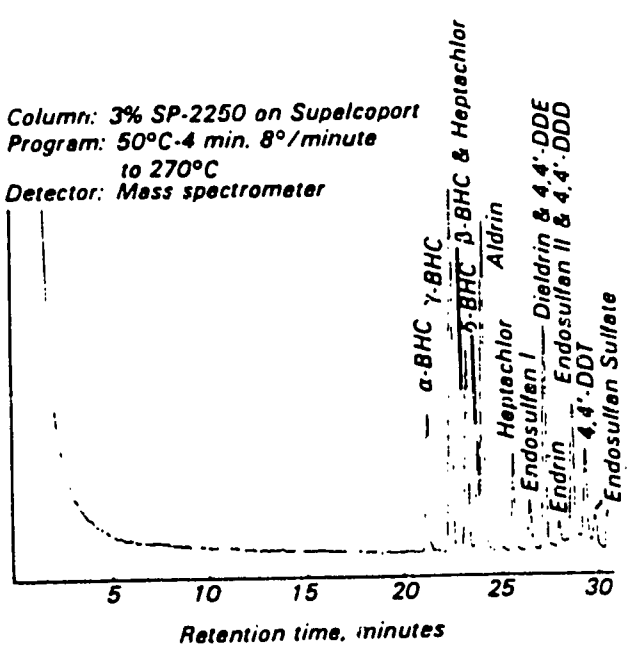


Figure 3. *Gas chromatogram of pesticide fraction.*

Column 3% SP-2250 on Supelcoport
Program 50°C 4 min., 8° per min. to 270°C.
Detector Mass spectrometer

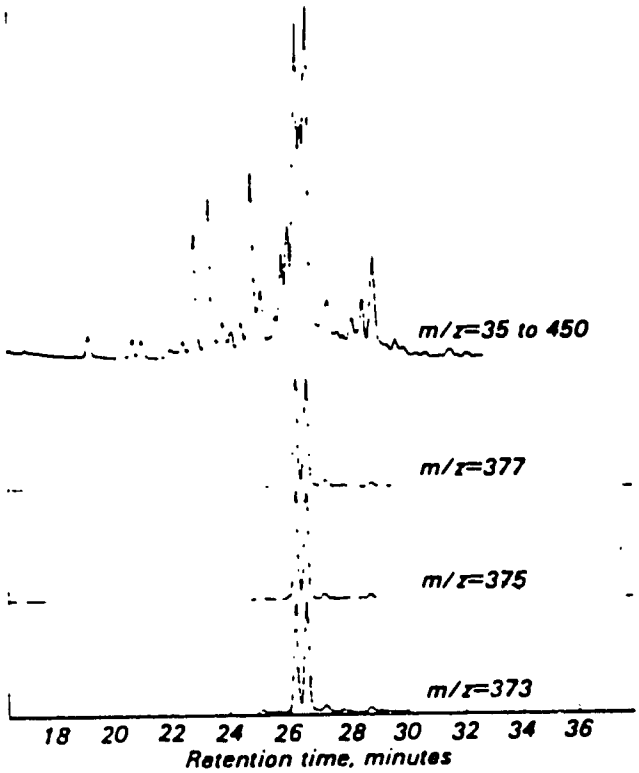


Figure 4. *Gas chromatogram of chlordane.*

Column: 3% SP-2250 on Supelcoport
Program 50°C. 4 min., 8° per min. to 270°C.
Detector: Mass spectrometer

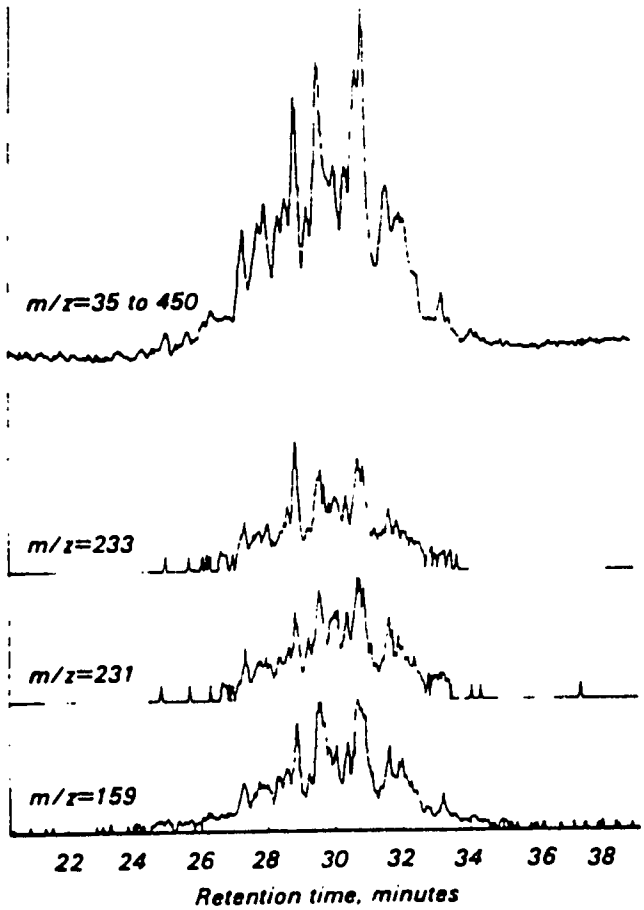


Figure 5. *Gas chromatogram of toxaphene.*

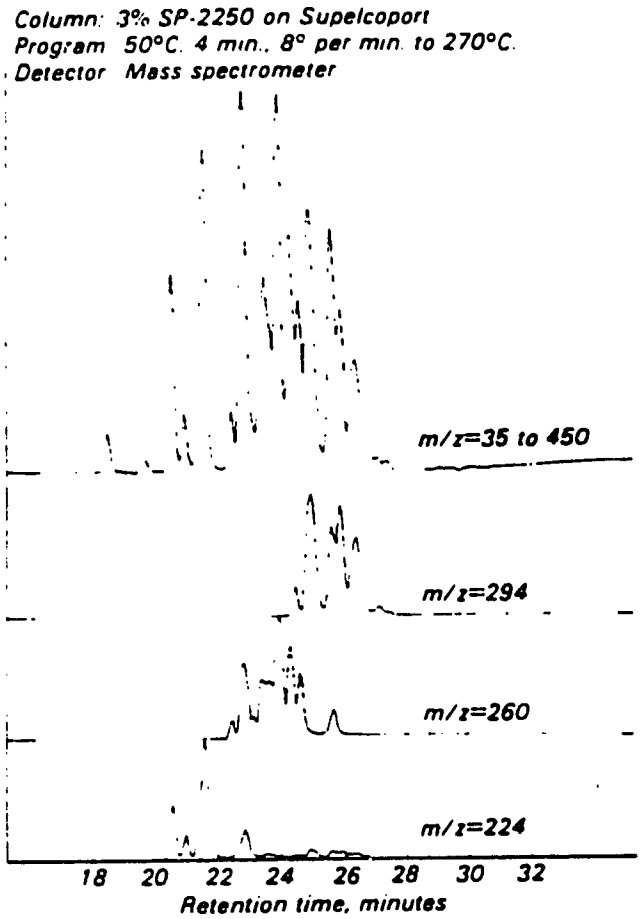


Figure 6. *Gas chromatogram of PCB-1016.*

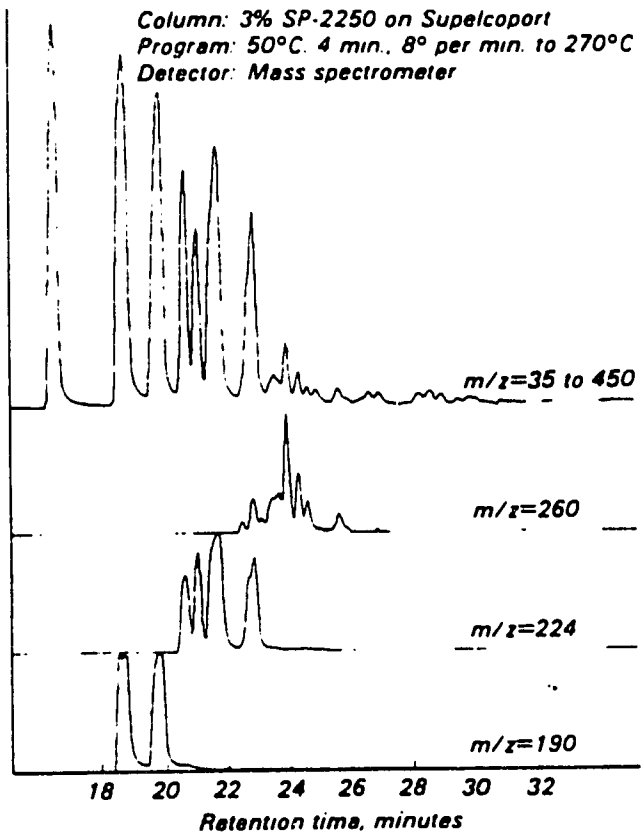


Figure 7. *Gas chromatogram of PCB-1221.*

385

Column 3% SP-2250 on Supelcoport
Program: 50°C. 4 min., 8° per min. to 270°C.
Detector Mass spectrometer

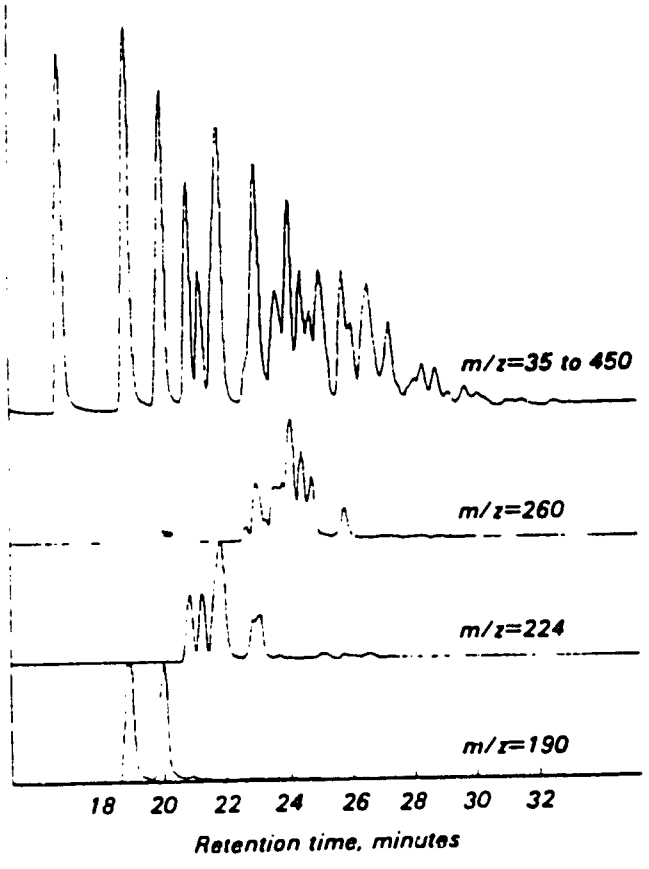


Figure 8. *Gas chromatogram of PCB-1232.*

Column: 3% SP-2250 on Supelcoport
Program. 50°C. 4 min., 8° per min. to 270°C
Detector: Mass spectrometer

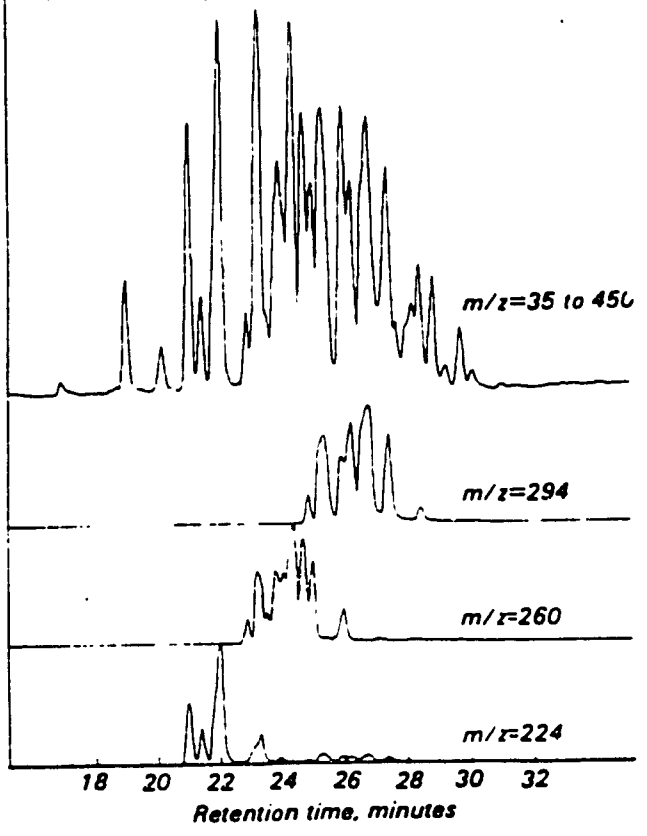


Figure 9. *Gas chromatogram of PCB-1242.*

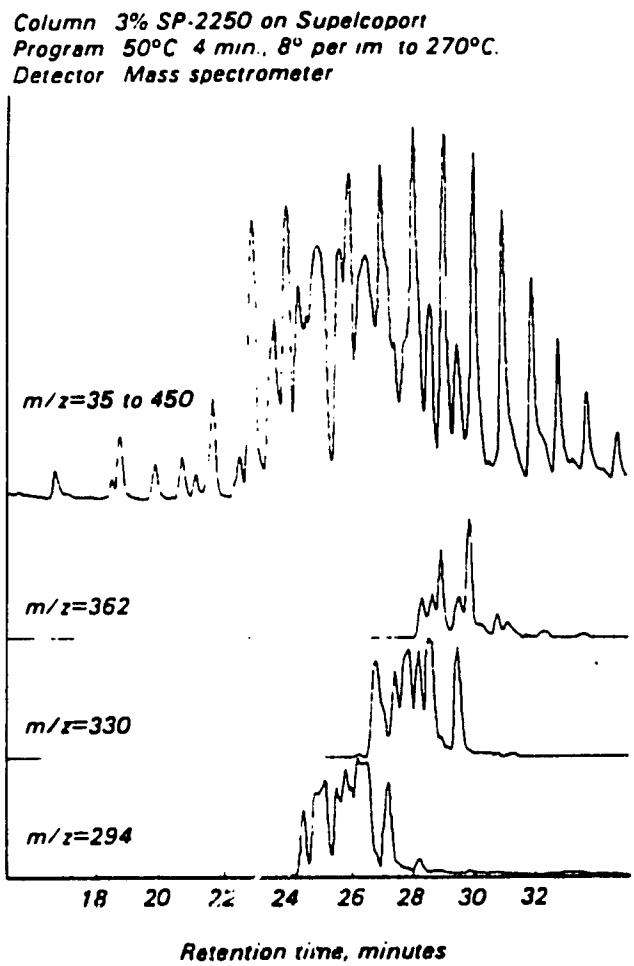


Figure 10. *Gas chromatogram of PCB-1248.*

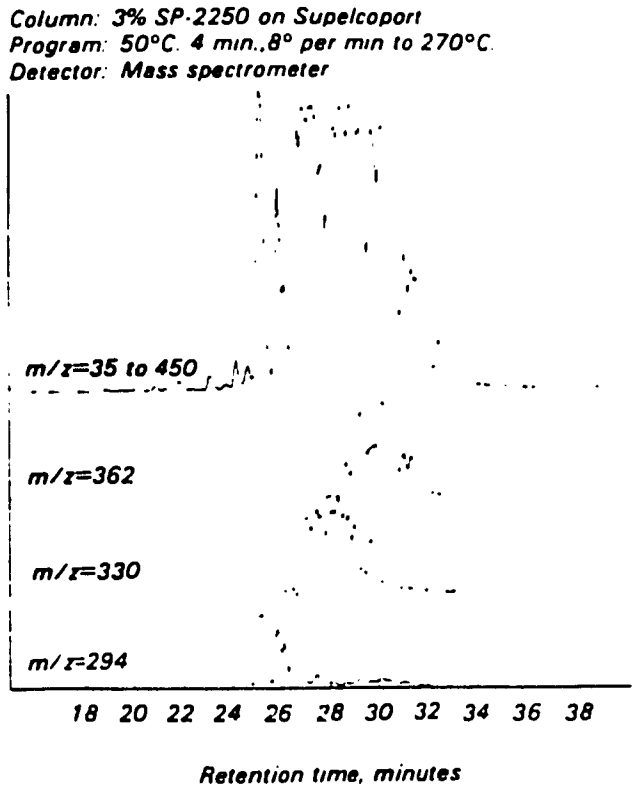


Figure 11. *Gas chromatogram of PCB-1254.*

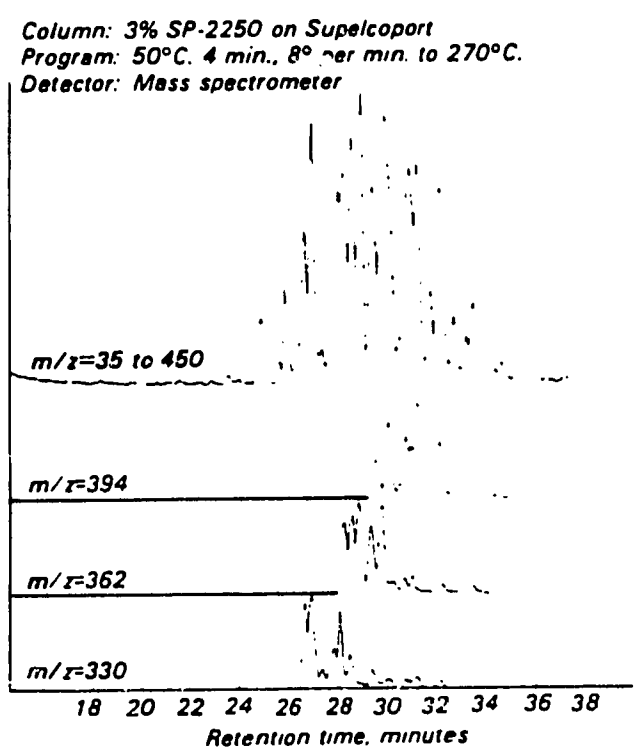
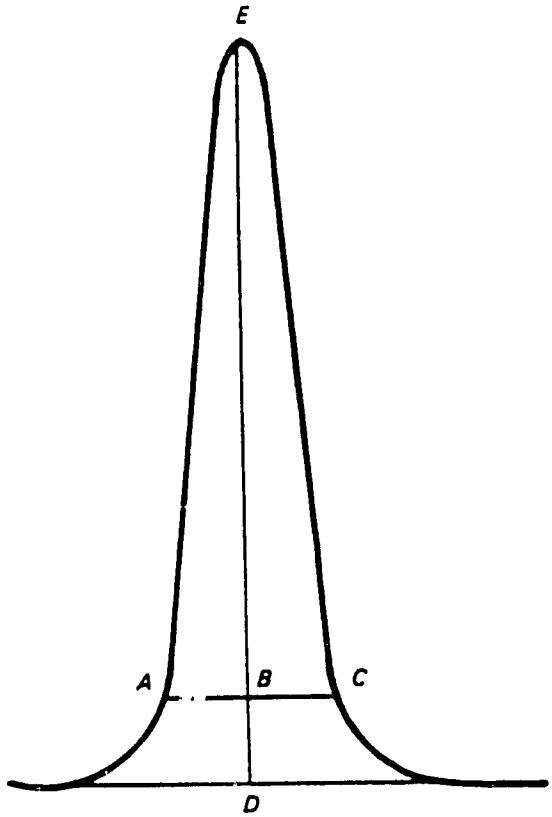


Figure 12. *Gs chromatogram of PCB-1260.*



$$\text{Tailing Factor} = \frac{BC}{AB}$$

Example calculation:

Peak Height = DE = 100mm
10% Peak Height = BD = 10 mm
Peak Width at 10% Peak Height = AC = 23 mm
AB = 11 mm
BC = 12 mm

Therefore: Tailing Factor $= \frac{12}{11} = 1.1$

Figure 13. *Tailing factor calculation.*

Appendix A

Definition and Procedure for the Determination of the Method Detection Limit

The method detection limit (MDL) is defined as the minimum concentration of a substance that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero and determined from analysis of a sample in a given matrix containing analyte

Scope and Application

This procedure is designed for applicability to a wide variety of sample types ranging from reagent (blank) water containing analyte to wastewater containing analyte. The MDL for an analytical procedure may vary as a function of sample type The procedure requires a complete, specific and well defined analytical method. It is essential that all sample processing steps of the analytical method be included in the determination of the method detection limit

The MDL obtained by this procedure is used to judge the significance of a single measurement of a future sample

The MDL procedure was designed for applicability to a broad variety of physical and chemical methods. To accomplish this, the procedure was made device- or instrument-independent.

Procedure

1 Make an estimate of the detection limit using one of the following

(a) The concentration value that corresponds to an instrument signal/noise ratio in the range of 2.5 to 5 If the criteria for qualitative identification of the analyte is based upon pattern recognition techniques, the least abundant signal necessary to achieve identification must be considered in making the estimate

(b) The concentration value that corresponds to three times the standard deviation of replicate instrumental measurements for the analyte in reagent water

(c) The concentration value that corresponds to the region of the standard curve where there is a significant change in sensitivity at low analyte concentrations, i e , a break in the slope of the standard curve

(d) The concentration value that corresponds to known instrumental limitations

It is recognized that the experience of the analyst is important to this process However, the analyst must include the above considerations in the estimate of the detection limit

2. Prepare reagent (blank) water that is as free of analyte as possible. Reagent or interference free water is defined as a water sample in which analyte and interferent concentrations are not detected at the method detection limit of each analyte of interest. Interferences are defined as systematic errors in the measured analytical signal of an established procedure caused by the presence of interfering species (interferent). The interferent concentration is presupposed to be normally distributed in representative samples of a given matrix.

3. (a) If the MDL is to be determined in reagent water (blank), prepare a laboratory standard (analyte in reagent water) at a concentration which is at least equal to or in the same concentration range as the estimated MDL. (Recommend between 1 and 5 times the estimated MDL) Proceed to Step 4

38

(b) If the MDL is to be determined in another sample matrix, analyze the sample. If the measured level of the analyte is in the recommended range of one to five times the estimated MDL, proceed to Step 4.

If the measured concentration of analyte is less than the estimated MDL, add a known amount of analyte to bring the concentration of analyte to between one and five times the MDL. In the case where an interference is coanalyzed with the analyte.

If the measured level of analyte is greater than five times the estimated MDL, there are two options.

(1) Obtain another sample of lower level of analyte in same matrix if possible.
(2) The sample may be used as is for determining the MDL if the analyte level does not exceed 10 times the MDL of the analyte in reagent water. The variance of the analytical method changes as the analyte concentration increases from the MDL, hence the MDL determined under these circumstances may not truly reflect method variance at lower analyte concentrations.

4. (a) Take a minimum of seven aliquots of the sample to be used to calculate the MDL and process each through the entire analytical method. Make all computations according to the defined method with final results in the method reporting units. If blank measurements are required to calculate the measured level of analyte, obtain separate blank measurements for each sample aliquot analyzed. The average blank measurement is subtracted from the respective sample measurements.

(b) It may be economically and technically desirable to evaluate the estimated MDL before proceeding with 4a. This will: (1) prevent repeating this entire procedure when the costs of analyses are high and (2) insure that the procedure is being conducted at the correct concentration. It is quite possible that an incorrect MDL can be calculated from data obtained at many times the real MDL even though the background concentration of analyte is less than five times the calculated MDL. To insure that the estimate of the MDL is a good estimate, it is necessary to determine that a lower concentration of analyte will not result in a significantly lower MDL. Take two aliquots of the sample to be used to calculate the MDL and process each through the entire method, including blank measurements as described above in 4a. Evaluate these data:

(1) If these measurements indicate the sample is in the desirable range for determining the MDL, take five additional aliquots and proceed. Use all seven measurements to calculate the MDL.
(2) If these measurements indicate the sample is not in the correct range, reestimate the MDL, obtain new sample as in 3 and repeat either 4a or 4b.

5. Calculate the variance (S^2) and standard deviation (S) of the replicate measurements, as follows:

$$S^2 = \frac{1}{n-1}\left[\sum_{i=1}^{n} X_i^2 - \left(\sum_{i=1}^{n} X_i\right)^2 \Big/ n\right]$$

$$S = (S^2)^{1/2}$$

where: the x_i, $i = 1$ to n are the analytical results in the final method reporting units obtained from the n sample aliquots and $\sum_{i=1}^{n} X_i^2$ refers to the sum of the X values from $i = 1$ to n.

6. (a) Compute the MDL as follows:

$$MDL = t_{(n-1,\ 1-\alpha = .99)}(S)$$

where

MDL = the method detection

$t_{(n-1, 1-\alpha = .99)}$ = the students' t value appropriate for a 99% confidence level and a standard deviation estimate with n-1 degrees of freedom. See Table.

S = standard deviation of the replicate analyses.

(b) The 95% confidence limits for the MDL derived in 6a are computed according to the following equations derived from percentiles of the chi square over degrees of freedom distribution (X^2/df) and calculated as follows:

$$MDL_{LCL} = 0.69\ MDL$$
$$MDL_{UCL} = 1.92\ MDL$$

where MDL_{LCL} and MDL_{UCL} are the lower and upper 95% confidence limits respectively based on seven aliquots.

7 Optional iterative procedure to verify the reasonableness of the estimated MDL and calculated MDL of subsequent MDL determinations

(a) If this is the initial attempt to compute MDL based on the estimated MDL in Step 1, take the MDL as calculated in Step 6, spike in the matrix at the calculated MDL and proceed through the procedure starting with Step 4.

(b) If the current MDL determination is an iteration of the MDL procedure for which the spiking level does not permit qualitative identification, report the MDL as that concentration between the current spike level and the previous spike level which allows qualitative identification.

(c) If the current MDL determination is an iteration of the MDL procedure and the spiking level allows qualitative identification, use S^2 from the current MDL calculation and S^2 from the previous MDL calculation to compute the F ratio.

$$\text{if } \frac{S_A^2}{S_B^2} < 3.05$$

then compute the pooled standard deviation by the following equation:

$$S_{pooled} = \left[\frac{6S_A^2 + 6S_B^2}{12}\right]^{1/2}$$

if $\frac{S_A^2}{S_B^2} > 3.05$, respike at the last calculated MDL and process the samples through the procedure starting with Step 4.

(c) Use the S_{pooled} as calculated in 7b to compute the final MDL according to the following equation:

$$MDL = 2.681\ (S_{pooled})$$

where 2.681 is equal to $t_{(12,\ 1-\alpha = .99)}$.

(d) The 95% confidence limits for MDL derived in 7c are computed according to the following equations derived from percentiles of the chi squared over degrees of freedom distribution.

$$MDL_{LCL} = 0.72\ MDL$$
$$MDL_{UCL} = 1.65\ MDL$$

where LCL and UCL are the lower and upper 95% confidence limits respectively based on 14 aliquots.

Reporting

The analytical method used must be specifically identified by number or title and the MDL for each analyte expressed in the appropriate method reporting units. If the analytical method permits options which affect the method detection limit, these conditions must be specified with the MDL value. The sample matrix used to

determine the MDL must also be identified with the MDL value. Report the mean analyte level with the MDL. If a laboratory standard or a sample that contained a known amount analyte was used for this determinatic .. report the mean recovery, and indicate if the MDL determination was iterated.

If the level of the analyte in the sample matrix exceeds 10 times the MDL of the analyte in reagent water, do not report a value for the MDL.

Reference

Glaser, J. A., Foerst, D. L., McKee, G. D., Quave, S. A., and Budde, W. L., "Trace Analysis for Wastewaters," *Environmental Science and Technology, 15,* 1426 (1981).

Table of Students' t Values at the 99 Percent Confidence Level

Number of Replicates	*Degrees of Freedom (n-1)*	$t_{(n-1,\ 1-\alpha\ =\ .99)}$
7	6	3.143
8	7	2.998
9	8	2.896
10	9	2.821
11	10	2.764
16	15	2.602
21	20	2.528
26	25	2.485
31	30	2.457
61	60	2.390
∞	∞	2.326

• U.S. GOVERNMENT PRINTING OFFICE 1982-559-092/0463

796

EPA-600/4-79-019

HANDBOOK FOR ANALYTICAL QUALITY CONTROL IN WATER AND WASTEWATER LABORATORIES

March 1979

ENVIRONMENTAL MONITORING AND SUPPORT LABORATORY
U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
CINCINNATI, OHIO 45268

393

DISCLAIMER

The mention of trade names or commercial products in this handbook is for illustration purposes and does not constitute endorsement or recommendation for use by the U.S. Environmental Protection Agency.

394

ABSTRACT

One of the fundamental responsibilities of water and wastewater management is the establishment of continuing programs to insure the reliability and validity of analytical laboratory and field data gathered in water treatment and wastewater pollution control activities.

This handbook is addressed to laboratory directors, leaders of field investigations, and other personnel who bear responsibility for water and wastewater data. Subject matter of the handbook is concerned primarily with quality control (QC) for chemical and biological tests and measurements. Chapters are also included on QC aspects of sampling, microbiology, biology, radiochemistry, and safety as they relate to water and wastewater pollution control. Sufficient information is offered to allow the reader to inaugurate or reinforce programs of analytical QC that emphasize early recognition, prevention, and correction of factors leading to breakdowns in the validity of water and wastewater pollution control data.

395

ACKNOWLEDGMENTS

This handbook was prepared by the Environmental Monitoring and Support Laboratory (EMSL) of the United States Environmental Protection Agency. The contributions of the following individuals in preparing the handbook are gratefully acknowledged:

J. B. Anderson
D. G. Ballinger
E. L. Berg
R. L. Booth
R. H. Bordner
P. W. Britton
J. F. Kopp
H. L. Krieger
J. J. Lichtenberg
L. B. Lobring
J. E. Longbottom
C. I. Weber
J. A. Winter

Technical editing and preparation of the final manuscript were performed under contract to John F. Holman & Co. Inc., 1346 Connecticut Avenue, N.W., Washington, D.C. 20036.

Inquiries regarding material contained in the handbook should be made to Environmental Protection Agency, Environmental Research Center, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

TABLE OF CONTENTS

		Page
	PREFACE	iii
	ACKNOWLEDGMENTS	v
Chapter		
1	IMPORTANCE OF QUALITY CONTROL	1-1
	1.1 General	1-1
	1.2 Quality Assurance Programs	1-1
	1.3 Analytical Methods	1-2
	1.4 Reference	1-4
2	LABORATORY SERVICES	2-1
	2.1 General	2-1
	2.2 Distilled Water	2-1
	2.3 Compressed Air	2-5
	2.4 Vacuum	2-5
	2.5 Hood System	2-5
	2.6 Electrical Services	2-6
	2.7 References	2-6
3	INSTRUMENT SELECTION	3-1
	3.1 Introduction	3-1
	3.2 Analytical Balances	3-1
	3.3 pH/Selective-Ion Meters	3-3
	3.4 Conductivity Meters	3-6
	3.5 Turbidimeters (Nephelometers)	3-7
	3.6 Spectrometers	3-8
	3.7 Organic Carbon Analyzers	3-13
	3.8 Gas Chromatographs	3-14
	3.9 References	3-14
4	GLASSWARE	4-1
	4.1 General	4-1
	4.2 Types of Glassware	4-2
	4.3 Volumetric Analyses	4-3
	4.4 Federal Specifications for Volumetric Glassware	4-4
	4.5 Cleaning of Glass and Porcelain	4-5
	4.6 Special Cleaning Requirements	4-6
	4.7 Disposable Glassware	4-7
	4.8 Specialized Glassware	4-7
	4.9 Fritted Ware	4-8
	4.10 References	4-9
5	REAGENTS, SOLVENTS, AND GASES	5-1
	5.1 Introduction	5-1
	5.2 Reagent Quality	5-1

5.3 Elimination of Determinate Errors 5-4
5.4 References 5-4

6 QUALITY CONTROL FOR ANALYTICAL PERFORMANCE 6-1
6.1 Introduction 6-1
6.2 The Industrial Approach to QC 6-1
6.3 Applying Control Charts in Environmental Laboratories 6-2
6.4 Recommended Laboratory Quality Assurance Program 6-9
6.5 Outline of a Comprehensive Quality Assurance Program 6-10
6.6 Related Topics 6-13
6.7 References 6-13

7 DATA HANDLING AND REPORTING 7-1
7.1 Introduction 7-1
7.2 The Analytical Value 7-1
7.3 Glossary of Statistical Terms 7-3
7.4 Report Forms 7-5
7.5 References 7-11

8 SPECIAL REQUIREMENTS FOR TRACE ORGANIC ANALYSIS . . . 8-1
8.1 Introduction 8-1
8.2 Sampling and Sample Handling 8-1
8.3 Extract Handling 8-4
8.4 Supplies and Reagents 8-5
8.5 Quality Assurance 8-7
8.6 References 8-10

9 SKILLS AND TRAINING 9-1
9.1 General 9-1
9.2 Skills 9-2
9.3 Training 9-4

10 WATER AND WASTEWATER SAMPLING 10-1
10.1 Introduction 10-1
10.2 Areas of Sampling 10-2
10.3 References 10-6

11 RADIOCHEMISTRY 11-1
11.1 Introduction 11-1
11.2 Sample Collection 11-1
11.3 Laboratory Practices 11-2
11.4 Quality Control 11-4
11.5 References 11-5

12 MICROBIOLOGY 12-1
12.1 Background 12-1
12.2 Specific Needs in Microbiology 12-1
12.3 Intralaboratory Quality Control 12-2
12.4 Interlaboratory Quality Control 12-2
12.5 Development of a Formal Quality Assurance Program 12-3
12.6 Documentation of a Quality Assurance Program 12-3

12.7 Chain-of-Custody Procedures for Microbiological Samples 12-3
12.8 References 12-10

13 AQUATIC BIOLOGY 13-1
13.1 Summary of General Guidelines 13-1
13.2 Discussion 13-2
13.3 Reference 13-4

14 LABORATORY SAFETY 14-1
14.1 Law and Authority for Safety and Health 14-1
14.2 EPA Policy on Laboratory Safety 14-5
14.3 Laboratory Safety Practices 14-7
14.4 Report of Unsafe or Unhealthful Condition 14-15
14.5 References 14-15

Appendix A—Suggested Checklist for the Safety Evaluation of EPA Laboratory Areas A-1

LIST OF FIGURES

Figure No.		Page
4-1	Example of Markings on Glassware	4-5
6-1	Essentials of a Control Chart	6-2
6-2	Shewhart Control Chart for Percent Recovery Data	6-5
6-3	Procedure for Evaluating QC Data From a Monitoring Study	6-12
7-1	Example of Bench Sheet	7-8
7-2	Example of Summary Data Sheet	7-9
7-3	Example of STORET Report Form	7-10
7-4	Flow Chart of the Sequence of Events During a Controlled Series of Laboratory Measurements	7-12
12-1	Example of Chain-of-Custody Sample Tag (*a*) Front, (*b*) Back	12-5
12-2	Example of a Sample Log Sheet	12-6
12-3	Example of a Chain-of-Custody Record	12-7
14-1	Health and Safety Inspection Checklist	14-8
14-2	Report of Unhealthful or Unsafe Condition	14-16
14-3	Notice of Unhealthful or Unsafe Condition	14-17

LIST OF TABLES

Table No.		Page
2-1	Water Purity	2-1
2-2	Requirements for Reagent Water	2-2
2-3	Comparison of Distillates From Glass and Metal Stills	2-3
3-1	Performance Characteristics of Typical pH/Selective-Ion Meter	3-5
3-2	Electrical Conductivity of Potassium Chloride Reference Solutions	3-7
4-1	Tolerances for Volumetric Glassware	4-4
4-2	Fritted Ware Porosity	4-8
6-1	Analyses of Total Phosphate-Phosphorus Standards, in Milligrams Per Liter of Total PO_4-P	6-4
6-2	Estimates of the Range ($R = \|A - B\|$) and the Industrial Statistic ($I = \|A - B\|/(A + B)$) of Three Different Parameters for Various Concentration Ranges	6-7
6-3	Shewhart Upper Control Limits UCL and Critical Range R_c Values for the Differences Between Duplicate Analyses Within Specific Concentration Ranges for Three Parameters	6-8
6-4	Critical Range Values for Varying Concentration Levels	6-9
9-1	Skill-Time Rating of Standard Analytical Operations	9-3

400

10-1 Guidance for Water/Wastewater Sampling 10-2

11-1 Sample Handling, Preservation, Methodology, and Major Instrumentation Required 11-2

Chapter 1

IMPORTANCE OF QUALITY CONTROL

1.1 General

The analytical laboratory provides qualitative and quantitative data for use in decision-making. To be valuable, the data must accurately describe the characteristics and concentrations of constituents in the samples submitted to the laboratory. In many cases, because they lead to faulty interpretations, approximate or incorrect results are worse than no result at all.

Ambient water quality standards for pH, dissolved oxygen, heavy metals, and pesticides are set to establish satisfactory conditions for drinking water, fishing, irrigation, power generation, or other water uses. The laboratory data define whether conditions are being met and whether the water can be used for its intended purposes. In wastewater analyses, the laboratory data identify the characteristics of the treatment plant influent and the final load imposed upon receiving water resources, as well as the effectiveness of steps in the treatment process. Decisions on process changes, plant modifications, or the construction of new facilities may be based upon the results of water laboratory analyses. The financial implications of such decisions suggest that extreme care be taken in analysis.

Effective research in water pollution control also depends upon a valid laboratory data base, which in turn may contribute to sound evaluations of both the progress of the research itself and the viability of available water pollution-control alternatives.

The analytical data from water and wastewater laboratories may also be used to determine the extent of compliance of a polluting industry with discharge or surface water standards. If the laboratory results indicate a violation of a standard, remedial action is required by the responsible parties. Both legal and social pressures can be brought to bear to protect the environment. The analyst should realize not only that he has considerable responsibility for providing reliable laboratory descriptions of the samples at issue, but also that his professional competence, the validity of the procedures used, and the resulting values reported may be challenged (perhaps in court). For the analyst to meet such challenges, he should support the laboratory data with an adequate documentation program that provides valid records of the control measures applied to all factors bearing on the final results of investigations.

1.2 Quality Assurance Programs

Because of the importance of laboratory analyses in determining practical courses of action that may be followed, quality assurance programs to insure the reliability of the water and wastewater data are essential. Although all analysts practice quality control (QC) in amounts depending upon their training, professional pride, and the importance of their particular projects, under actual working conditions sufficiently detailed QC may be neglected. An established, routine, quality assurance program applied to each analytical test can relieve analysts of the necessity of originating individual QC efforts.

482

Quality assurance programs have two primary functions in the laboratory. First, the programs should continually monitor the reliability (accuracy and precision) of the results reported; i.e., they should continually provide answers to the question "How good (accurate and precise) are the results obtained?" This function is the *determination* of quality. The second function is the *control* of quality (to meet the program requirements for reliability). As an example of the distinction between the two functions, the processing of spiked samples may be a determination of measurement quality, but the use of analytical grade reagents is a control measure.

Each analytical method has a rigid protocol. Similarly, QC associated with a test must include definite required steps for monitoring the test and insuring that its results are correct. The steps in QC vary with the type of analysis. For example, in a titration, standardization of the titrant on a frequent basis is an element of QC. In any instrumental method, calibration and checking out of instrumental response are also QC functions. All of the experimental variables that affect the final results should be considered, evaluated, and controlled.

In summary, laboratory data, in quantitative terms, e.g., in milligrams per liter, are reported by the analyst. These values are interpreted by industrial plant engineers to show compliance or noncompliance with permits for discharge, by state pollution control agencies to define the need for additional sampling and analysis to confirm violations, or by EPA to demonstrate that prescribed waste treatment was sufficient to protect the surface waters affected by the discharge.

This handbook discusses the basic factors of water and wastewater measurements that determine the value of analytical results and provides recommendations for the control of these factors to insure that analytical results are the best possible. Quality assurance programs initiated from, and based upon, these recommendations should increase confidence in the reliability of the reported analytical results.

Because ultimately a laboratory director must assume full responsibility for the reliability of the analytical results submitted, the laboratory director must also assume full responsibility in both design and implementation for the corresponding quality assurance program.

1.3 Analytical Methods

Many analytical methods for common water pollutants have been in use for many years and are used in most environmental laboratories. Examples are tests for chloride, nitrate, pH, specific conductance, and dissolved oxygen. Widespread use of an analytical method in water and wastewater testing usually indicates that the method is reliable, and therefore tends to support the validity of the reported test results. Conversely, the use of little-known analytical techniques forces the water and wastewater data user to rely on the judgment of the laboratory analyst, who must then defend his choice of analytical technique as well as his conclusions. Present Federal regulations, notably section 304(h) of Public Law 92-500 (Federal Water Pollution Control Amendments of 1977) and the Interim Drinking Water Regulations specifically require the use of EPA-approved methods of analysis.

Uniformity of methodology within a single laboratory as well as among a group of cooperating laboratories is required to remove methodology as a variable when there are many data users. Uniformity of methodology is particularly important when several

403

laboratories provide data to a common data bank (such as STORET*) or cooperate in joint field surveys. A lack of uniformity of methodology may raise doubts as to the validity of the reported results. If the same constituents are measured by different analytical procedures within a single laboratory, or by a different procedure in different laboratories, it may be asked which procedure is superior, why the superior method is not used throughout, and what effects the various methods and procedures have on the data values and their interpretations.

Physical and chemical measurement methods used in water or wastewater laboratories should be selected by the following criteria:

a. The selected methods should measure desired constituents of water samples in the presence of normal interferences with sufficient precision and accuracy to meet the water data needs.

b. The selected procedures should use equipment and skills ordinarily available in the average water pollution control laboratory or water supply laboratory.

c. The selected methods should be sufficiently tested to have established their validity.

d. The selected methods should be sufficiently rapid to permit repetitive routine use in the examination of large numbers of water samples.

The restriction to the use of EPA methods in all laboratories providing data to EPA permits the combination of data from different EPA programs and supports the validity of decisions made by EPA.

Regardless of which analytical methods are used in a laboratory, the methodology should be carefully documented. In some reports it is stated that a standard method from an authoritative reference (such as ref. 1) was used throughout an investigation, when close examination has indicated, however, that this was not strictly true. Standard methods may be modified or entirely replaced because of recent advances in the state of the art or personal preferences of the laboratory staff. Documentation of measurement procedures used in arriving at laboratory data should be clear, honest, and adequately referenced; and the procedures should be applied exactly as documented.

Reviewers can apply the associated precision and accuracy of each specific method when interpreting the laboratory results. If the accuracy and precision of the analytical methodology are unknown or uncertain, the data user may have to establish the reliability of the result he or she is interpreting before proceeding with the interpretation.

The necessarily strict adherence to accepted methods in water and wastewater analyses should not stifle investigations leading to improvements in analytical procedures. Even with accepted and documented procedures, occasions arise when the procedures must be modified; e.g., to eliminate unusual interferences, or to yield increased sensitivities. When a

*STORET is the acronym used to identify the computer-oriented U.S. Environmental Protection Agency water quality control information system; STORET stands for STOrage and RETrieval of data and information.

404

modification of a procedure is necessary, it should be carefully formulated. Data should then be assembled using both the original and the modified procedures to show the superiority of the latter. Such results can be brought to the attention of the organizations responsible for standardization of procedures. To increase the benefit, the modified procedures should be written in a standard format for routine use as applicable. The standard format usually includes scope and application, principle, equipment, reagents, procedure, calculation of results, and expected precision and accuracy.

Responsibility for the results obtained from use of a nonstandard procedure (i.e., one that has not become accepted through wide use) rests with the analyst and his supervisor.

In field operations, because it may be difficult to transport samples to the laboratory, or to examine large numbers of samples (e.g., for gross characteristics), the use of rapid field methods yielding approximate answers is sometimes required. Such methods should be used only with a clear understanding that the results obtained are not as reliable as those obtained from standard laboratory procedures. The fact that such methods have been used should be documented, and the results should not be reported in the same context with more reliable analytical information. When only approximate values are available, perhaps obtained for screening purposes in the field only, the data user would then be so informed.

1.4 Reference

1. Standard Methods for the Examination of Water and Wastewater, 14th Edition, American Public Health Association, New York (1975).

Chapter 2

LABORATORY SERVICES

2.1 General

Quality control of water and wastewater laboratory analyses involves consideration and control of the many variables that affect the production of reliable data. The quality of the laboratory services available to the analyst must be included among these variables. An abundant supply of distilled water, free from interferences and other undesirable contaminants, is an absolute necessity. An adequate source of clean, dry, compressed air is needed. Electrical power for routine laboratory use and voltage-regulated sources for delicate electronic instrumentation must be provided. This chapter, therefore. will be devoted to describing methods of maintaining the quality of these services, as used in water and wastewater laboratory operations.

2.2 Distilled Water

Distilled or demineralized water is used in the laboratory for dilution, preparation of reagent solutions, and final rinsing of glassware. Ordinary distilled water is usually not pure. It may be contaminated by dissolved gases and by materials leached from the container in which it has been stored. Volatile organics distilled over from the feed water may be present, and nonvolatile impurities may occasionally be carried over by the steam, in the form of a spray. The concentration of these contaminants is usually quite small, and distilled water is used for many analyses without further purification. However, it is highly important that the still, storage tank, and any associated piping be carefully selected, installed, and maintained in such a way as to insure minimum contamination.

Water purity has been defined in many different ways, but one generally accepted definition states that high-purity water is water that has been distilled or deionized, or both, so that it will have a specific resistance of 500,000 Ω or greater (or a conductivity less than 2.0 μmho/cm). This definition is satisfactory as a base to work from, but for more critical requirements, the breakdown shown in table 2-1 has been suggested to express degrees of purity (1).

Table 2-1
WATER PURITY

Degree of Purity	Maximum Conductivity (μmho/cm)	Approximate Concentration of Electrolyte (mg/l)
Pure	10	2-5
Very Pure	1	0.2-0.5
Ultrapure	0.1	0.01-0.02
Theoretically Pure	0.055	0.00

406

The American Society for Testing and Materials (ASTM) specifies four different grades of water for use in methods of chemical analysis and physical testing. The method of preparation of the various grades of reagent water determines the limits of impurities. The various types of reagent water and ASTM requirements are listed in table 2-2.

Type I grade water is prepared by the distillation of feed water having a maximum conductivity of 20 μmho/cm at 25°C followed by polishing with a mixed bed of ion-exchange materials and a 0.2-μm membrane filter.

Type II grade water is prepared by using a still designed to produce a distillate having a conductivity of less than 1.0 μmho/cm at 25°C. This may be accomplished by double distillation or the use of a still incorporating special baffling and degassing features.

Type III grade water is prepared by distillation, ion exchange, or reverse osmosis, followed by polishing with the 0.45-μm membrane filter.

Type IV grade water is prepared by distillation, ion exchange, reverse osmosis, or electrodialysis.

Properly designed metal stills from reputable manufacturers offer convenient and reliable sources of distilled water. These stills are usually constructed of copper, brass, and bronze. All surfaces that contact the distillate should be heavily coated with pure tin to prevent metallic contamination. The metal storage tank should be of sturdy construction with a tight-fitting cover, and have a filter in the air vent to remove airborne dust, gases, and fumes.

For special purposes, an all-glass distillation unit may be preferable to the metal still. These stills are usually smaller, and of more limited capacity than the metal stills. An actual comparison in which the distillates from an all-glass still and a metal still were analyzed spectrographically for certain trace metal contaminants is given in table 2-3. It can be seen that the all-glass still produced a product that had substantially lower contamination from zinc, copper, and lead.

All stills require periodic cleaning to remove solids that have been deposited from the feed water. Hard water and high-dissolved-solids content promote scale formation in the

Table 2-2
REQUIREMENTS FOR REAGENT WATER

Grade of Water	Maximum Total Matter (mg/l)	Maximum Electrical Conductivity at 25°C (μmho/cm)	Minimum Electrical Resistivity* at 25°C (MΩ · cm)	pH at 25°C	Minimum Color Retention Time of $KMnO_4$ (min)
Type I	0.1	0.06	16.67	–	60
Type II	0.1	1.0	1.0	–	60
Type III	1.0	1.0	1.0	6.2-7.5	10
Type IV	2.0	5.0	0.2	5.0-8.0	10

*See reference 2.

457

Table 2-3
COMPARISON OF DISTILLATES FROM GLASS AND METAL STILLS

Source	Element and Concentration (μg/l)							
	Zn	B	Fe	Mn	Al	Cu	Ni	Pb
All-Glass Still	<1	12	1	<1	<5	5	<2	<2
Metal Still	9	13	2	<1	<5	11	<2	26

evaporator, and cleaning frequency will thus depend on the quality of the feed water. The boiler of an all-glass still should be drained daily and refilled with clean water. Buildup of scale is easily detected, and the boiler and condenser coils should be cleaned at frequent intervals. Metal stills usually incorporate a constant bleeder device that retards scale formation to some extent. Nevertheless, these units should be dismantled and cleaned at regular intervals. Cleaning should always be in accordance with the manufacturer's instructions.

Pretreatment of the incoming feed water will often improve still performance and raise the quality of the distillate. For example, preliminary softening of hard water removes calcium and magnesium prior to distillation. This reduces scale formation in the boiler and condenser, thereby reducing maintenance service. These softeners employ the ion-exchange principle using a sodium chloride cycle, and are relatively inexpensive to operate. A carbon filtration system, installed at the feed-water intake, will remove organic materials that might subsequently be carried over in the distillate. If trace concentrations of ions are a major concern, the distillate may be passed through a mixed-bed ion exchanger.

At least two commercially manufactured systems are available for production of high-purity water by ion exchange. The Millipore Super-Q System (Millipore Corp., Bedford, Mass.) consists essentially of disposable cartridges for prefiltration, organic absorption, deionization, and Millipore filtration. The company claims it can produce 10-MΩ water, containing no particulate matter larger than 0.45 μm in size, from tap water, at the rate of 20 gal/h. Continental Water Conditioning Corp., El Paso, Tex., advertises a system that can be tailored to the needs of the customer. Performance specifications include minimum flow rates of 45 gal/h and total dissolved solids of less than 0.1 mg/l when required.

Specific conductance is a rapid and simple measurement for determining the inorganic quality of distilled water. Stills of the types previously discussed are capable of producing a distillate with a specific conductance of less than 2.0 μmho/cm at 25°C. This is equivalent to 0.5 to 1.0 mg/l of ionized material. Frequent checks should be made to determine that optimum performance is being maintained. A purity meter installed between the still and the storage reservoir will monitor the conductivity of the distillate, in terms of the equivalent in milligrams per liter of sodium chloride. If the reading on the meter begins to rise above the present limit of conductivity, effective action should be taken to eliminate the source of contamination. Organic quality is more difficult to monitor, but the total organic carbon determination is a simple and rapid test of organic contaminants.

A piping system for delivering distilled water to the area of use within the laboratory is a convenient feature. In this case, special care should be taken that the quality of the water is not degraded between the still and the point of use. Piping may be of tin, tin-lined brass, stainless steel, plastic, or chemically resistant glass, depending on the quality of the water desired, its intended use, and on available funds. Tin is best, but is also very expensive. As a compromise, plastic pipe or glass pipe with Teflon* O-rings at all connecting joints is satisfactory for most purposes. The glass pipe has an obvious advantage when freedom from trace amounts of organic materials is important.

When there is no piped-in supply, distilled water will probably be transported to the laboratory and stored in polyethylene or glass bottles of about 5-gal capacity. If stored in glass containers, distilled water will gradually leach the more soluble materials from the glass and cause an increase in dissolved solids. On the other hand, polyethylene bottles contain organic plasticizers, and traces of these materials may be leached from the container walls. These are of little consequence, except in some organic analyses. Rubber stoppers often used in storage containers contain leachable materials, including significant quantities of zinc. This is usually no problem, because the water is not in direct contact with the stopper. However, the analyst should be aware of the potential for contamination, especially when the supply is not replenished by frequent use.

The delivery tube may consist of a piece of glass tubing that extends almost to the bottom of the bottle, and that is bent downward above the bottle neck, with a 3- to 4-ft piece of flexible tubing attached for mobility. Vinyl tubing is preferable to latex rubber, because it is less leachable; however, a short piece of latex tubing may be required at the outlet for better control of the pinchcock. The vent tube in the stopper should be protected against the entrance of dust.

Ordinary distilled water is quite adequate for many analyses, including the determination of major cations and anions. Certain needs may require the use of double- or even triple-distilled water. Redistillation from an alkaline permanganate solution can be used to obtain a water with low organic background. When determining trace organics by solvent extraction and gas chromatography, distilled water with sufficiently low background may be extremely difficult to obtain. In this case, preextraction of the water with the solvent used in the respective analysis may be helpful in eliminating undesirable peaks in the blank. Certain analyses require special treatment or conditioning of the distilled water, and these will now be discussed.

2.2.1 Ammonia-Free Water

Removal of ammonia can be accomplished by shaking ordinary distilled water with a strong cation exchanger, or by passing distilled water through a column of such material. For limited volumes of ammonia-free water, use of the Quikpure (Box 254, Chicago, Ill.) 500-ml bottle is highly recommended. The ion-free water described in section 2.2.3 is also suitable for use in the determination of ammonia.

2.2.2 Carbon-Dioxide-Free Water

Carbon-dioxide-free water may be prepared by boiling distilled water for 15 min and cooling to room temperature. As an alternative, distilled water may be vigorously aerated with a

*Trademark of E. I. duPont de Nemours & Co.

409

stream of inert gas for a period sufficient to achieve saturation and CO_2 removal. Nitrogen is most frequently used. The final pH of the water should lie between 6.2 and 7.2. It is not advisable to store CO_2-free water for extended periods.

2.2.3 Ion-Free Water

A multipurpose high purity water, free from trace amounts of the common ions, may be conveniently prepared by slowly passing distilled water through an ion-exchange column containing one part of a strongly acidic cation-exchange resin in the hydroxyl form. Resins of a quality suitable for analytical work must be used. Ion-exchange cartridges of the research grade, available from scientific supply houses, have been found satisfactory. By using a fresh column and high-quality distilled water, a water corresponding to the ASTM designation for type I reagent water (2) (maximum 0.1 mg/l of total matter and maximum conductivity of 0.06 mho/cm) can be obtained. This water is suitable for use in the determination of ammonia, trace metals, and low concentrations of most cations and anions. It is not suited to some organic analyses, however, because this treatment adds organic contaminants to the water by contact with the ion-exchange materials.

2.3 Compressed Air

The quality of compressed air required in the laboratory is usually very high, and special attention should be given to producing and maintaining clean air until it reaches the outlet. Oil, water, and dirt are undesirable contaminants in compressed air, and it is important to install equipment that generates dry, oil-free air. When pressures of less than 50 psi are required, a rotary-type compressor, using a water seal and no oil, eliminates any addition of oil that would subsequently have to be removed from the system. Large, horizontal, water-cooled compressors will usually be used when higher pressures are required.

Compression heats air, thus increasing its tendency to retain moisture. An aftercooler is therefore necessary to remove water. Absorption filters should be used at the compressor to prevent moisture from entering the piping system. Galvanized steel pipe with threaded, malleable-iron fittings, or solder-joint copper tubing should be used for piping the air to the laboratory.

When the compressed air entering the laboratory is of low quality, an efficient filter should be installed between the outlet and the point of use to trap oil, moisture, and other contaminants. As an alternative, high-quality compressed air of the dry grade is commercially available in cylinders when no other source exists.

2.4 Vacuum

A source of vacuum in the chemical laboratory, while not an absolute necessity, can be a most useful item. While used primarily as an aid in filtration, it is also sometimes used in pipetting and in speeding up the drying of pipets.

2.5 Hood System

An efficient hood system is a requirement for all laboratories. In addition to removing the various toxic and hazardous fumes that may be generated when using organic solvents, or that may be formed during an acid digestion step, a hood system may also be used to remove toxic gases that may be formed during atomic absorption analyses or other

410

reactions. A regular fume hood should have a face velocity of 100 ft/min (linear) with the sash fully open.

2.6 Electrical Services

An adequate electrical system is indispensable to the modern laboratory. This involves having both 115- and 230-V sources in sufficient capacity for the type of work that must be done. Requirements for satisfactory lighting, proper functioning of sensitive instruments, and operation of high-current devices must be considered. Any specialized equipment may present unusual demands on the electrical supply.

Because of the special type of work, requirements for a laboratory lighting system are quite different from those in other areas. Accurate readings of glassware graduations, balance verniers, and other measuring lines must be made. Titration endpoints, sometimes involving subtle changes in color or shading, must be observed. Levels of illumination, brightness, glare, and location of light sources should be controlled to facilitate ease in making these measurements and to provide maximum comfort for the employees.

Such instruments as spectrophotometers, flame photometers, atomic absorption equipment, emission spectrographs, and gas chromatographs have complicated electronic circuits that require relatively constant voltage to maintain stable, drift-free instrument operation. If the voltage of these circuits varies, there is a resulting change in resistance, temperature, current, efficiency, light output, and component life. These characteristics are interrelated, and one cannot be changed without affecting the others. Voltage regulation is therefore necessary to eliminate these conditions.

Many instruments have built-in voltage regulators that perform this function satisfactorily. In the absence of these, a small, portable, constant-voltage transformer should be placed in the circuit between the electrical outlet and the instrument. Such units are available from Sola Basic Industries, Elk Grove Village, Ill., and are capable of supplying a constant output of 118 V from an input that varies between 95 and 130 V. When requirements are more stringent, special transformer-regulated circuits can be used to supply constant voltage. Only the instrument receiving the regulated voltage should be operated from such a circuit at any given time. These lines are in addition to, and separate from, the ordinary circuits used for operation of equipment with less critical requirements.

Electrical heating devices provide desirable heat sources, and should offer continuously variable temperature control. Hot plates and muffle furnaces wired for 230-V current will probably give better service than those that operate on 115 V, especially if the lower voltage circuit is only marginally adequate. Water baths and laboratory ovens with maximum operating temperatures of about 200°C perform well at 115 V. Care must be taken to ground all equipment that could constitute a shock hazard. The three-pronged plugs that incorporate grounds are best for this purpose.

2.7 References

1. Applebaum, S. B., and Crits, G. J., "Producing High Purity Water," Industrial Water Engineering (Sept./Oct. 1964).
2. "Water," Part 31 of 1977 Book of ASTM Standards, p. 20, American Society for Testing and Materials, Philadelphia (1977).

411

Chapter 3

INSTRUMENT SELECTION

3.1 Introduction

The modern analytical laboratory depends very heavily upon instrumentation. This statement may be completely obvious, but it should be remembered that the exceptional emphasis on electronic equipment has really begun since the development of the transistor and the computer. To a certain extent, analytical instrumentation is always in the development stage, with manufacturers continually redesigning and upgrading their products, striving for miniaturization, better durability and sensitivity, and improved automation. For laboratory supervisors and staff members the net result is a bewildering stream of advertising brochures, announcements, and catalogs of newly available equipment. Consequently, the selection of analytical equipment is always difficult.

The instruments commonly used in water and wastewater analysis include the following:

Analytical balance
pH/selective-ion meter
Conductivity meter
Turbidimeter
Spectrometers (visible, ultraviolet (UV), infrared (IR), and atomic absorption (AA))
Total carbon analyzer
Gas chromatograph (GC)
Gas chromatograph/mass spectrometer (GC/MS)
Temperature devices (such as ovens and water baths)
Recorders

These devices represent basic equipment used in routine work and should be the subject of careful consideration before purchase. Further, their operation and maintenance ought to be primary considerations in sustained production of satisfactory data. Obviously, fundamental understanding of instrument design will assist the analyst in the correct use of instruments and in some cases will aid in detecting instrumental failures. Calibration of all laboratory instruments with primary standards is encouraged whenever practical. This normally involves a National Bureau of Standards standard reference material or calibration and certification procedures. Calibration checks with secondary standards, made in each laboratory or available from private sources, are encouraged on a frequent basis if not required by the analytical method each time an analysis is made.

In the pages that follow an attempt is made to discuss basic instrument design and to offer some remarks about desirable instrumental features.

3.2 Analytical Balances

The most important piece of equipment in any analytical laboratory is the analytical balance. The degree of accuracy of the balance is reflected in the accuracy of all data related to weight-prepared standards. Although the balance should therefore be the most protected and cared-for instrument in the laboratory, proper care of the balance is frequently overlooked.

412

There are many fine balances on the market designed to meet a variety of needs. Types of balances include top-loading, two-pan, microanalytical, electroanalytical, semianalytical, analytical, and other special-purpose instruments. Each type of balance has its own place in the scheme of laboratory operation, but analytical single-pan balances are by far the most important in the production of reliable data.

Single-pan analytical balances range in capacity from the 20-g to the popular 200-g models with sensitivities from 0.01 to 1 mg. Features of single-pan balances may include mechanical and electronic switching of weights, digital readout, automatic zeroing of the empty balance, and automatic preweighing and taring capabilities. Even with all the design improvements, however, modern analytical balances are still fragile instruments, the operation of which is subject to shock, temperature, and humidity changes, to mishandling, and to various other insults. Some of the precautions to be observed in maintaining and prolonging the dependable life of a balance are as follows:

a. Analytical balances should be mounted on a heavy, shockproof table, preferably one with an adequately large working surface and with a suitable drawer for storage of balance accessories. The balance level should be checked frequently and adjusted when necessary.

b. Balances should be located away from laboratory traffic and protected from sudden drafts and humidity changes.

c. Balance temperatures should be equilibrated with room temperature; this is especially important if building heat is shut off or reduced during nonworking hours.

d. When the balance is not in use, the beam should be raised from the knife edges, the weights returned to the beam, objects such as the weighing dish removed from the pan, and the weighing compartment closed.

e. Special precautions should be taken to avoid spillage of corrosive chemicals on the pan or inside the balance case; the interior of the balance housing should be kept scrupulously clean.

f. Balances should be checked and adjusted periodically by a company service man or balance consultant; if service is not available locally, the manufacturer's instructions should be followed as closely as possible. Service contracts, including an automatic preventive maintenance schedule, are encouraged.

g. The balance should be operated at all times according to the manufacturer's instructions.

Standardized weights to be used in checking balance accuracy, traceable to the National Bureau of Standards, may be purchased from various supply houses. A complete set of directions for checking the performance of a balance is contained in part 41 of ASTM Standards (1).

Because all analytical balances of the 200-g capacity suitable for water and wastewater laboratories have about the same design specifications with reference to sensitivity, precision, convenience, and price, it is safe to assume that there is no clear preference for a certain model, and selection can be made on the basis of availability of service.

43

3.3 pH/Selective-Ion Meters

The concept of pH as a means of expressing the degree of *effective* acidity or alkalinity instead of *total* acidity or alkalinity was developed in 1909 by Sorenson (2). It was not until about 1940 that commercial instruments were developed for routine laboratory measurement of pH.

A basic meter consists of a voltage source, amplifier, and scale or digital readout device. Certain additional refinements produce varying performance characteristics between models. Some models incorporate expanded scales for increased readability, solid state circuitry for operating stability and extreme accuracy, and temperature and slope adjustment to correct for asymmetric potential of glass electrodes. Other features are scales that facilitate use of selective-ion electrodes, recorder output, and interfacing with complex data-handling systems.

In routine pH measurements the glass electrode is used as the indicator and the calomel electrode as the reference. Glass electrodes have a very fast response time in highly buffered solutions. However, accurate readings are obtained slowly in poorly buffered samples, and particularly so when changing from buffered to unbuffered samples. Electrodes, both glass and calomel, should be well rinsed with distilled water after each reading, and should be rinsed with, or dipped several times into, the next test sample before the final reading is taken. Weakly buffered samples should be stirred during measurement. When not in use, glass electrodes should not be allowed to become dry, but should be immersed in an appropriate solution consistent with the manufacturer's instructions. The first steps in calibrating an instrument are to immerse the glass and calomel electrodes into a buffer of known pH, set the meter to the pH of the buffer, and adjust the proper controls to bring the circuit into balance. The temperature-compensating dial should be set at the temperature of the buffer solution. For best accuracy, the instrument should be calibrated against two buffers that bracket the expected pH of the samples.

The presence of a faulty electrode is indicated by failure to obtain a reasonably correct value for the pH of the second reference buffer solution after the meter has been standardized with the first reference buffer solution. A cracked glass electrode will often yield pH readings that are essentially the same for both standards. The response of electrodes may also be impaired by failure to maintain the KCl level in the calomel electrode, by improper electrode maintenance, or by certain materials such as oily substances and precipitates that may coat the electrode surface. Faulty electrodes can often be restored to normal by an appropriate cleaning procedure. Complete and detailed cleaning methods are given in part 31 of ASTM Standards (3), and are also usually supplied by the electrode manufacturer.

Because of the asymmetric potential of the glass electrode, most pH meters are built with a slope adjustment that enables the analyst to correct for slight electrode errors observed during calibration with two different pH buffers. Exact details of slope adjustment and slope check may vary with different models of instruments. The slope adjustment must be made whenever electrodes are changed, subjected to vigorous cleaning, or refilled with fresh electrolyte. The slope adjustment feature is highly desirable and recommended for consideration when purchasing a new meter.

Most pH meters now available are built with transistorized circuits rather than vacuum tubes, which greatly reduces the warmup time and increases the stability of the meters.

Also, many instruments are designed with a switching circuit so that the entire conventional 0 to 14 scale of pH may be used to read a single pH unit with a corresponding increase in accuracy.

This expanded-scale feature is of definite value when the meter is used for potentiometric titrations and selective-ion work. It is of dubious value, however, in routine analyses, because pH readings more precise than ±0.1 are seldom required. Primary standard buffer salts are available from the National Bureau of Standards* and should be used in situations where extreme accuracy is necessary. Preparation of reference solutions from these salts requires some special precautions and handling (3,4) such as the use of low-conductivity dilution water, drying ovens, and carbon-dioxide-free purge gas. These solutions should be replaced at least once each month.

Secondary standard buffers may be prepared from NBS salts or purchased as solutions from commercial vendors. Routine use of these commercially available solutions, which have been validated by comparison to NBS standards, is recommended.

The electrometric measurement of pH varies with temperature because of two effects. The first effect is the change in electrode output with temperature. This interference can be controlled by use of instruments having temperature compensation or by calibrating the instrument system including the electrode at the temperature of the samples. The second effect is the change of pH of the sample with temperature. This error is sample dependent and cannot be controlled; it should therefore be noted by reporting both the pH and temperature at the time of analysis.

Typical characteristics of a conventional expanded-scale meter are shown in table 3-1.

3.3.1 pH Electrodes

A wide variety of special- and general-purpose pH electrodes are now available to meet all applications in the general analytical laboratory. A survey through any laboratory supply catalog may confuse more than clarify the selection process. A rugged, full-range, glass- or plastic-bodied combination electrode is a good choice for routine use. An added convenience is an electrode that contains solid geltype filling materials not requiring the normal maintenance of an electrode containing liquid filling solutions.

3.3.2 Selective-Ion Electrodes

Electrodes have been developed to measure almost every common inorganic ion normally measured in the water and wastewater laboratory. Application of these electrodes has progressed at a much slower pace and currently only three are approved for EPA monitoring applications.

Reference 5 includes methods for use of fluoride, ammonia, and dissolved oxygen electrodes. Various techniques for use of these and other electrodes are reviewed in references 6 through 9. A major problem in measuring the total parameter with electrodes is that of relating the ion activity to ion concentration. Because the electrodes only measure

*NBS, Office of Standard Reference Materials, Institute for Materials Research, Washington, D.C. 20234.

415

Table 3-1
PERFORMANCE CHARACTERISTICS OF TYPICAL pH/SELECTIVE-ION METERS

Scale	Range		Smallest Scale Division		Accuracy		Reproducibility		Temperature Compensation[1] (°C)	Input Impedance (Ω)	Power Requirements	
	(pH)	(mV)	(pH)	(mV)	(pH)	(mV)	(pH)	(mV)			(V)	(Hz)
Normal	0-14	±1,400	0.1	10	±0.05	±5	±0.02	±2	0-100	>10	[2] 115/220	50/60
Expanded	0-1	±100	.005	.5	±0.002	([3])	±0.002	±0.2	0-100	>10	[2] 115/220	50/60

[1] Manual or automatic.
[2] May also be powered by self-contained batteries.
[3] ±2 percent of reading.

activity, the challenge is to put all of the parameters of interest into the same measurable ionic form and then to modify the activity to be proportional to the concentration. The technique of known addition (spiking of samples) is recommended when unproven electrode methods are being used or when sample matrix problems are suspected or not controlled by prior distillation or separation techniques.

3.4 Conductivity Meters

Solutions of electrolytes conduct an electric current by the migration of ions under the influence of an electric field. For a constant applied EMF, the current flowing between opposing electrodes immersed in the electrolyte will vary inversely with the resistance of the solution. The reciprocal of the resistance is called conductance and is expressed in reciprocal ohms (mhos). For natural water samples where the resistance is high, the usual reporting unit is micromhos.

Most conductivity meters on the market today use a cathode-ray tube, commonly known as the "magic eye," for indicating solution conductivity. A stepping switch for varying resistances in steps of 10X facilitates reading conductivities from about 0.1 to about 250,000 μmho. The sensing element for a conductivity measurement is the conductivity cell, which normally consists of two thin plates of platinized metal, rigidly supported with a very precise parallel spacing. For protection, the plates are mounted inside a glass tube with openings in the side walls and submersible end for access of sample. Variations in designs have included use of hard rubber and plastics for protection of the cell plates. Glass may be preferable, in that the plates may be visually observed for cleanliness and possible damage, but the more durable encasements have the advantage of greater protection and reduced cell breakage. Selection of various cell designs is normally based on personal preference with consideration of sample type and durability requirements.

In routine use, cells should be frequently examined to insure that (a) the platinized coating of plates is intact; (b) plates are not coated with suspended matter; (c) plates are not bent, distorted, or misalined; and (d) lead wires are properly spaced.

Temperature has a pronounced effect on the conductance of solutions, and must be corrected for when results are reported. The specified temperature for reporting data used by most analytical groups (and all EPA laboratories) is 25°C. Data correction may be accomplished by adjusting sample temperatures to 25°C, or by use of mathematical or electronic adjustment.

Instrumental troubles are seldom encountered with conductivity meters because of the design simplicity. When troubles occur, they are usually in the cell, and for most accurate work the following procedures should be used:

a. Standardize the cell and establish a cell factor by measuring the conductivity of a standard potassium chloride solution (standard conductivity tables may be found in various handbooks).

b. Rinse the cell by repeated immersion in distilled water.

c. Again, immerse the cell in the sample several times before obtaining a reading.

417

d. If the meter is equipped with a magic eye, determine the maximum width of the shadow at least twice, once by approaching the endpoint from a low reading upward, and once from a high reading downward.

Because the cell constants are subject to slow change even under ideal conditions, and sometimes to more rapid change under adverse conditions, it is recommended that the cell constant be periodically established. Table 3-2 can be used for this operation.

For instruments reading in mhos, the cell constant is calculated as follows:

$$L = \frac{K_1 + K_2}{10^6 K_x}$$

where

L = cell constant

K_1 = conductivity, in micromhos per centimeter, of the KCl solution at the temperature of measurement

K_2 = conductivity, in micromhos per centimeter, of the KCl solution at the same temperature as the distilled water used to prepare the reference solution

K_x = measured conductance, in mhos

Many different manufacturers produce conductivity meters that perform well on water and wastewater samples. Selection should be made consistent with sampling requirements, availability of service and sales, and individual personal preference.

3.5 Turbidimeters (Nephelometers)

Many different instrument designs have been used for the optical measurement of turbidity by measurement of either transmission or reflection of light. An equal or even greater

Table 3-2
ELECTRICAL CONDUCTIVITY OF POTASSIUM CHLORIDE REFERENCE SOLUTIONS

Solution	Normality	Method of Preparation	Temperature (°C)	Conductivity (μmho/cm)
A	0.1	7.4365 g/l KCl at 20°C	0	7,138
			18	11,167
			25	12,856
B	0.01	0.7440 g/l KCl at 20°C	0	773
			18	1,220
			25	1,408
C	0.001	Dilute 100 ml of B to 1.0 l at 20°C	25	147

418

number of materials have been used or proposed as calibration standards. As described in reference 10, EPA has standardized on the instrument design and the standard turbidity suspension of Formazin for instrument calibration.

Both the analyst and the user of turbidity data should keep in mind that a turbidity measurement is not a substitute for particle weight or residue analysis. Turbidity instruments can be calibrated to give gravimetric data on specific sample types, but the influence of particle geometry, specific gravity, refractive index, and color make estimates of total weight impractical on a variety of sample types.

For production of data with maximum accuracy and precision, the following precautions should be observed:

a. Protect the sample cuvette from scratches and fingerprints.

b. Use a constant orientation of the sample cuvette while calibrating the instrument and analyzing samples.

c. Use a well-mixed sample in the sample cuvette; do not take readings until finely dispersed bubbles have disappeared.

d. Dilute samples containing excess turbidity to some value below 40 nephelometric turbidity units (NTU); take reading; and multiply results by correct dilution factor.

3.6 Spectrometers

Because a large portion of routine quantitative measurements are performed colorimetrically, the spectrometer or filter photometer is usually the workhorse of any analytical laboratory. Indeed, the versatility of such instruments and the number of demands imposed upon them have resulted in a variety of designs and price ranges. Systematic listing and detailed discussion of all instrumental types are beyond the scope of this chapter; however, ultraviolet, visible, infrared, and atomic absorption instruments will be discussed.

A spectrometer is an instrument for measuring the amount of light or radiant energy transmitted through a solution or solid material as a function of wavelength. A spectrometer differs from a filter photometer in that it uses continuously variable, and more nearly monochromatic, bands of light. Because filter photometers lack the versatility of spectrometers, they are used most profitably where standard methodologies are used for routine analysis.

The essential parts of a spectrometer include the following:

a. A source of radiant energy

b. Monochromator or other device for isolating narrow spectral bands of light

c. Cells (cuvettes) or sample holders for containing samples under investigation

d. A photodetector (a device to detect and measure the radiant energy passing through the sample)

Each of the essential features listed, especially the monochromator and the photodetector system, varies in design principles from one instrument to another. Some of the characteristics of the commonly used Perkin-Elmer model 124 double-beam grating spectrometer are the following:

Light source	
Visible region	Tungsten
Lamp UV region	Deuterium lamp
Wavelength accuracy	±0.5 nm
Spectral bandwidth	0.5, 1.0, and 2.0 nm
Photometric presentation	
Linear transmittance	0 to 100 percent
Linear absorbance	0 to 1*A* or 0 to 2*A*
Photodetector R-136	190 to 800 nm
Sample cells	1.0 to 10 cm

3.6.1 Visible Range

Desirable features on a visible-range spectrometer are determined by the anticipated use of the instrument. Simple, limited programs requiring use of only a few parameters can probably be supported by inexpensive but reliable filter photometers. On the other hand, if the laboratory programs require a wide variety of measurements on diverse samples at low concentrations, more versatile instruments may be needed. One of the prime considerations is adaptability to various sample cell sizes from 1.0 to 10.0 cm.

3.6.2 Ultraviolet Range

A UV spectrometer is similar in design to a visible-range instrument except for differences in the light source and in the optics. The UV light source is a hydrogen- or deuterium-discharge lamp, which emits radiation in the UV portion of the spectrum, generally from about 200 nm to the low-visible-wavelength region. The optical system and sample cells must be constructed of UV-transparent material, which is usually quartz. A grating used in a UV system may be specially cut (blazed) in the UV region for greater sensitivity.

3.6.3 Infrared Range

A number of instrumental modifications are required in the construction of spectrometers for measurements in the IR region because materials such as glass and quartz absorb IR radiant energy, and ordinary photocells do not respond to it. Most IR spectrometers use front-surfaced mirrors to eliminate the necessity for the transmission of radiant energy through quartz, glass, or other lens materials. These mirrors are usually parabolic to focus the diffuse IR energy. Such instruments must be protected from high humidities and water vapor to avoid deterioration of the optical system and the presence of extraneous absorption bands in the IR.

420

The energy or light source for an IR instrument may be a Nernst glower or a Globar. Each of these sources has certain characteristics that recommend it for use, but the more rugged Globar is more commonly used because it also has a more stable emission. The receiving or detection unit may be a thermocouple, bolometer, thermistor, or photoconductor cell. The type of analysis being performed dictates the degree of sophistication required in the IR instrumentation selected to acquire usable data.

3.6.4 Proper Use of Spectrometers

The spectrometer manufacturer's instructions for proper use should be followed in all cases. Several safeguards against misuse of the instruments, however, are mandatory.

Instruments should be checked for wavelength alinement. If a particular colored solution is to be used at a closely specified wavelength, considerable loss of sensitivity can be encountered if the wavelength control is misalined. In visible-range instruments, an excellent reference point is the maximum absorption for a diluted solution of potassium permanganate, which has dual peaks at 526 and 546 nm. On inexpensive instruments with less resolution the permanganate peak appears at 525 to 550 nm as a single, flat-topped peak.

For both UV and IR instruments, standard absorption curves for many organic materials have been published so that reference material for standard peaks is easily available. Standard films of styrene and other transparent plastics are available for IR wavelength checks. A very good discussion of factors that affect the proper performance of spectrometers and standard reference materials available to calibrate them can be found in publications of the National Bureau of Standards.* Use of certified standards is encouraged whenever practical.

Too much emphasis cannot be placed on care of absorption cells. All absorption cells should be kept scrupulously clean, free of scratches, fingerprints, smudges, and evaporated film residues. Matched cells should be checked to see that they are equivalent, and any differences should be accounted for during use or in the final data. Directions for cleaning cells are given in chapter 4.

Generally speaking, trained technicians may operate any of the spectrometers successfully; however, because interpretation of data from both the UV and IR instruments is becoming increasingly complex, mere compliance with the operations manual may not be sufficient for completely accomplishing the special techniques of sample preparation, instrument operation, and interpretation of absorption curves.

3.6.5 Atomic Absorption

There are a number of differences in the basic design and accessories for atomic absorption (AA) equipment that require consideration before purchase and during subsequent use. These choices concern the light sources, nebulizer burners, optical systems, readout devices, and mode conversions. Because some of these choices are not readily obvious, the purchaser or user must be familiar with the types and numbers of samples to be analyzed and the

*NBS, Office of Standard Reference Materials, Institute for Materials Research, Washington, D.C. 20234.

specific elements to be measured before making the choice. For a program analyzing a wide variety of samples for a number of elements at varying concentrations, an instrument of maximum versatility would be best. Most of the discussion that follows applies to use of instrumentation in absorption, emission, or fluorescence modes.

3.6.5.1 Lamps

Hollow-cathode (HC) lamps or electrodeless-discharge lamps (EDL) are available for over 70 elements with single-element or multielement capability. Multielement lamps are considerably cheaper per element than single-element lamps, but the savings may not be realized if the lamps are not used strategically, because all the elements in the cathode deteriorate when the lamp is used, regardless of which element is measured. The deterioration phenomena result from the different volatilities of metals used in the cathode. One metal volatilizes (sputters) more rapidly than the others and redeposits upon an area of the other cathode metals. Thus, with continual use, a drift in signal will be noted with at least one metal increasing and the other (or others) decreasing. If one can ignore the dubious cost savings of multielement lamps, use of single-element lamps could result in more precise and accurate data.

The line intensities of one element in a multielement HC lamp will usually be less than those of a lamp containing a pure cathode of the same element because this element must share the discharge energy with the other elements present. However, this reduction should not affect the output by a factor of more than one-sixth to one-half, depending on the particular combination and the number of elements combined. The output can be even greater in some multielement lamps because alloying may permit a higher operating current than for the case of the pure cathode. All HC lamps have life expectancies that are related to the volatility of the cathode metal, and for this reason the manufacturer's recommendations for operating should be closely followed.

Recent improvements in design and manufacture of HC lamps and EDLs have resulted in lamps with more constant output and longer life. Under normal conditions an HC lamp may be expected to operate satisfactorily for several years. HC lamps used to be guaranteed for a certain minimum ampere-hour period, but this has been changed to a 90-day warranty. It is good practice to date newly purchased lamps and to inspect them immediately upon receipt. The operating current and voltage indicated on the lamp should not be exceeded during use. An increase in background noise or a loss of sensitivity are signs of lamp deterioration.

A basic design feature of AA spectrometers is the convenience of the HC lamp changeover system. Some instruments provide for as many as six lamps in a rotating turret, all electronically stabilized and ready for use by simply rotating the lamp turret. Other instruments provide for use of only one lamp at a time in the lamp housing, and require manual removal and replacement whenever more than one element is to be measured. A quick-changeover system enables frequent lamp changes during the period of operation. If necessary lamp changes are infrequent, however, multilamp mounts do not represent a great convenience.

After the proper lamp has been selected, the HC current should be adjusted according to the manufacturer's recommendations and allowed to electronically stabilize (warm up) before use. During this 15-min period, the monochromator may be positioned at the correct wavelength, and the proper slit width may be selected. For those instruments employing a multilamp turret, a warmup current is provided to those lamps not in use, thereby

422

minimizing the warmup period after the turret is rotated. In a single-lamp instrument, the instability exhibited during warmup is minimized by the use of a double-beam optical system.

3.6.5.2 Burner Types

The most difficult and inefficient step in the AA process is converting the metal in the sample from an ion or a molecule to the neutral atomic state. It is the function of the atomizer/burner to produce the desired neutral atomic condition of the elements. With minor modifications burners are the same as those used for flame photometry.

Basically there are two different types of burners. They are the total-consumption, or surface-mix burner, and the laminar-flow or premix burner. There are many variations of these two basic types, such as the Boling, the high-solids, the turbulent-flow, the triflame, the nitrous oxide burners, and many others. As one might expect, there are many similarities among the various burners, the different names resulting from the different manufacturers. The element being determined and the type of sample solution dictate the type of burner to be used. Generally, all types and makes of burner can be adjusted laterally, rotationally, and vertically for selection of the most sensitive area of the flame for the specific element sought.

Nonflame techniques have gained wide acceptance in AA analysis because of the extreme sensitivity and the capability to directly introduce very diverse sample matrices. These systems, which replace the conventional flame burner, come in various designs using electrical resistance to produce temperatures as high as 3,500°C.

3.6.5.3 Single- and Double- (Split-) Beam Instruments

There is a great deal of existing uncertainty among instrument users about the relative merits of single- and double-beam instruments. Neither system is appropriate for all cases.

With a single-beam instrument the light beam from the source passes directly through the flame to the detector. In a double-beam system the light from the source is divided by a beam splitter into two paths. One path, the reference beam, goes directly to the detector. The second path, the sample beam, goes through the flame to the detector. The chopper alternately reflects and passes each beam, creating two equal beams falling alternately upon the detector. If the beams are equal, they cancel the alternate impulses reaching the detector, and no signal is generated. If the beams are different, the resulting imbalance causes the detector to generate a signal that is amplified and measured. The difference between the reference and sample beams is then determined as a direct function of absorbed light. The advantages of the double-beam design are that any variations in the source are of reduced importance, and smaller dependence is placed upon the stability of the power supply. Conversely, stabilization of the power supply can eliminate the apparent need for the split-beam system. Furthermore, the beam splitter requires additional mirrors or optical accessories that cause some loss of radiant energy.

A single-beam system does not monitor source variations, but offers certain other advantages. It allows use of low-intensity lamps, smaller slit settings, and smaller gain. As a consequence, the single-beam instrument, properly designed, is capable of operating with lower noise and better signal-to-noise ratio, and therefore with better precision and improved sensitivity. Because the simplified optical system conserves radiant energy,

especially in the shorter wavelengths, it facilitates operation in the low-wavelength range. With this advantage, it should be possible to obtain better sensitivity for those elements with a strong resonance line below 350 nm and particularly those below 300 nm. Background correction techniques are also available in single-beam systems.

Double-beam instruments, however, offer the opportunity to perform more sophisticated techniques like background correction, two-channel multielement analysis, use of internal standards, and element rationing. If one of these techniques is necessary, a double-beam instrument must be considered.

3.6.5.4 Readout Devices

Readout devices in even the lower cost AA instruments include digital concentration display. Using high-speed digital electronics, data-handling techniques encompass multiple calibration standards, regression analysis to characterize the calibration curves, mean variance and standard deviation statistical programs for sample calculations, and various forms of printout reports in addition to recorder output. Choice of a readout system is predicated largely upon laboratory needs and availability of budget. In general, any step toward automation is desirable, but the degree of automation should be compatible with the laboratory program.

3.6.5.5 Miscellaneous Accessories

A number of instruments contain a mode selector, making an instrument usable for either absorption or emission. The conversion to emission may be a desirable feature because certain elements are more amenable to analysis by this method. Some models offer an option of atomic fluorescence and can also be used as a UV/visible spectrometer.

Automatic sample changers are offered for almost all instruments on the market, and as has been previously stated, any automation feature is desirable. However, unless a laboratory program performs a large number of repetitious measurements daily, an automatic sample changer is not required. As a practical measure, other commonly used sample-changing devices, although not expressly designed for AA use, can be interfaced with most AA instruments.

3.7 Organic Carbon Analyzers

A number of instruments designed to measure total organic carbon (TOC) in waters and wastes have appeared on the market in the past several years. The first of these units involved pyrolysis followed by IR measurement of the carbon dioxide formed. Sample injection of 20 to 200 μl in a carrier gas of air or oxygen was performed with a syringe. Combustion at 800°C to 900°C followed by IR analysis was performed automatically with final output on an analog recorder. Systems using these principles are still produced and represent a large part of the TOC market.

Other techniques of TOC analysis that modify every phase of the original TOC instruments have been introduced. Sample presentation in small metallic boats and purging of CO_2 from solution are two new techniques. Wet chemical oxidation, either external to the instrument or within the instrument, using various oxidants including ultraviolet irradiation is now in wide use. Measurement of the CO_2 by reduction to methane (CH_4) and quantitation with a flame ionization detector are also available. Various methods of data handling are now used,

424

ranging from recorder output to direct readings and printouts of concentration. Techniques are also available for measuring materials like soil and sludges, and also the volatile component of the TOC. Sensitivity on some systems has been extended down to the microgram per liter level. The major problems associated with TOC measurements are interference from forms of inorganic carbon and the difficulty of obtaining a representative sample in the presence of particulate matter. Each system has its own procedure for sample pretreatment or for accounting for these problems. When choosing a TOC instrument, consideration should be given to the types of samples to be analyzed, the expected concentration range, and the forms of carbon to be measured.

3.8 Gas Chromatographs

Because GC's are available from a large number of manufacturers, selection of a particular manufacturer may be based on convenience. No single multipurpose GC instrument permits analysis of a wide range of compounds. In this case, a GC/MS could be considered (11). If, however, relatively few types of environmentally significant compounds are being surveyed, an inexpensive system equipped with a glass-lined injection port, electrolytic conductivity detector, and analog recorder is a good choice. A review of the organic methods (12) to be used will give the analyst all the necessary information on the specific instrument, apparatus, and materials necessary for each type or class of compounds. A discussion of various quality control considerations in GC analysis is given in chapter 8 of this manual.

Data handling requirements vary widely, and the need to automate GC data collection is determined by the extent of the sample load. In a routine monitoring laboratory, GC systems incorporating their own microprocessers and report generating capabilities would be useful in solving this problem. Because such systems greatly increase the cost, the overall economy of this choice must be considered.

3.9 References

1. "Single Arm Balances Testing," Part 41 of 1976 Book of ASTM Standards, American Society for Testing and Materials, Philadelphia (1976).
2. Sorenson, S. P. L., Biochem. Z., *21*, 201 (1909).
3. "pH of Water and Wastewater," from Part 31 of 1976 Book of ASTM Standards, American Society for Testing and Materials, Philadelphia (1976).
4. Catalog of NBS Standard Reference Materials, NBS Special Publication 260, National Bureau of Standards (1975-76).
5. Methods for Chemical Analysis of Water and Wastes, U.S. EPA, Office of Research and Development, EMSL, Cincinnati (1978).
6. Rechnitz, G. A., "Ion-Selective Electrodes," Chem. Eng. News (June 12, 1967), p. 146.
7. Riseman, Jean M., "Measurement of Inorganic Water Pollutants by Specific Ion Electrode," American Laboratory (July 1969), p. 32.
8. Koryta, Jiri, "Theory and Application of Ion-Selective Electrodes," Anal. Chim. Acta, *61*, 329-411 (1972).
9. Covington, A. L., "Ion-Selective Electrodes," CRC Critical Review in Anal. Chem. (Jan. 1974), pp. 355-406.
10. Black, A. P., and Hannah, S. A., "Measurement of Low Turbidities," J. Am. Water Works Assoc., *57*, 901 (1965).
11. EPA GC/MS Procedural Manual, Budde, W. L., and Eichelberger, J. W., Editors, 1st Edition, Vol. 1, U.S. EPA, Office of Research and Development, EMSL, Cincinnati (in press).

105

12. Methods for Organic Analysis of Water and Wastes, U.S. EPA, EMSL, Environmental Research Center, Cincinnati (in preparation).

Chapter 4

GLASSWARE

4.1 General

The measurement of trace constituents in water demands methods capable of maximum sensitivity. This is especially true for metals and trace organics such as pesticides, as well as for the determination of ammonia and phosphorus. In addition to sensitive methods, however, there are other areas that require special consideration. One such area is that of the cleanliness of laboratory glassware. Obviously, the very sensitive analytical systems are more sensitive to errors resulting from the improper use or choice of apparatus, as well as to contamination effects due to an improper method of cleaning the apparatus. The purpose of this chapter is to discuss the kinds of glassware available, the use of volumetric ware, and various cleaning requirements.

4.2 Types of Glassware

Laboratory vessels serve three functions: storage of reagents, measurement of solution volumes, and confinement of reactions. For special purposes, vessels made from materials such as porcelain, nickel, iron, aluminum, platinum, stainless steel, and plastic may be employed to advantage. Glass, however, is the most widely used material of construction. There are many grades and types of glassware from which to choose, ranging from student grade to others possessing specific properties such as super strength, low boron content, and resistance to thermal shock or alkali. Soft glass containers are not recommended for general use, especially for storage of reagents because of the possibility of dissolving of the glass (or of some of the constituents of the glass). The mainstay of the modern analytical laboratory is a highly resistant borosilicate glass, such as that manufactured by Corning Glass Works under the name "Pyrex" or by Kimble Glass Co. as "Kimax." This glassware is satisfactory for all analyses included in reference 1.

Depending on the particular manufacturer, various trade names are used for specific brands possessing special properties such as resistance to heat, shock, and alkalies. Examples of some of these special brands follow:

a. Kimax- or Pyrex-brand glass is a relatively inert all-purpose borosilicate glass.

b. Vycor-brand glass is a silica glass (96 percent) made to withstand continuous temperatures up to 900°C and can be down-shocked in ice water without breakage.

c. Corning-brand glass is claimed to be 50 times more resistant to alkalies than conventional ware and practically boron-free (maximum 0.2 percent).

d. Ray-Sorb- or Low-Actinic-brand glass is used when the reagents or materials are light sensitive.

e. Corex-brand labware is harder than conventional borosilicates and therefore better able to resist clouding and scratching.

The use of plastic vessels, containers, and other apparatus made of Teflon, polyethylene, polystyrene, and polypropylene has increased markedly over recent years. Some of these

427

materials, such as Teflon, are quite expensive; however, Teflon stopcock plugs have practically replaced glass plugs in burets, separatory funnels, etc., because lubrication to avoid sticking or "freezing" is not required. Polypropylene, a methylpentene polymer, is available as laboratory bottles, graduates, beakers, and even volumetric flasks. It is crystal clear, shatterproof, autoclavable, and chemically resistant.

The following are some points to consider in choosing glassware or plasticware:

a. The special types of glass listed above, other than Pyrex or Kimax, generally are not required to perform the analyses given in "Methods for Chemical Analysis of Water and Wastes" (1).

b. Unless instructed otherwise, borosilicate or polyethylene bottles may be used for the storage of reagents and standard solutions.

c. Dilute metal solutions are prone to plate out on container walls over long periods of storage. Thus, dilute metal standard solutions must be prepared fresh at the time of analysis.

d. For some operations, disposable glassware is entirely satisfactory. One example is the use of disposable test tubes as sample containers for use with the Technicon automatic sampler.

e. Plastic bottles of polyethylene and Teflon have been found satisfactory for the shipment of water samples. Strong mineral acids (such as sulfuric acid) and organic solvents will readily attack polyethylene and are to be avoided.

f. Borosilicate glassware is not completely inert, particularly to alkalies; therefore, standard solutions of silica, boron, and the alkali metals are usually stored in polyethylene bottles.

For additional information the reader is referred to the catalogs of the various glass and plastic manufacturers. These catalogs contain a wealth of information such as specific properties, uses, and sizes.

4.3 Volumetric Analyses

By common usage, accurately calibrated glassware for precise measurements of volume has become known as volumetric glassware. This group includes volumetric flasks, volumetric pipets, and accurately calibrated burets. Less accurate types of glassware including graduated cylinders and serological and measuring pipets also have specific uses in the analytical laboratory when exact volumes are unnecessary.

The precision of volumetric work depends in part upon the accuracy with which volumes of solutions can be measured. There are certain sources of error that must be carefully considered. The volumetric apparatus must be read correctly; that is, the bottom of the meniscus should be tangent to the calibration mark. There are other sources of error, however, such as changes in temperature, which result in changes in the actual capacity of glass apparatus and in the volume of the solutions. The capacity of an ordinary glass flask of 1000-ml volume increases 0.025 ml/deg with rise in temperature, but if the flask is made of borosilicate glass, the increase is much less. One thousand milliliters of water or of most 0.1*N* solutions increases in volume by approximately 0.20 ml/deg increase at room

428

temperature. Thus solutions must be measured at the temperature at which the apparatus was calibrated. This temperature (usually 20°C) will be indicated on all volumetric ware. There may also be errors of calibration of the apparatus; that is, the volume marked on the apparatus may not be the true volume. Such errors can be eliminated only by recalibrating the apparatus or by replacing it.

Volumetric apparatus is calibrated to contain or to deliver a definite volume of liquid. This will be indicated on the apparatus with the letters "TC" (to contain) or "TD" (to deliver). Volumetric flasks are calibrated to contain a given volume and are available in various shapes and sizes.

Volumetric pipets are calibrated to deliver a fixed volume. The usual capacities are 1 through 100 ml although micropipets are also available. Micropipets are most useful in furnace work and are available in sizes ranging from 1 to 100 μl.

In emptying volumetric pipets, they should be held in a vertical position and the outflow should be unrestricted. The tip of the pipet is kept in contact with the wall of the receiving vessel for a second or two after the free flow has stopped. *The liquid remaining in the tip is not removed*; this is most important.

Measuring and serological pipets should also be held in a vertical position for dispensing liquids; however, the tip of the pipet is only touched to the wet surface of the receiving vessel after the outflow has ceased. For those pipets where the small amount of liquid remaining in the tip is to be blown out and added, indication is made by a frosted band near the top.

Burets are used to deliver definite volumes. The more common types are usually of 25- or 50-ml capacity, graduated to tenths of a milliliter, and are provided with stopcocks. For precise analytical methods in microchemistry, microburets are also used. Microburets generally are of 5- or 10-ml capacity, graduated in divisions of hundredths of a milliliter. Automatic burets with reservoirs are also available ranging in capacity from 10 to 100 ml. Reservoir capacity ranges from 100 to 4,000 ml.

General rules in regard to the manipulation of a buret are as follows: Do not attempt to dry a buret that has been cleaned for use, but rinse it two or three times with a small volume of the solution with which it is to be filled. Do not allow alkaline solutions to stand in a buret because the glass will be attacked, and the stopcock, unless made of Teflon, will tend to freeze. A 50-ml buret should not be emptied faster than 0.7 ml/s, otherwise too much liquid will adhere to the walls and as the solution drains down, the meniscus will gradually rise, giving a high false reading. It should be emphasized that improper use or reading of burets can result in serious calculation errors.

In the case of all apparatus for delivering liquids, the glass must be absolutely clean so that the film of liquid never breaks at any point. Careful attention must be paid to this fact or the required amount of solution will not be delivered. The various cleaning agents and their use are described later.

4.4 Federal Specifications for Volumetric Glassware

Reference 2 contains a description of Federal specifications for volumetric glassware. The National Bureau of Standards no longer accepts stock quantities of volumetric apparatus

from manufacturers or dealers for certification and return for future sale to consumers. This certification service is still available, but apparatus will be tested only when submitted by the ultimate user, and then only after an agreement has been reached with the Bureau concerning the work to be done.

Consequently, the various glass manufacturers have discontinued the listing of NBS-certified ware. In its place catalog listings of volumetric glass apparatus that meet the Federal specifications are designated as class A and all such glassware is permanently marked with a large "A." These NBS specifications are listed in table 4-1. The glassware in question includes the usual burets, volumetric flasks, and volumetric pipets.

In addition to the "A" marking found on calibrated glassware and the temperature at which the calibration was made, other markings also appear. These include the type of glass, such

Table 4-1
TOLERANCES FOR VOLUMETRIC GLASSWARE[1]

Type of Glassware	Capacity[2] (ml)	Limit of Error (ml)
Graduated flask	25	0.03
	50	0.05
	100	0.08
	200	0.10
	250	0.11
	300	0.12
	500	0.15
	1,000	0.30
	2,000	0.50
Transfer pipet	2	0.006
	5	0.01
	10	0.02
	25	0.025
	30	0.03
	50	0.05
	100	0.08
	200	0.10
Buret[3]	5	0.01
	10	0.02
	30	0.03
	50	0.05
	100	0.10

[1] Abridged from reference 3.
[2] Less than and including.
[3] Limits of error are of total or partial capacity. Customary practice is to test the capacity at five intervals.

430

as Pyrex, Corex, or Kimax; the stock number of the particular item; and the capacity of the vessel. If the vessel contains a ground-glass connection, this will also be included along with the TD or TC symbol. An example of the markings usually found on volumetric ware is shown in figure 4-1. Class A glassware need not be recalibrated before use. However, if it should become necessary to calibrate a particular piece of glassware, directions may be found in texts (4) on quantitative analysis.

4.5 Cleaning of Glass and Porcelain

The method of cleaning should be adapted to both the substances that are to be removed, and the determination to be performed. Water-soluble substances are simply washed out with hot or cold water, and the vessel is finally rinsed with successive small amounts of distilled water. Other substances more difficult to remove may require the use of a detergent, organic solvent, dichromate cleaning solution, nitric acid, or aqua regia (25 percent by volume concentrated HNO_3 in concentrated HCl). In all cases it is good practice to rinse a vessel with tap water as soon as possible after use. Material allowed to dry on glassware is much more difficult to remove.

Volumetric glassware, especially burets, may be thoroughly cleaned by a mixture containing the following: 30 g of sodium hydroxide, 4 g of sodium hexametaphosphate (trade name, Calgon), 8 g of trisodium phosphate, and 1 l of water. A gram or two of sodium lauryl sulfate or other surfactant will improve its action in some cases. This solution should be used with a buret brush.

Dichromate cleaning solution (chromic acid) is a powerful cleaning agent; however, because of its destructive nature upon clothing and upon laboratory furniture, extreme care must be taken when using this mixture. If any of the solution is spilled, it must be cleaned up immediately. Chromic acid solution may be prepared in the laboratory by adding 1 l of concentrated sulfuric acid slowly, with stirring, to a 35-ml saturated sodium dichromate solution. This mixture must be allowed to stand for approximately 15 min in the vessel that is being cleaned and may then be returned to a storage bottle. Following the chromic acid wash, the vessels are rinsed thoroughly with tap water, then with small successive portions of distilled water. The analyst should be cautioned that when chromium is included in the

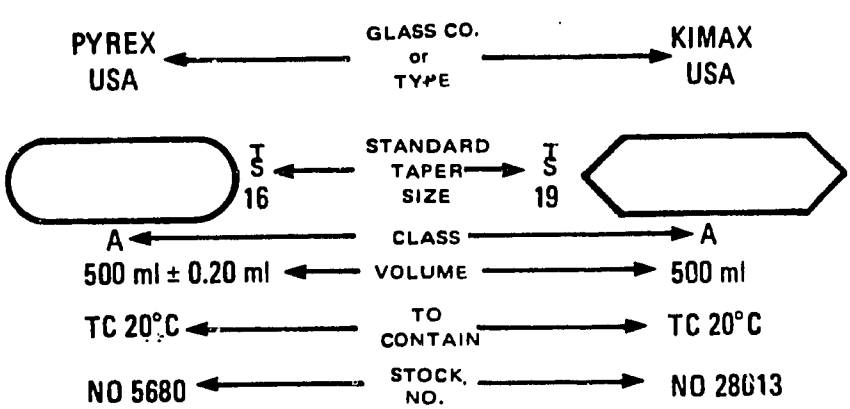


Figure 4-1. Example of markings on glassware.

431

scheme of analysis, it is imperative that the last traces of dichromate be removed from the apparatus. To this end, a substitute for dichromate cleaning solution, called Nochromix,* is available and may be used to advantage. Fuming nitric acid is another powerful cleaning agent, but is disagreeable to handle. As with dichromate, when the acid becomes dilute, the cleaning mixture is no longer effective. A mixture of concentrated sulfuric and fuming nitric acids is even more efficient but is also hazardous to use. A persistent greasy layer or spot may be removed by acetone or by allowing a warm solution of sodium hydroxide, about 1 g per 50 ml of water, to stand in the vessel for 10 to 15 min; after rinsing with water, dilute hydrochloric acid, and water again, the vessel is usually clean. Alcoholic potassium hydroxide is also effective in removing grease. To dry glass apparatus, rinse with acetone and blow or draw air through it.

4.6 Special Cleaning Requirements

Absorption cells, used in spectrophotometers, should be kept scrupulously clean, free of scratches, fingerprints, smudges, and evaporated film residues. The cells may be cleaned with detergent solutions for removal of organic residues, but should not be soaked for prolonged periods in caustic solutions because of the possibility of etching. Organic solvents may be used to rinse cells in which organic materials have been used. Nitric acid rinses are permissible, but dichromate solutions are not recommended because of the adsorptive properties of dichromate on glass. Rinsing and drying of cells with alcohol or acetone before storage is a preferred practice. Matched cells should be checked to see that they are equivalent by placing portions of the same solution in both cells and taking several readings of the transmittance (T, percent) or optical density (OD) values.

For certain determinations, especially trace metals, the glassware should also be rinsed with a 1:1 nitric acid-water mixture. This operation is followed by thoroughly rinsing with tap water and successive portions of distilled water. This may require as many as 12 to 15 rinses, especially if chromium is being determined. The nitric acid rinse is also especially important if lead is being determined.

Glassware to be used for phosphate determinations should not be washed with detergents containing phosphates. This glassware must be thoroughly rinsed with tap water and distilled water. For ammonia and Kjeldahl nitrogen, the glassware must be rinsed with ammonia-free water. (See ch. 2.)

Glassware to be used in the determination of trace organic constituents in water, such as chlorinated pesticides, should be as free as possible of organic contaminants. A chromic acid wash of at least 15 min is necessary to destroy these organic residues. Rinse thoroughly with tap water and, finally, with distilled water. Glassware may be dried for immediate use by rinsing with redistilled acetone. Otherwise glassware may be oven dried or drip dried. Glassware should be stored immediately after drying to prevent any accumulation of dust and stored inverted or with mouth of glassware covered with foil.

Bottles to be used for the collection of samples for organic analyses should be rinsed successively with chromic acid cleaning solution, tap water, distilled water, and, finally, several times with a redistilled solvent such as acetone, hexane, petroleum ether, or chloroform. Caps are washed with detergent, rinsed with tap water, distilled water, and

*Available from Godax Laboratories, 6 Varick Street, New York, N.Y. 10013.

432

solvent. Liners are treated in the same way as the bottles and are stored in a sealed container.

4.7 Disposable Glassware

When the risk of washing a pipet for reuse becomes too great, as in the case of use with toxic materials, or when the cost of washing glassware becomes prohibitive, disposable vessels may be the answer, provided they meet the necessary specification. Various types are available including bacteriological, serological, and microdilution pipets. Disposable glassware generally is made of soft glass although plastic vessels and pipets are also available.

4.8 Specialized Glassware

The use of vessels and glassware fitted with standard-taper, ground-glass, and ball-and-socket joints has increased because of certain advantages such as less leakage and fewer freezeups. Standard-taper, interchangeable ground joints save time and trouble in assembling apparatus. They are precision-ground with tested abrasives to insure an accurate fit and freedom from leakage. Ball and socket joints increase flexibility of operation and eliminate the need for exact alinements of apparatus. Symbols and their meaning as applied to standard joints, stoppers, and stopcocks are shown below.

4.8.1 Standard Taper (Ŧ)

The symbol Ŧ is used to designate interchangeable joints, stoppers, and stopcocks that comply with the requirements of reference 5. All mating parts are finished to a 1:10 taper.

The size of a particular piece appears after the appropriate symbol. Primarily because of greater variety of apparatus equipped with Ŧ fittings, a number of different types of identifications are used:

a. For joints—a two-part number as Ŧ 24/40, with 24 being the approximate diameter in millimeters at the large end of the taper and 40 the axial length of taper, also in millimeters

b. For stopcocks—a single number, as Ŧ 2, with 2 mm being the approximate diameter of the hole or holes through the plug

c. For bottles—a single number, as Ŧ 19, with 19 mm being the appropriate diameter at top of neck. However, there are differences in dimensions between the bottle and flask stoppers

d. For flasks and similar containers—a single number, as Ŧ 19, with 19 mm being the appropriate diameter of the opening at top of neck

4.8.2 Spherical Joints (§)

The designation § is for spherical (semiball) joints complying with reference 5. The complete designation of a spherical joint also consists of a two-part number, as 12/2, with 12 being the approximate diameter of the ball and 2 the bore of the ball and the socket, also in millimeters.

433

4.8.3 Product Standard (§)

The symbol § is used for stopcocks with Teflon plugs, the mating surfaces being finished to a 1:5 taper. As with ⅊ stopcocks, a single number is used. Thus, § 2 means a Teflon stopcock with a hole of approximately 2-mm diameter in the plug.

4.9 Fritted Ware

For certain laboratory operations the use of fritted ware for filtration, gas dispersion, absorption, or extractions may be advantageous.

There are six different porosities of fritted ware available, depending on its intended use. Porosity is controlled in manufacture, and disks are individually tested and graded into these classifications. The extra-coarse and coarse porosities are held toward the maximum pore diameter as listed. The medium, fine, very fine, and ultrafine are held toward the minimum pore diameter as listed in table 4-2.

Pore sizes are determined by the method specified in reference 6.

4.9.1 Recommended Procedures for Maximum Filter Life

a. New Filters. Wash new filters by suction with hot hydrochloric acid, followed by a water rinse.

b. Pressure Limits. The maximum, safe, differential pressure on a disk is 15 lb/in^2.

c. Thermal Shock. Fritted ware has less resistance to thermal shock than nonporous glassware. Hence, excessive, rapid temperature changes and direct exposure to a flame should be avoided. Heating in a furnace to 500°C may be done safely, provided the heating and cooling are gradual. Dry ware may be brought to constant weight by heating at 105°C to 110°C.

Never subject a damp filter of ultrafine porosity to a sudden temperature change. Steam produced in the interior may cause cracking.

Table 4-2
FRITTED-WARE POROSITY

Porosity Grade	Designation	Pore Size (μm)	Principal Uses
Extra Coarse	EC	170-220	Coarse filtration; gas dispersion, washing, and absorption
Coarse	C	40-60	Coarse filtration; gas dispersion, washing, and absorption
Medium	M	10-15	Filtration and extraction
Fine	F	4-5.5	Filtration and extraction
Very Fine	VF	2-2.5	General bacterial filtration
Ultrafine	UF	0.9-1.4	General bacterial filtration

434

4.9.2 Cleaning of Used Filters

In many cases, precipitates can be removed by rinsing with water, passed through from the underside, with the pressure not exceeding 15 lb/in^2. The suggestions that follow will be helpful in dealing with material that will not be removed by such a reverse water-wash:

Material	*Removal Agent*
Albumen	Hot ammonia or hydrochloric acid
Aluminous and siliceous residues	Hydrofluoric acid (2 percent) followed by concentrated sulfuric acid; rinse immediately with water until no trace of acid can be detected.
Copper or iron oxides	Hot hydrochloric acid plus potassium chlorate
Fatty materials	Carbon tetrachloride
Mercuric sulfide	Hot aqua regia
Organic matter	Hot, concentrated cleaning solution, or hot, concentrated sulfuric acid with a few drops of sodium nitrite
Silver chloride	Ammonium or sodium hyposulfite

4.10 References

1. Methods for Chemical Analysis of Water and Wastes, U.S. EPA, Office of Research and Development, EMSL (1978).
2. Hughes, J. C., Testing of Glass Volumetric Apparatus, NBS Circular 602, National Bureau of Standards (1959).
3. Peffer, E. L., and Mulligan, G. C., Testing of Glass Volumetric Apparatus, NBS Circular 434, National Bureau of Standards (1941).
4. Willard, H. H., and Furman, N. H., Elementary Quantitative Analysis–Theory and Practice, D. Van Nostrand Co., Inc., New York (1947).
5. Interchangeable Ground Glass Joints, Commercial Standard CS-21-30, National Bureau of Standards (Aug. 25, 1930).
6. Maximum Pore Diameter and Permeability of Rigid Porous Filters for Laboratory Use, E-128, American Society for Testing and Materials, Philadelphia (1968).

435

Chapter 5

REAGENTS, SOLVENTS, AND GASES

5.1 Introduction

The objective of this chapter is to provide general information and suggestions that will serve to keep the analyst conscious of his responsibilities in analytical quality control, as they relate to reagents, solvents, and gases. While the material presented here will assist the analyst in producing high-quality data, it is by no means complete. It is incumbent on the analyst to obtain details of special precautions required to insure proper selection, preparation, and storage of reagents, solvents, and gases from the descriptions of individual methods.

5.2 Reagent Quality

Chemical reagents, solvents, and gases are available in a wide variety of grades of purity, ranging from technical grade to various ultrapure grades. The purity of these materials required in analytical chemistry varies with the type of analysis. The parameter being measured and the sensitivity and specificity of the detection system are important factors in determining the purity of the reagents required. For many analyses, including most inorganic analyses, analytical reagent grade is satisfactory. Other analyses, such as trace organic and radiological, frequently require special ultrapure reagents, solvents, and gases. In methods where the purity of reagents is not specified it is intended that analytical reagent grade be used. Reagents of lesser purity than that specified by the method should not be used. The labels on the container should be checked and the contents examined to verify that the purity of the reagents meets the needs of the particular method involved. The quality of reagents, solvents, and gases required for the various classes of analyses—inorganic, metals, radiological, and organic—are discussed in this section.

Reagents must always be prepared and standardized with the utmost of care and technique against reliable primary standards. They must be restandardized or prepared fresh as often as required by their stability. Stock and working standard solutions must be checked regularly for signs of deterioration: e.g., discoloration, formation of precipitates, and change of concentration. Standard solutions should be properly labeled as to compound, concentration, solvent, date, and preparer.

Primary standards must be obtained from a reliable source, pretreated (e.g., dried, under specified conditions), accurately prepared in calibrated volumetric glassware, and stored in containers that will not alter the reagent. A large number of primary standards are available from the National Bureau of Standards (NBS). A complete listing of available standards is given in reference 1. Primary standards may also be obtained from many chemical supply companies. Suppliers for special quality reagents, solvents, and gases are noted in later discussions of the various classes of analyses. Reagents and solvents of all grades are available from many chemical supply houses.

There is some confusion among chemists as to the definition of the terms "Analytical Reagent Grade," "Reagent Grade," and "ACS Analytical Reagent Grade." A review of the literature and chemical supply catalogs indicates that the three terms are synonymous. Hereafter, in this document, the term "Analytical Reagent Grade" (AR) will be used. It is

426

intended that AR-grade chemicals and solvents shall conform to the current specifications of the Committee on Analytical Reagents of the American Chemical Society (2).

References 3 through 5 devote several chapters to problems related to preparation, standardization, and storage of reagents. The information provided therein is particularly appropriate to inorganic determinations. The type of volumetric glassware to be used, the effect of certain reagents on glassware, the effect of temperature on volumetric measurements, purity of reagents, absorption of gases and water vapor from the air, standardization of solutions, instability, and the need for frequent standardization of certain reagents are among the topics discussed. It is recommended that the analyst become thoroughly familiar with these publications.

5.2.1 General Inorganic Analyses

In general, AR-grade reagents and solvents are satisfactory for inorganic analyses. Primary standard reagents must, of course, be used for standardizing all volumetric solutions. Commercially prepared reagents and standard solutions are very convenient and may be used when it is demonstrated that they meet the method requirements. All prepared reagents must be checked for accuracy.

The individual methods specify the reagents that require frequent standardization, or other special treatment, and the analyst must follow through with these essential operations. To avoid waste, the analyst should prepare a limited volume of such reagents, depending on the quantity required over a given period of time. Examples and brief discussions of the kind of problems that occur are given in section 5.3.

As far as possible, distilled water used for preparation of reagent solutions must be free of measurable amounts of the constituent to be determined. Special requirements for distilled water are given in chapter 3 of this manual and in individual method descriptions.

Compressed gases, such as oxygen and nitrogen, used for total organic carbon determination may be of commercial grade.

5.2.2 Metals Analyses

All standards used for atomic absorption and emission spectroscopy should be of spectroquality. It is recommended that other reagents and solvents also be of spectroquality, although AR grade is sometimes satisfactory. Standards may be prepared by the analyst in the laboratory, or spectrographically standardized materials may be purchased commercially. Standards required for determination of metals in water are not generally available from the National Bureau of Standards.

Analytical-reagent-grade nitric and hydrochloric acids must be specially prepared by distillation in borosilicate glass and diluted with deionized distilled water. All other reagents and standards are also prepared in deionized water.

In general, fuel and oxidant gases used for atomic absorption can be of commercial grade. Air supplied by an ordinary laboratory compressor is quite satisfactory, if adequate pressure is maintained and necessary precautions are taken to filter oil, water, and possible trace metals from the line. For certain determinations such as aluminum, AR-grade nitrous oxide is required.

5.2.3 Radiological Analyses

The great sensitivity of radioactive counting instruments requires that scintillation-grade reagents and solvents, or equivalent, be used for all radioactivity determinations. Some of the reagents, for example, strontium carbonate and yttrium oxide carriers used for the determination of strontium-90 and yttrium-90, must be stable, that is, free of radioactivity. Barium sulfate, used for coprecipitation of radium, must be free from all traces of radium. These reagents and solvents are commercially available from chemical supply houses.

Calibrated standard sources of specific radioactive materials with known count and date of counting are available from various suppliers. No single company supplies all standards.

Gases used for radioactive counting must be of high purity and extra dry. Gases such as helium and air are aged for about 30 days to allow radioactive background to decay. All gases are checked for background before use. Some cylinders contain inherent radioactivity that is imparted to the gas. When this background is above normal, the gas should not be used for radioactivity determinations.

5.2.4 Organic Chemical Analyses

The minimum purity of reagents that can be used for organic analyses is AR grade. Reference-grade standards should be used whenever available. Special note should be taken of the assay of standard materials. Owing to the great sensitivity (nanogram and subnanogram quantities) of gas chromatography (GC), which is often used to quantitate organic results, much greater purity is frequently required (6). The specificity of some GC detectors requires that reagents and solvents be free of certain classes of compounds. For example, analyses by electron capture require that reagents and solvents be free of electronegative materials that would interfere with the determination of specific compounds in the sample. Similarly, use of the flame photometric detector requires that reagents and solvents be free from sulfur and phosphorus interference. Pesticide-quality solvents, available from several sources, are required when doing low-level work. AR-grade solvents are permissible when analyzing industrial waste samples.

However, the contents of each container must be checked to assure its suitability for the analyses. Similarly, all analytical reagents and other chemicals must also be checked routinely.

The quality of gases required for GC determinations varies somewhat with the type of detector. In general, the compressed gases are a prepurified dry grade. Grade A helium from the U.S. Bureau of Mines has always been satisfactory. The Dohrmann nitrogen detection system requires the use of ultrapure hydrogen for satisfactory results. Argon-methane used for electron-capture work should be oxygen free and should have an oxygen trap in the supply line. The use of molecular-sieve, carrier-gas filters and drying tubes is required on combustion gases and is recommended for use on all other gases. It is recommended that the analyst familiarize himself with an article by Burke (7) on practical aspects of GC.

All reagents, solvents, and adsorbents used for thin-layer chromatography must be checked to be certain that there are no impurities present that will react with the chromogenic reagent or otherwise interfere with subsequent qualitative or quantitative determinations. Glass-backed layers prepared in the laboratory or precoated layers supplied by a manufacturer may be used; however, precoated layers are more difficult to scrape. It is

recommended, therefore, that layers prepared in the laboratory be used when zones are to be scraped to recover isolated compounds. Plastic-backed layers are generally unsatisfactory for this type of analysis.

Adsorbents most commonly used for column chromatographic cleanup of sample extracts are Florisil,* silica gel, and alumina. These must be preactivated according to the method specifications and checked for interfering constituents.

5.3 Elimination of Determinate Errors

To produce high-quality analytical data, determinate errors must be eliminated or at least minimized. For purposes of this discussion, we assume that a competent analyst and reliable equipment in optimum operating condition are available. Thus, determinate errors that might result from an inexperienced or careless analyst and poor equipment are eliminated. The remaining sources of error are the reagents, solvents, and gases that are used throughout the analyses. The quality of these materials, even though they are AR grade or better, may vary from one source to another, from one lot to another, and even within the same lot. Therefore, the analyst must predetermine that all of these materials are free of interfering substances under the conditions of the analyses. To do this he must have a regular check program. Materials that do not meet requirements are replaced or purified so that they can be used.

5.3.1 Reagent Blank

The first step the analyst must take is to determine the background or blank of each of the reagents and solvents used in a given method of analysis. The conditions for determining the blank must be identical to those used throughout the analysis, including the detection system. If the reagents and solvents contain substances that interfere with a particular analysis, they should be treated so that they can be used, or satisfactory reagents and solvents must be found.

5.3.2 Method Blank

After determining the individual reagent or solvent blanks, the analyst must determine the method blank to see if the cumulative blank interferes with the analyses. The method blank is determined by following the procedure step by step, including all of the reagents and solvents, in the quantity required by the method. If the cumulative blank interferes with the determination, steps must be taken to eliminate or reduce the interference to a level that will permit this combination of solvents and reagents to be used. If the blank cannot be eliminated, the magnitude of the interference must be considered when calculating the concentration of specific constituents in the samples being analyzed.

A method blank should be determined whenever an analysis is made. The number of blanks to be run is determined by the method of analysis and the number of samples being analyzed at a given time. In some methods, such as the AutoAnalyzer procedures, the method blank is automatically and continuously compensated for because a continuous flow of the reagents passes through the detector. In other procedures, such as the gas chromatographic determination of pesticides, a method blank is run with each series of samples analyzed. Usually this is one blank for every nine samples.

---

*Trademark of Floridin Co.

4'39

5.3.3 Elimination of Interferences and Other Sources of Error

Procedures for eliminating or at least minimizing impurities that produce specific interferences or high general background vary with the reagent and method involved. These procedures may include the following: recrystallization, precipitation, distillation, washing with an appropriate solvent, or a combination of these. Examples of procedures used for various types of analyses are given below. For complete information, the analyst should consult the individual methods.

5.3.3.1 General Inorganic Analyses

Analytical-reagent-grade chemicals and solvents usually present no interference problems in inorganic analyses. However, some reagents do not always meet methods requirements. An example is potassium persulfate used in phosphorus and nitrogen determinations. This reagent is frequently contaminated with ammonia. Therefore, it is routinely purified by passing air through a heated water solution of the reagent. The purified potassium persulfate is recovered by recrystallization.

A problem more commonly encountered in inorganic analyses is the rapid deterioration of the standard reagents and other ingredients. To minimize or eliminate this problem, some reagents, for example, ferrous ammonium sulfate, must be standardized daily. Others, such as sodium thiosulfate used for dissolved oxygen determination, may require a substitute reagent such as phenylarsine oxide. Solid phenol, which readily oxidizes and acquires a reddish color, can be purified by distillation. Starch indicator used for idiometric titrations may be prepared for each use or preserved by refrigeration or by addition of zinc chloride or other suitable compounds.

5.3.3.2 Metals Analyses

In general, spectrograde chemicals, solvents, and gases present no interference problems in atomic absorption or emission spectrographic determinations. However, standards that do not meet the requirements of the method are sometimes obtained. Ordinarily, no effort is made to purify them. They are simply replaced by new reagents of sufficient purity. Some reagents may form precipitates on standing. Such reagents will reduce the accuracy of quantitative analyses and should not be used.

5.3.3.3 Radiological Analyses

In general, reagents that do not meet the purity requirements for radiological determinations are replaced with reagents that are satisfactory. However, in some instances (for example, barium sulfate used for coprecipitation of radium) it may be necessary to perform repeated recrystallization to remove all forms of radium, and reduce the background count to a usable level. In some instances, solvents that do not meet requirements may be distilled to produce adequate purity. In some cases, gases having background counts may be usable after aging as described earlier. If not, they should be replaced with gases that are satisfactory.

5.3.3.4 Organic Analyses

Many AR-grade chemicals and solvents, and at times pesticide-quality solvents, do not meet the specifications required for the determination of specific organic compounds. Impurities

440

that are considered trace, or insignificant, for many analytical uses are often present in greater quantities than the organic constituents being measured. Coupled with the several-hundred-fold concentration of the sample extract that is usually required, such impurities can cause very significant interferences in trace organic analyses. Reagents and solvents found to be unsatisfactory, under the conditions of the analyses, must be replaced or cleaned up so that they are usable. Some useful cleanup procedures are—

a. Washing the inorganic reagents with each solvent that the reagent contacts during the analysis

b. Washing the adsorbents, such as silica gel G and Florisil, with the solvents that are used for a specified column or thin-layer chromatographic procedure, or reactivating the Florisil by firing to 630°C

c. Preextracting distilled water with solvents used for the particular analysis involved

d. Preextracting aqueous reagent solutions with the solvents involved

e. Redistilling solvents in all-glass systems using an efficient fractionating column

f. Recrystallizing reagents and dyes used in colorimetric or thin-layer determinations

If the reagents and solvents thus produced are not of sufficient purity, they should be replaced.

Dirty gases (quality less than specified) are particularly troublesome in gas chromatographic analyses. They may reduce the sensitivity of the detector, and produce a high or noisy baseline. If this occurs, the cylinder should be replaced immediately. Similarly, if cylinders of compressed gases are completely emptied in use, the end volumes of the gas may produce a similar and often more severe effect. Oils and water may get into the system and foul the detector. When this occurs the system must be dismantled and cleaned. Overhaul of the detector may be required. To reduce chances of this, it is recommended that all gas cylinders be replaced when the pressure falls to 100 to 200 psi. Filter driers are of little help in coping with this type of contamination.

5.3.4 Storing and Maintaining Quality of Reagents and Solvents

Having performed the tasks of selecting, preparing, and verifying the suitability of reagents, solvents, and gases, the analyst must properly store them to prevent contamination and deterioration prior to their use. Borosilicate glass bottles with ground-glass stoppers are recommended for most standard solutions and solvents. Plastic containers such as polyethylene are recommended for alkaline solutions. Plastic containers must not be used for reagents or solvents intended for organic analyses. However, plastic containers may be used for reagents not involved with organic analyses if they maintain a constant volume, and if it is demonstrated that they do not produce interferences and do not absorb constituents of interest. It is important that all containers be properly cleaned and stored prior to use. (Refer to ch. 4 for details.)

Standard reagents and solvents must always be stored according to the manufacturer's directions. Reagents or solvents that are sensitive to the light should be stored in dark bottles and in a cool, dark place. It is particularly important to store materials used for

radiological determinations in dark bottles, because photoluminescence will produce high background if light-sensitive detectors are used for counting. Some reagents require refrigeration.

Adsorbents for thin-layer and column chromatography are stored in the containers that they are supplied in, or according to the requirements of individual methods. When new stock solutions are necessary, dilutions of the old and new standard should be compared to determine their accuracy.

The analyst should pay particular attention to the stability of the standard reagents. Standards should not be kept longer than recommended by the manufacturer or in the method. Some standards are susceptible to changes in normality because of absorption of gases or water vapor from the air. Provisions for minimizing this effect are given in reference 4.

The concentration of the standards will change as a result of evaporation of solvent. This is especially true of standards prepared in volatile organic solvents. Therefore, the reagent bottles should be kept stoppered, except when actually in use. The chemical composition of certain standards may change on standing. Certain pesticides, for instance, will degrade if prepared in acetone that contains small quantities of water. Thus, it is essential that working standards be frequently checked to determine changes in concentration or composition. Stock solutions should be checked before preparing new working standards from them.

5.4 References

1. Catalog of Standard Reference Materials, NBS Special Publication 260, National Bureau of Standards (June 1975).
2. "Reagent Chemicals," American Chemical Society Specifications, 5th Edition, American Chemical Society, Washington, D.C. (1974).
3. Manual on Industrial Water and Industrial Waste Water, 2nd Edition, ASTM Special Publication 148-H, American Society for Testing and Materials (1965), p. 869.
4. "Standard Methods for Preparation, Standardization, and Storage of Standard Solutions for Chemical Analysis," from Part 31 of 1976 Book of ASTM Standards, American Society for Testing and Materials, Philadelphia (1977).
5. Standard Methods for the Examination of Water and Wastewater, 13th Edition, American Public Health Association, New York (1971).
6. Methods for Organic Pesticides in Water and Wastewater, U.S. EPA, Environmental Research Center, Cincinnati (1971).
7. Burke, J., "Gas Chromatography for Pesticide Residue Analysis; Some Practical Aspects," J. Assoc. Off. Anal. Chem., *48*, 1037 (1965).

442

Chapter 6

QUALITY CONTROL FOR ANALYTICAL PERFORMANCE

6.1 Introduction

Previous chapters discussed basic elements of quality control (QC) pertaining to laboratory services, instrumentation, glassware, reagents, solvents, and gases; the reader should refer to the appropriate sections to determine necessary specifications and requirements for QC. Assuming that these basic variables are under QC, that approved methods are being used, and that the complete system is initially under QC, valid precision and accuracy data must initially be developed for each method and analyst. Then, to insure that valid data continue to be produced, systematic daily checks must show that the test results remain reproducible, and that the methodology is actually measuring the quantity in each sample. In addition, QC must begin with sample collection and must not end until the resulting data have been reported. QC of analytical performance within the laboratory is thus but one vital link in the dissemination of valid data to the public. Understanding and conscientious use of QC among all field sampling personnel, analytical personnel, and management personnel is imperative. Technical approaches are discussed in the following sections.

6.2 The Industrial Approach to QC

In the 1920's, Dr. Walter A. Shewhart of Bell Telephone Laboratories, Inc. developed the theory of control charts as a basic method for evaluating the quality of products from manufacturing processes His book (1) on statistical QC grew out of this original work. Later, acceptance of his concepts and related statistical techniques within industry led to refined, quantitative evaluations of product quality in manufacturing. Dr. Shewhart's work on production processes assumed a uniform product manufactured in large numbers and inspected on a continuous basis through the periodic analysis of samples of n production units. The resulting data, $x_1, x_2, \ldots x_n$, were then used to estimate precision, as the standard deviation S or range R, and accuracy, as the arithmetic mean $\bar{X}$. These statistics were calculated as follows:

$$S = \sqrt{\frac{\sum_{i=1}^{n} X_i^2 - \left(\sum_{i=1}^{n} X_i\right)^2 / n}{n-1}}$$

$$R = \text{the largest of the } X_i - \text{the smallest of the } X_i$$

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

These statistics were evaluated by plotting them on control charts developed from similar statistics taken while the process was under properly controlled operation. The elements common to such control charts are represented in figure 6-1. They include an expected value (the central line) and an acceptable range of occurrence (the region between upper and lower control limits).

443

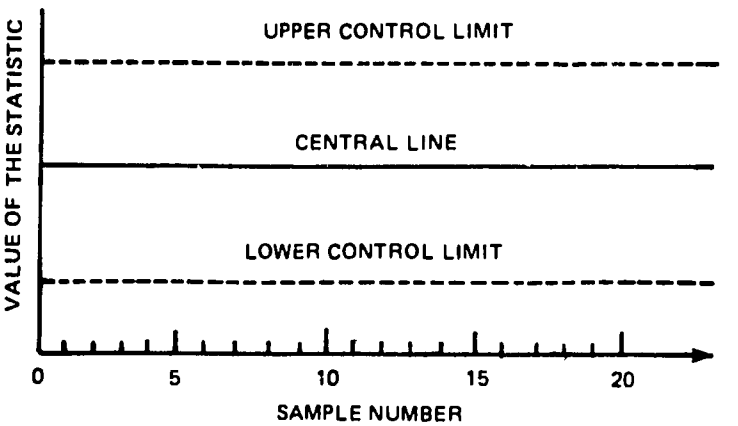


Figure 6-1. Essentials of a control chart.

There are many reference sources available that discuss in great detail the classic Shewhart control charts and related statistics that have since been developed for specific industrial applications (2-4). In addition, many authors have discussed applications of a related type of control chart called a cumulative-summation (cusum) chart (2,4). Rather than evaluating each sample independently, the cusum chart evaluates the cumulative trend of the statistics from a series of samples. Because each successive point is based upon a cumulative data trend, cusum charts are often considered more effective than control charts in recognizing process changes and, therefore, may minimize losses from production of unacceptable units; however, cusum charts require the more difficult calculations, and optimally designed Shewhart techniques have been found to be almost as effective (2,4), so there is no universal agreement on the choice between them.

6.3 Applying Control Charts in Environmental Laboratories

In industrial applications, separate control charts are recommended for each product, each machine, and each operator. Analogous system variables in an environmental laboratory are the parameter, the instrument, and the analyst. However, environmental laboratories routinely have to contend with a variable that has no industrial counterpart—the true concentration level of the investigated parameter, which may vary considerably among samples. Unfortunately, the statistics that work well for industry are sensitive to the variability in true concentration that is common in environmental analysis; e.g., the classic $\bar{X}$ and R statistic values increase substantially as concentration increases. This variability in true concentration means there are no expected values for randomly selected samples, so that the accuracy of testing methodology must be evaluated indirectly through the recovery of standards and spikes. As a result, it has been difficult for environmental laboratories to satisfactorily apply industrial QC techniques.

There are two possible approaches to the solution of the problem of variation in the true concentration level; either use of a statistic that is not sensitive to this variation or application of the industrial techniques within restricted concentration ranges. Obviously, the former should be preferred because it actually solves the problem and does not require the development and maintenance of a series of charts for each parameter.

6.3.1 Quality Control Charts for Accuracy

Two replacements for the Shewhart $\bar{X}$ control chart have been suggested for evaluating the recovery of a series of different standards or spikes. One of these, a cusum chart using the

444

square of the difference between the observed and true values, is described in an EPA Region VI QC manual (5). The other alternative uses the classic Shewhart technique to evaluate the percent recovery instead of $\bar{X}$. It is recommended that the percent recovery be calculated as

$$P = 100 \frac{\text{observed}}{\text{known}}$$

for standards, or

$$P = 100 \frac{\text{observed} - \text{background}}{\text{spike}}$$

for recovery of spikes into natural water backgrounds. An example of the linear relationship between percent recovery and the known concentration of standards and spikes is demonstrated in the accuracy plots of a recent EPA method study report on analysis of mercury (6). Both approaches are being used on a daily basis by various environmental laboratories.

The data in table 6-1 were used in the EPA Region VI manual (5) to illustrate the development of a cusum chart. The actual data have been reordered here to appear in ascending order of the known values. Note that the mean and the range of the d_i^2 values increase with increasing concentration levei, and this violates a basic premise for acceptable control chart statistics. Because the percent recovery data do not show any such trend, it is the recommended control chart statistic for controlling accuracy.

From the data in table 6-1, a Shewhart control chart for percent recovery can be calculated in the following way:

Average percent recovery

$$\bar{P} = \frac{\sum_{i=1}^{23} P_i}{23}$$

$$= \frac{2{,}310}{23}$$

$$= 100.4$$

The standard deviation for percent recovery

$$S_P = \sqrt{\frac{\sum_{i=1}^{23} P_i^2 - \left(\sum_{i=1}^{23} P_i\right)^2 / 23}{22}}$$

$$= \sqrt{\frac{234{,}074 - (2{,}310)^2/23}{22}}$$

$$= \sqrt{94.0751}$$

$$= 9.70$$

Therefore, the upper control limit becomes the following:

$$UCL = \bar{P} + 3S_P$$

$$= 100.4 + 3(9.70)$$

$$= 129.5$$

Table 6-1
ANALYSIS[1] OF TOTAL PHOSPHATE-PHOSPHORUS STANDARDS, IN mg/l
TOTAL PO_4-P

Point	Known	Obtained	Difference d_i	d_i^2	Percent Recovery P_i	P_i^2
1	0.34	0.33	0.01	0.0001	97	9,409
2	0.34	0.34	0.00	0.0000	100	10,000
3	0.40	0.40	0.00	0.0000	100	10,000
4	0.49	0.49	0.00	0.0000	100	10,000
5	0.49	0.49	0.00	0.0000	100	10,000
6	0.49	0.63	-0.14	0.0196	129	16,641
7	0.50	0.47	0.03	0.0009	94	8,836
8	0.50	0.53	-0.03	0.0009	106	11,236
9	0.50	0.56	-0.06	0.0036	112	12,544
10	0.52	0.59	-0.07	0.0049	113	12,769
11	0.66	0.70	-0.04	0.0016	106	11,236
12	0.66	0.60	0.06	0.0036	91	8,281
13	0.67	0.65	0.02	0.0004	97	9,409
14	0.68	0.65	0.03	0.0009	96	9,216
15	0.83	0.80	0.03	0.0009	96	9,216
16	0.98	0.75	0.23	0.0529	77	5,929
17	1.3	1.2	0.10	0.0100	92	8,464
18	1.3	1.3	0.00	0.0000	100	10,000
19	1.6	1.7	-0.10	0.0100	106	11,236
20	2.3	2.3	0.00	0.0000	100	10,000
21	2.3	2.4	-0.10	0.0100	104	10,816
22	3.3	3.3	0.00	0.0000	100	10,000
23	4.9	4.6	0.30	0.0900	94	8,836
Totals					2,310	234,074

[1] Using a colorimetric method with persulfate digestion.

446

and the lower control limit becomes

$$LCL = 100.4 - 29.1$$

$$= 71.3$$

The completed control chart is shown in figure 6-2.

Following normal procedures, the control chart must indicate the conditions under which it was developed; i.e., laboratory name, parameter, method of analysis, date of preparation, and any other information unique to the initializing data, such as range of concentration and identification of analyst(s). A control chart is not generally applicable under other conditions.

To verify the control chart, the initializing data should be checked to be sure that none of the values exceeds these new control limits. In addition, if its distribution is proper, about 68 percent of the initializing data should fall within the interval $\bar{P} \pm S_p$. It has been suggested that the control chart is not valid if less than 50 percent of the initializing data falls within this interval.

In applying the control chart, either of the following two conditions would indicate an out-of-control situation:

a. Any point beyond the control limits

b. Seven successive points on the same side of the value $\bar{P}$ of the central line

When an out-of-control situation occurs, analyses must be stopped until the problem has been identified and resolved, after which the frequency should be increased for the next few percent-recovery QC checks. The problem and its solution must be documented, and all analyses since the last in-control point must be repeated or discarded.

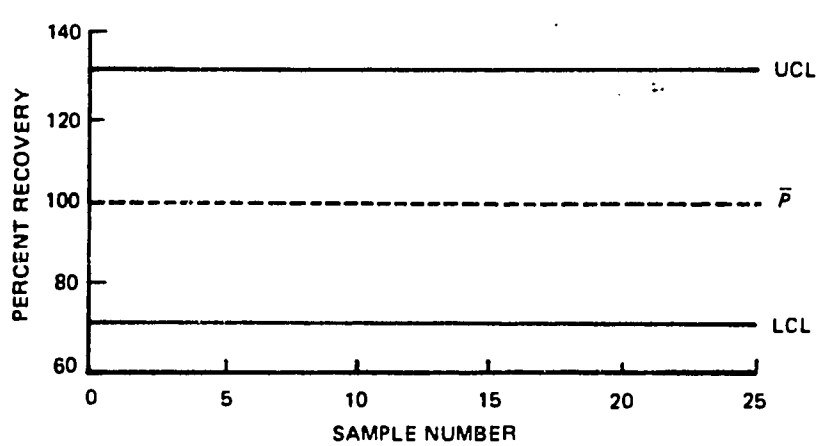


Figure 6-2. Shewhart control chart for percent recovery data.

447

A final note of caution regarding use of a single percent-recovery P control chart over a broad concentration range is necessary. As noted earlier for the analysis of mercury, a good linear relationship of the form

$$\bar{X} = P\,(\text{known concentration}) + K$$

where K is a constant, seems appropriate for many parameters. However, to justify use of a single percent-recovery control chart, K must be small enough relative to the P(known concentration) term that it has little or no practical effect upon the value of $\bar{X}$. This will usually be true for moderate or high concentration levels, but may not be true at very low concentration levels. As a result, for some parameters it may be necessary to develop a separate percent-recovery or Shewhart $\bar{X}$ chart for each standardized low concentration level sample.

6.3.2 Quality Control Charts for Precision

Because the characteristics of the range statistic change as concentration changes, two alternatives to Shewhart's R chart have been used in environmental laboratories to evaluate the precision of routine sample analyses.

One alternative is a cusum chart using the sum of the squared difference between duplicate determinations on randomly selected routine samples (5). Because the range R for duplicate analyses is equal to the difference between them, the cusum statistic equals the sum of squared ranges $\sum R^2$. However, if R changes significantly as concentration level changes, then R^2 is affected even more and, therefore, is *not* as good a criterion for judging whether precision of the system is within acceptable limits.

The other alternative uses a chart similar to the R chart, but the chart statistic is either the percent relative standard deviation ($100S/\bar{X}$), the coefficient of variation (CV or $S/\bar{X}$), or the industrial statistic I. For the duplicate determinations A and B, I equals the absolute value of their difference divided by their sum, or $|A - B|/(A + B)$, and can be shown to be equivalent to the other two statistics:

$$\begin{aligned}
100(\text{CV}) &= 100\frac{S}{\bar{X}} \\
&= 100\frac{R/\sqrt{2}}{(A+B)/2} \\
&= 100\frac{2}{\sqrt{2}}\frac{R}{A+B} \\
&= \frac{200}{\sqrt{2}}\frac{|A-B|}{A+B} \\
&= \frac{200I}{\sqrt{2}}
\end{aligned}$$

For the sake of computational ease, I seems to be a logical alternative to R.

445

The next concern is whether I is independent of changes in concentration level. Based upon experience with duplicates on routine samples taken during the last 2 years by EPA Region VII, I appears to decrease substantially as concentration increases. In recognition of this possible dependency, control charts for I should only be developed from and applied to results within a limited concentration range. Note that control charts for R could be applied under similar limitations.

As an illustration of the concentration dependency of these precision statistics, table 6-2 provides estimates of R and I for different concentration ranges of three parameters. These parameters were selected because approximately 100 sets of duplicates were available that were well distributed over a reasonably broad concentration range. The ranges for the sum of duplicates $A + B$ used in table 6-2 were selected because they were convenient and the data tended to be well distributed among them. Data judged to be out of control were discarded before any calculations were made.

Table 6-2 indicates the concentration dependence of both the range R and the industrial statistic I for these three parameters. Because I is not independent of concentration and is

Table 6-2
ESTIMATES OF THE RANGE ($R = |A - B|$) AND THE INDUSTRIAL STATISTIC [$I = |A - B|/(A + B)$] OF THREE DIFFERENT PARAMETERS FOR VARIOUS CONCENTRATION RANGES[1]

Parameter	Range of $A + B$	No. of Sets of Duplicates	[2]$A + B$	[2]R	[2]I
BOD, 5-day (mg/l)	2 to <20	21	11.7	1.04	0.0888
	20 to <50	30	35.2	1.94	0.0552
	50 to <100	27	72.2	3.33	0.0462
	100 to <300	29	204.1	6.52	0.0319
	300 to <600	17	394.4	11.1	0.0282
	600 to <2,000	12	1,041	12.1	0.0116
	2,000 up	3	6,683	177	0.0264
Chromium (μg/l)	10 to <20	32	12.3	0.32	0.0306
	20 to <50	15	33.4	0.57	0.0170
	50 to <100	16	72.4	1.12	0.0155
	100 to <300	15	170.3	3.80	0.0223
	300 to <1,000	8	480.3	5.25	0.0109
	1,000 up	5	6,340	76.0	0.0120
Copper (μg/l)	10 to <30	16	22.2	0.93	0.0617
	30 to <50	23	38.2	1.35	0.0368
	50 to <100	21	70.8	1.14	0.0169
	100 to <200	26	131.9	2.33	0.0177
	200 to <400	10	268.0	2.81	0.0105
	400 up	3	702.0	4.56	0.0065

[1] From EPA Surveillance and Analysis Laboratory, Region VII.
[2] Average values.

449

Table 6-3
SHEWHART UPPER CONTROL LIMITS (UCL) AND CRITICAL RANGE R_c VALUES FOR THE DIFFERENCES BETWEEN DUPLICATE ANALYSES WITHIN SPECIFIC CONCENTRATION RANGES FOR THREE PARAMETERS[1]

Parameter	Concentration Range[2]	UCL	R_c
BOD, 5-day (mg/l)	1 to <10	3.40	3.5
	10 to <25	6.34	6
	25 to <50	10.9	11
	50 to <150	21.3	21
	150 to <300	36.3	36
	300 to <1,000	39.6	[3]40
	1,000 up	579	[3]579
Chromium (μg/l)	5 to <10	1.05	1
	10 to <25	1.86	2
	25 to <50	3.66	4
	50 to <150	12.4	12
	150 to <500	17.2	[3]17
	500 up	249	[3]249
Copper (μg/l)	5 to <15	3.04	3
	15 to <25	4.41	4
	25 to <50	3.73	5
	50 to <100	7.62	8
	100 to <200	9.19	[3]9
	200 up	14.9	[3]15

[1] From EPA Surveillance and Analysis Laboratory, Region VII.
[2] Equal to half of the range of $A + B$ given in table 6-2.
[3] Based on fewer than 15 sets of duplicate analyses.

more difficult to calculate and develop control charts for, the use of R charts for a series of sequential concentration ranges for each parameter seems practical. However, because the primary concern when using any range chart is whether the upper control limit has been exceeded, an even more practical approach would be to develop a table of these limits for all concentration levels of each parameter. As an example, table 6-3 contains the calculated Shewhart upper control limits for the range R from duplicate analyses within the various concentration levels for the three parameters in table 6-2. These limits were calculated, as usual, from the Shewhart factor D_4 for ranges based upon duplicate analyses and the appropriate average value of the range R given in table 6-2. For example, the UCL for 25 to 50 mg/l of BOD was calculated as follows:

$$\text{UCL} = D_4 R$$

$$= 3.27(3.33)$$

$$= 10.9$$

450

Table 6-3 also contains a critical range R_c column. Because the data from EPA Region VII were almost always whole units with only a very occasional half unit reported, the R_c value is the UCL value rounded to the nearest whole unit at higher concentration levels and to the nearest half unit for the lowest concentration level. However, there is an exception to this rule among the low-concentration R_c values for copper that demonstrates an advantage beyond the simplicity of using such tables. The UCL value for copper at 25 to 50 μg/l is inconsistent with the UCL values for adjacent concentration levels, and the R_c value has been adjusted to resolve this inconsistency. Without the table, such inconsistencies could very easily go unnoticed.

The examples in table 6-4 illustrate how to use the R_c values in table 6-3. This technique, consisting of the development and use of a table of critical-range R_c values at different concentration levels, is recommended to control precision. Normal control chart procedures should be followed as in section 6.3.1 regarding identification and verification of the table. The table should be updated periodically as additional, or more current, data become available, or whenever the basic analytical system undergoes a major change. If any difference between duplicate analyses exceeds the critical-range value for the appropriate concentration level, then analyses must be stopped until the problem is identified and resolved, and the frequency should be increased for the next few precision checks. After resolution, the problem and its solution must be documented, and all analyses since the last in-control check must be repeated or discarded.

6.4 Recommended Laboratory Quality Assurance Program

A minimum laboratory quality assurance program should include control procedures for each parameter as described in the following sections.

6.4.1 Standard Curves

A new standard curve should be established with each new batch of reagents, using at least seven concentration levels.

6.4.2 Quality Control Checks for Each Analytical Run

With each batch of analyses, the following tests should be run:

a. One blank on water and reagents

Table 6-4
CRITICAL RANGE VALUES FOR VARYING CONCENTRATION LEVELS

Parameter	Duplicates	R	R_c	$R \leq R_c$	Condition of System
BOD (mg/l)	20 and 24	4	6	Yes	Normal
Chromium (μg/l)	60 and 75	15	12	No	Out-of-control
Copper (μg/l)	46 and 51	5	[1]5	Yes	Normal

[1] This R_c value is used because (46 + 51)/2 = 48.5, which is between 25 and 50.

451

b. One midpoint standard

c. One spike to determine recovery

d. One set of duplicate analysis

The results from b through d should be compared with previous in-control data by using the appropriate technique recommended in section 6.3.

6.4.3 Interlaboratory QC

An interlaboratory QC program would require each laboratory to do the following:

a. Analyze reference-type samples to provide independent checks on the analytical system. These may be available from EPA as QC samples, from the National Bureau of Standards as standard reference materials, or from commercial sources. If performance limits are not provided, the results should fall within the routine limits of each laboratory for a standard at a level comparable to the specified true value.

b. Participate in performance evaluation and method studies as available from EPA, from the American Society for Testing and Materials, and from other agencies.

6.5 Outline of a Comprehensive Quality Assurance Program

In the following discussion the symbols used represent the results of analysis according to the scheme:

A_1 = first replicate of sample A

A_2 = second replicate of sample A

B = sample taken simultaneously with sample A

B_{SF} = field spike into sample B

B_{SL} = laboratory spike into sample B

D_F = field spike into distilled water

D_L = laboratory spike into distilled water

T = true value for all spikes

The laboratory spikes B_{SL} and D_L are the only analyses that may not be necessary. All other analyses *must* be done simultaneously.

6.5.1 Steps for the Field Personnel

A comprehensive quality assurance program would include the following steps for each parameter in the monitoring study:

a. Take independent simultaneous samples A and B at the same sampling point. Depending on the parameter, this might involve side-by-side grab samples or composite samplers mounted in parallel.

452

b. Split sample A into the equal-volume samples A_1 and A_2.

c. Split sample B into equal volumes and add a spike T to one of them; the latter sample becomes sample B_{SF}. As with all spikes, the addition of T should approximately double the anticipated concentration level.

d. Add the same spike T to a distilled water sample furnished by the laboratory and designate this sample as D_F.

These QC samples must be treated in the same way as routine samples; i.e., the volume, type of container, preservation, labeling, and transportation must be the same for all.

6.5.2 Steps for the Laboratory

The laboratory personnel should perform the following steps for quality assurance:

a. Analyze the blank and midpoint standard recommended in section 6.4. If results are unsatisfactory, resolve problems before continuing.

b. Analyze sample D_F. If the percent recovery of T is unsatisfactory (see section 6.3.1), create a similarly spiked, distilled-water sample D_L and analyze to test for a systematic error in the laboratory or fundamental problems with the spike. If the percent recovery of T from D_L is satisfactory, any systematic error occurred before the samples reached the laboratory.

c. Analyze samples B and B_{SF}. If B is below the detection limit, or if B is greater than $10T$ or less than $0.1T$, disregard the remainder of this step and proceed to step d. If the percent recovery of T from B_{SF} is unsatisfactory (see section 6.3.1), spike an aliquot of sample B the same way in the laboratory so that a similar recovery can be anticipated. Analyze this sample B_{SL} to test for immediate sample interferences or a bad background result B. If the percent recovery from B_{SL} is satisfactory, then the interference must require a longer delay before analysis, or other special conditions not present in the laboratory, in order to have a noticeable effect upon recovery of the spike.

d. Analyze A_1 and A_2. If the absolute (unsigned) difference between these results exceeds the critical value (see section 6.3.2), then precision is out of control.

e. Calculate the absolute difference between A_1 and B. If it is unsatisfactory (see section 6.3.2), the field sampling procedure did not provide representative samples.

If initial results at each of the laboratory steps were satisfactory, then the validity of the related data has been indisputably established. If results at any step are unsatisfactory, resolution depends upon the problem identified. Laboratory problems may just require that the analyses be repeated, but field problems will usually require new samples. Figure 6-3 is intended to clarify the interdependence of the preceding laboratory steps b through e.

In figure 6-3 it must be noted that there is no way to identify *additive* sample interferences; i.e., those that have an equal effect upon the background-plus-spike results (B_{SF} or B_{SL}) and the background result B. Recovery of a spike will not show such interferences.

453

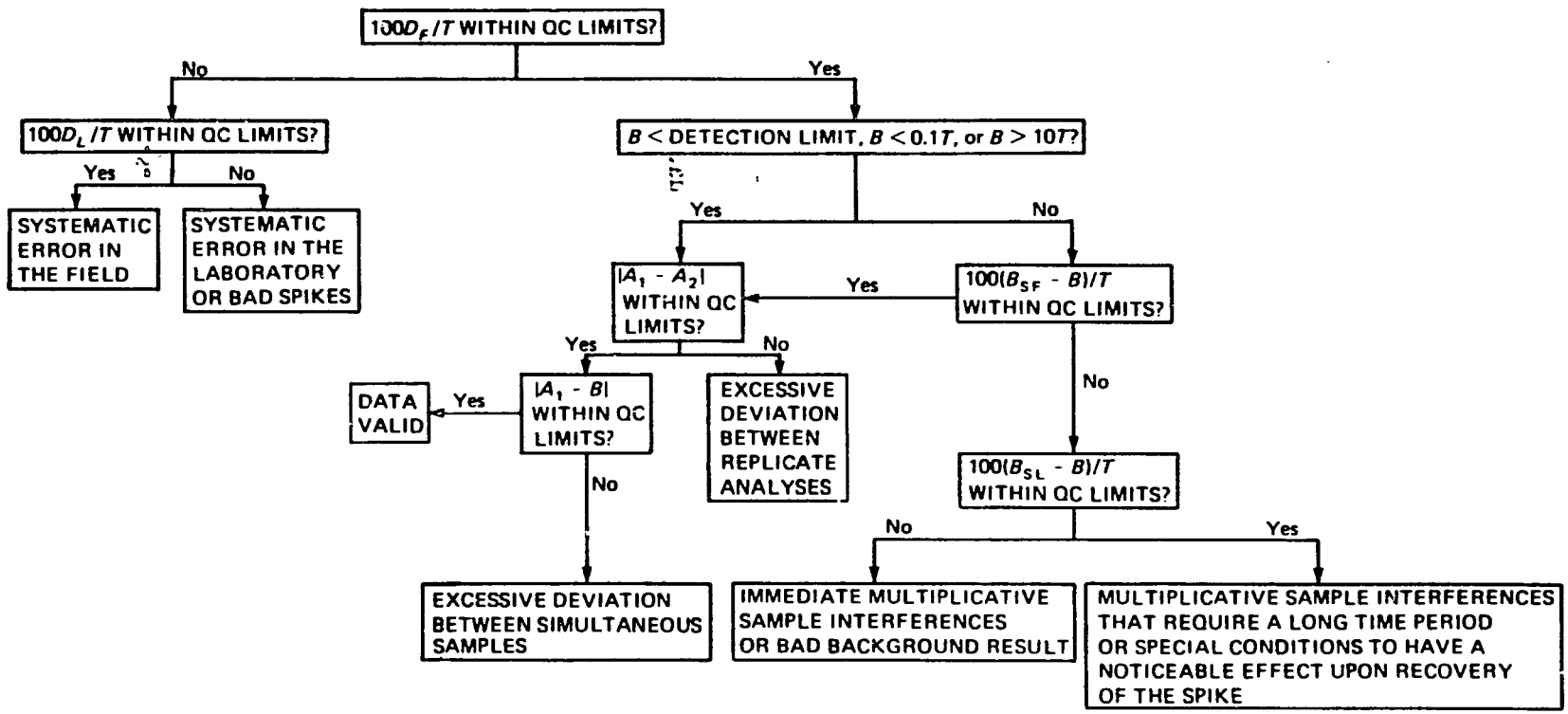


Figure 6-3. Procedure for evaluating QC data from a monitoring study.

Problems causing systematic errors that may occur in the field include the following:

a. Contaminated preservative, distilled water, or containers

b. Contamination by sampling personnel

c. Deterioration through excessive holding time or use of an ineffectual preservation technique

d. Use of a bad field-spiking procedure

6.6 Related Topics

6.6.1 Advanced Laboratory Automation and Its Effect on QC

Advanced laboratory automation systems under development analyze samples automatically and use a control computer to interpret the resulting data and produce an analytical report. The primary benefits of such a system are not only that the data-recording and calculation errors common to manual analyses have been inherently eliminated, but also that extensive QC can be accomplished quite easily and cheaply. The computer can be programed to automatically recognize different kinds of QC samples and to establish or recall appropriate control limits. Thus the QC overhead is reduced considerably and QC procedures previously too costly or complex become practical.

As an example of a QC procedure that is considered impractical for manual use, regression could be used to determine the relationship between concentration change and the accuracy and precision statistics discussed earlier. Using these relationships, very responsive, single accuracy and precision charts could be developed for each parameter. As computer-assisted analysis becomes common, automated laboratories will very likely replace the manual procedures recommended earlier in this chapter with evaluation criteria based upon regressions.

6.6.2 Method Comparability (Equivalency)

Requirements for method comparability are under development for proposed alternatives to the methodology specified in Public Law 92-500, section 304(g). A final version of these requirements should be available from EPA at a later date.

6.7 References

1. Shewhart, W. A., Economic Control of Quality of Manufactured Product, Van Nostrand Reinhold Co., Princeton, N.J. (1931).
2. Duncan, A. J., Quality Control and Industrial Statistics, 3d Edition, Ch. 18, R. D. Irwin, Inc., Homewood, Ill. (1965).
3. Grant, E. L., and Leavenworth, R. S., Statistical Quality Control, 4th Edition, McGraw-Hill Book Co., Inc., New York (1972).
4. Davies, O. L., and Goldsmith, P. L., Statistical Methods in Research and Production, 4th Edition, Hafner Publishing Co., New York (1972).
5. Laboratory Quality Control Manual, 2d Edition, U.S. EPA, Region VI, Surveillance and Analysis Division, Ada, Okla. (1972).

455

6. Winter, J., Britton, P., Clements, H., and Kroner, R., "Total Mercury in Water," EPA Method Study 8, pp. 35-36, U.S. EPA, Office of Research and Development, EMSL, Cincinnati (Feb. 1977).

456

Chapter 7

DATA HANDLING AND REPORTING

7.1 Introduction

To obtain meaningful data on water quality, the sample collector must obtain a representative sample and then deliver it unchanged for analysis. The analyst must perform the proper analysis in the prescribed fashion, complete calculations, and convert results to final form for permanent recording of the analytical data in meaningful, exact terms. These results are transferred to a storage facility for future interpretation and use.

The following sections discuss processing of actual values, recording and reporting of data in the proper way, some means of quality control of data, and the storage and retrieval of data.

7.2 The Analytical Value

7.2.1 Significant Figures

The term "significant figure" is used, sometimes rather loosely, to describe a judgment of the reportable digits in a result. When the judgment is not soundly based, meaningful digits are lost or meaningless digits are reported. On the other hand, proper use of significant figures gives an indication of the reliability of the analytical method used.

The following discussion describes the process of retention of significant figures.

A number is an expression of quantity. A figure or digit is any of the characters 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, which, alone or in combination, serve to express a number. A significant figure is a digit that denotes the amount of the quantity in the particular decimal place in which it stands. Reported analytical values should contain only significant figures. A value is made up of significant figures when it contains all digits known to be true and one last digit in doubt. For example, if a value is reported as 18.8 mg/l, the 18 must be firm while the 0.8 is somewhat uncertain, but presumably better than one of the values 0.7 or 0.9 would be.

The number zero may or may not be a significant figure depending on the situation.

Final zeros after a decimal point are always meant to be significant figures. For example, to the nearest milligram, 9.8 g is reported as 9.800 g.

Zeros before a decimal point with nonzero digits preceding them are significant. With no preceding nonzero digit, a zero before the decimal point is not significant.

If there are no nonzero digits preceding a decimal point, the zeros after the decimal point but preceding other nonzero digits are not significant. These zeros only indicate the position of the decimal point.

Final zeros in a whole number may or may not be significant. In a conductivity measurement of 1,000 μmho/cm, there is no implication by convention that the conductivity is 1,000 ± 1 μmho. Rather, the zeros only indicate the magnitude of the number.

457

A good measure of the significance of one or more zeros interspersed in a number is to determine whether the zeros can be dropped by expressing the number in exponential form. If they can, the zeros may not be significant. For example, no zeros can be dropped when expressing a weight of 100.08 g in exponential form; therefore the zeros are significant. However, a weight of 0.0008 g can be expressed in exponential form as 8×10^{-4} g, so the zeros are not significant. Significant figures reflect the limits in accuracy of the particular method of analysis. It must be decided whether the number of significant digits obtained for resulting values is sufficient for interpretation purposes. If not, there is little that can be done within the limits of the given laboratory operations to improve these values. If more significant figures are needed, a further improvement in method or selection of another method will be required.

Once the number of significant figures obtainable from a type of analysis is established, data resulting from such analyses are reduced according to set rules for rounding off.

7.2.2 Rounding Off Numbers

Rounding off of numbers is a necessary operation in all analytical areas. It is automatically applied by the limits of measurement of every instrument and all glassware. However, when it is applied in chemical calculations incorrectly or prematurely, it can adversely affect the final results. Rounding off should be applied only as described in the following sections.

7.2.2.1 Rounding-Off Rules

If the figure following those to be retained is less than 5, the figure is dropped, and the retained figures are kept unchanged. As an example, 11.443 is rounded off to 11.44.

If the figure following those to be retained is greater than 5, the figure is dropped, and the last retained figure is raised by 1. As an example, 11.446 is rounded off to 11.45.

If the figure following those to be retained is 5, and if there are no figures other than zeros beyond the five, the figure 5 is dropped, and the last-place figure retained is increased by one if it is an odd number or it is kept unchanged if an even number. As an example, 11.435 is rounded off to 11.44, while 11.425 is rounded off to 11.42.

7.2.2.2 Rounding Off Arithmetic Operations

When a series of numbers is added, the sum should be rounded off to the same number of decimal places as the addend with the smallest number of places. However, the operation is completed with all decimal places intact, and rounding off is done afterward. As an example,

$$\begin{array}{r} 11.1 \\ 11.12 \\ +11.13 \\ \hline 33.35 \end{array}$$

The sum must be rounded off to 33.4.

458

When one number is subtracted from another, rounding off should be completed after the subtraction operation, to avoid possible invalidation of the operation.

When two numbers are to be multiplied, all digits are carried through the operation, then the product is rounded off to the number of significant digits of the multiplier with the fewer significant digits.

When two numbers are to be divided, the division is carried out on the two numbers using all digits. Then the quotient is rounded off to the number of significant digits of the divisor or dividend, whichever has the fewer.

When a number contains n significant digits, its root can be relied on for n digits, but its power can rarely be relied on for n digits.

7.2.2.3 Rounding Off the Results of a Series of Arithmetic Operations

The preceding rules for rounding off are reasonable for most calculations; however, when dealing with two nearly equal numbers, there is a danger of loss of all significance when applied to a series of computations that rely on a relatively small difference in two values. Examples are calculation of variance and standard deviation. The recommended procedure is to carry several extra figures through the calculations and then to round off the final answer to the proper number of significant figures.

7.3 Glossary of Statistical Terms

To clarify the meanings of statistical reports and evaluations of water quality data, the following statistical terms are introduced. They are derived in part from usage (1,2) of the American Society for Quality Control.

Accuracy—The difference between an average value and the true value when the latter is known or assumed.

Arithmetic mean—The arithmetic mean (or average) of a set of n values is the sum of the values divided by n:

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

Bias—A systematic error due to the experimental method that causes the measured values to deviate from the true value.

Confidence limit, 95 percent—The limits of the range of analytical values within which a single analysis will be included 95 percent of the time,

$$95 \text{ percent CL} = \bar{X} \pm 1.96S$$

where CL is the confidence level and S is the estimate of the standard deviation.

459

Constant—A nonvarying qualitative or quantitative characteristic of the population.

Geometric mean—A measure of central tendency for data from a positively skewed distribution (log normal),

$$\bar{X}_g = \sqrt[n]{(X_1)(X_2)\ldots(X_n)}$$

$$\bar{X}_g = \text{antilog}\,\frac{\sum_{i=1}^{n} \log X_i}{n}$$

Interference—A biological or chemical attribute of a test sample that positively or negatively offsets the measured result from the true value. If interference that is not segregated and identified is present, it enlarges or reduces the method bias.

Median—Middle value of all data ranked in ascending order. If there are two middle values, the median is the mean of these values.

n—The number of values X_i reported for a sample.

N—The total number of values X_i of the entire population or universal set of data.

Population—The total set of units, items, or measurements under consideration.

Precision—Relative to the data from a single test procedure, the degree of mutual agreement among individual measurements made under prescribed conditions.

Precision data—Factors that relate to the variations among the test results themselves; i.e., the scatter or dispersion of a series of test results, without assumption of any prior information.

Range—The difference between the highest and lowest values reported for a sample.

Relative deviation (coefficient of variation)—The ratio of the standard deviation S of a set of numbers to their mean $\bar{X}$ expressed as percent. It relates standard deviation (or precision) of a set of data to the size of the numbers:

$$\text{CV} = \text{RD (percent)} = 100\frac{S}{\bar{X}}$$

Relative error—The mean error of a series of measured data values as a percentage of the true value X_t,

$$\text{RE (percent)} = 100\frac{|X - \bar{X}|}{X_t}$$

Sample—Groups of units or portions of material, taken from a larger collection of units or quantity of material, that provide information to be used for judging the quality of the total collection or entire material as a basis for action on them or on their production processes.

460

Series—A number of test results with common properties that identify them uniquely.

Skewness—A measure of the asymmetry of a frequency distribution,

$$K = \frac{(X_i - \bar{X})^3}{n\sigma^3}$$

This measure is a pure signed number. If the data are perfectly symmetrical, the skewness is zero. If K is negative, the long tail of the distribution is to the left. If K is positive, the long tail extends to the right.

Standard deviation—The square root of the variance of the universe,

$$\sigma = \sqrt{\frac{\sum_{i=1}^{N} X_i^2 - \left(\sum_{i=1}^{N} X_i\right)^2 / N}{N}}$$

Standard deviation estimate—The most widely used measure to describe the dispersion of a set of data. Normally $\bar{X} \pm S$ will include 68 percent, and $\bar{X} \pm 2S$ will include about 95 percent of the data from a study:

$$S = \sqrt{\frac{\sum_{i=1}^{n} X_i^2 - \left(\sum_{i=1}^{n} X_i\right)^2 / n}{n - 1}}$$

Standard deviation, single analyst—A measure of dispersion for data from a single analyst that is calculated here using an equation developed by Youden for his nonreplicate study design (3),

$$S_r = \sqrt{\frac{\sum_{i=1}^{n} (D_i - \bar{D})^2}{2(n - 1)}}$$

where D = the difference in paired values obtained from a single analyst.

Universe—The total set of items or measurements.

Variable—An experimentally determined estimate denoted X for a particular quality or trait of the population.

7.4 Report Forms

The analytical information reported should include the measured parameters; the details of the analysis such as burette readings, absorbance, wavelength, normalities of reagents, correction factors, blanks; and the reported data values.

461

To reduce errors in manipulation of numbers a general rule is to reduce handling and transposition of data to an absolute minimum. Ideally, a report form includes preliminary information about the sample and its analysis, and *the same* form is used for the final entering of data into a computer; however, such report forms are not yet common. Rather, a variety of methods is used to record data.

7.4.1 Loose Sheets

Reporting of data onto loose or ring-binder forms is a means of recording data that allows easy addition of new sheets, removal of older data, or collection of specific data segments. However, the easy facility for addition or removal also permits loss or misplacement of sheets, mixups in date sequence, and ultimately questionable status of the data for formal display or presentation as courtroom evidence.

7.4.2 Bound Books

The use of bound books is an improvement in data recording that tends to result in a chronological sequence of data insertion. Modification beyond a simple lined book improves its effectiveness with little additional effort. Numbering of pages encourages use of data in sequence and also aids in referencing data through a table of contents ordered according to time, type of analysis, kind of sample, and identity of analyst.

Validation can be easily accomplished by requiring the analyst to date and sign each analysis on the day completed. This validation can be strengthened further by providing space for the laboratory supervisor to witness the date and the completion of the analyses.

A further development of the bound notebook is the commercially available version designed for research-type work. These notebooks are preprinted with book and page numbers, and spaces for title of project, project number, analyst signature, witness signature, and dates. Each report sheet has a detachable duplicate sheet that allows up-to-date review by management without disruption of the notebook in the laboratory. The cost of research-type bound notebooks is about four times that of ordinary notebooks.*

Use of bound notebooks has been limited to research and development work where an analysis is part of a relatively long-term project, and where the recording in the notebook is the prime disposition of the data until an intermediate or final report is written.

However, bound notebooks can and should be used in routine analytical laboratories such as those concerned with water quality. The need for repeated information on sampling and analyses can be answered by use of preprinted pages in the bound notebooks.

7.4.3 Preprinted Report Forms

Most field laboratories and installations repetitively analyzing fixed parameters develop their own system of compiling laboratory data that may include bound notebooks, but a means of forwarding data is also required. Usually, laboratories design forms to fit a related group

*Scientific Notebook Co., 719 Wisconsin Ave., Oak Park, Ill. 60304.

462

of analyses or to report a single type of analysis for a series of samples. As much information as possible is preprinted to simplify use of the form. With loose-sheet, multicopy forms (using carbon or NCR paper) information can be forwarded on the desired schedule while also allowing retention of data in the laboratory. Still, the most common means for recording data in rough form are internal bench sheets or bound books. The bench sheet or book never leaves the laboratory but serves as the source of information for transfer of data to appropriate report forms. (See fig. 7-1.)

In most instances the supervisor and analyst wish to look at the data from a sampling point or station in relation to other sampling points or stations on a particular river or lake. This review of data by the supervisor prior to release is a very important part of the QC program of the laboratory; however, such reviews are not easily accomplished with bench sheets. For review purposes, a summary sheet can be prepared that displays a related group of analyses from a number of stations such as shown in figure 7-2. Because the form contains space for all of the information necessary for reporting data, the completed form can also be used to complete the data forms forwarded to the computer storage and retrieval system.

The forms used to report data to storage systems provide spaces for identification of the sampling point, the parameter code, the type of analysis used, and the reporting terminology. Failure to provide the correct information can result in rejection of the data, or insertion of the data into incorrect parameter fields. As sample analyses are completed, the data values are usually reported in floating decimal form along with the code numbers for identifying the parameter data fields and the sampling point data fields. Figure 7-3 shows an example of a preprinted report form used for forwarding data to keypunch.

7.4.4 Plastic-Coated Labels and Forms

A recent addition to good sample handling and data management is the availability of plastic-coated (blank or preprinted) labels, report forms, and bound report books. These materials are waterproof, do not disintegrate when wet or handled, can be written on while wet, and retain pencil or waterproof ink markings though handled when wet.

7.4.5 Digital Readout

Instrumental analyses, including automated, wet-chemistry instruments such as the Technicon AutoAnalyzer, the atomic absorption spectrophotometer, the pH meter, and the selective electrode meter, provide digital readout of concentrations, which can be recorded directly onto report sheets without further calculation. Programmed calculators can be used to construct best-fit curves, to perform regression analyses, and to perform series of calculations leading to final reported values.

7.4.6 Keypunch Cards and Paper Tape

Because much of the analytical data generated in laboratories is first recorded on bench sheets, then transferred to data report forms, keypunched, and manipulated on small terminal computers (or manipulated and stored in a larger data storage system), there is a danger of transfer error that increases with each data copy. The analyst can reduce this error by recording data directly from bench sheets onto punch cards that can be retained or forwarded immediately to the data storage system. Small hand-operated keypunch machines are available.

NL-C-88
(1-88)

Spectrographic Analyses Bench Data

Sample # ____ Date _____ Source _____ TDS _____

_____ ml. conc. to _____ ml. Factor _____

Test Count	Sec
1. _____	_____
2. _____	_____
3. _____	_____

	Count			Rerun Count			Av. Count	PPM In Conc. Sample	Less Than	(μg/l) PPB Orig. Sample
1. Zn										
2. Cd										
3. As										
4. B										
5. P										
6. Fe										
7. Mo										
8. Mn										
9. Al										
10. Be										
11. Cu										
12. Ag										
13. Ni										
14. Co										
15. Pb										
16. Cr										
17. V										
18. Ba										
19. Sr										

Figure 7-1. Example of bench sheet.

464

MINERALS ANALYSES OF ZONE B, OHIO RIVER SAMPLES, CONC., mg/l.

STATION	Storet Number	Date	Alkalinity	Hardness	Chloride	Sulfate	Fluoride	SOLIDS		
								Total	Diss.	Susp.
Ohio at Ironton	200152									
Ohio at Greenup Dam	200001									
Ohio at Portsmouth	200139									
Scioto at Lucasville	381710									
Ohio at Maysville	200153									
Ohio at Meldahl Dam	383070									
Little Miami at Cincinnati	380090									
Ohio at Cincinnati	380037									
Licking at 12th Street	200523									
Ohio at Miami Fort	383072									
Ohio at Markland Dam	200521									
Kentucky at Dam I	200522									
Ohio at Madison	174304									
Great Miami at Eldean										
Great Miami at Sellars Road	383047									
Great Miami at Liberty-Fairfield Road	383015									
Great Miami at American Materials Bridge	383007									
Whitewater at Suspension										
Great Miami at Lawrenceburg (Lost Bridge)	383071									

Figure 7-2. Example of summary data sheet.

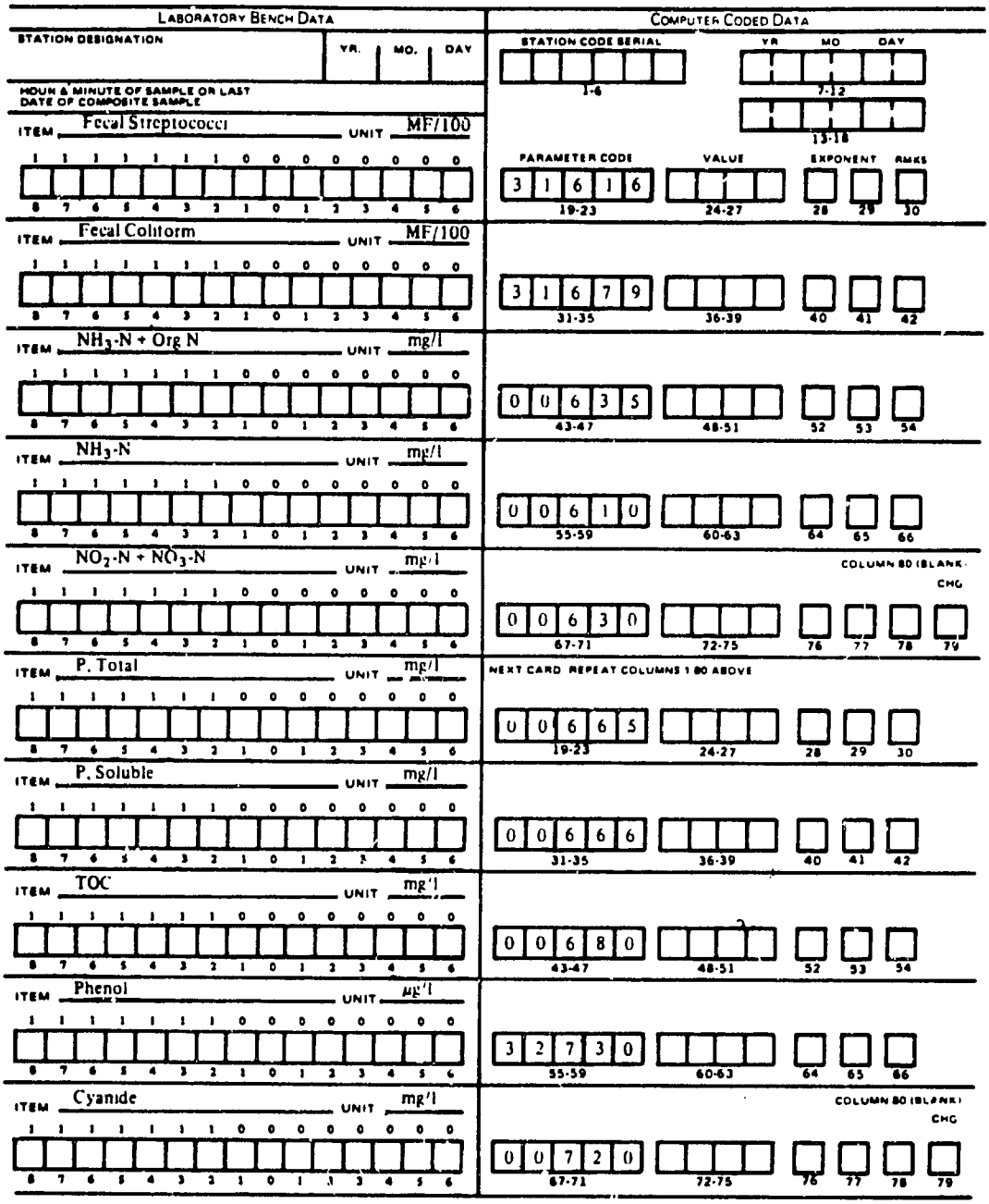


Figure 7-3. Example of STORET report form.

7.4.7 STORET—Computerized Storage and Retrieval of Water Quality Data

Because of their ability to record, store, retrieve, and manipulate huge amounts of data, the use of computers is a natural outgrowth of demands for meaningful interpretation of the great masses of data generated in almost all technical activities.

In August 1961, numerous ideas were brought together concerning the basic design of a system called STORET for storage and retrieval of water pollution control data. A

refinement of this system is now operated by the Technical Data and Information Branch, Division of Applied Technology, Office of Water Programs, EPA.

This is a State/Federal cooperative activity that provides State water pollution control agencies with direct, rapid access into a central computer system for the storage, retrieval, and analysis of water quality-control information.

If properly stored, the data can be retrieved according to such descriptors as the point of sampling, the date, and the specific parameters stored, or all data at a sample point or series of points can be extracted as a unit.

Full details on use of the STORET system are given in the recently revised STORET handbook (4).

7.4.8 Automated Laboratory Systems

The use of digital readout, keypunch cards, and paper tape have been overshadowed by the development of customized, fully automated online computer systems that make measurements, calculate results, perform quality control, and report analytical data simultaneously from a full range of laboratory instruments. (See fig. 7-4.) Such systems can contain the following functions:

a. Manual or automatic sampling and testing of a series of samples, standards, replicates, and check samples

b. Detection of the measurement signals from the series of samples

c. Conversion of signals to concentrations, generation of a standard curve, and calculation of sample values in final units

d. Calculation of the deviation and recovery values of the results and indication of acceptance or nonacceptance based on limits established by the analyst

e. Provision of the output in a form designated by the analyst: dial, paper recording chart, digital readout, cathode ray tube, or printed report form

The degree of hands-on operation required in the system is specified by the analyst.

If an automated system is properly designed and operated, most calculation and transposition errors are avoided and the proper level of quality control is automatically exerted. Laboratory automation systems for water analyses are being developed and coordinated by EMSL-Cincinnati for use in a number of EPA laboratories (5).

7.5 References

1. "Guide for Measure of Precision and Accuracy," Anal. Chem. *33*, 480 (1961).
2. "Glossary of General Terms Used in Quality Control," Quality Progress, Standard Group of the Standards Committee, American Society for Quality Control, *II*(7), 21-2 (1969).
3. Youden, W. J., Statistical Techniques for Collaborative Tests, Association of Official Analytical Chemists, Washington, D.C. (1967).

465

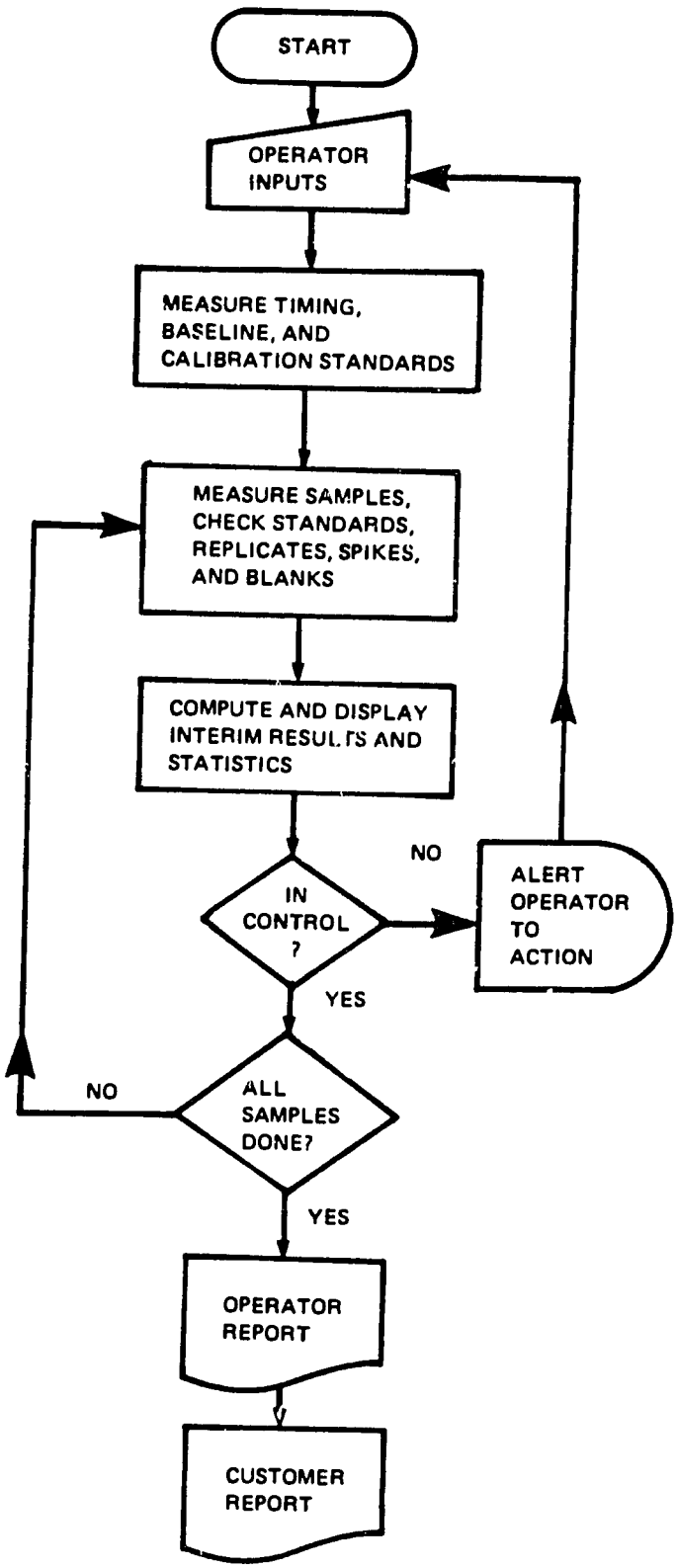


Figure 7-4. Flow chart of the sequence of events during a controlled series of laboratory measurements.

468

4. "STORET, EPA's Computerized Water Quality Data Base, the Right Answer," U.S. EPA, Office of Water and Hazardous Materials, Washington, D.C. (1977).
5. Budde, W. L., Almich, B. P., and Teuschler, J. M., The Status of the EPA Laboratory Automation Project, EPA-600/4-77-025, U.S. EPA, Office of Research and Development, EMSL, Cincinnati (1977).

409

Chapter 8

SPECIAL REQUIREMENTS FOR TRACE ORGANIC ANALYSIS

8.1 Introduction

The high sensitivity of the instrumentation used in trace organic chemical analysis and the low concentration of organic compounds being investigated dictate that special attention must be given to this analytical field. Contamination of the sample from any possible source must be diligently guarded against, and interferences in the sample must be carefully controlled. Strict attention to method and highly refined technique are required to produce valid quantitative and qualitative results.

Two manuals have been developed by the Environmental Monitoring and Support Laboratory (EMSL) in Cincinnati covering the two broad areas of quantitative and qualitative trace organic analysis. Reference 1 contains both general and specific quality assurance programs designed to insure the production of acceptable measurements when using the methods contained in that manual. Reference 2 contains specific, detailed quality assurance programs covering both the performance of the instrument and the interpretation of the resulting spectra.

This chapter is organized with three sections devoted to general material applicable to all organic analysis, and one section abstracting highly specialized materials from each of the two manuals cited.

8.2 Sampling and Sample Handling

Regardless of the intent, all numbers generated by a water quality laboratory are ultimately represented as the concentration levels in the sample matrix at the time of collection. Such numbers tend, automatically, to endorse the sample collection, preservation, and shipment procedures. Thus, quality assurance programs limited to the care of the sample beginning with its receipt by the laboratory are inadequate. The laboratory must share responsibility for the preservation and shipment of all samples that it will accredit with concentration values. Two approaches are available that will generally protect the laboratory from generating numbers that may not reflect actual conditions of the sample at the time of collection. The best, but usually least practical solution, is for the laboratory personnel to collect all samples. The alternative is for the laboratory to adopt a policy of sample rejection based on minimum standards of sample identification and age. Guidelines for establishing these standards are discussed in this section. It is recommended that copies of this material be supplied to all sample collectors along with an understanding of the specific policy of the laboratory toward rejecting samples that do not meet these criteria.

In all of the cases to be discussed, it is the responsibility of the project director to (a) coordinate his sampling, preservation, and shipment with the laboratory, (b) obtain clean sample containers from the laboratory, (c) provide adequate sample identification and compositing instructions, and (d) provide duplicates and blanks as required by the laboratory. Additional prearrangements should be made with the laboratory if sample splitting is desired, to create separate supernatant and settleable matter samples, or if calculations on a wet-weight basis in addition to the standard dry-weight calculations are desired.

4P

Bottles and caps are to be supplied by the laboratory because rigorous cleaning is required even for new bottles. New and recycled bottles should be washed as described in reference 1. Samples received in bottles of unknown origin or questionable cleanliness should be rejected by the laboratory. For water samples that are to be analyzed by solvent extraction methods, narrow-mouthed, screw-cap bottles such as Boston rounds are preferred because they have less tendency to leak and are easy to handle in the laboratory. One-liter bottles are generally more expensive than quart bottles, thus most laboratories use the clear 32-oz containers. The bottle should be sealed with Teflon (du Pont) lined caps. For water samples of intermediate pH, aluminum-foil-lined caps may be used when Teflon (du Pont) is not available. Oil and grease samples can be collected with Polyseal caps—the conical liner provides an excellent seal against pressure during shipment and sample extraction. Screw-cap, widemouthed glass bottles are preferred for sediment samples because they are easier to handle in the field and in the laboratory. Precleaned, 16- or 32-oz bottles with Teflon (du Pont) or foil-lined screw-cap closures should be provided by the laboratory. Masking tape or other suitable labels should be applied to the dry bottles.

Sampling for purgeable organics requires special consideration and equipment. The sample container should consist of a 45-ml, screw-cap vial fitted with a Teflon (du Pont) faced, silicone septum.* The vials, septa, and caps should be washed in hot detergent water and thoroughly rinsed with tapwater and organic-free water, then dried at 105°C for 1 h. The vials should then be cooled to room temperature in a contaminant-free area. When cool, the vials should be sealed with the septa, Teflon (du Pont) side down, and screw cap and maintained in this sealed condition until filled with sample.

The bottles used for collecting water samples for solvent extraction should not be overfilled or prerinsed with sample before filling because oil and other material that can cause erroneously high results may remain in the sample bottle after rinsing. Bottles should be filled with sample to about 90 percent of capacity, and the level should be marked to determine if leakage occurs. Because full sample bottles are difficult to pour from during extractions, complete filling should be avoided. In the collection of sediment samples, nonrepresentative debris such as large stones or wood should be discarded.

Multiple samples are usually required for purgeable organics analysis because of leakage and because the measurement process is destructive to the sample. All vials should be identified with waterproof labels. The water sample vials are filled to overflowing from a bubble-free source so that a convex meniscus is formed at the top. They are sealed by carefully placing the septum, Teflon (du Pont) side down on the opening of the vial and screwing the cap firmly in place.

Shipment and receipt of samples must be coordinated with the laboratory to minimize time in transit because it is the prerogative of the laboratory to reject samples where delays in shipment have caused them to age beyond acceptable holding times. To avoid the need to resample, the sampler should determine in advance the most efficient and reliable form of transportation for the samples. All samples for organic analysis should arrive at the laboratory on the same day collected, or should be shipped and maintained at less than 4°C for arrival by the next day. The samples are usually shipped in insulated ice chests. Water samples should be prechilled before packing to reduce the ice requirements during shipment.

---

*Vial and septum are available from Pierce Chemical Co., P.O. Box 117, Rockford, Ill. 61105. Vial: No. 13074; septum: No. 12722.

471

The bottles should be stabilized in the container with styrofoam and covered with ice. The information needed to identify the samples should be attached to the outside of the ice chest.

A part of the quality assurance program for a laboratory must be the development of a clear policy for accepting or rejecting samples. Because organic analyses are expensive in terms of manpower and supplies, it is poor management to commit such resources just to obtain data of questionable validity. Water samples that clearly have not met the preservation criteria during shipment (e.g., in the case of a spill) should be accepted only if resampling is impossible. Results from such samples must be qualified in the laboratory report.

Upon receipt, the samples should be checked for adequate identification, sample temperature or presence of ice in the chest, and leakage. Samples for volatile organics should be checked for air bubbles, although it is extremely difficult to avoid the development of very small bubbles regardless of the type of sample bottle employed. The samples are logged in with the receipt time noted. Unless the condition of the samples fails to meet the criteria for acceptance by the laboratory, required preservatives are added immediately and the samples are all refrigerated.

The laboratory staff should be alerted to the arrival of the samples, so that the required analysis can begin as soon as possible. When sediment samples are to be reported on a wet-weight basis, or when a water sample is to be filtered or divided into supernatant and settleable subsamples for separate analysis, the sample processing should begin promptly.

When analysis of water samples is to be restricted to the water phase only, filtration is required. This may be accomplished with 4.7-cm, glass-fiber filter disks that have been preextracted with acetone and allowed to dry. The filter disks must not contain organic binder. The disks are placed on a membrane filter holder and up to a liter of water is filtered. The filtrate is transferred without rinsing to a clean sample container and treated as a normal, whole-water sample.

When separate reporting is required for the settleable and supernatant phases, the water sample (or a large portion of it) is allowed to settle overnight in a closed glass container at 4°C. If phase separation occurs, the supernatant phase is carefully decanted or siphoned into a graduated cylinder without disturbing the surface of the settled material. After the volume of supernatant is noted, the supernatant is filtered as just described into a clean glass container and stored at 4°C. The volume of settled material is determined either by marking the slurry level on the side of the sample container for later calibration, or by transferring the slurry without washing into a calibrated vessel. A portion of well-mixed slurry is removed for a determination of percent solids. The remaining slurry is stored in a sealed glass container at 4°C.

When both dry-weight and wet-weight results are required for sediments and sludges, a percent-solids determination should be performed soon after receipt of the sample. A representative portion (ideally 10 to 25 g) of well-mixed sample is weighed into a tared Erlenmeyer flask and dried at 105°C to a constant weight. Then percent solids are calculated for the sample.

Routine laboratory management involves detailed recordkeeping beginning with the initial contact with the sample collector. A master flowsheet should be prepared for each sample, listing parameters to be determined and pretreatment operations to be performed. The

472

master flowsheet is kept at a fixed location and is designed to handle dated entries indicating when operations are completed. Methods requiring extraction permit double entry on the master flowsheet so laboratory throughput times can be calculated in terms of receipt-to-extraction and receipt-to-completion times. A separate set of forms follows the sample through the laboratory. Each sample form records information for a class of parameters and details cleanup operations and other method options. Calculations and cross references to chromatograph files are entered on the sample form also.

A chain-of-custody program lends significant legal support to the results generated. The program begins when the laboratory dispatches the sample collectors. Each time a sample is collected, a form is initiated stating where and when the sample was collected, with cross reference to a number on the sample container. The sample collectors return to the laboratory with the samples in their guarded possession and deliver them personally to the responsible person in the laboratory. The person (custodian) receiving the samples in turn signs each ledger to acknowledge receipt and locks the sample in a refrigerator. The analyst then comes to the custodian and signs for the sample that he is taking for analysis. In this way full documentation of the sample handling is maintained from sample collection through completion of the analysis.

8.3 Extract Handling

Each method in reference 1 is prepared in sections identified by titles. This style of presentation is aimed at presenting the analyst logical places to interrupt his analysis. Often, because of the length of the method, the analyst is unable to complete an analysis in a single day. When planning a partial analysis, certain factors must be considered. Because the organics are generally more stable in solvent than in water, it is always preferable to extract a sample and hold the extract rather than to hold the sample. Extracts to be held overnight or longer should first be dried by treatment with sodium sulfate.

The methods include several transfers of the solvent extract from one piece of glassware to another. These quantitative transfers are made using several small portions of solvent to wash the walls of the previous container. A 5-ml, luer-lock glass syringe with a 2-in., 20-gage needle is convenient for this purpose. Solvents tend to creep up the outside of a container such as an ampoule, while pouring. To minimize contamination during transfer, solvent extracts are poured as rapidly as possible. Extracts can become contaminated not only from oils from the skin of the handler but also from other extracts handled at the same time where deposits on the outside of the container are unintentionally transferred from one container to another. Instead of using labels, contamination problems from this source can be reduced by etching permanent numbers on ampoules. Sample log sheets should be used to index the extract with a numbered ampoule, eliminating the need for tape or wax pencils.

Of the several ways to dry a solvent extract with sodium sulfate, passing it through a chromatographic column packed with 2 to 3 in. of anhydrous crystals is the most convenient and quantitative. When a wet extract is being transferred from a separatory funnel to a Kuderna-Danish (K-D) concentrator, it should be drained directly through the column to eliminate the need for an intermediate piece of glassware and the resulting transfer step. Prewashing the sodium sulfate column with extracting solvent is recommended, although interferences can be controlled by preheating the salt in a shallow tray at 400°C for 30 min. After the extract has passed through the column, 20 to 30 ml of extracting solvent are used to wash the residual extract from the column.

473

The standard K-D concentration apparatus consists of a three-ball Snyder column, a 500-ml flask, and a calibrated ampoule. It is designed to concentrate 100- to 300-ml volumes of extract to a final volume of 5 to 10 ml. To use the apparatus, one 10/20 mesh boiling chip (previously rinsed with solvent and heated for 1/2 h) is added, and the assembly is supported above a concentric-ring water bath with the tip of the ampoule below the surface of the water. The lower ground-glass joint must be kept above the water. The water temperature should be adjusted for mild distillation, with no chamber flooding or splashing (about 10°C to 20°C above the boiling point of the solvent). When the volume of liquid reaches 1 to 2 ml (checked frequently), the assembly should be removed and allowed to cool. The chambers and flask will drain to a final volume near 10 ml. The flask and the lower ground-glass joint are rinsed with a minimum of solvent. The evaporation can be continued with the microscale K-D concentrator if further concentration is required.

The microscale K-D concentrator is designed to concentrate extracts from 5 to 10 ml to 1.0 ml. A fresh boiling chip must be added, and a two-ball micro-Snyder column is attached to the ampoule. The ampoule is supported above the water bath, and the extract is concentrated to about 0.7 ml. The column and ground-glass joint are rinsed with a minimum of solvent, and the volume is adjusted to 1.0 ml.

The K-D concentrator can be used to exchange solvents. When the sample is dissolved in a solvent unsuitable for a cleanup operation or for gas chromatography (GC), it can be displaced by a suitable higher boiling solvent. The actual volumes that should be used to effect the exchange vary with the solvent pairs depending upon the difference in boiling points and azeotrope formations, but a general procedure is to concentrate the extract to 10 ml, add 20 ml of the higher boiling solvent, and reconcentrate to 10 ml.

After extractions and subsequent K-D concentrations, solubilities of some materials may be exceeded. High sulfur levels are a particular problem encountered in sediment extractions. Extracts should be decanted from ampoules where sulfur has crystallized. In some samples the extractable organic levels are so high the extract tends to solidify and will not concentrate further. When this occurs, a small aliquot of the extract should be taken and diluted as appropriate for final analysis.

A significant source of the variation in GC analysis can be attributed to the injection of a portion of the extract into the analytical system. Manual injections of 2 to 3 μl with the use of a 10-μl syringe will introduce variance even when the injection volumes are determined to the nearest 0.05 μl. Of a variety of injection techniques in use, the solvent flush technique has been found to be acceptable for quantitative work. This technique is described in detail in reference 1.

8.4 Supplies and Reagents

Reference compounds of materials should be assayed and of 98 percent purity or higher. If the purity is less than 98 percent, the appropriate correction factor must be included in all calculations of standard concentration. The reference materials should be cataloged, dated, and stored in a refrigerator.

Stock solutions of these reference materials should be prepared in a high-boiling, inert solvent, if possible, to minimize errors due to evaporation or solvent-induced decomposi-

474

tion. The laboratory should have an accurate six-place balance for preparing small quantities of reference standards. The following method of preparing stock solutions is recommended: Weigh 10 mg (to the nearest 0.01 mg) of reference standard into a small aluminum weighing pan.* Drop the entire pan into a 10-ml volumetric flask. Dissolve the reference material in about 5 ml of solvent, then dilute to volume. Label the solution with compound name, concentration, solvent used, date prepared, and initials of preparer. Store the volumetric in a refrigerator, except when preparing dilutions. When the standard is a replacement for an existing stock solution, the two solutions should be compared, and the results as well as the suspected reason for any variation should be permanently recorded in the laboratory files.

Working standards are prepared from one or more stock solutions after they have warmed to room temperature. As these working standards usually represent three to six orders of magnitude of dilution of the stock standards, it is obviously necessary to take great care in preparing them. Serial dilutions are recommended with a maximum of 1:100 dilution for each step. Although 10-μl volumes can be read within 1 percent with a 10-μl syringe, the inherent problems with the dead volume in the syringe make the use of such equipment less desirable for preparing working standards than volumetric pipets. New working standards should be prepared frequently unless long-term stability has been demonstrated. When several compounds are combined into a single standard for simultaneous GC, they must be closely monitored for chemical interactions.

Pesticide-quality solvents are usually required, and each new lot should be checked for interferences prior to use. The solvent check, representing approximately 10 percent more solvent than required for any method, should be concentrated and analyzed for method interferences under all GC conditions applicable to that solvent. If interfering peaks or a broad solvent front are observed, the solvent should be redistilled in an all-glass distillation system, with a distillation column.** If interferences persist, the solvent lot should be discarded. This preliminary lot check does not eliminate the need for routine solvent blanks to monitor for purity changes over a period of time.

Diethyl ether must be shown to be free of peroxides before use. Peroxide test strips† can be used for a quick, convenient test. The alumina column procedure for removing peroxides, described in literature supplied with the test strips, has been used successfully to remove all peroxides from the solvent. The solvent should always be stabilized with 2 percent volume/volume ethyl alcohol. Chromatographic elution patterns are based on ether containing this alcohol.

Granular sodium sulfate should be purchased in glass containers. If purchased in a large container (5-lb bottles or larger), it should be transferred to smaller bottles for daily use. Before sodium sulfate is used for chromatographic work, it should be heated to 400°C for 30 min and shown not to be contaminated. When the sodium sulfate is used to dry extracts before concentration, the heating is usually unnecessary because impurities will be removed by preelution of the drying column with solvent.

---

*Available from The Perkin-Elmer Corp., Norwalk, Conn.; No. 219-0041.

**Available from Lab Glass, Inc., North West Blvd., Vineland, N.J. 08360; Widner No. LG-5930.

†Available from Scientific Products, 1430 Waukegan Rd., McGaw Park, Ill. 60085; Quant peroxide test strip, No. P1126-8.

475

Florisil (Floridin Co.) is purchased from the manufacturer preactivated at 630°C and transferred to glass containers with Teflon (du Pont) or foil-lined lids. Prior to use the Florisil (Floridin Co.) is heated in a shallow open dish for 5 h in a 130°C oven. The Florisil (Floridin Co.) can then be transferred into a sealed, glass bottle and stored indefinitely at 130°C until needed. Cleanup methods using Florisil (Floridin Co.) require that a lauric acid value be determined for each lot before use. In addition, the determination of a pesticide elution pattern is recommended. These procedures are described in detail in reference 1.

Carrier gases are a very important part of the chromatographic system; therefore, special care should be taken in their selection and handling. Only high-purity or equivalent carrier gases should be purchased, and they should be filtered, online, through a 5-Å molecular sieve. Porous polymer and other column packings degrade at elevated temperatures in the presence of trace quantities of oxygen. Oxygen in the carrier gas also adversely affects the performance of electron-capture detectors. Therefore, some type of online oxygen-removal system is recommended for these applications. The chemical traps should be changed or regenerated with each new cylinder of carrier gas. One purifier* has been found to last through many cylinders without replacement. A better grade of gas is required for temperature programing than for isothermal operation. Combustion gases may be of lower quality but should be at least equivalent to dry air or purified hydrogen. Regulators should have stainless steel internal parts and be of two-stage design. External tubing should be of good quality, such as refrigeration tubing. Such tubing should be rinsed with solvent and heated at 200°C under gas flow before use in the analytical system.

The purchase of precoated GC column packings is strongly recommended over the preparation of coated materials in the laboratory. The commercial products are, generally, of higher quality and consistency than those prepared by the average analyst. All tubing should be cleaned before packing by passing a series of solvents (e.g., hexane, chloroform, and acetone) through it. Glass columns should also be silylated. (Instructions are included with the purchase of silylating agents.) Dry the tubing thoroughly before packing it.

A vibrator should never be used to settle the packing material in the column. Such vibration may fracture the solid support material, expose uncoated active sites, and produce inferior chromatographic separations. When vacuum alone is inadequate and further settling is required, the column may be tapped with a pencil or similar object while the vacuum is being applied. Unless otherwise stated, stainless-steel tubing should be packed before coiling it to fit the GC apparatus.

8.5 Quality Assurance

8.5.1 Measurements

Most of the quality assurance programs suggested in chapter 6 of this manual cannot easily be adapted to the methods for organic compounds. The reasons for this, and the suggested approach for a suitable program for the organic analytical laboratory are discussed in detail in reference 1 and only summarized here.

Reference 1 suggests that quality assurance for organic analysis be divided into three separate categories. The first category represents the determination of purgeable com-

*Available from Matheson Gas Products, P.O. Box E, Lyndhurst, N.J. 07071: Hydrox Purifier model No. 8301.

pounds. This determination is performed in a closed analytical system; the complete analysis can be performed in 1 h; and the number of theoretically possible interferences is somewhat limited. The second category represents liquid/liquid partition methods in a regulatory situation. Here a very limited number of compounds are being measured; there is a high occurrence of positive results; and it is important to establish that the method works satisfactorily on the particular sample matrix. The third category represents liquid/liquid partition methods in a monitoring situation. Here a large number of compounds are often being measured simultaneously; there is a low occurrence of positive results; and each sample matrix may be different. Quality assurance is aimed at establishing that the laboratory is using the method correctly.

The purgeable methods are unique among organic methods because the standards are treated in exactly the same way as the samples, and there is no inherent method bias. The methods are amenable to a variety of quality assurance programs. The approach that has been found applicable to all types of samples and provides the maximum data for the expended effort consists of the addition of one or more internal standards to the matrix before purging. Data generated in this program provide a continuous monitoring of the equipment and establishes matrix applicability for the test.

For liquid/liquid extraction methods in a regulatory situation, the emphasis is placed on duplicates and dosed samples. Both field duplicates and laboratory duplicates are used in the program to establish sampling and subsampling validity. The dosing of samples to establish method accuracy for the matrix is an integral part of this program. Where the analytical program will extend over a long period of time the construction of control charts is recommended.

When the liquid/liquid extraction methods are used for monitoring, the emphasis is placed on an external control series. A standard laboratory matrix is developed. With each series of samples the matrix is dosed and analyzed with the samples. Data generated over a period of time can be used to monitor the performance of the equipment and the analyst, with relatively tight specifications to define problems that arise. Control charts can be constructed to alert the analyst to problems, but there is no provision for rejection of results for samples of this type.

8.5.2 Identifications

The combined gas chromatography/mass spectrometer (GC/MS) has emerged as the most important single instrument at the disposal of the environmental analytical chemist. It alone can provide both the sensitivity and the high degree of certainty necessary for an identification culled from a complex environmental matrix. The instrument has generated an aura of well-deserved respect, and its results are seldom questioned. For these reasons it is mandatory that strict quality assurance programs be followed in both the generation and the interpretation of mass spectra. The EMSL, with the cooperation of many other EPA GC/MS users, has produced a procedural manual (2) generally for use with a Finnigan quadrupole instrument. A detailed quality assurance program constitutes an integral part of the manual. The actual detailed program is beyond the scope of this manual but has been summarized in the following paragraphs.

To insure that a quadrupole mass spectrometer generates quality spectra, the program provides for at least daily performance evaluation with a reference compound, and

477

readjustment of the instrument as necessary. The operator prepares a solution of decafluorotriphenylphosphine (DFTPP).* When this compound is injected into the GC/MS using any of several compatible GC columns, the resulting elaborate spectrum can be evaluated using criteria developed at EMSL-Cincinnati (3).

Decafluorotriphenylphosphine key ions and the ion-abundance criteria that are used for determining whether the mass spectrometer is generating high-quality spectra are as follows:

Abundance Criteria

Mass (amu)	*Ion Abundance Criteria*
51	30 to 60 percent of mass 198
68	Less than 2 percent of mass 69
70	Less than 2 percent of mass 69
127	40 to 60 percent of mass 198
197	Less than 1 percent of mass 198
198	Base peak, 100 percent relative abundance
199	5 to 9 percent of mass 198
275	10 to 30 percent of mass 198
365	1 percent (or greater) of mass 198
441	Less than mass 443
442	Greater than 40 percent of mass 198
443	17 to 23 percent of mass 442

If these specifications are not met, the guidelines provided for adjusting the instrument (2,3) must be considered.

Having produced quality spectra, the analyst must always ascertain, by analyzing a method blank under exactly the same analytical and instrumental conditions, that the spectra are relevant. Most commercial systems have a software program, extracted ion current profile (EICP), that permits the sample and the blank to be overlayed on a graphic display device when each is scanned for a particular mass. When large numbers of spectra result from a sample and the blank must be checked for a match for each one, this technique simplifies the screening process.

If the spectra are found to be unique to the sample, it is normally processed through a mass-spectral search-and-match system. The computerized version of such a system consists

*Available from PCR Research Chemicals, Inc., P.O. Box 1778, Gainsville, Fla. 32602; No. 11898-4.

478

of an organized collection of many thousands of compound spectra stored in a large central computer accessed by the analyst through telephone linkups. On the basis of 8 or 10 major masses the computer can rapidly search the complete system for similar spectra. These spectra are ranked for similarity to the unknown using a mathematical algorithm. The resulting similarity index (SI), which can be further refined to a quality index (2), is a measure of the degree of confidence the analyst may place in the identification. If a match is not found, the analyst must revert to manual interpretation of the spectra and deduce the structure of the compound by its fragmentation patterns.

After a tentative identification is made, several other types of supporting experiments become possible. Retention-time GC/MS data of a pure compound (standard) may be compared with analogous data from the sample component. Similarly, the mass spectrum of the standard, obtained under the same conditions that were used for the sample, may be compared with the sample component spectrum. The standard may be dissolved in water at an appropriate concentration, isolated, and measured. The recovery of this spike in the same fraction in which the suspected component appeared and the observation of equivalent mass spectra for the spike and the sample component constitute strong evidence for confirmation of the identification.

8.6 References

1. Methods for Organic Analysis of Water and Wastes, U.S. EPA, EMSL, Environmental Research Center, Cincinnati (in preparation).
2. EPA GC/MS Procedural Manual, Budde, W. L., and Eichelberger, J. W., editors, 1st Edition, Vol. 1, U.S. EPA, Office of Research and Development, EMSL, Cincinnati (in press).
3. Eichelberger, J. W., Harris, L. E., and Budde, W. L., Anal. Chem., *47*, 995 (1975).

Chapter 9

SKILLS AND TRAINING

9.1 General

Analytical operations in the laboratory can be graded according to the degree of complexity. Some analyses require no sample treatment, and the measurement can be performed in minutes on a simple instrument. Other determinations require extensive sample preparation prior to complex instrumental examination. Consequently, work assignments in the laboratory should be clearly defined. Each analyst should be completely trained and should fully understand all the assignments of his job before being given new responsibilities. In this regard, all analysts, subprofessional or professional, should be thoroughly instructed in basic laboratory operations, according to the degree of professional maturity. Some of the basic operations that should be reviewed periodically with laboratory personnel follow.

9.1.1 Sample Logging

Routine procedure for recording of samples entering the laboratory and assigning primary responsibility should be emphasized. The information that is required and the routing of the sample to the analyst is then established. The stability, preservation, and storage of samples prior to analyses are then discussed.

9.1.2 Sample Handling

The analyst should understand thoroughly at which points in his procedures the sample is to be settled, agitated, pipetted, etc., before he removes it from the original container.

9.1.3 Measuring

The analysts, especially new employees and subprofessionals, should be instructed in the use of volumetric glassware. The correct use of pipettes and graduates should be emphasized as discussed in chapter 4 of this manual.

9.1.4 Weighing

Because almost every measuring operation in the analytical laboratory is ultimately related to a weighing operation, the proper use of the analytical balance should be strongly emphasized. Maintenance of the balance, including periodic standardization, should be repeatedly emphasized to all personnel. The correct use and maintenance of balances is discussed in chapter 3 of this manual.

9.1.5 Glassware

All glassware should be washed and rinsed according to the requirements of the analysis to be performed. Not only must the personnel assigned to these tasks be instructed, but also all lab personnel should know the routine for washing and special requirements for particular uses of glassware. In addition, the precision tools of the laboratory such as pipets, burets,

graduates, and Nessler tubes should be inspected before use for cleanliness, broken delivery tips, and clarity of marking. Defective glassware should be discarded or segregated.

9.1.6 Instrumentation

Operation and maintenance of analytical instrumentation is of primary consideration in the production of valid data. All instruments must be properly calibrated, quality-control checks documented, and standard curves verified on a routine basis. Details on instrumental quality control are presented in chapter 3 of this manual.

9.1.7 Data Handling and Reporting

As with sample logging, the routine procedure for recording results of analyses and pertinent observations, including quality control checks, should be emphasized. Analytical data should be permanently recorded in meaningful, exact terms and reported in a form that permits future interpretation and unlimited use. Details are discussed in chapter 7 of this manual.

9.1.8 Quality Control

The need to continuously assess precision and recovery values of methodology is a prime responsibility of the analyst. Self-evaluation through the analyses of replicates and recovery of spikes from samples representative of the daily workload provides confidence and documentation of the quality of the reported data.

9.1.9 Safety

Laboratory safety should be discussed on a continuing basis with all employees, but it should be emphasized when an employee is assigned to perform new duties.

9.1.10 Improvement

In summary, quality control begins with basic laboratory techniques. Individual operator error and laboratory error can be minimized if approved techniques are consistently practiced. To insure the continued use of good technique, laboratory supervisors should periodically review the basic techniques and point out areas of needed improvement with each analyst.

Continuing improvement of technical competence by all laboratory personnel is, of course, the final responsibility of the laboratory supervisor. In a well-organized laboratory, however, a big-brother attitude of higher ranking to lower grade personnel should be encouraged; each person should be eager to share experience, tricks of the trade, special skills, and special knowledge with subordinates. Obviously, efficiency and results will improve.

9.2 Skills

The cost of data production in the analytical laboratory is based largely upon two factors: the pay scale of the analyst, and the number of data units produced per unit of time. However, because of the large variety of factors involved, estimates of the number of measurements that can be made per unit of time are difficult. If the analyst is pushed to

48

Table 9-1
SKILL-TIME RATING OF STANDARD ANALYTICAL OPERATIONS

Measurement	Skill Required (Rating No.)[1]	Number Per Day
Simple Instrumental:		
pH	1	100-125
Conductivity	1	100-125
Turbidity	1	75-100
Color	1	60-75
Dissolved Oxygen (Probe)	1,2	100-125
Fluoride (Probe)	1,2	100-125
Simple Volumetric:		
Alkalinity (Potentiometric)	1	50-75
Acidity (Potentiometric)	1	50-75
Chloride	1	100-125
Hardness	1	100-125
Dissolved Oxygen (Winkler)	1,2	75-100
Simple Gravimetric:		
Solids, Suspended	1,2	20-25
Solids, Dissolved	1,2	20-25
Solids, Total	1,2	25-30
Solids, Volatile	1,2	25-30
Simple Colorimetric:		
Nitrite N (Manual)	2	75-100
Nitrate N (Manual)	2	40-50
Sulfate (Turbidimetric)	2	70-80
Silica	2	70-80
Arsenic	2,3	20-30
Complex, Volumetric, or Colorimetric:		
BOD	2,3	[2]15-20
COD	2,3	25-30
TKN	2,3	25-30
Ammonia	2,3	25-30
Phosphorus, Total	2,3	50-60
Phenol (Distillation Included)	2,3	20-30
Oil and Grease	2,3	15-20
Fluoride (Distillation Included)	2,3	25-30
Cyanide	2,3	8-10
Special Instrumental:		
TOC	2,3	75-100
Metals (by AA), No Preliminary Treatment	2,3	150
Metals (by AA), With Preliminary Treatment	2,3	60-80
Organics (by GC), Pesticides, Without Cleanup	3,4	3-5
Organics (by GC), Pesticides, With Cleanup	3,4	2-4

[1] Skill-required rating numbers are defined as follows:
1—aide who is a semiskilled subprofessional with minimum background or training, comparable to GS-3 through GS-5. (Continued)

482

produce data at a rate beyond his capabilities, unreliable results may be produced. On the other hand, the analyst should be under some compulsion to produce a minimum number of measurements per unit of time, lest the cost of data production become prohibitive. In table 9-1, estimates are given for the number of determinations that an analyst should be expected to perform on a routine basis. The degree of skill required for reliable performance is also indicated.

The time limits presented in the table are based on use of approved methodology. A tacit assumption has been made that multiple analytical units are available for measurements requiring special equipment, as for cyanides, phenols, ammonia, nitrogen, and COD. For some of the simple instrumental or simple volumetric measurements, it is assumed that other operations such as filtration, dilution, or duplicate readings are required; in such cases the number of measurements performed per day may appear to be fewer than one would normally anticipate.

9.3 Training

For more experienced, higher grade personnel, formal training in special fields, possibly leading to specialization, should be almost mandatory. Such training can be fostered through local institutions and through the training courses provided by the EPA. Regional policies on after-hours, Government-supported training should be properly publicized.

Formalized training for lower grade personnel, comparable to GS-3 to GS-5, is relatively scarce. However, skills can be most efficiently improved at the bench level on a personal, informal basis by more experienced analysts working in the same area. Exposure of personnel to pertinent literature should also be a definite program policy.

(Continued)

2—aide with special training or professional with minimum training with background in general laboratory techniques and some knowledge of chemistry, comparable to GS-5 through GS-7.

3—experienced analyst capable of following complex procedures with good background in analytical techniques, professional, comparable to GS-9 through GS-12.

4—experienced analyst specialized in highly complex procedures, professional, comparable to GS-11 through GS-13.

[2] Rate depends on type of samples.

Chapter 10

WATER AND WASTEWATER SAMPLING

10.1 Introduction

The quality of data resulting from water and wastewater sampling surveys is dependent upon the following six major activities: (a) formulating the particular objectives of the water sampling program, (b) collecting representative water samples, (c) maintaining the integrity of the water samples through proper handling and preservation, (d) adhering to adequate chain-of-custody and sample identification procedures, (e) practicing quality assurance in the field, and (f) properly analyzing the pollutants in the water samples. These areas are equally important for insuring that environmental data are of the highest validity and quality.

The present section addresses aspects of quality control (QC) concerned with the collection of environmental samples and data in the field. It includes a capsule summary of the specific areas mentioned previously and a list of references (table 10-1) that provide specific guidance in these areas, rather than a collection of guidelines on sampling procedures.

10.2 Areas of Sampling

The specific areas that comprise an overall water sampling program are as follows.

10.2.1 Objectives of the Particular Sampling Program

The objectives of the sampling program affect all the other aspects of the sampling program.

Sampling program objectives are determined by the following activities: (a) planning (areawide or basin), (b) permitting, (c) compliance, (d) enforcement, (e) design, (f) process control, and (g) research and development. The types of water sampling programs to be employed, depending on suitability to program objectives, include reconnaissance surveys; point-source characterization; intensive surveys; fixed-station-network monitoring; ground-water monitoring; and special surveys involving chemical, biological, microbiological, and radiological monitoring.

Factors that must be considered in meeting the objectives of the sampling program are the extent of the manpower resources, the complexity of the parameters of interest, the duration of the survey, the number of samples, the frequency of sampling, the type of samples (grab or composite), and the method of sample collection (manual or automatic).

10.2.2 Collection of Representative Samples

The objective of all water and wastewater sampling is to obtain a representative portion of the total environment under investigation. The techniques for obtaining representative water samples may vary with the length, width, and depth of a body of water, its physical and chemical parameters, and its type to be sampled (such as municipal or industrial effluents, surface waters and bottom sediments, agricultural runoff, and sludges). In collecting representative samples, the following factors should be considered.

484

Table 10-1
GUIDANCE FOR WATER/WASTEWATER SAMPLING

Sampling Subject Area	Reference Numbers											
	.	2	3	4	5	6	7	8	9	10	11	12
Objectives of a Sampling Program	X					X	X					
Collection of Representative Samples:	X	X										
Site Selection	X	X					X	X				
Sample Types[1]	X	X				X	X					
Automatic Samplers	X	X		X	X	X	X					X
Flow Measurement	X	X				X	X		X	X		X
Statistical Approach to Sampling:	X											
Frequency of Sampling	X					X						
Number of Samples	X					X						
Probability of Exceeding Standard	X					X						
Special Sampling Procedures:												
Municipal Wastewaters	X											X
Industrial Wastewaters	X											
Agricultural Wastewaters	X											
Surface Waters	X											
Bottom Sediments	X											
Sludges	X											
Biology	X		X				X					
Microbiology	X					X		X				
Radiation	X	X					X	X				
Sample Preservation and Handling:	X	X				X	X	X			X	X
Volume of Sample	X	X									X	
Container Type and Cleaning	X	X										
Sample Identification	X	X						X				
Chain of Custody	X	X						X				
Quality Assurance in the Field		X										

[1] Grab, composite, manual, etc.

10.2.2.1 Site Selection

The location of the sampling site is critical in obtaining representative data. Preferably, water sampling sites for point sources of pollution from municipal and industrial effluents are located at points of highly turbulent flow to insure good mixing; however, inaccessibility, lack of site security, or power unavailability may preclude use of the best sites, but these impediments should not be used as reasons for collecting samples at unacceptable locations. Locations of sampling sites for streams, lakes, impoundments, estuaries, and coastal areas vary, but in general occur in the following bodies: (a) in water bodies for sensitive uses (swimming and drinking water supply), (b) in major impoundments or reservoirs near the mouths of major tributaries and in the rivers entering and leaving the impoundments, (c) in water bodies polluted by man's activities, (d) in rivers upstream and downstream from tributaries, and (e) where hydrological conditions change significantly.

10.2.2.2 Types

The basic types of water and wastewater sampling methods are grab sampling and composite sampling. Composite sampling may be conducted manually or automatically. The six methods for forming composite samples, all of which depend on either a continuous or periodic sampling mode, are the following: (a) constant sample pumping rates, (b) sample pumping rates proportional to stream flow rates, (c) constant sample volumes and constant time intervals between samples, (d) constant sample volumes and time intervals between samples proportional to stream flow rates, (e) constant time intervals between samples and sample volumes proportional to total stream flow volumes since last sample, and (f) constant time intervals between samples, and sample volumes proportional to total stream flow rates at time of sampling. The choice of using the grab sampling method or one of the six compositing sampling methods is determined by program objectives and the parameters to be sampled.

10.2.2.3 Automatic Samplers

The use of automatic samplers eliminates errors caused by the human element in manual sampling, reduces personnel cost, provides more frequent sampling than practical for manual sampling, and eliminates the performance of routine tasks by personnel. Criteria for brand selection of automatic samplers include evaluations of the intake device, intake pumping rates, sample transport lines, sample gathering systems (including pumps and scoops), power supplies and power controls, sample storage systems, and additional desirable features to fit particular sampling conditions. There are many commercially available automatic samplers: however, because no single automatic sampler is ideally suited for all situations, the user carefully selects the automatic sampler most suited for the particular water or wastewater to be characterized. Precautions must be taken in regard to using certain types of samples in potentially explosive atmospheres.

10.2.2.4 Flow Measurement

An essential part of any water or wastewater sampling survey as well as a necessary requirement of the National Pollution Discharge Elimination System (NPDES) permit program is accurate flow measurement, which can be divided into four categories:

a. Flow measurement in completely filled pipes under pressure—common devices employed are orifices, Venturi tubes, flow nozzles, Pitot tubes, magnetic flow meters, ultrasonic flow meters, and elbow meters.

b. Trajectory methods, either full or partially full, measured at the end of the pipe—common flow measurement methods are the California and Purdue pipe methods. These methods are normally considered as estimates rather than accurate measurements.

c. Flow measurement in open channels and sewers—common methods are the velocity-area measurement, time-of-passage measurement, and level measurement methods using weirs and flumes.

d. Miscellaneous flow measurement methods—common methods include use of Manning formula, tracer and salt dilution techniques, water meters, pump rates, and measurements of level changes in tanks and calibrated vessels.

Flow measurement data may be instantaneous or continuous. For continuous measurements, a typical system consists of primary devices such as weirs and flumes and secondary devices such as flow sensors, transmitting equipment, recorders, and totalizers. The improper installation or design of a primary device or malfunction of any part of a secondary device results in erroneous flow data. The accuracy of flow measurement data also varies widely, depending principally on the accuracy of the primary device and the particular flow measurement method used. In any case, an experienced investigator should be able to measure flow rates within ±10 percent of the true values.

10.2.2.5 Statistical Approach to Sampling

Four factors must be established for every sampling program: (a) number of samples, (b) frequency of sampling, (c) parameters to be measured, and (d) sampling locations. These factors are usually determined in varying degrees by details of the pertinent discharge permits or are more arbitrarily set by the program resource limitations. Nevertheless, the nature of the statistical methods selected and scientific judgment should be used to establish the best procedures.

10.2.2.6 Special Sampling Procedures

Special sampling procedures should be employed for municipal, industrial, and agricultural waters, surface waters as well as bottom sediments and sludges, and for biological, microbiological, and radiological studies.

10.2.3 Sample Preservation and Handling

During and after collection, if immediate analysis is not possible, the sample must be preserved to maintain its integrity. The only legally binding reference EPA has for sample preservation methods is the NPDES permit program specified in reference 13. However, these sample preservation procedures serve as a guide for other program objectives.

Proper handling of the samples helps insure valid data; consideration must also be given to care of the field container material and cap material, cleaning, structure of containers, container preparation for determination of specific parameters, container identification, and volumes of samples.

10.2.4 Chain-of-Custody Procedures

All programs involved in water and wastewater surveys should document and implement a chain of possession and custody of any sample collected, whether or not the resulting data are to be used in enforcement cases. Such procedures insure that the samples are collected, transferred, stored, analyzed, and destroyed only by authorized personnel. See section 12.7 for detailed procedures that can be used on all sample types.

10.2.5 Quality Assurance in the Field

Quality assurance programs for sampling equipment and for field measurement procedures (of such parameters as temperature, dissolved oxygen, pH, and conductance) are necessary to insure data of the highest quality. A field quality assurance program administered by a quality assurance coordinator should contain the following documented elements:

a. The analytical methodology; the special sample handling procedures; and the precision, accuracy, and detection limits of all analytical methods used.

b. The basis for selection of analytical and sampling methodology. For example, all analytical methodology for NPDES permits shall be that specified in reference 13, or shall consist of approved alternative test procedures. Where methodology does not exist, the quality assurance plan should state how the new method will be documented, justified, and approved for use.

c. The amount of analyses for quality control (QC), expressed as a percentage of overall analyses, to assess the validity of data. Generally, the complete quality assurance program should approximate 15 percent of the overall program with 10 and 5 percent assigned to laboratory QC and field QC, respectively. The plan should include a shifting of these allocations or a decrease in the allocations depending upon the degree of confidence established for collected data.

d. Procedures for the recording, processing, and reporting of data; procedures for review of data and invalidation of data based upon QC results.

e. Procedures for calibration and maintenance of field instruments and automatic samplers.

f. A performance evaluation system, administered through the quality assurance coordinator, allowing field sampling personnel to cover the following areas:

   (1) Qualifications of field personnel for a particular sampling situation.

   (2) Determination of the best representative sampling site.

   (3) Sampling technique including location of the points of sampling within the body of water, the choice of grab or composite sampling, the type of automatic sampler, special handling procedures, sample preservation, and sample identification.

   (4) Flow measurement, where applicable.

(5) Completeness of data, data recording, processing, and reporting.

(6) Calibration and maintenance of field instruments and equipment.

(7) The use of QC samples such as duplicate, split, or spiked samples to assess the validity of data.

g. Training of all personnel involved in any function affecting the data quality.

Quality assurance in sample collection should be implemented to minimize such common errors as improper sampling methodology, poor sample preservation, and lack of adequate mixing during compositing and testing. The checks listed in the following sections will help the quality assurance coordinator to determine when the sample collection system is out of control.

10.2.5.1 Duplicate Samples

At selected stations on a random time frame duplicate samples are collected from two sets of field equipment installed at the site, or duplicate grab samples are collected. This provides a check of sampling equipment and technique for precision.

10.2.5.2 Split Samples

A representative subsample from the collected sample is removed and both are analyzed for the pollutants of interest. The samples may be reanalyzed by the same laboratory or analyzed by two different laboratories for a check of the analytical procedures.

10.2.5.3 Spiked Samples

Known amounts of a particular constituent are added to an actual sample or to blanks of deionized water at concentrations at which the accuracy of the test method is satisfactory. The amount added should be coordinated with the laboratory. This method provides a proficiency check for accuracy of the analytical procedures.

10.2.5.4 Sample Preservative Blanks

Acids and chemical preservatives can become contaminated after a period of use in the field. The sampler should add the same quantity of preservative to some distilled water as normally would be added to a wastewater sample. This preservative blank is sent to the laboratory for analysis of the same parameters that are measured in the sample and values for the blank are then subtracted from the sample values. Liquid chemical preservatives should be changed every 2 weeks—or sooner, if contamination increases above predetermined levels.

10.2.5.5 Precision, Accuracy, and Control Charts

A minimum of seven sets each of comparative data for duplicates, spikes, split samples, and blanks should be collected to define acceptable estimates of precision and accuracy criteria for data validation.

489

10.2.5.6 Calibration of Field Equipment

Plans should be developed and implemented for calibrating all field analysis test equipment and calibration standards to include the following: (a) calibration and maintenance intervals, (b) listing of required calibration standards, (c) environmental conditions requiring calibration, and (d) a documented record system. Written calibration procedures should be documented and should include mention of the following:

a. To what tests the procedure is applicable.

b. A brief description of the calibration procedure. (A copy of the manufacturer's instructions is usually adequate.)

c. A listing of the calibration standard, the reagents, and any accessory equipment required.

d. Provisions for indicating that the field equipment is labeled and contains the calibration expiration date.

10.3 References

1. Huibregtse, K. R., and Moser, J. H., Handbook for Sampling and Sample Preservation of Water and Wastewater, EPA-600/4-76-049 (Sept. 1976).
2. Olson, D. M., Berg, E. L., Christensen, R., Otto, H., Ciancia J., Bryant, G., Lair, M. D., Birch, M., Keffer, W., Dahl, T., and Wehner, T., Compliance Sampling Manual, Enforcement Division, Office of Water Enforcement, Compliance Branch (June 1977).
3. Weber, C. I., Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents, EPA-670/4-73-001, U.S. EPA (July 1973).
4. Lauch, R. P., A Survey of Commercially Available Automatic Wastewater Samplers, EPA-600/4-76-051, U.S. EPA (Sept. 1976).
5. Shelly, P. E., and Kirkpatrick, G. A., An Assessment of Automatic Sewer Flow Samplers, EPA-600/2-75-065, U.S. EPA (Dec. 1975).
6. Handbook for Monitoring Industrial Wastewater, U.S. EPA, Technology Transfer (Aug. 1973).
7. Areawide Assessment Procedures Manual, Vol. II, App. D, EPA-600/9-76-014, U.S. EPA (July 1976).
8. Winter, J. A., Bordner, R., and Scarpino, P., Microbiological Methods for Monitoring the Environment, Part I–"Water and Wastes" U.S. EPA, EMSL, Cincinnati (1977).
9. Water Measurement Manual, 2d Edition (Revised), U.S. Department of the Interior, Bureau of Reclamation (1974).
10. Kulin, G., and Compton, P., A Guide to Methods and Standards for Measurement of Water Flow, Special Publication 421, National Bureau of Standards (May 1975).
11. Methods for Chemical Analysis of Water and Wastewater, U.S. EPA Technology Transfer (1974).
12. Harris, D. J., and Keffer, W. J., Wastewater Sampling Methodologies and Flow Measurement Techniques, EPA-907/9-74-005 (Sept. 1974).
13. Guidelines Establishing Test Procedures for the Analysis of Pollutants, Federal Register, *41*, No. 232 (Dec. 1976).

490

Chapter 11

RADIOCHEMISTRY

11.1 Introduction

The objective of this chapter is to provide general information and suggestions that enable the analyst to execute his responsibilities in analytical quality control (QC) as they relate to radiochemical analyses. Because chemical and radiochemical responsibilities should be considered together, the following requirements could be included with the items specified in the preceding chapters as tasks for the radiochemist: verifying the validity of laboratory data, recommending methodology, interpreting the results, and examining the need for standard procedures.

Environmental radiation measurements are made daily by Federal, State, local, and private agencies. The data obtained from these measurements are used by the U.S. EPA and other agencies for such purposes as estimating doses, describing health effects, establishing standards and guides, and conducting regulatory activities. It is imperative to insure the precision and accuracy of the data, so that policy decisions concerning environmental quality are based on valid and comparable data.

A radiation QC program should be designed to encourage the development and implementation of QC procedures at all levels of sample collection, analysis, data processing, and reporting. It should enable the analyst to verify his analyses and document the validity of the data. In addition, such a program allows the determination of the precision and accuracy of environmental radiochemical analyses.

11.2 Sample Collection

Analytical results can be no more meaningful than the integrity of the samples that are analyzed. Representative samples must be collected so the data for any aliquot can be related to a well-defined pollution source. For most analyses (table 11-1) the sample should be preserved at the sampling site to maintain its integrity and to minimize activity losses from absorption on container walls. If at all possible, analyses should be performed soon after receipt of the samples at the laboratory.

Sample container types and descriptions have already been discussed. Both plastic and glass containers have been recommended, and each has its particular merit. Cost most likely determines which of the many types of plastic containers will be used; in general, those more expensive should be more resistant to adsorption losses. Because sample analyses are recommended soon after collection, less expensive plastic ware can be tolerated. In any event, containers should be discarded after use to prevent contamination of subsequent samples.

Glass bottles are popular items, readily available in all sizes. Although the possibility of breakage either in handling or shipping is obviously great, the very small sizes for radioactive tracer standards and calibration sources can withstand breakage. Use of the larger size bottles for these purposes should therefore be avoided. It is poor economics to ship radioactive samples in fragile glass containers when unbreakable types serve the purpose much better.

49'

Table 11-1

SAMPLE HANDLING, PRESERVATION, METHODOLOGY, AND MAJOR INSTRUMENTATION REQUIRED[1]

Parameter	Sample Preservation[2]	Container[3]	Instrumentation
Gross Alpha Activity	Concentrated HNO_3 to pH < 2	P or G	Low-background proportional counter
Gross Beta Activity	Concentrated HNO_3 to pH < 2	P or G	Low-background proportional counter
Strontium-89	Concentrated HNO_3 to pH < 2	P or G	Low-background proportional counter
Strontium-90	Concentrated HNO_3 to pH < 2	P or G	Low-background proportional counter
Radium-226	Concentrated HNO_3 to pH < 2	P or G	Scintillation cell system
Radium-228	Concentrated HNO_3 to pH < 2	P or G	Low-background proportional counter
Cesium-134	Concentrated HCl to pH < 2	P or G	Low-background proportional counter
Iodine-131	None	P or G	Low-background proportional counter
Tritium	None	G	Liquid scintillation counter
Uranium	Concentrated HNO_3 to pH < 2	P or G	Fluorometer
Photon Emitters	Concentrated HNO_3 to pH < 2	P or G	Gamma spectrometer

[1] See reference 1 for method.

[2] Preservative to be added at the time of collection unless the sample is to be characterized for suspended solids activity.

[3] P = hard or soft plastic; G = hard or soft glass.

492

11.3 Laboratory Practices

11.3.1 Laboratory Safety

The general principles of laboratory safety are covered in chapter 14. The hazards to be avoided are listed, and the importance of good housekeeping practices is stressed. These practices reduce the potential hazard of the many chemical operations being performed. In the radiochemical laboratory they eliminate the probability of radiological cross-contamination from sample to sample and from sample to glassware. Many of the materials used in the laboratory are potentially hazardous because of their chemical properties or their radioactivity and therefore should be handled with the utmost care and respect.

In the radiochemical laboratory, the prevention of contamination by radioactive materials requires attention to radiation-protection practices, an ongoing personnel-monitoring program, and the designation of a segregated storage area for radioactive sources following use in radiochemical analyses. Adequate labeling of work areas, of samples for analysis, of aliquots, and of separated fractions helps to control radiation hazards and to insure personnel safety.

The handling of radioactive materials involves safety hazards of a type not usually associated with a chemical laboratory. Special precautions and instruments should be used to insure the greatest personal safety. It is imperative to wear monitoring devices (personal film badges) at all times.

A number of health and contamination hazards are to be considered. Many radioactive materials are dangerous even in extremely minute quantities if inhaled or ingested. All radioactive materials are capable of contaminating laboratories, instruments, and clothing. All radioactive materials in large concentrations are dangerous because of the effects of their radiation external to their containers. To control these hazards, the following rules should be in effect at all times.

a. For the case of radioactive materials that are capable of being volatilized or airborne, perform all work in a closed area or hood. Perform distillations, evaporations, and other such processes in a well-ventilated hood.

b. Do not bring food or liquid refreshments into a laboratory engaged in work with radioactive materials. The same applies to the counting rooms.

c. Do not smoke when handling radioactive materials.

d. After working with radioactive materials, wash hands thoroughly before eating or handling uncontaminated materials.

11.3.2 Laboratory Analyses

Standard radiochemical procedures or their equivalent are needed to comply with sensitivity detection limits for each nuclide as designated by the quality assurance program (2). Radiochemical procedures have been compiled for a multitude of nuclides in a multitude of media (3-9) and descriptions of specific separations are to be found in the scientific literature. As laboratory techniques become more sophisticated and as more sensitive instrumentation is developed, these procedures will be improved.

Depending on the media, the radioactivity levels, and the nuclide composition, there are several approaches that can be made when a sample is received for QC determination. The requirements for an acceptable QC program are described in section 11.4.

Before starting such a program, the laboratory should be already set up for radiochemical analyses and the analysts should have the prescribed education and experience.

11.3.3 Laboratory Radiation Instruments

The types of radiation counting systems (described in ref. 2) needed to comply with the requirements are set forth in the following paragraphs. Only those instruments needed for analyzing specific radionuclides are required. Such instruments should meet the specifications discussed in the next sections.

11.3.3.1 Liquid Scintillation System

A liquid scintillation system must measure tritium with the sensitivity required by the National Interim Primary Drinking Water Regulations. Efficiency of the system should be greater than 57 percent for tritium. The tritium figure of merit $(E_\tau)^2/B$ should be greater than 100.

11.3.3.2 Gas-Flow Proportional Counting System or Alternative

A gas-flow proportional counter, or the alternative described later, is required for measurement of gross alpha- and gross beta-particle activities, radium-228, strontium-89, cesium-134, and iodine-131. The detector may be either windowless (internal proportional counter) or of the thin-window type. A minimum shielding equivalent of 5 cm of lead must surround the detector. A cosmic (guard) detector should be operated in anticoincidence with the main detector. The main detector should have an efficiency greater than 20 percent for polonium-210 and carbon-14 and greater than 40 percent for strontium-90. The detector background should be less than 1.3 counts per minute.

The detector plateau should be less than 1.5 percent per 100 V and should be at least 100 V wide for carbon-14 and less than 2 percent per 100 V for strontium-90.

A scintillation system designed for alpha- and beta-particle counting may be substituted for the gas-flow proportional counter described. In such a system a Mylar disk coated with a phosphor (silver-activated zinc sulfide) is either placed directly on the sample (for alpha measurements) or on the face of a photomultiplier tube, enclosed within a light-tight container along with the appropriate electronics (high-voltage supply, amplifier(s), timer, and scaler). Radiation shielding, although desirable, is not required for this system.

11.3.3.3 Gamma Spectrometer System

A sodium iodide (NaI) detector connected to a multichannel analyzer is required for determination of manmade photon emitters. A 7.5- by 7.5-cm NaI crystal is satisfactory; however, a 10- by 10-cm crystal is recommended. The crystal detector must be shielded with a minimum of 10 cm of iron or equivalent.

It is recommended but not required that the distance from the center of the crystal detector to any part on the shield should not be less than 30 cm. The multichannel analyzer, in

addition to the appropriate electronics (high-voltage supply, preamplifier, and linear amplifier), must contain a memory of not less than 200 channels and at least one readout device.

11.3.3.4 Scintillation Cell System

A scintillation system must be designed to accept scintillation flasks (Lucas cells) for measurement of radium-226 by the radon-emanation method. The system consists of a light-tight enclosure for the scintillation flasks, a detector (phototube), and the appropriate electronics (high-voltage supply, amplifier, timers, and scalers). The scintillation flasks required for this measurement may either be purchased from commercial suppliers or constructed according to published specifications.

11.4 Quality Control

The following requirements are recommended for all laboratories:

a. All QC data should be available for inspection to determine validity of laboratory results.

b. Each laboratory should participate at least twice each year in EPA laboratory intercomparison studies (10).

c. Each laboratory should participate once each year in an appropriate EPA-administered performance study on unknowns. Results must be within the control limits established by EPA for each analysis.

d. Counting-instrument operating manuals and calibration protocols should be available to analysts and technicians.

e. Calibration data and maintenance records on all radiation instruments and analytical balances must be maintained in a permanent record.

f. Minimum daily QC

(1) To verify precision of methods, a minimum of 10 percent of the samples shall be duplicates. Checks must be within ±2 standard deviations of the mean range.

(2) If less than 20 samples per day are analyzed, a performance standard and a background sample must be measured. If 20 or more samples are analyzed per day, a performance standard and a background sample must be measured with each 20 samples. Checks must be within ±2 standard deviations of the mean range.

(3) Quality control performance charts or performance records must be maintained.

11.5 References

1. Federal Register *41*, No. 133 (July 9, 1976).
2. Jarvis, A. N., et al., The Status and Quality of Radiation Measurements of Water, EPA-600/4-76-017 (Apr. 1976).

495

3. Krieger, H. L., Interim Radiochemical Methodology for Drinking Water, EPA-600/4-75-008 (Revised), U.S. EPA, Office of Research and Development, Cincinnati (Mar. 1976).
4. Doublas, G. S., Editor, Radioassay Procedures for Environmental Samples, Environmental Health Series, Publication 999-RH-27, U.S. DHEW, Public Health Service, National Center for Radiological Health (Jan. 1967).
5. Harley, J. H., HASL Procedures Manual, HASL-300, U.S. ERDA, Health and Safety Laboratory, New York (1972).
6. Kleinberg, J., Editor, Collected Radiochemical Procedures, LA-1721 (Revised), Los Alamos Scientific Laboratory, Los Alamos (Nov. 1955).
7. Krieger, H. L., et al., Radionuclide Analysis of Environmental Samples, Technical Report R59-6 (Revised), U.S. DHEW, Public Health Service (Feb. 1966).
8. Nuclear Science Series, Reports NAS-NS-3001 to NAS-NS-3115, National Academy of Sciences, National Research Council Technical Information Center, Office of Information Services (1960).
9. "Water," Part 31 of 1977 Annual Book of ASTM Standards, American Society for Testing and Materials, Philadelphia (1977).
10. Jarvis, A. N., et al., Environmental Radioactivity Laboratory Intercomparison Studies Program 1975, EPA-680/4-75-002b, U.S. EPA, Office of Research and Development, Las Vegas (1975).
11. Ziegler, L. H., and Hunt, H. M., Quality Control for Environmental Measurements Using Gamma-Ray Spectrometry, U.S. EPA, EPA-600/7-77-144 (Dec. 1977).
12. Kanipe, L. G., Handbook for Analytical Quality Control in Radioanalytical Laboratories, U.S. EPA, EPA-600/7-77-088 (Aug. 1977).

496

Chapter 12

MICROBIOLOGY

12.1 Background

The quality assurance program described in this chapter is a synopsis of a detailed program prescribed in parts IV and V of the EPA microbiological methods manual (1). Quality assurance is a program of integration of all intralaboratory and interlaboratory quality control (QC), methods standardization, and QC management practices into a formal, coordinated, continuing effort.

12.2 Specific Needs in Microbiology

A quality assurance program for microbiological analyses must emphasize the control of laboratory operations and analytical procedures because the tests measure living organisms that continually change in response to their environment. Further, because true values cannot be provided for the microbial parameters, microbiologists do not yet have the advantages of analytical standards, QC charts, and spiked samples available to other disciplines for measurement of accuracy. Because known values cannot be applied, it is important that careful and continuous control be exerted over sampling, personnel, analytical methodology, materials, supplies, and equipment.

12.3 Intralaboratory Quality Control

Intralaboratory QC is the orderly application *within a single laboratory* of laboratory practices necessary to eliminate or reduce systematic error and to control random error. This within-laboratory program must be practical, integrated, and time-efficient or it will be bypassed. When properly administered, such a program helps to insure high-quality data without interfering with the primary analytical functions of the laboratory. This within-laboratory program should be supplemented by participation of the laboratory in an interlaboratory quality assurance program such as that conducted by EPA.

Intralaboratory QC for microbiology should cover the following areas:

a. Laboratory operations

   (1) Sample collection and handling
   (2) Laboratory facilities
   (3) Laboratory personnel
   (4) Laboratory equipment and instrumentation
   (5) Laboratory supplies
   (6) Culture media
   (7) Analytical methodology

b. Analytical QC

   (1) Sterility checks
   (2) Positive and negative controls

497

(3) Duplicate analyses
(4) Single-analyst precision
(5) Comparison of results between analysts
(6) Verification of membrane filter analyses
(7) Completion of most probable number analyses
(8) Data handling

12.4 Interlaboratory Quality Control

An interlaboratory quality assurance program is an agreed-upon system of minimal requirements necessary to maintain a quality standard among a group of laboratories. Such a program may be voluntary or compulsory for participants. It may include the following activities:

a. Selection and approval of uniform sampling methodology and analytical methodology

b. Collaborative studies to establish the precision and accuracy of selected methodology

c. Preparation of guidelines to set minimal group standards for personnel, equipment, instrumentation, facilities, and intralaboratory QC programs

d. Onsite inspection of laboratory capabilities

e. Periodic evaluation of laboratory performance on unknown samples

f. Followup on problems identified in onsite inspections and performance evaluations

As a part of its interlaboratory quality assurance program, EPA has selected microbiological methodology and standards for laboratory operations (1). EMSL-Cincinnati is currently conducting research on the development of QC samples for use in performance testing and method-validation studies.

12.5 Development of a Formal Quality Assurance Program

Unless records are kept of the QC checks and procedures, there is no proof of performance, no value in future reference, and for practical purposes, no quality assurance program in operation. To insure a viable quality assurance program, management must first recognize the need and require the development of a formal program, and then commit 15 percent of the laboratory man-years to QC activities. The laboratory manager holds meetings with supervisors and staff workers to establish levels of responsibility and functions of management, supervisor, and analyst in the quality assurance program. Laboratory personnel participate in planning and structuring the program.

Once the quality assurance program is functioning, supervisors review laboratory operations and QC with analysts on a frequent (weekly) basis. Supervisors use the results of the regular meetings with laboratory personnel to inform management of the status of the program on a regular (monthly) basis. These meetings identify problems through participation of laboratory personnel and provide the backing of management for actions required to correct problems.

148

12.6 Documentation of a Quality Assurance Program

A laboratory operating manual should be prepared that describes operation, maintenance, and QC of laboratory operations and analyses as practiced. The review mechanisms and the frequency of review in the quality-assurance program are included.

12.6.1 Sampling

A sample log is used to record information on samples received in the laboratory including details of sample identification and origin, the necessary chain-of-custody information, analyses performed, and final results.

12.6.2 Laboratory Operations

A QC record is maintained on media preparation, instrument calibration, purchase of supplies, QC checks on materials, supplies, equipment, instrumentation, facilities, and analyses.

12.6.3 Analytical Quality Control

A record of analytical QC checks is maintained on positive and negative controls, sterility checks, single-analyst precision, precision between analysts, and use-test results from comparison of lots of media, membrane filters, and other supplies.

12.7 Chain-of-Custody Procedures for Microbiological Samples

12.7.1 General

A regulatory agency must demonstrate the reliability of its evidence by proving the chain of possession and custody of any samples that are offered for evidence or that form the basis of analytical test results introduced into evidence in any water pollution case. It is imperative that the office and the laboratory prepare procedures to be followed whenever evidence samples are collected, transferred, stored, analyzed, or destroyed.

The primary objective of these procedures is to create an accurate written record that can be used to trace the possession of the sample from the moment of its collection through its introduction into evidence. A sample is in custody if it is in any one of the following states:

a. In actual physical possession

b. In view, after being in physical possession

c. In physical possession and locked up so that no one can tamper with it

d. In a secured area, restricted to authorized personnel

Personnel should receive copies of study plans prior to the study of a water pollution case. Prestudy briefings should then be held to apprise participants of the objectives, sample locations, and chain-of-custody procedures to be followed. After the chain-of-custody samples are collected, a debriefing is held in the field to verify the adherence to the chain-of-custody procedures and to determine whether additional samples are required.

199

12.7.2 Rules for Sample Collection

An agency or laboratory engaged in sample study activities should follow these rules:

a. Involve a minimum number of trained persons in sample collection and handling.

b. Establish guidelines for particular procedures to be used for each type of sample collection, preservation, and handling.

c. Handle samples as little as possible.

d. Obtain stream and effluent samples using the appropriate sampling techniques.

e. Attach sample tag or label securely (see fig. 12-1) to the sample container at the time the sample is collected. The tag should contain the following items as a minimum: the serial number of the tag, the station number and location, the date and time taken, the type of sample, the sequence number (e.g., first sample of the day–sequence No. 1), the preservative used, the analyses required, and the name of the sample collector. Tags should be completed legibly in waterproof ink.

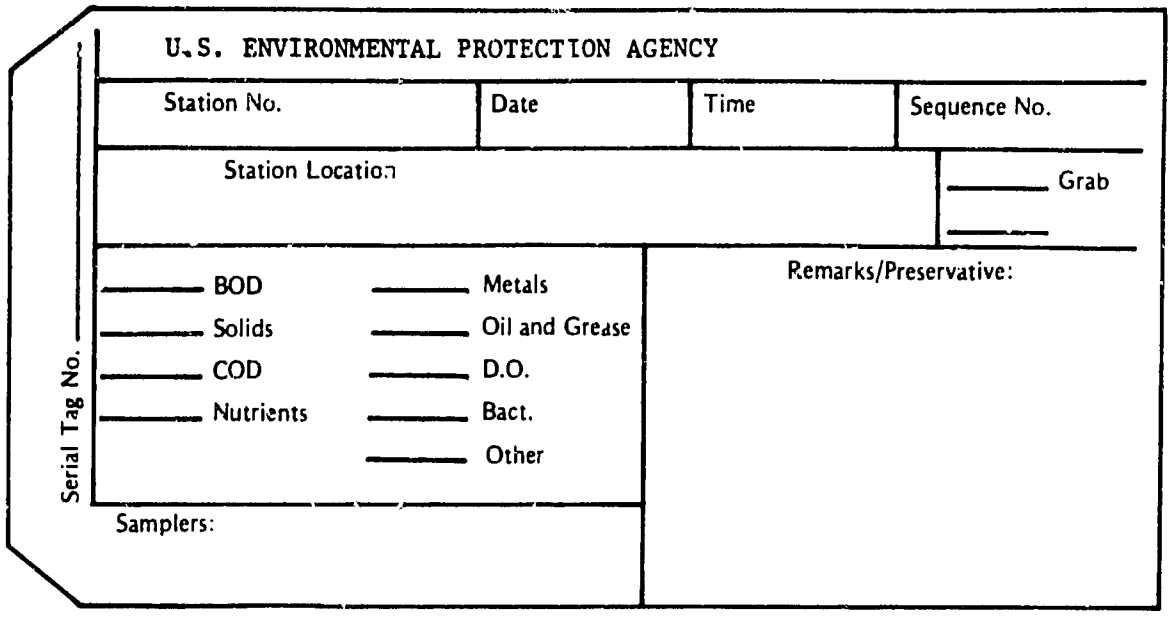
f. Use bound field notebooks to record field measurements and other pertinent information necessary to reconstruct the sample collection processes in the event of a later enforcement proceeding. Maintain a separate set of field notebooks for each study and store them in a safe place where they can be protected and accounted for at all times. Establish a sample log sheet with a standard format to minimize field entries and include the serial number of the sheet, the date, time, survey, type of samples taken, volume of each sample, type of analyses, (unique) sample numbers, sampling location, field measurements (such as temperature, conductivity, dissolved oxygen (DO), and pH), and any other pertinent information or observation. (See fig. 12-2.) The entries should be signed by the sample collector. The responsibility for preparing and retaining field notebooks during and after a study should be assigned to a study coordinator or his designated representative.

g. The sample collector is responsible for the care and custody of the samples until the samples are properly dispatched to the receiving laboratory or given to an assigned custodian. The sample collector must insure that each container is in his physical possession or in his view at all times, or stored in a locked place where no one can tamper with it.

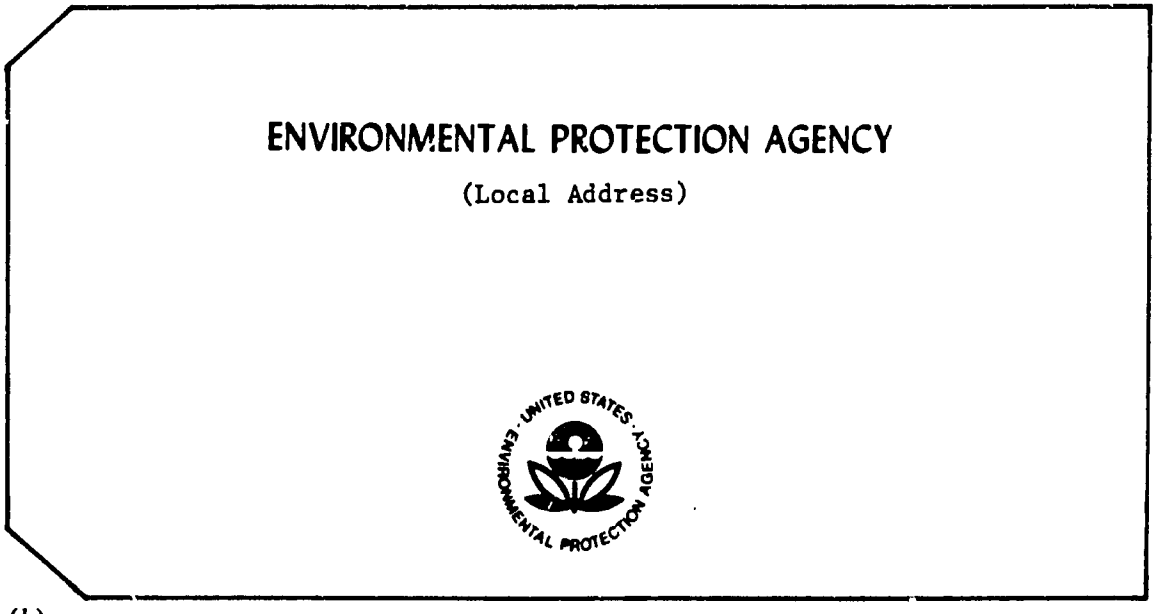
h. Take color slides or photographs of the sample locations and any visible water pollution. Sign and indicate time, date, and site location on the back of the photo. To prevent alteration, handle such photographs according to the established chain-of-custody procedures.

12.7.3 Transfer of Custody and Shipment

In transfer-of-custody procedures, each custodian or sampler must sign, record, and date the transfer. Most environmental regulatory agencies develop chain-of-custody procedures tailored to their needs. These procedures may vary in format and language but contain the same essential elements. Historically, sample transfer under chain of custody has been on a



(*a*)



(*b*)

Figure 12-1. Example of chain-of-custody sample tag. (*a*) Front. (*b*) Back.

sample-by-sample basis, which is awkward and time consuming. However, the EPA National Enforcement Investigation Center (NEIC) at Denver has set precedent with its bulk transfer of samples. Bulk transfer is speedier and reduces paperwork and the number of sample custodians. The following description of bulk transfer of custody is essentially that of the Office of Enforcement (2).

501

FOR _____ SURVEY SAMPLE LOG SHEET NO. _____ DATE _____

TYPE OF SAMPLE _____ SAMPLER: (Signature) _____

ANALYSES REQUIRED

STATION NUMBER	STATION DESCRIPTION	TOTAL VOLUME	TYPE CONTAINER	PRESERVATIVE	NUTRIENTS	BOD	COD	TOC	TOTAL SOLIDS	SUSPENDED SOLIDS	ALKALINITY	DO	pH*	CONDUCTIVITY*	TEMPERATURE*	TOTAL COLIFORM	FECAL COLIFORM	TURBIDITY	OIL AND GREASE	METALS	BACTI	PESTICIDES	HERB	TRACE ORGANICS	PHENOL	CYANIDE

REMARKS

*Field Measurements

US EPA, NEIC-Denver

Figure 12-2. Example of sample log sheet.

502

a. Samples must be accompanied by a chain-of-custody record that includes the name of the study, collectors' signatures, station number, station location, date, time, type of sample, sequence number, number of containers, and analyses required. (See fig. 12-3.) When turning over possession of samples, the transferor and transferee sign, date, and time the record sheet. This record sheet allows transfer of custody of a

CHAIN OF CUSTODY RECORD

				SAMPLERS (Signature)					
STATION NUMBER	STATION LOCATION	DATE	TIME	SAMPLE TYPE Water Comp	Water Grab	Air	SEQ NO	NO OF CONTAINERS	ANALYSIS REQUIRED

Relinquished by: (Signature)	Received by: (Signature)	Date/Time
Relinquished by: (Signature)	Relinquished by: (Signature)	Date/Time
Relinquished by: (Signature)	Received by: (Signature)	Date/Time
Received by: (Signature)	Received by Mobile Laboratory for field analysis: (Signature)	Date/Time
Dispatched by: (Signature) Date/Time	Received for Laboratory by:	Date/Time
Method of Shipment:		

Distribution Orig – Accompany Shipment
1 Copy – Survey Coordinator Field Files

Figure 12-3. Example of chain-of-custody record.

-503

group of samples in the field to the mobile laboratory or to the central laboratory. When a custodian transfers a portion of the samples identified on the sheet to the mobile laboratory, the individual samples must be noted in the column with the signature of the person relinquishing the samples. The laboratory person receiving the samples acknowledges receipt by signing in the appropriate column.

b. If the custodian has not been assigned, the field custodian or field sampler has the responsibility of packaging and dispatching samples to the laboratory for analysis. The dispatch portion of the chain-of-custody record must be filled out, dated, and signed.

c. To avoid breakage, samples must be carefully packed in shipment containers such as ice chests. The shipping containers are padlocked for shipment to the receiving laboratory.

d. Packages must be accompanied by the chain-of-custody record showing identification of the contents. The original must accompany the shipment. A copy is retained by the survey coordinator.

e. If sent by mail, register the package with return receipt requested. If sent by common carrier, a Government bill of lading should be obtained. Receipts from post offices and bills of lading will be retained as part of the permanent chain-of-custody documentation.

f. If delivered to the laboratory when appropriate personnel are not there to receive them, the samples must be locked in a designated area within the laboratory, so that no one can tamper with them or must be placed in a secure area. The recipient must return to the laboratory, unlock the samples, and deliver custody to the appropriate custodian.

12.7.4 Laboratory Custody Procedures

Suitable laboratory procedures during custody of samples include the following:

a. The laboratory shall designate a sample custodian and an alternate custodian to act in his absence. In addition, the laboratory shall set aside a sample storage security area. This should be a clean, dry, isolated room with sufficient refrigerator space that can be securely locked from the outside.

b. Samples should be handled by the minimum possible number of persons.

c. Incoming samples shall be received only by the custodian who will indicate receipt by signing the chain-of-custody record sheet accompanying the samples and retaining the sheet as a permanent record. Couriers picking up samples at the airport or post office shall sign jointly with the laboratory custodian.

d. Immediately upon receipt, the custodian places the samples in the sample room, which will be locked at all times except when samples are removed or replaced by the custodian. To the maximum extent possible, only the custodian shall be permitted in the sample room.

504

e. The custodian shall insure that microbiological samples are properly stored and maintained at 4°C.

f. Only the custodian will distribute samples to personnel who are to perform tests.

g. The analyst records in his laboratory notebook or analytical worksheet, identifying information describing the sample, the procedures performed, and the results of the testing. The notes shall be dated, shall indicate who performed the tests, and should include any abnormalities that occurred during the testing procedure. The notes shall be retained as a permanent record in the laboratory. In the event that the person who performed the tests is not available as a witness at the time of a trial, the Government may be able to introduce the notes in evidence under the Federal Business Records Act.

h. Approved methods of laboratory analyses shall be used as required by Public Laws 92-500, 93-523, 92-532, and amendments.

i. Laboratory personnel are responsible for the care and custody of a sample once it is handed to them and should be prepared to testify that the sample was in their possession and view or secured in the laboratory at all times from the moment it was received from the custodian until the tests were run.

j. The laboratory area shall be maintained as a secured area and shall be restricted to authorized personnel.

k. Once the sample analyses are completed, the unused portion of the sample, together with identifying labels and other documentation, must be returned to the custodian. The returned, tagged sample should be retained in the custody room until permission to destroy the sample is received by the custodian.

l. Samples should be destroyed only upon the order of the laboratory director, in consultation with previously designated enforcement officials, or when it is certain that the information is no longer required, or that the samples have deteriorated. The same destruction procedure is true for tags and laboratory records.

12.7.5 Evidentiary Considerations

Reducing chain-of-custody procedures and promulgated analytical procedures to writing will facilitate the admission of evidence under Rule 803 (6) of the Federal Rules of Evidence (Public Law 93-575). Under this statute, written records of regularly conducted business activities may be introduced into evidence as an exception to the hearsay rule without the testimony of the person(s) who made the record. Although it would be preferable, it is not always possible for the individuals who collected, kept, and analyzed samples to testify in court. In addition, if the opposing party does not intend to contest the integrity of the sample or testing evidence, admission under Rule 803(6) can save a great deal of trial time. For these reasons, it is important that the procedures followed in the collection and analyses of evidentiary samples be standardized and described in an instruction manual, which, if need be, can be offered as evidence of the regularly conducted business activity followed by the laboratory or office in generating any given record.

In criminal cases, however, records and reports of matter observed by police officers and other law enforcement personnel are not included under the business record exceptions to the hearsay rule previously cited. (See Rule 803(8), Public Law 93-595.) It is arguable that those portions of the compliance inspection report dealing with matters other than sampling and analysis results come within this exception. For this reason, in criminal actions, records and reports of matter observed by field investigators may not be admissible, and the evidence may still have to be presented in the form of oral testimony by the person(s) who made the record or report, even though the materials come within the definition of business records. In a criminal proceeding, the opposing counsel may be able to obtain copies of reports prepared by witnesses (even if the witness does not refer to the records while testifying), which may be used for cross-examination purposes.

Admission of records is not automatic under either of these sections. The business records section authorizes admission "unless the source of information or the method or circumstances of preparation indicate lack of trustworthiness," and the caveat under the public records exception reads "unless the sources of information or other circumstances indicate lack of trustworthiness."

Thus, whether or not the inspector anticipates that a report will be introduced as evidence, the inspector should make certain that the report is as accurate and objective as possible.

12.8 References

1. Winter, J. A., Bordner, R., and Scarpino, F., Microbiological Methods for Monitoring the Environment, Part I–"Water and Wastes," U.S. EPA, EMSL, Cincinnati (1978).
2. NPDES Compliance Sampling Manual, U.S. EPA, Office of Water Enforcement (June 1977).

Chapter 13

AQUATIC BIOLOGY

Quality assurance guidelines for aquatic biology programs (fully described in ref. 1) are summarized in the following section.

13.1 Summary of General Guidelines

Successful quality assurance programs in aquatic biology are based on the following essential elements:

a. An understanding and acceptance of the importance of quality control (QC) and a commitment on the part of the biology staff to fully integrate QC practices into field and laboratory operations

b. A staff with adequate formal training and experience and proper specialization to meet program needs

c. Adequate field equipment, storage and laboratory space, instrumentation, and taxonomic references

d. Careful advance preparation and design of field and laboratory studies

e. Strict adherence to approved methodology, where available, and careful consideration of the technical defensibility of the methods and their application

f. Use of replication in sample collection and analysis where feasible, and determination of the accuracy and precision of the data

g. Frequent calibration of field and laboratory instruments

h. Proper sample identification and handling to prevent misidentification or intermixing of samples

i. Use of blind, split, or other control samples to evaluate performance

j. Development and regular use of in-house reference specimen collections, and use of outside taxonomic experts to confirm or provide identifications for problem specimens

k. Meticulous, dual-level review of the results of manual arithmetical data manipulations and transcriptions before the data are used in reports or placed in BIOSTORET (2)

l. Participation in EPA formal interlaboratory aquatic biology methods studies, and use of EPA biological reference materials

m. Documentation of methodology and QC practices employed in the program

507

13.2 Discussion

A brief description of each of the areas mentioned in section 13.1 is provided here to indicate the scope of the quality assurance program.

13.2.1 Staff Commitment

To establish and maintain an effective quality assurance program in aquatic biology, the supervisor must actively support and frequently monitor the use of QC practices in all aquatic biology activities. This will require the commitment of 10 to 15 percent of the total manpower resources. The supervisor must review field and laboratory operations with his staff frequently (weekly) to insure that QC practices are followed and properly documented.

13.2.2 Staff

The quality and reliability of the data rest heavily on the competence of the staff. The range of aquatic organisms studied by biological programs is very broad, and each community requires unique skills in sample collection and analysis, and in data interpretation. Several disciplines, therefore, must be represented on the staff to deal effectively with the taxonomy and ecology of the major groups of aquatic organisms, which include the phytoplankton, zooplankton, periphyton, macrophyton, macroinvertebrates, and fish.

13.2.3 Facilities

The quality of the data also depends upon the availability and performance of laboratory equipment. Such items as sampling gear, current meters, spectrophotometers, and microscopes must be available and must meet performance standards related to the biological parameters measured. Laboratory instrumentation must provide the sensitivity and accuracy required by the state of the art in sample analysis. Adequate laboratory and storage space must also be provided.

13.2.4 Advance Planning

Thorough advance planning of field and laboratory projects is necessary to maintain the required control over the technical aspects of the project and to insure the collection of meaningful data. Factors taken into consideration include the objectives of the study, the parameters to be measured, station selection, the sampling frequency and replication, seasonal cycles in the properties of communities of aquatic organisms, and QC measures to be incorporated into the various phases of the project.

13.2.5 Use of Approved Methodology

Methods in the EPA manual "Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents" (1) should be employed where applicable. The manual contains concensus methods selected by a committee of senior Agency aquatic biologists as the preferred methods for use within EPA. If program activities require methods for parameters not covered by reference 1, methods may be selected from other sources if their application is technically defensible.

13.2.6 Replication

Comparisons of biological parameters measured in control and affected regions of water bodies, and in laboratory experiments, may be meaningless if the precision of the results is not known. Preliminary measurements must be made in each study to determine the scatter in the data and to establish the number of replicate samples required to achieve the level of precision required to detect differences in the responses measured.

13.2.7 Instrument Calibration and Maintenance

Sampling equipment and laboratory instrumentation with mechanical metering devices and electronic components are calibrated on a regularly scheduled basis to insure the accuracy of the data. Standards are obtained, such as NBS-certified thermometers for temperature-measuring devices, class-S weights for balances, and absorption filters for spectrophotometers. Records of calibrations, regular performance checks, and service for each device are maintained in bound log books in such a manner that the history of performance of the instruments may be easily reviewed. Analytical reagents are labeled and dated when received, and are protected from deterioration if labile. The expected shelf life of each reagent is recorded on the label, and the material is not used after the expiration date.

13.2.8 Sample Labeling

Samples are securely labeled in the field and recorded in a bound log. Information on the label should include the station, date, time of day, depth, and other relevant information. A unique lot number is assigned to the sample and recorded on the label. Waterproof paper and ink must be used for the labels, and are recommended for the field logs. Depending on requirements, labels are placed inside samples such as macroinvertebrates.

13.2.9 Quality Control Samples

The accuracy of the data from routine analyses such as counts and identification of organisms and chlorophyll and biomass measurments is determined by introducing blind, split, or reference samples in the sample processing stream. These samples are either prepared by the supervisory aquatic biologist, laboratory quality control officer, or analytical QC coordinator, or are obtained from EMSL-Cincinnati. The results are discussed at regularly scheduled staff meetings and any problems identified are discussed and corrected.

13.2.10 Organism Identification and Reference Specimens

Accurate identification of aquatic organisms to the species level is essential to the interpretation of biological data. A set of reference specimens is established within each laboratory, to be used as taxonomic (identification) standards in processing samples and in training new personnel. The set is representative of the aquatic organisms collected by the program, and each specimen embodies the morphological characteristics essential to the identification of that taxon. The identity of these specimens is verified by outside taxonomic experts, who also examine organisms that pose unusually difficult identification problems in routine sample analysis.

13.2.11 Data Records, Editing (Proofing), Review

Data collected manually are entered in a bound log or on specialized bench sheets that fully describe the origin and nature of the sample and that are maintained in a binder or file. Source data such as organism abundance, metabolic rates, and chlorophyll, which are manually or electronically manipulated or transcribed from one record to another, are doublechecked by a second person. All manual calculations and all electronic calculations, where data are manually keyboarded, are performed twice, except where the source (input) data are included in the output and can be proofed. Keyboarded data are carefully proofed before they are submitted for computer manipulation.

13.2.12 Interlaboratory Methods Studies

The aquatic biology programs participate in formal interlaboratory biological methods studies performed by EMSL-Cincinnati. Studies on chlorophyll and macroinvertebrate identification methods have been completed, and additional studies on phytoplankton and periphyton identification methods are planned in the future.

13.2.13 Documentation for the Quality Assurance Program

A laboratory operations manual is available (1) that describes the scope of the program, organizational structure, qualifications of the staff, available space and equipment, methodology employed for sample collection and analysis, and QC procedures.

13.3 References

1. Weber, C. I., Editor, Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents, 2d Edition, EPA-670/4-73-001, U.S. EPA (July 1973).
2. Nacht, L., and Weber, C. I., BIO-STORET Final Design Specification, U.S. EPA (1976).

510

Chapter 14

LABORATORY SAFETY

14.1 Law and Authority for Safety and Health

Public Law 91-596 is the Occupational Safety and Health Act of 1970 (1). The rpose of this act is the following:

> To assure safe and healthful working conditions for working men and women; by authorizing enforcement of the standards developed under the act; by assisting and encouraging the States in their efforts to assure safe and healthful working conditions; by providing for research, information, education, and training in the field of occupational safety and health; and for other purposes.

The intent of the act (1) is "to assure so far as possible every working man and woman in the Nation safe and healthful working conditions and to preserve our human resources."

The responsibility for promulgating and enforcing occupational safety and health standards rests with the Department of Labor. The Department of Health, Education, and Welfare is responsible for conducting research on which new standards can be based, and for implementing education and training programs for producing an adequate supply of manpower to implement the purposes of the act. These responsibilities are performed by the National Institute for Occupational Safety and Health (NIOSH).

Section 19(a) of Public Law 91-596 states the following (1):

> It shall be the responsibility of the head of each Federal agency to establish and maintain an effective and comprehensive occupational safety and health program that is consistent with the standards promulgated under section 6. The head of each agency shall (after consultation with representatives of the employees thereof)–
>
> (1) provide safe and healthful places and conditions of employment, consistent with the standards set under section 6;
>
> (2) acquire, maintain, and require the use of safety equipment, personal protective equipment, and devices reasonably necessary to protect employees;
>
> (3) keep adequate records of all occupational accidents and illnesses for proper evaluation and necessary corrective action;
>
> (4) consult with the Secretary with regard to the adequacy as to form and content of records kept pursuant to subsection (a)(3) of this section; and
>
> (5) make an annual report to the Secretary of Labor with respect to occupational accidents and injuries and the agency's program under this section. Such report shall include any report submitted under section 7902(e)(2) of title 5, United States Code.

511

Executive Order 11807 was issued in the fall of 1974 to provide general direction to the heads of Federal agencies and to the Secretary of Labor in establishing occupational safety and health programs in the Federal Government. The executive order details the following duties:

a. Appointment of a Safety and Health Official.

b. Establishment of a management information system.

c. Establishment of an occupational safety and health program.

   (1) Adoption of safety and health standards as effective as the Secretary of Labor's standards.

   (2) Institution of procedures for processing reports from employees on hazardous conditions.

   (3) Institution of periodic inspection of facilities.

   (4) Provision for abatement of hazards in facilities.

d. Provision for training of agency personnel.

   (1) Training of supervisors at all levels.

   (2) Training of those responsible for conducting inspections of facilities.

   (3) Training of other employees. Attention is called to the list of OSHA training requirements.

e. Assistance of the Secretary of Labor.

   (1) Compliance with the recordkeeping and reporting requirements.

   (2) Observation of the guidelines issued in 28 CFR 1960.

   (3) Cooperation with the Secretary of Labor in the performance of his responsibilities.

f. Issuance of guidelines for a safety and health program.

g. Prescription of recordkeeping and reporting requirements.

h. Provision of consulting services to Federal agencies in adoption of standards, training agency personnel, and in other matters.

i. Upon request and subject to reimbursement, performance of such services as evaluation of safety and health conditions in the agency, recommendation on the adoption of standards, inspection of facilities for safety and health hazards, and training of agency personnel in safety and health matters.

j. Evaluation of agency programs and report of the condition to the President. This item in the executive order gives to the Secretary of Labor a degree of enforcement of safety and health rules and regulations within Federal agencies.

k. Section 4 continues the Federal Advisory Council on Occupational Safety and Health. Note that at least one-third of the members are to be labor representatives.

14.1.1 Occupational Safety and Health Administration Regulations

The Occupational Safety and Health Act of 1970 covers laboratory workplaces as well as industrial and manufacturing workplaces (1). Many large laboratories have already come under the scrutiny of the Occupational Safety and Health Administration (OSHA); small and medium-sized laboratories can expect direct involvement in the future.

The 1970 act set up NIOSH within the U.S. Department of Health, Education, and Welfare. NIOSH provides OSHA with the scientific information it needs to effectively perform its regulatory function.

The initial OSHA regulations were, and in some measure still are, a collection of many well-established standards taken from industry and from standards-making groups. The American National Standards Institute, the National Safety Council, the National Fire Protection Association, and the American Society for Testing and Materials were some of the prime sources for these OSHA standards. Certain existing State health and safety regulations that predated OSHA were used to develop the Federal OSHA regulations.

Efforts are continually underway to refine and quantify current OHSA regulations through court decisions resulting from appeals of compliance citations, through continuing reviews of standards by OSHA safety compliance officers, through the work of NIOSH, and through internal reviews.

Because of the continual publication of revisions of the regulations consisting of refinements and clarifications, as well as dissemination of newly issued regulations, laboratory operators must expend great efforts to keep strictly up to date. What was permitted yesterday may not be permitted today.

The first place to start in keeping up to date on OSHA regulations is to obtain a copy of the Federal or State regulations that apply. In States not operating their own OSHA function, copies of applicable regulations are usually available without charge from the local office of the U.S. Department of Labor. In other States, copies of their regulations are available from similar State offices and boards either free or at a nominal charge. In either case, appropriate steps must be taken to keep up to date on revisions and reissues. Usually this means getting on a mailing list for automatic receipt of this material as it is published. Because entire sections of the regulations may be reissued to incorporate only a few changes, it is usually necessary to completely study these reissues to determine the actual change that has been made.

As OSHA moves more into the complicated areas of chemical and biological laboratory workplaces, the level of the compliance inspector's knowledge must be increased accordingly.

14.1.2 Federal and State OSHA

There is no completely identical OSHA standard in force throughout the United States.

A provision of the act of 1970 permits Federal OSHA to certify individual State OSHA operations when they are satisfied that applicable State regulations and compliance enforcement methods fulfill the provisions of the Federal act (1).

While many State OSHA applications are in process, the following States and territories are certified or at least provisionally certified to permit their own OSHA operation:

Alaska
Arizona
California
Colorado
Connecticut
Hawaii
Indiana
Iowa
Kentucky
Maryland
Michigan
Minnesota
Nevada
North Carolina
Oregon
South Carolina
Tennessee
Utah
Vermont
Washington
Wyoming
Virgin Islands

All other states and territories are under Federal OSHA jurisdiction.

Even though the Federal Government supports 50 percent of the cost of State OSHA programs, these States apparently would prefer that the Federal Government take on the entire cost burden for them.

An important difference between Federal and State OSHA operations lies in their jurisdictions. While State OSHAs are responsible for State-operated workplaces, Federal OSHA can only inspect Federal workplaces. It cannot enforce compliance. There is, however, a current effort by the General Accounting Office to urge Congress to remove this restriction on Federal OSHA's effectiveness in dealing with Federal workplaces.

While Federal OSHA is not permitted to offer a consulting service, most State OSHAs can and do. Some who were unaware of this difference have called local Federal OSHA offices for advice. All they succeeded in doing was to invite an inspection. Normally, however,

54

inspections of facilities, be they manufacturing or laboratories, are triggered by an employee's complaint or an accident report, either as required by law or through police, fire, or hospital channels.

Reactions to OSHA operations vary widely. Many laboratories have been cited for violations and have been heavily fined. Yet Dr. David Pettit, Technical Director of Kelco's operations in San Diego, says, "We feel that OSHA standards are not unrealistic, just good laboratory practice."

14.2 EPA Policy on Laboratory Safety

EPA has issued an Occupational Safety and Health Manual (3) in which policy, responsibilities, accident reporting, inspections, standards, and training are described. The Agency has also issued a Safety Management Manual (4) that contains information on safety protection plans at EPA facilities and air, water, and road safety.

The EPA Occupational Safety and Health Manual (3) establishes policy, responsibilities, and procedures for the conduct of the EPA safety and health program.

The policy of EPA is to administer its programs in a manner that assures adequate protection of its own employees and property, and that for which it has a responsibility. *Every* manager, supervisor, and employee is responsible for identifying risks, hazards, and unsafe situations or practices and for taking steps to insure adequate safety in the activities under his supervison.

A facility safety officer designated for each unit must be responsible for assisting the officer-in-charge in developing, organizing, directing, and evaluating the safety and health program and coordinating illness and inquiry reporting and recordkeeping requirements; analyzing accidents and injuries for prevention and control; and providing technical advice in the implementation of program standards and policy.

Safety in any laboratory requires continuing attention. Use of new or different techniques, chemicals, and equipment requires careful reading, instruction, and supervision, and may require consultation with other people with special knowledge or experience.

Prevention of laboratory accidents requires positive attitudes toward safety and training, and suitable information for understanding laboratory and chemical hazards and their consequences.

Responsibility for safety within the laboratories for an organization may be considered to exist at three different levels–individual, supervisory (or instructional), and organizational (or institutional).

The division of responsibility needs to be clearly assigned and accepted, steps need to be taken to see that the responsibilities are exercised, and the assignments need to be reassessed if unexpected problems develop.

Each individual who works in a laboratory is responsible for learning the health and safety hazards of the chemicals he will be using or producing, and the hazards that may occur from

515

the equipment and techniques he will employ, so that he can design his setup and procedures to limit the effects of any accident. The individual should investigate any accident that occurs, and record and report the apparent causes and the preventive measures that may be needed to prevent similar accidents.

The supervisor has the responsibility for giving all the necessary directions, including the safety measures to be used, and the responsibility of seeing that employees carry out their individual responsibilities. Whoever directs the activities of others has a concurrent responsibility to prevent accidental injuries from occurring as a result of the activities.

The organization of which the laboratories are part has a fundamental responsibility to provide the facilities, equipment, and maintenance for a safe working environment and to provide an organized program to make the improvements necessary for a safe working environment. Unless the organization fulfills its responsibilities, it cannot expect its supervisors, employees, or students to fulfill their responsibilities for laboratory safety.

The Environmental Protection Agency has a designated safety and health official who is responsible for assuring that formal safety and health inspections are conducted at all EPA workplaces. He will notify the Administrator regarding uncorrected safety and health deficiencies.

14.2.1 Formal Safety Inspections

Formal safety and health inspections at workplaces where there is an increased risk of accident, injury, or illness because of the nature of the work performed, as in the case of chemical operations and material-handling or material-loading operations, must be made by a safety and health specialist. A "Safety and Health Specialist" is defined in 29 CFR 1960.2(h) as a person who meets the Civil Service standards for the position of Safety Manager/Specialist GS-018, Safety Engineer GS-803, Fire Protection Engineer GS-804, Industrial Hygienist GS-690, Fire Protection Specialist/Marshall GS-081, or Health Physicist GS-1306, or who is an employee of an equally qualified military agency or nongovernment organization.

Formal safety and health inspections need not be made by a safety and health specialist at workplaces where there is little risk involved, but should be conducted by a person having sufficient training and experience in the safety and health needs of the workplaces involved to adequately perform the duties of an inspector as set forth in Executive Order 11807. Inspectors should be accompanied on formal safety and health inspections by representatives of the officer in charge of the reporting unit being inspected and representatives of the employees of such establishments. Management and employee representatives should be familiar with and maintain OSHA standards.

To insure safe and healthful working conditions for EPA employees, safety and health inspectors are authorized to enter without delay and at reasonable times, any building, installation, facility, construction site, or other area, workplace, or environment where work is performed by employees of the Agency, to inspect and investigate during regular working hours and at other reasonable times, and within reasonable limits and in a reasonable manner, any such place of employment and all pertinent conditions, structures, machines,

apparatus, devices, equipment, and materials therein; and to question privately any employee, or any supervisory employee, or any officer in charge of a reporting unit.

14.2.2 Informal Safety Inspections

An informal safety and health inspection is performed on either a scheduled or unscheduled basis by the facility safety officer, regional safety officer, supervisory management, or members of a safety and health committee. EPA Form 1440-2, Health and Safety Inspection Checklist (fig. 14-1), shall be used by the inspector or spokesman of a safety and health committee conducting the inspection to note safety and health deficiencies identified during the inspection process.

A more detailed checklist for safety evaluation of the laboratory is given in appendix A. This inspection list contains many specific recommendations and guidelines for laboratory safety.

14.3 Laboratory Safety Practices*

14.3.1 Introduction

14.3.1.1 Safe Use, Handling, and Storage of Chemicals

Chemicals in any form can be safely stored, handled, and used if their hazardous physical and chemical properties are fully understood and the necessary precautions, including the use of proper safeguards and personal protective equipment are observed.

The management of every unit within a manufacturing establishment must give wholehearted support to a well-integrated safety policy.

14.3.1.2 General Rules for Laboratory Safety

Supervisory personnel should think "safety." Their attitude toward fire and safety standard practices is reflected in the behavior of their entire staff.

A safety program is only as strong as the worker's will to do the correct things at the right time.

The fundamental weakness of most safety programs lies in too much lip service to safety rules and not enough action in putting them into practice.

Safety practices should be practical and enforceable.

Accident prevention is based on certain common standards of education and training of personnel, and provision of safeguards against accidents.

---

*This description was prepared by Paul F. Hallbach, Chemist, National Training and Operational Technology Center, U.S. EPA, Cincinnati, Ohio 45268.

517

HEALTH AND SAFETY INSPECTION CHECKLIST

INSPECTION CONDUCTED BY	TITLE	DATE

NAME/NUMBER OF BUILDING INSPECTED *(Use separate form for each building)*	REPORTING UNIT

PART I. PHYSICAL CONDITIONS *(Check each applicable item)*

ITEM	SAT	UNSAT	ITEM	SAT	UNSAT
1 HOUSEKEEPING AND SANITATION			18 ELECTRICAL AND ELECTRONIC EQUIPMENT		
2 NON HAZARDOUS MATERIALS STORAGE			19 WATER *(anti siphone and cross connections)*		
3 HAZARDOUS MATERIALS STORAGE			20 ELECTRICAL *(fuses, grounding, etc.)*		
4 MATERIALS HANDLING EQUIPMENT			21 COMPRESSED GAS CYLINDERS		
5 AISLES AND WALKWAYS			22 STRUCTURAL CONDITION OF BUILDING		
6 LADDERS AND STAIRS			23 PARKING AREA		
7 FLOORS PLATFORMS AND RAILINGS			24 MOTOR VEHICLES		
8 EGRESS			25 PORTABLE TOOLS		
9 LIGHTING			26 PROTECTIVE CLOTHING AND EQUIPMENT		
10 VENTILATION			27 HAZARDOUS WARNING SIGNS		
11 FLAMMABLE OR NOXIOUS DUST OR VAPORS			28 EMISSION OF POLLUTANTS *(air, fluid, solids)*		
12 HAZARDOUS BIOLOGICAL AGENTS			29 OCCUPATIONAL NOISE EXPOSURE		
13 FIRE DETECTION DEVICES			30 MEDICAL SERVICES INCLUDING FIRST AID		
14 FIRE ALARM SYSTEM			31 PROVISIONS FOR HANDICAPPED		
15 FIRE SUPRESSION *(including extinguishers)*			32 OTHER		
16 MECHANICAL EQUIPMENT					
17 MACHINE GUARDS AND SAFETY DEVICES					

PART II PROCEDURES AND INSTRUCTIONS *(Check each applicable item)*

33 MATERIALS HANDLING			45 FIELD OPERATIONS OTHER		
34 MOTOR VEHICLE OPERATION			46 SAFETY COMMITTEE ACTIVITY		
35 BUILDING MAINTENANCE			47 FEDERAL SAFETY COUNCIL PARTICIPATION		
36 EQUIPMENT MAINTENANCE			48 EMPLOYMENT REPRESENTATION IN SAFETY AND HEALTH PROGRAM		
37 USE OF PROTECTIVE CLOTHING AND EQUIPMENT			49 JOB ORIENTATION		
38 HAZARD MONITORING EQUIPMENT *(carbon monoxide, radiation, etc.)*			50 JOB TRAINING INCLUDING SAFETY AND HEALTH		
39 FACILITY SELF PROTECTION PLAN			51 SAFETY PROMOTIONAL AND MOTIVATIONAL ACTIVITIES		
40 LABORATORY OPERATIONS					
41 BOATING OPERATIONS			52 PERIODIC PHYSICAL EXAMINATIONS		
42 DIVING OPERATIONS			53 OTHER		
43 AVIATION OPERATIONS					
44 CRAFTS AND SHOPS OPERATIONS					

REMARKS *(Continue on back if necessary)* (NOTE *Use EPA Form 1440-6 to document in detail each unhealthful or unsafe condition*)

EPA Form 1440-2 (Rev. 5-77) PREVIOUS EDITION MAY BE USED UNTIL SUPPLY IS EXHAUSTED

Figure 14-1. Health and safety inspection checklist.

14.3.2 Laboratory Design and Equipment

14.3.2.1 Type of Construction

The construction of the laboratory should generally be fire resistant or noncombustible.

58

Multiple story buildings should have adequate means of exit.

Stairways should be enclosed with brick or concrete walls.

Laboratories should have adequate exit doors to permit quick, safe escape in an emergency and to protect the occupants from fires or accidents in adjoining rooms. Each room should be checked to make sure there is no chance of a person being trapped by fire, explosions, or release of dangerous gases.

Laboratory rooms in which most of the work is performed with flammable liquids or gases should be provided with explosion-venting windows.

14.3.2.2 Arrangement of Furniture and Equipment

Furniture should be arranged for maximum use of available space and should provide working conditions that are efficient and safe.

Aisles between benches should be at least 4 ft wide to provide adequate room for passage of personnel and equipment.

Desks should be isolated from benches or adequately protected.

Every laboratory should have an eyewash station and a safety shower.

14.3.2.3 Hoods and Ventilation

Adequate hood facilities should be installed where highly toxic or highly flammable materials are used.

Hoods should be ventilated separately and the exhaust should be terminated at a safe distance from the building.

Makeup air should be supplied to rooms or to hoods to replace the quantity of air exhausted through the hoods.

Hood ventilation systems are best designed to have an airflow of not less than 60 ft/min (linear) across the face of the hood with all doors open, and 150 ft/min (linear) if toxic materials are involved.

Exhaust fans should be sparkproof if exhausting flammable vapors and corrosive resistant if handling corrosive fumes.

Controls for all services should be located at the front of the hood and should be operable when the hood door is closed.

All laboratory rooms should have the air changed continuously at a rate depending on the materials being handled.

Recent California OSHA regulations require the presence of a means of visual indication of the existence of the airflow in the hood and specify the height and type of hood exhaust permitted.

14.3.2.4 Electrical Services

Electrical outlets should be placed outside of hoods to afford easy access and thus protect them from spills and corrosion by gases.

Noninterchangeable plugs should be provided for multiple electrical services.

Adequate outlets should be provided and should be of the three-pole-type to provide for adequate grounding.

Rubber or nonconductive composition shoe soles should be required (except when flammable vapors are present). Shoe soles should not be of a type that readily absorbs water or other liquids.

14.3.2.5 Storage

Laboratories should provide for adequate storage space for mechanical equipment and glassware that will be used regularly.

Flammable solvents should not be stored in glass bottles over 1 l in size. Large quantities should be stored in metal safety cans. Quantities requiring containers larger than 1 gal should be stored outside the laboratory.

Explosionproof refrigerators should be used for the storage of highly volatile and flammable solvents.

Cylinders of compressed or liquefied gases should not be stored in the laboratory.

Alphabetized storage of chemicals should be avoided to prevent the unintentional mixing of two incompatible chemicals in an accident situation.

An appropriate antidote must be readily available for every stored chemical compound for which an antidote is specified.

14.3.2.6 Housekeeping

Housekeeping plays an important role in reducing the frequency of laboratory accidents. Rooms should be kept in a neat and orderly condition. Floors, shelves, and tables should be kept free from dirt and from all apparatus and chemicals not in use.

A cluttered laboratory is a dangerous place to work. Maintenance of a clean and orderly work space is indicative of interest, personal pride, and safetymindedness.

Passageways should be kept clear to all building exits and stairways.

Metal containers should be provided for the disposal of broken glassware and should be properly labeled.

Separate approved waste disposal cans should be provided for the disposal of waste chemicals.

Flammable liquids not miscible with water and corrosive materials or compounds that are likely to give off toxic vapors should never be poured into the sink.

Laboratory operators must be sure that unguarded rotating equipment such as belt-driven vacuum pumps is provided with guards all the way around and that the guards are always in place.

Whenever heavy laboratory equipment must be moved frequently, rollers should be provided. In other cases, proper lifting equipment should be available.

14.3.2.7 Fire Protection

Laboratory personnel should be adequately trained regarding pertinent fire hazards associated with their work.

Personnel should know rules of fire prevention and methods of combating fires.

Fire extinguishers (CO_2 type) should be provided at convenient locations and personnel should be instructed in their use.

Automatic sprinkler systems are effective for the control of fires in chemical laboratories.

14.3.2.8 Alarms

An *approved* fire-alarm system should be provided.

Wherever a hazard of accidental release of toxic gases exists, a gas alarm system to warn occupants to evacuate the building should be provided.

Gas masks of oxygen or compressed-air-type should be located near exits and selected personnel trained to use them.

14.3.3 Handling Glassware

14.3.3.1 Receiving, Inspection, and Storage

Packages containing glassware should be opened and inspected for cracked or nicked pieces, pieces with flaws that may become cracked in use, and badly shaped pieces.

Glassware should be stored on well-lighted stockroom shelves designed and having a coping of sufficient height around the edges to prevent the pieces from falling off.

14.3.3.2 Laboratory Practice

Select glassware that is designed for the type of work planned.

To cut glass tubing or a rod, make a straight, clean cut with a cutter or file at the point where the piece is to be severed. Place a towel over the piece to protect the hands and fingers, then break away from the body.

Large size tubing is cut by means of a heated nichrome wire looped around the piece at the point of severance.

When it is necessary to insert a piece of glass tubing or a rod through a perforated rubber or cork stopper, select the correct bore so that the insertion can be made without excessive strain.

Use electric mantels for heating distillation apparatus, etc.

To remove glass splinters, use a whisk broom and a dustpan. Very small pieces can be picked up with a large piece of wet cotton.

14.3.4 Gases and Flammable Solvents

14.3.4.1 Gas Cylinders

Large cylinders must be securely fastened so that they cannot be dislodged or tipped in any direction.

Connections, gages, regulators, or fittings used with other cylinders must not be interchanged with oxygen cylinder fittings because of the possibility of fire or explosion from a reaction between oxygen and residual oil in the fitting.

Return empty cylinders promptly with protective caps replaced.

14.3.4.2 Flammable Solvents

Store in well-ventilated designated areas.

Flash point: the temperature at which a liquid gives off vapor sufficient to form an ignitible mixture with the air near the surface of the liquid or within the vessel used.

Ignition temperature: the minimum temperature required to initiate or cause self-sustained combustion independently of the heating or heated element.

Explosive or flammable limits: for most flammable liquids, gases, and solids there is a minimum concentration of vapor in air or oxygen below which propagation of flame does not occur on contact with a source of ignition. There is also a maximum proportion of vapor or gas in air above which propagation of flame does not occur. These limit mixtures of vapor or gas with air, which if ignited will just propagate flame, are known as the "lower and higher explosive or flammable limits."

Explosive range: the difference between the lower and higher explosive or flammable limits, expressed in terms of percentage of vapor or gas in air by volume.

Vapor density: the relative density of the vapor as compared with air.

Underwriter's Laboratories classification: a standard classification for grading the relative hazard of the various flammable liquids. This classification is based on the following scale:

Ether class	100
Gasoline class	90 to 100
Alcohol (ethyl) class	60 to 70
Kerosene class	30 to 40
Paraffin oil class	10 to 20

Extinguishing agents that are appropriate for each of the four classes of fires are required.

14.3.5 Chemical Hazards

14.3.5.1 Acids and Alkalies

Some of the most hazardous chemicals are the strong or mineral acids such as hydrochloric, hydrofluoric, sulfuric, and nitric.

Organic acids are less hazardous because of their comparatively low ionization potentials; however, such acids as phenol (carbolic acid), hydrocyanic, and oxalic are extremely hazardous because of their toxic properties.

Classification of acids is according to mineral or organic composition. Acids should be stored together, except that perchloric acid should not be placed next to glacial acetic acid. Picric acid should be stored separately.

14.3.5.2 Oxidizing Materials

Oxidizing agents, in contact with organic matter, can cause explosions and fire. They are exothermic and decompose rapidly, liberating oxygen, which reacts with organic compounds.

Typical hazardous oxidizing agents are–

Chlorine dioxide
Sodium chlorate; chlorates
Potassium chromate
Chromium trioxide
Perchloric acid; perchlorates

14.3.5.3 Explosive Power

Many chemicals are explosive or form compounds that are explosive and should be treated accordingly.

A few of the more common examples of this class of hazardous materials are–

Acetylides
Silver fulminate

523

Peroxides
Peracetic acid
Nitroglycerine
Picric acid
Chlorine and ethylene
Sodium metal
Calcium carbide

14.3.5.4 Toxicity

Laboratory chemicals improperly stored or handled can cause injury to personnel by virtue of their toxicity.

There are four types of exposure to chemicals:

a. Contact with the skin and eyes

b. Inhalation

c. Swallowing

d. Injection

Special classes of toxic agents:

a. Carcinogens—laboratory operators should recheck the OSHA 1974 regulations on carcinogens.

b. Mercury—complete cleaning of spills is essential for compliance with OSHA limits.

14.3.6 Precautionary Measures

14.3.6.1 Clothing and Personal Protective Equipment

Chemical laboratories should have special protective clothing and equipment readily available for emergency use and for secondary protection of personnel working with hazardous materials.

Equipment should be provided for adequate—

a. Eye protection

b. Body protection

c. Respiratory protection

d. Foot protection

e. Hand protection

14.3.6.2 Bodily Injury

Burns, eye injuries, and poisoning are the injuries with which laboratory people must be most concerned.

First emphasis in the laboratory should be on preventing accidents. This means observing all recognized safe practices using necessary personal protective equipment and exercising proper control over poisonous substances at the source of exposure.

So that a physician can be summoned promptly, every laboratory should post the names, telephone numbers, and addresses of doctors to be called in an emergency requiring medical care.

A consulting physician should specify the type and extent of first aid materials required for the laboratory.

14.4 Report of Unsafe or Unhealthful Condition

In EPA a procedure has been established for reporting an unsafe or unhealthful condition by the employee or supervisor. The procedure also provides for communication between the employees; supervisors; safety official; head of the unit; and, in the case of an unresolved report, the Department of Labor.

A sample of an unsafe or unhealthful condition reporting form is shown in figure 14-2, and a sample notice of an unhealthful or unsafe condition is shown in figure 14-3.

14.5 References

1. Public Law 91-596, Occupational Safety and Health Act of 1970 (Dec. 29, 1970).
2. Occupational Safety and Health Manual, U.S. EPA (Jan. 8, 1976).
3. Safety Management Manual, U.S. EPA, TN 1440.1 (Dec. 4, 1972).

RNN1440 011

REPORT OF UNHEALTHFUL OR UNSAFE CONDITION

TO	FROM

BRIEF DESCRIPTION OF UNHEALTHFUL OR UNSAFE CONDITION

OCCUPATIONAL SAFETY AND HEALTH STANDARD VIOLATED *(If known)*	LOCATION *(Include organization, Facility and Building)*

ACTION TAKEN BY SUPERVISOR

SIGNATURE	EMPLOYING ORGANIZATION	DATE

EPA Form 1440-6 (Rev. 5-77) PREVIOUS EDITION MAY BE USED UNTIL SUPPLY IS EXHAUSTED. NOTE A COPY OF THIS FORM MUST BE RETAINED BY THE ORGINATOR

Figure 14-2. Report of unhealthful or unsafe condition.

526

NOTICE OF UNHEALTHFUL OR UNSAFE WORKING CONDITION

TO *(Name of Officer-in-Charge of Reporting Unit)*	FROM *(Name of Inspector)*
REPORTING UNIT	Occupational Health and Safety Office Washington D.C. 20460

An inspection conducted by me on_____ at _____ revealed the following violation(s) of EPA Occupational Health and Safety Standards. These Standards have been adopted in compliance with the Occupational Safety and the Health Act of 1970, PL 91-956, Section 19.

ITEM NO	STANDARD, REGULATION OR SECTION VIOLATED	DESCRIPTION OF VIOLATION	LOCATION OF VIOLATION	NO. OF WORKING DAYS BY WHICH VIOLATION MUST BE CORRECTED AND DATE

Subpart D of 29CFR 1960.33, Safety and Health Provisions for Federal Employees, requires that a copy of this Notice shall be posted *immediately* in a prominent place at or near each place that the violation(s) referred to in the Notice occurred. The Notice must remained posted until all violations cited therein are corrected, or for three (3) working* days, whichever period is longer. A copy of this Notice shall be sent to the Health and Safety Committee of the establishment or Reporting Unit and to any person(s) who made a report of the unhealthful or unsafe condition which precipitated this inspection pursuant to the provisions of 29CFR 1960.31.

Subpart D of 29CFR 1960.34 requires the Officer-in-Charge of the Reporting Unit to *immediately* submit an abatement plan to the Designated Agency Safety and Health Official, if, in his judgment, the correction of the violation will not be possible within thirty (30) working days*. Such plan shall contain an explanation of the circumstances of the delay in abatement, a proposed timetable for the abatement, and a summary of steps being taken in the interim to protect employees. A copy of the plan shall be sent to the Health and Safety Committee of the establishment or Reporting Unit and to any person(s) who made a report of the unhealthful or unsafe condition which precipitated this inspection pursuant to the provisions of 29CFR 1960.31.

*Under the Occupational Safety and Health Act, the term "Working Day" means Monday through Friday but does not include Saturday, Sunday, or Federal Holidays.

SIGNATURE OF HEALTH AND SAFETY OFFICER	DATE

EPA Hq Form. 1440 B (8 77)

Figure 14-3. Notice of unhealthful or unsafe condition.

527

APPENDIX A

SUGGESTED CHECKLIST FOR THE SAFETY EVALUATION OF EPA LABORATORY AREAS

528

Organization: _____ Date: _____

Location: _____ Building: _____

Room(s): _____ By: _____

Item Inspected	Yes	No	N.A.	Comments
Fire Prevention				
1. Is fire alarm facility available? [.36(b)(7)]				
2. Are all exits maintained to provide free and unobstructed egress from all parts of building? [.36(b)(4)]				
3. Are all exits free of locks or fastening devices that could prevent free escape? [.36(b)(4)]				
4. a. Is the fire detection system in working order? b. Is the sprinkler system in working order? c. Are fire doors in working order? [.36(d)(2)]				
5. Are corridors and hallways at least 44 in wide? [.37(h)(1)]				
6. Do all exits discharge directly to a street, yard, court, or other open space? [.37(h)(1)]				
7. Are all exits marked by proper sign and illuminated? Are letters in sign not less than 6 in high, 3/4 in wide? [.37(q)(8)]				
8. Is access to exits marked in all cases where the exit or the way to reach it is not immediately visible? [.37(q)(5)]				
9. Is care taken to insure that no exit signs are obscured by decorations, furniture, or equipment? [.37(q)(3)]				
10. Is exit access arranged so that it is not necessary to travel toward any high hazard area to escape? [.37(f)(5)]				

Note: Adapted from a safety inspection work sheet developed by the Center for Disease Control (CDC). The pertinent section of the Code of Federal Regulations, title 29, part 1910 is given within brackets at the end of each item.

Listed below are explanations given by OSHA to questions that are not immediately clear on the safety checklist for laboratory areas. Each explanation is numbered according to the corresponding question on the facing page.

1. Self-explanatory.

2. Self-explanatory.

3. At no time should an exit door be locked or fastened in a manner that prevents it from being *immediately* opened from the inside of the building in the event of emergency. Safety inspectors should check all doors marked "EXIT" to insure that they can be readily opened.

4a,b. The building manager should conduct periodic tests (as recommended by the manufacturer or as required by local code) to insure proper working order of the fire detection system and the automatic sprinkler system.

4c. Fire doors are designed to be closed in the event of fire. Automatic fire doors normally remain open; however, the heat produced by a fire will cause them to close. Regular fire doors are designed to stay closed at all times, except for the passage of personnel. *No fire door should ever be blocked open as this will interfere with its function.* Fire doors are typically used to enclose stairways and to separate buildings and corridors.

5. Self-explanatory.

6. An exit should never discharge into a location that could potentially trap personnel. For example, an exit should not discharge into a closed courtyard.

7. Self-explanatory.

8. A sign reading "EXIT" or similar designation, with an arrow indicating the direction, shall be placed in every location where the direction of travel to reach the nearest exit is not immediately apparent.

9. Self-explanatory.

10. In designing and maintaining exit routes from a building, care should be taken to avoid routing people through or near a high hazard area. An example of a high hazard area is a corridor or room where flammable liquids are stored.

Item Inspected	Yes	No	N.A.	Comments
11. Are aisles maintained clear and unobstructed for movement of personnel and fire-fighting equipment? [.22(b)(1)]				
12. Is all fire protection equipment and apparatus identified with the color red? [.144(a)(1)]				
13. Are portable fire extinguishers maintained fully charged and operable and kept in designated places at all times? [.157(a)(1)]				
14. Are fire extinguishers conspicuously located, readily accessible, and available along normal paths of travel? [.157(a)(2)]				
15. Are extinguishers and locations conspicuously marked to indicate intended usage? [.157(a)(4)]				
16. Are extinguishers mounted so that the top is not more than 5 ft above floor; not more than 3½ ft if weight equals more than 40 lb? [.157(a)(6)]				
17. Are all extinguishers mounted in cabinets placed so that the instructions face outward? [.157(a)(7)]				
18. Are extinguishers available suited to the class of fire anticipated in each area? [.157(b)(1)]				
19. Are extinguishers placed according to distances for proper coverage? Within 75 ft–class A. Within 50 in–class B. [.157(c)]				
20. Are extinguishers inspected, maintained, and replaced by spares when they are discharged or missing? [.157(d)]				
21. Are laboratory rooms with potential fire hazards equipped with proper extinguishers for emergency situations? [.157(b)]				
22. If flammable liquids are used in a laboratory, is the mechanical ventilation sufficient to remove vapors before they reach a hazardous concentration? [.106(e)(2)(iii)]				

531

11. Self-explanatory.

12. Self-explanatory.

13. Self-explanatory. During a safety inspection *all* fire extinguishers should be checked aginst the requirements of this question.

14. Fire extinguishers should *never* be located in places where they are concealed from general view.

15. Fire extinguishers must be clearly labeled to indicate the type of fire they are capable of fighting. The following code is used to classify types of fires:

    Class A—fires in ordinary combustible materials such as wood, cloth, paper, and rubber
    Class B—fires in flammable liquids, gases, and greases
    Class C—fires that involve energized electrical equipment where the electrical nonconductivity of the extinguishing medium is of importance; when electrical equipment is deenergized, extinguishers for class A or B fires may be safely used
    Class D—fires in combustible metals such as magnesium, titanium, zirconium, sodium, and potassium

16. Inspector should make certain that no fire extinguishers (other than wheeled-type extinguishers) are placed on the floor.

17. Self-explanatory.

18. See explanation to question No. 15 for classification of fire hazards. Fire extinguishers (of more than one type, if necessary) should be selected and located according to anticipated fire hazards.

19. Self-explanatory.

20. Self-explanatory.

21. In certain instances, laboratories can be fire traps. When this is the case, fire extinguishers should be provided *inside* the laboratory so that occupants can fight their way out of a fire emergency. Safety inspectors should check for escape routes to the nearest corridor when considering this question.

22. Self-explanatory.

Item Inspected	Yes	No	N.A.	Comments
23. Are hose outlets within easy reach of persons standing on floor and not over 6 ft from floor? [.158(b)(1)(i)]				
24. Do all connections on dry standpipes have a sign reading "DRY STANDPIPE–FOR FIRE DEPARTMENT USE ONLY"? [.158(b)(7)]				
25. Are automatic sprinkler systems provided with at least one fire department connection with at least 4-in pipe size? [.159(b)(1)]				
26. Are fire department connections designated "AUTO SPKR" or "OPEN SPKR"? [.159(b)(4)(v)]				
27. Is there a water flow detection device on the sprinkler system that will activate the fire alarm? [.159(c)(1)]				
28. Are fire alarm boxes readily accessible and within normal path distance of 200 ft? [.163(b)(3)]				
29. Are all fire alarm systems inspected and tested at weekly intervals? [.163(c)]				
30. Are "NO SMOKING" signs posted in prohibited areas? [.106(d)(7)(iii)]				
Flammable Liquid Storage				
31. Are drums which contain flammable liquids constructed of noncombustible materials? [.106(b)(1)(i)]				
32. Are storage drums vented? [.106(b)(2)(iv)]				
33. Are flammable liquids stored in proper containers? [.106(d)(2)(i)]				
34. Are safety cans and portable containers of flammable liquids painted red with yellow band or name of contents? [.144(a)(1)(ii)]				
35. Are storage cabinets being used for storing flammable liquids? [.106(d)(3)]				
36. Are storage cabinets labeled "FLAMMABLE–KEEP FIRE AWAY"? [.106(d)(3)(ii)]				

23. This question applies to buildings equipped with water and hose standpipe systems. Hose outlets are normally located in stairways or in the corridor immediately outside the stairway.

24. Self-explanatory. The building manager should be able to answer this question.

25. Fire department connections to automatic sprinkler systems are normally located on the outside of the building being protected. The size of the connection is usually embossed on a metal plate or on the connection itself.

26. The designations "AUTO-SPKR" or "OPEN-SPKR" are found embossed on a plate at the fire department connection. Failure to display one of the designations is a violation of OSHA.

27. This question is self-explanatory. The building manager should know if the facility is equipped with a sprinkler waterflow detection device.

28. Self-explanatory.

29. This procedure should be part of a regular maintenance program.

30. The laboratory supervisor shall designate areas where smoking is prohibited and "NO SMOKING" signs shall be placed in these areas.

31. Flammable liquid storage drums should be constructed of steel or some other noncombustible material.

32. Self-explanatory.

33. Flammable liquids may be safely stored in glass containers if the total capacity of the container is 1 gal or less. Larger quantities of flammable liquids should be stored in safety cans approved by a recognized testing laboratory.

34. Self-explanatory.

35. Self-explanatory.

36. Self-explanatory.

Item Inspected	Yes	No	N.A.	Comments
37. Is the storage area provided with either a gravity or mechanical exhaust ventilation system? [.106(d)(4)(iv)]				
38. Are extinguishers available where flammable or combustible liquids are stored? [.106(d)(7)]				
39. Is at least one portable fire extinguisher of rating not less than 12-B units located outside of, but not more than 10 ft from, door opening into room used for storage? [.106(d)(7)(i)(a)]				
40. Is at least one portable fire extinguisher with rating not less than 12-B units located not less than 10 ft nor more than 25 ft from flammable liquid storage located outside of a storage room but inside of a building? [.106(d)(7)(i)(b)]				
41. Are "NO SMOKING" signs posted in the flammable or combustible liquid storage areas? [.106(d)(7)(iii)]				
Electrical Hazards				
42. Are all new electrical installations and all replacements, modifications, or repairs made and being maintained in accordance with the National Electrical Code? [.309]				
43. Does the interior wiring system have a grounded conductor? i.e., three-wire system? [.309–NEC(200-2)]				
44. Do all electrical appliances have Underwriter's Laboratories Inc. approval, or that of some other nationally recognized testing laboratory? [.309–NEC(90-8)]				
45. Are the cords of all electrical equipment in good condition, not frayed or spliced, etc.? [.309–NEC(400-5)]				
46. Are cords used properly (not run under rugs)? [.309–NEC(400-4)]				
47. Is there only one plug-in per socket outlet; i.e., no multiple plug-ins to one socket? [.309–NEC(240-2)]				
48. Are the lighting levels such that good illumination is provided in all walking, working, and service areas to insure safety? [.309–NEC(110-16(e))]				

935

37. The building manager or engineering department should know the answer to this question.

38. Self-explanatory. Fire extinguishers approved for fighting class B fires should be available in flammable liquids storage areas.

39. This question is self-explanatory but should be reread several times for full comprehension. Fire extinguisher ratings are often stamped on the title plate along with other specifications. *Note:* This question is to be used *only* when inspecting flammable-liquid storage rooms.

40. This question is self-explanatory, but should be reread several times for full comprehension. *Note:* This question is to be used only when inspecting flammable liquid storage *outside* of a special designated room, but inside of a building.

41. Self-explanatory.

42. Prior to adding any new electrical facilities to a laboratory, it is the responsibility of the laboratory supervisor or building manager to check with the electrical contractor to insure that the National Electrical Code is observed. This rule is retroactive to Mar. 15, 1972.

43. All convenience outlets shall be of the three-pronged type. These outlets should be checked with a grounding tester to insure proper wiring.

44. Approved appliances will bear the label of a recognized testing laboratory.

45. Self-explanatory.

46. Self-explanatory.

47. Multiple outlet (cube) adaptors or extension cords with multiple outlet ends should not be used to plug more than one appliance into a single socket.

48. Self-explanatory.

536

Item Inspected	Yes	No	N.A.	Comments
49. Are circuit breaker panels and cutoff switches located so as to be readily accessible? [.309–NEC(240-25(b)) and (1926.400(e))]				
50. Are all circuit breaker switches marked or labeled? [.309–NEC(240-25(e))]				
Aisles, Exits, Floors, and Stairways				
51. Are all floors kept clean and dry? [.22(a)(2)]				
52. Are all floors, areas, and passageways free from protruding nails, splinters, holes, and loose boards? [.22(a)(3)]				
53. Are aisles and passageways clear of all obstructions and in good repair? [.22(b)(1)]				
54. Are aisles and passageways wide enough to operate equipment safely? [.22(b)(1)]				
55. Is floor loading within *posted* maximum limits? [.22(d)]				
56. Does every floor opening having standard guarded railings? [.23(a)(1)]				
57. Do fixed stairs make an angle to the horizontal between 30° to 50°? [.24(e)]				
58. Are all opensided floors, platforms, ramps, 4 ft above adjacent floor or ground, guarded with railing? [.23(c)(1)]				

537

49. Circuit breaker cabinets should *never* be locked.

50. Each circuit breaker switch shall be labeled as to the lights, outlets, or appliances it controls.

51. Self-explanatory.

52. Self-explanatory.

53. Self-explanatory.

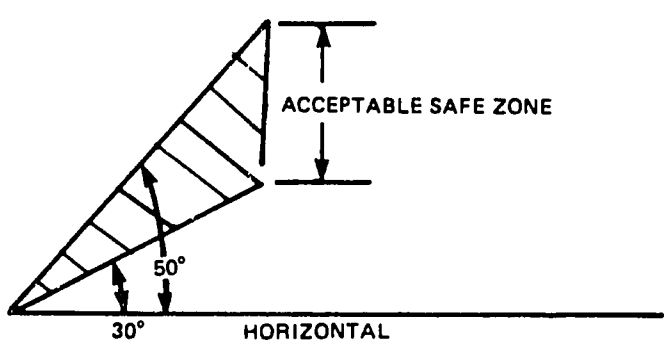
54. Self-explanatory.

55. Safe floor loadings must be *posted* and *observed* in all buildings and on all floors.

56. *Specifications for standard railing:* A standard railing shall consist of a top rail, intermediate rail, and posts. It shall have a vertical height of 42 in nominal from upper surface top rail to floor, platform, runway, or ramp level. The top rail shall be smooth surfaced throughout its length. The intermediate rail shall be approximately halfway between the top rail and the floor, platform, runway, or ramp.

    Specifications for standard railing to be used on stairways: A stair railing shall be of construction similar to a standard railing (for a floor opening), but the vertical height shall not be more than 34 in nor less than 30 in from upper surface of top rail to surface of tread in line with face of visor at forward edge of tread.

57. All fixed stairways must rise at an angle within the limits shown:



58. Self-explanatory.

538

Item Inspected	Yes	No	N.A.	Comments
59. Are flights of stairs having four or more risers equipped with railings or handrails? [.23(d)(1)]				
60. Are stair treads reasonably uniform and slip resistant? [.24(f)]				
61. Are all places kept clean and orderly and in a sanitary condition? [.141(a)(1)(i)]				
62. Are trash receptacles of approved use? [.141(a)(3)]				
63. Is every enclosed work place so constructed and maintained to prevent entrance and harborage of rodents, insects, and vermin? [.141(a)(4)]				
64. Is drinking water potable and available within 200 ft of location at which employees work? [.141(b)(1)(i)]				
65. Are toilet facilities adequate for both sexes and in accordance with regulations listed? [.141(c)(1)]				
66. Are toilet rooms constructed so that each water closet occupies a separate compartment equipped with door, latch, and clothes hanger? [.141(c)(2)(i)]				
67. Does door to toilet room have a self-closing device, and is entrance screened so interior is not visible from outside? [.141(e)(2)(iii)]				
68. Does every water closet have a hinged open-front seat made of nonabsorbent material? [.141(c)(3)(ii)]				
69. Are suitable washing facilities available and maintained in a sanitary condition? [.141(d)(1)]				
70. If employees are allowed to lunch on the premises, is an adequate space provided for that purpose? [.141(g)(2)]				
71. Are adequate waste disposal containers provided? [.141(g)(2)]				
72. Are change rooms provided for each sex where it is necessary to change clothes? [.141(e)(1)]				
73. Are change rooms provided with separate storage facilities for street clothes and protective clothing? [.141(e)]				
74. Are noise levels acceptable? [.95]				

59. Self-explanatory.

60. Stair treads should be free of cracks and other uneven areas.

61. Self-explanatory.

62. Trash receptacles used for garbage disposal should be equipped with tight-fitting lids.

63. Self-explanatory.

64. Self-explanatory.

65. Self-explanatory.

66. Self-explanatory.

67. Self-explanatory.

68. Self-explanatory.

69. Suitable washing facilities consist of a lavatory with hot and cold water, a suitable cleansing agent, and individual hand towels.

70. Self-explanatory. Employees are *not* permitted to eat in *any* laboratory that uses agents that are dangerous to health if ingested. It is the responsibility of the laboratory supervisor to enforce this rule.

71. Self-explanatory.

72. Self-explanatory.

73. Self-explanatory.

74. If any employees are exposed to noise levels greater than 90 dB at any time during the day, a safety expert should be consulted to determine if corrective measures need to be taken.

540

Item Inspected	Yes	No	N.A.	Comments
75. Is necessary protective equipment provided, used, and maintained in a sanitary, safe, and reliable condition? [.132(a)]				
76. Are eye protectors provided where machines or operations present the hazard of flying objects, glare, liquids, radiation? [.133(a)]				
77. Are sufficient washing facilities (including eye washes and deluge showers) available for all persons required to handle liquids that may burn, irritate, etc.? [.151(c)]				
78. Are employees in the area exposed to air contaminants only in accordance with proper limits? [.134(a)(1)]				
79. Is a respiratory protection program used where needed? [.134(a)(2)]				
80. Are written standard operating procedures governing the selection and use of respirators established? [.134(b)(1)]				
81. Has the user of the respirator been instructed and trained in the proper use and limitations of the respirator? [.134(b)(3)]				
82. Are respirators regularly cleaned, disinfected, inspected, and stored in a convenient, clean, and sanitary location? [.134(b)(5)]				
83. Has the person assigned the task requiring a respirator been determined physically able to perform the work and use the equipment by the local physician? [.134(b)(10)]				
84. Are breathing gas containers marked in accordance with the American National Standards identifying contents? [.134(d)(4)]				
85. Can gas mask canisters be identified by properly worded labels and color code or atmospheric contaminant? [.134(g)(1)]				
86. Is the compressor for supplying air to respirators equipped with necessary safety and standby equipment? [.134(d)(2)(ii)]				

75. Protective equipment, including personal protective equipment, shall be provided, used, and maintained in a sanitary and reliable condition wherever work-associated hazards may cause injury or impairment in function of any part of the body through absorption, inhalation, or physical contact. Laboratory supervisors should recognize such hazards and take the action necessary to insure that employees use adequate personal protection to avoid injury.

76. Safety glasses or goggles may be obtained through the CDC Safety Office.

77. All laboratories using liquids that may burn or irritate must be equipped with some type of emergency eye wash equipment.

78. If laboratory supervisors have any questions concerning safety concentrations of air contaminants, they should contact the CDC Office of Biosafety.

79. Respirators that are applicable and suitable for the purpose intended shall be provided when such equipment is necessary to protect the health of employees. The CDC Office of Biosafety will provide the proper respirators for a given hazard.

80. Self-explanatory.

81. Self-explanatory.

82. Self-explanatory. Respirators should be cleaned and disinfected after each use.

83. Self-explanatory.

84. Self-explanatory.

85. Self-explanatory.

86. Compressors for breathing air should be equipped with receivers of sufficient capacity to enable the respirator wearer to escape from a contaminated atmosphere in the event of compressor failure; alarms to indicate compressor failure and overheating shall be installed in the system.

542

Item Inspected	Yes	No	N.A.	Comments
87. Is each employee who works in an area where radioactive material is used furnished and wearing film badge? [.96(d)(2)]				
88. Is radiation exposure of individuals to the body limited to 1¼ rems per calendar quarter? [.96(b)]				
89. Is each radiation area posted with the proper radiation caution sign? [.96(e)(2)]				
90. Are all radiation area employees instructed in the safety problems, precautions, and devices to minimize exposure? [.96(i)]				
91. Are records maintained of the radiation exposure of all employees who are monitored? [.96(n)]				
92. Are radioactive materials stored and disposed properly? Are storage containers labeled? [.96(j,k)]				
Compressed Gases				
93. Is each portable gas container for gases such as hydrogen legibly marked with the name of contents? [.252(a)(2)(i)(a)]				
94. Are compressed gas cylinders determined in safe condition by visual and other inspection required in regulations? [.101(a)]				
95. Does the compressed gas cylinder or tank have an installed pressure relief device? [.101(c)]				
96. Are all compressed gas cylinders stored and secured so they cannot fall? [.252(a)(2)(ii)(b)]				
97. Are protection caps in place on compressed gas cylinders except when in use? [.252(a)(2)(ii)(d)]				
98. Is each mobile hydrogen supply unit secured to prevent movement? [.103(b)(1)(iv)(e)]				
99. Is the hydrogen storage area permanently marked "HYDROGEN–FLAMMABLE GAS–NO SMOKING OR OPEN FLAMES"? [.103(b)(1)(v)]				

543

87. Self-explanatory.

88. Self-explanatory.

89. Self-explanatory.

90. Self-explanatory.

91. Self-explanatory.

92. Self-explanatory.

93. Self-explanatory.

94. Deep dents or heavy corrosion of a gas cylinder might indicate a dangerous situation.

95. Self-explanatory.

96. When either storing or moving a compressed gas cylinder, care must be taken to prevent the cylinder from falling. OSHA requires that compressed gas cylinders either be chained or strapped in an upright position at all times.

97. Self-explanatory.

Preface to Questions 98 through 107

A gaseous hydrogen system is one in which the hydrogen is delivered, stored, and discharged in the gaseous form to the consumer's piping. The system includes stationary or movable containers, pressure regulators, safety relief devices, manifolds, interconnecting piping, and controls. The system terminates at the point where hydrogen at service pressure first enters the consumer's distribution piping. Systems having a total hydrogen content of less than 400 ft^3 are *not* covered by the following questions.

98. Mobile supply units should be strapped or chained to prohibit movement.

99. Self-explanatory.

Item Inspected	Yes	No	N.A.	Comments
100. Is the hydrogen system in an adequately ventilated area? [.103(b)(2)(ii)(d)(1)]				
101. Is the hydrogen system 20 ft from stored flammable materials or oxidizing gases? [.103(b)(2)(ii)(d)(2)]				
102. Is it 25 ft from open flames, electrical equipment, or other sources of ignition? [.103(b)(2)(ii)(d)(3)]				
103. Is it 25 ft from concentrations of people? [.103(b)(2)(ii)(d)(4)]				
104. Is it 50 ft from intakes of ventilation or air-conditioning equipment and air compressors? [.103(b)(2)(ii)(d)(5)]				
105. Is it 50 ft from other flammable gas storage? [.103(b)(2)(ii)(d)(6)]				
106. Is it protected from damage due to falling objects or working activity in the area? [.103(b)(2)(ii)(d)(7)]				
107. Are safety relief devices arranged to discharge upward and unobstructed to the open air and prevent impingement of gas upon container, adjacent structure, or personnel? [.109(b)(1)(ii)(b)]				
Storage				
108. Is storage of material stable and secure against sliding, collapse, falls, or spills? [.176(b)]				
109. Are storage areas kept free from accumulation of materials that constitute hazards from tripping, fire, explosion, or pest harborage? [.176(e)]				
110. Are dangerous parts of machines or energized equipment, which may injure, colored orange where exposed? [.144(a)(2)]				
111. Is yellow the color used for designating physical hazards and caution in the particular environment? [.144(a)(3)]				
112. Is the color green used to designate "SAFETY" and location of first aid equipment? [.144(a)(4)]				
113. Is purple the color used to designate radiation hazards? [.144(a)(6)]				
114. Are the colors black and white used for traffic and housekeeping markings? [.144(a)(7)]				

545

100. Self-explanatory.

101. Self-explanatory.

102. Self-explanatory.

103. Self-explanatory.

104. Self-explanatory.

105. Self-explanatory.

106. Self-explanatory.

107. Self-explanatory.

108. Self-explanatory but very *important.*

109. Self-explanatory.

110. Self-explanatory.

111. Self-explanatory.

112. Self-explanatory.

113. Self-explanatory.

114. Self-explanatory.

Item Inspected	Yes	No	N.A.	Comments
115. Are all accident prevention signs used to minimize workplace hazards? [.145(e)(e)]				
116. Are all exposed steam and hot water pipes within 7 ft of floor or working platform covered with an insulating material or guarded? [.264(e)(4)(iii)]				
117. At all loading docks employing powered industrial trucks, are wheel chocks used to prevent trailers or railroad cars from rolling? [.178(k)(1)]				
118. Is medical help readily available through personnel and first aid supplies approved by consulting physician? [.151(b)]				

FINAL REPORT

Advanced Training for Tabbin Institute of Metallurgical Studies (TIMS), Cairo, Egypt

USAID CONTRACT #263-010-C-00-2038-00
PROJECT #263-0101

VOLUME IV

APPENDIX

MICROBIOLOGY

Prepared by
A. Curley,
Team Leader
Office of Research and Sponsored Programs
Clark Atlanta University
J.P. Brawley Drive at Fair Street, SW
Atlanta, GA 30314

548

TABLE OF CONTENTS

VOLUME IV

HANDOUTS FOR MICROBIOLOGY

APPENDIX	**Volumes 2 - 4**
Volume 4	Introduction to Microbial Ecology
	Microbes in the Environment
	General Operations
	Sample Collection, Preservation and Storage
	Laboratory Equipment, Techniques and Media
	Techniques for Isolation and Enumeration of Bacteria of Sanitary Significance
	Selection of Analytical Methods
	Standard Procedures for Analytical Methodology
	Total Coliform Methods
	Fecal Coliform Methods
	Quality Assurance
	Laboratory Operations

HANDOUTS

FOR

MICROBIOLOGY

550

MICROBIOLOGY LECTURES

1

Introduction to Microbial Ecology

Defining the Scope of Microbial Ecology

At the outset, we should define the meaning of *microbial ecology* and the scope of this field of science. The term "ecology" is derived from the Greek words *oikos* (household or dwelling) and *logos* (law). Thus, ecology is the "law of the household," or by its contemporary definition, the science that explores the interrelationships between organisms and their living (biotic) and nonliving (abiotic) environments. Microbial ecology is the science that specifically examines the relationships between microorganisms and their biotic and abiotic environments.

The term "ecology" was first used by the German biologist Ernest Haeckel in 1866, but "microbial ecology" came into frequent use only in the early 1960s. The current popularity of this term and the rapid development of this field in the last 25 years relate to a general awareness of ecology and the role of microorganisms in maintaining a high level of environmental quality.

The unprecedented spurt of technological and economic growth in industry that followed the conclusion of World War II, and an accompanying mood of boundless expectations, gradually gave way to a feeling of alarm over population explosion, deterioration of the environment, and rapid depletion of nonrenewable natural resources. Mankind, having acquired almost limitless powers to subdue and exploit the earth, appeared neither able to control its own population size, nor to manage the limited resources of "Spaceship Earth" in a wise and sustainable fashion. Grim, but quite credible, scenarios of impending disaster were drawn up by groups of scientists and economists who attempted to project recent population, consumption, and pollution trends into the near future. Proposed remedies stressed population control, limits to technological and economic growth, pollution abatement, and increased reliance on renewable resources for energy and raw materials. Such ideas were presented in books understandable to large segments of society and had notable effects on societal attitudes and on legislation. Changes in societal attitudes led to various forms of antipollution legislation, conservation and consumer activism, and to the formation of the United States Environmental Protection Agency (EPA) and other environmental regulatory bodies around the world. Various legislative actions on air pollution, water pollution, and strip mining were enacted to conserve natural resources and to protect human health against deteriorating environmental quality.

The desire of a responsive segment of society

551

ı harmony with nature, rather than to
'. changed the once rather esoteric con-
ology into a common household word
ılated a broad interest in this branch of
Microbial ecology emerged as an en-
ınd dynamic branch of science in this
ıd atmosphere because it was recognized
ıoorganisms occupy a key position in
ıly flow of materials and energy through
ıl ecosystem by virtue of their metabolic
to transform organic and inorganic
es.

environmental persistence of various
chemicals and plastics, biomagnification
ıints, eutrophication, acid mine drainage,
pollution of wellwater, methylation of
. ozone-depleting nitrous oxide in the
ere, and a plethora of other environmental
ıs reflect unfavorable and unintended in-
ıs of human activities with the microbial
ent of the global ecosystem. At the same
croorganisms are crucial in solving some
pressing environmental and economic
ıs, such as the proper disposal of various
ıl solid wastes, relief of nitrogen fertilizer
:, food, feed, and fuel production from
ıcts and waste materials, recovery of
from low-grade ores, biological control
. etc. These and many other practical
ıons make microbial ecology a highly
' and exciting subject for study.

ical Overview

:h the widespread use of the term "mi-
ecology" is rather recent, ecologically
ıl research on microbes was performed as
. their existence was realized. Much of
earch was identified as soil or aquatic
ıology, but general microbiology, too,
ıted much to the understanding of mi-
activities that are crucial to the balance
:e. We need recognize, however, that some microbiological approaches that are often considered fundamental to the study of microbes, such as the isolation of pure cultures and their cultivation on synthetic media, are not conducive to ecological observations of the type that the general ecologist can readily make on plants and animals in their natural environments. It is, therefore, not surprising that some important observations relating to microbial ecology either predated the pure culture technique, or were made later by investigators who unwittingly or of necessity dealt with mixed microbial populations rather than with pure cultures.

The Beginnings of Microbiology

Although microbes were probably the first living organisms on earth, it was not until the mid-seventeenth century that the advent of the microscope permitted their observation. During this period, Robert Hooke, an English experimental philosopher, described his microscopic observations of fungi and protozoa (Hooke, 1665). It was Antonie van Leeuwenhoek in the 1680s, however, who was the first to glimpse and record the multitude and diversity of the previously hidden world of microbes, including such small forms as yeasts and some bacteria (Dobell, 1932). Leeuwenhoek, an amateur scientist and microscope maker in Delft (Holland), used simple but powerful lenses to tediously observe the otherwise invisible microbes. His observations, including detailed and recognizable drawings (Figure 1.1) (Leeuwenhoek, 1683), were recorded in a series of letters he sent between 1674 and 1723 to the Royal Society in London. Through the *Proceedings of the Royal Society* his discoveries, which otherwise might have been lost, were rapidly disseminated. Leeuwenhoek's reports included descriptions of microbes in rainwater (microbes in their natural habitats) and the effects of pepper on microbes (environmental influences on microbes), thus providing not only the earliest descriptions of bacteria, but also the earliest studies on microbial ecology.

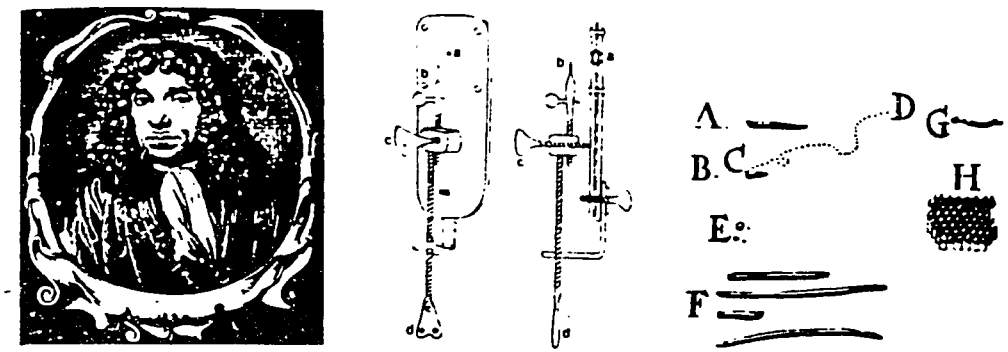


FIGURE 1.1 (a)
Antonie van Leeuwenhoek (1632–1723) developed simple, but powerful microscopes and published first sketches of microscopic observations of bacteria. (Courtesy of National Library of Medicine.)

FIGURE 1.1 (b)
Early microscopes of Antonie van Leeuwenhoek were very simple, consisting of (a) spherical glass lens held by two metal plates; (b) specimen holder; and (c) and (d) screws for positioning and focusing specimen. (From C.E. Dobell, 1932; reprinted by permission of Russell and Russell.)

FIGURE 1.1 (c)
Drawings of microorganisms by Antonie van Leeuwenhoek show several recognizable common bacterial shapes; A, F, rods of varying size; B, E, cocci; C, D, path of a short motile rod; G, spiral; H, cluster of cocci. (Courtesy of Royal Society of London.)

After Leeuwenhoek, little additional progress in the development of the science of microbiology took place until the turn of the eighteenth century, when the Italian naturalist Lazzarro Spallanzani, debating the then prevalent view of spontaneous generation, produced experimental evidence showing that putrefaction of organic substrates is caused by minute organisms that do not arise spontaneously, but multiply by cell division. Spallanzani showed that heating destroys these organisms and that sealing the containers after heating prevents spoilage indefinitely. Heating, however, did not always prevent putrefaction. The heat resistance of bacterial endospores, unknown at that time, contributed to inconsistent and confusing results (Kruif, 1926; Bulloch, 1938).

Despite the growing experimental evidence against their views, advocates of the spontaneous generation theory, who claimed that microorganisms arise spontaneously and as a result of putrefaction of organic substrances, were not convinced and tried to explain Spallanzani's experiments by the partial removal of air during the heat treatment. In retrospect the idea may seem rather strange to us, yet in the first half of the nineteenth century even such eminent scientists as the German chemist Justus Liebig firmly believed that yeasts and other microoganisms were products rather than the causative agents of fermentations (Liebig, 1839).

Using slightly different experimental protocols, Cagniard-Latour (1838), Theodor Schwann

(Friedrich Kuntzing (1837) sought to he theory of spontaneous generation, ntly reaching the conclusion that al- ermentations were caused by living nicroorganisms (yeasts). Experiments nn and later by Tyndall clearly impli- orne microorganisms as agents of sub- oilage in heat-sterilized media, but the sy dragged on until the classic exper- Pasteur silenced even the most obstinate of the spontaneous generation theory. Louis Pasteur, who, in a beautifully ies of experiments (1860–1862), utterly d the theory of spontaneous generation 1861), reportedly stating "No more shall of spontaneous generation ever rear ead again." By direct microscopic ob- as well as by cultural methods, he ted the presence of microorganisms in d their role in initiating fermentation e in presterilized media. Pasteur placed er, sugared yeast water, urine, sugar and pepper water into ordinary flasks. eshaped the necks of the flasks so that several curves in each neck, resembling neck." By using curved ends, Pasteur e the flasks open to the air, thus over- major criticism of previous experiments disproving spontaneous generation where ssential life force," had been excluded. oiled the liquids until they steamed he necks. Dust and microbes settled out ved necks of the flasks and thus, while to air, the broth did not become con- with microorganisms. Contrary to the f those who believed in spontaneous n, no change or alteration appeared in l. These flasks, still sterile, were later d may be seen at the Pasteur Institute

s efforts to disprove spontaneous gen- Pasteur found a powerful ally in the En- sicist, John Tyndall (1820–1893), who ated that "optically clear" (particle-free) not cause spoilage of sterile media and solved the nagging problem of heat resistant bacterial endospores by discontinuous heat treatments (Tyndall, 1877). This technique resulted in germination of endospores and their destruction upon a subsequent heating, thus accomplishing the reliable sterilization of all media at 100°C.

One year before Tyndall devised his sterilization method by intermittent heating, Ferdinand Julius Cohn (1876), professor of botany at the University of Breslau, Germany, described the endospores of *Bacillus subtilis* and their heat-resistant properties. He began efforts to establish the taxonomy of bacteria and was the first scientist to recognize and encourage the work of Robert Koch on the cause of anthrax. He recognized that microorganisms have constant and characteristic morphology, a view not shared by many uncritical investigators who, observing a succession of forms in their impure cultures, believed them to be one and the same organism.

The Pure Culture Period

The mainstream of microbiology from 1880 to the middle of the twentieth century was overwhelmingly influenced by the towering figures of Louis Pasteur and Robert Koch (Figure 1.2). A large portion of Pasteur's and Koch's work was oriented towards the most pressing practical problems of their time. Louis Pasteur was trained as a chemist and was prompted to begin his microbiological studies because of the problems of vine growers, brewers, distillers, and vinegar makers. His studies on fermentations between 1857 and 1876 clearly established microorganisms as the causative agents of the various fermentation processes. Both Pasteur and Koch also devoted much of their efforts to examining the role of microbes as causative agents of infectious diseases and to the prevention of such diseases through immunization and sanitation measures.

The ability to determine the etiology (cause) of disease depended on the development of appropriate techniques and methodological approaches. Koch and his coworkers developed

(a) (b)

FIGURE 1.2 (a)
Louis Pasteur (1822–1895), noted for numerous accomplishments in microbiology. He demolished the theory of spontaneous generation, demonstrated the microbial origin of various fermentations and diseases, and contributed to the development of immunization by use of attenuated microorganisms. (Courtesy of National Library of Medicine.)

FIGURE 1.2 (b)
Robert Koch (1843–1910) made major contributions to the development of pure culture technique and the field of medical microbiology. (Courtesy of National Library of Medicine.)

simple methods for the isolation and maintenance of pure microbial cultures on solid media (Koch, 1883). Previous attempts to obtain pure cultures by dilution or on slices of boiled potato were cumbersome, uncertain, and not generally applicable. Gelatin and later agar, which were utilized by Koch to solidify liquid media of any desired composition, were eminently suitable for the unequivocal identification of disease-causing agents. More broadly, it allowed microbiologists to separate out a microbial type from a heterogenous community and study its activities, removed from complex interactions, in an exacting and reproducible manner. The importance of this advance was monumental and transformed microbiology from a chancy art into an exact science. Many of the pure culture techniques devised by Koch are being used today, a hundred years later, without any substantial modification.

Koch's pure culture techniques established a trend in microbiology that has had a profound influence on the approach used by microbiologists—first isolate a pure culture and then see what it does. The terse statement by Oscar Brefeld (1881) "work with impure cultures yields nothing but nonsense and *Penicillium glaucum*" is a good

ary of this attitude, and few microbiologists d argue with its wisdom even today. Unately this approach provides little opporfor the observation of interactions between organisms and their biotic and abiotic surings, that is, this approach has limited value robial ecology. We should remember that hibition zone around a chance *Penicillium* minant on a *Staphylococcus* culture, perl and correctly interpreted by Sir Alexander ng, sparked the line of investigations that ually yielded penicillin and other life-saving otics (Grainger, 1958; Lechevalier and Sovsky, 1965). Microbial ecology depends the development of methods for underng just such interactions.

Development of Microbial Ecology

ure culture orientation of microbiologists ubtedly contributed to the relative neglect ological research on microorganisms, but ter type of research also had to overcome ndous methodological difficulties. Small size, morphological detail, and low behavioral ntiation of microorganisms are formidable les to conventional ecological observation search.

vertheless, work by microbiologists in the enth century began to establish the role of bial metabolic activities in global ecological ses. Pasteur's work clearly established the microorganisms in biodegradation of orsubstances. He anticipated but failed to transformations of inorganic substances roorganisms in the conversion of ammoto nitrate (nitrification process), but proof h transformations was forthcoming from nvestigators. In 1839 DeSaussure reported servation on the capacity of soil to oxidize en gas. Since this activity was eliminated ting, by adding a 25 percent NaCl solution dding a 1 percent sulfuric acid solution, rectly concluded that the oxidation was ial, though hydrogen-oxidizing bacteria were isolated only during the first decade of the twentieth century. J. J. Schloesing and A. Müntz reported in 1877–1879 that ammonium in sewage was oxidized to nitrate during passage through a sand column. The nitrification activity of the column was eliminated by treatment with chloroform vapors but was restored by inoculation with a soil suspension. Again, microbial nitrifying activity was the only logical explanation for this experiment, which also pointed to the presence of nitrifying organisms in soil (Schloesing and Müntz, 1879).

All the above early information was obtained without the aid of the pure culture technique, but soon thereafter Sergei Winogradsky (Figure 1.3), a Russian scientist working at various European universities and later in his life at the Pasteur Institute in Paris, successfully isolated nitrifiers (1890). Winogradsky, during his long and fruitful career, also described the microbial oxidation of hydrogen sulfide and sulfur (1887), the oxidation of ferrous iron (1888), and correctly developed the concept of microbial chemoautotrophy even though one of his principal models (*Beggiatoa*) could not be grown as a chemoautotroph in pure culture until very recently. He also described the anaerobic nitrogen-fixing bacteria (1893) and contributed to the studies of reduction of nitrate and symbiotic nitrogen fixation. He originated the nutritional classification of soil microorganisms into the autochthonous (humus-utilizing) and zymogenous (opportunistic) groups, and is regarded by many as the founder of soil microbiology (Waksman, 1953; Grainger, 1958; Doetsch, 1960).

FIGURE 1.3
Sergei Winogradsky (1856–1953) established the concept of microbial chemoautotrophy and made major contributions to development of enrichment culture technique and to soil microbiology. (Courtesy of Waksman Institute of Microbiology, Rutgers University.)

Equally important was the work of the Dutch microbiologist, M. W. Beijerinck (Figure 1.4) at the University of Delft. In 1905, half a century before the emergence of microbial ecology as an area of specialization, Beijerinck stated: "The way I approach microbiology . . . can be concisely stated as the study of microbial ecology, i.e., of the relation between environmental conditions and the special forms of life corresponding to them." Beijerinck isolated the agents of symbiotic (1888) and non-symbiotic aerobic (1901) nitrogen fixation, and also isolated the sulfate reducers. He recognized the virtual ubiquity of most microbial forms and the selective influence of the environment that favors the development of certain types. Based on his principle "everything is everywhere, the environment selects" Beijerinck, with contributions from Winogradsky, developed the immensely useful and adaptable technique of enrichment culture. Tailoring culture conditions to favor microbes with a particular metabolic ability usually leads to the rapid enrichment and isolation of the desired organism even if its original numbers were very low in the sample (Van Iterson et al., 1940). With additional reports on nitrate reduction (Deherain, 1897), methanogenesis and methanotrophy (Söhngen, 1906), and the isolation of hydrogen bacteria (Kaserer, 1906), the outlines of microbial cycling processes were clear (Doetsch, 1960; Brock, 1961; Doelle, 1974).

FIGURE 1.4
Martinius Beijerinck (1851–1931) made major contributions to the knowledge of nitrogen and sulfur cycling by microorganisms including studies on symbiotic nitrogen fixation. (Courtesy of Waksman Institute of Microbiology, Rutgers University.)

The above rather incomplete list of fundamental discoveries pertaining to the role of microorganisms in cycling of materials was accomplished by relatively few microbiologists who had an interest in unusual metabolic patterns. Further progress in our understanding of microbial metabolism as related to cycling processes and the unifying metabolic features among micro- and macroorganisms emerged from the work of A. J. Kluyver, C. B. van Niel, and Roger Stanier; these microbiologists were notable for the breadth, comparativeness, and ecological implications of

work in microbial physiology. Kluyver
e 1.5) succeeded Beijerinck as the leading
biologist at the University of Delft, the
town of Leeuwenhoek. He took a broad
st in microbial physiology and with his stu-
and coworkers studied a variety of oxidative,
ntative and chemoautotrophic microor-
ms, including unusual metabolic types. His
st contribution was perhaps his comparative
ach which stressed the unifying metabolic
es within the diverse microbial world (Kamp
, 1959).

A student of Kluyver, C. B. van Niel (1897–
came from the same famous school of Dutch
biologists. Settling in the United States early
career (1929), he was instrumental in trans-
ing his school's tradition of comparative and
gically oriented microbial physiology to his
country. Among his numerous contributions,
vestigations on bacterial phototrophy have
al importance. He pointed out the striking
gy of hydrogen sulfide (H_2S) and water (H_2O)

RE 1.5
Kluyver (1888–1956) emphasized the unifying fea-
of microbial metabolism. (Courtesy of Waksman
ute of Microbiology, Rutgers University.)

in the photoprocesses of photosynthetic sulfur bacteria and green plants, respectively. Roger Stanier, a student of van Niel, continued the tradition of unraveling the role of microbial metabolism in ecological processes; his work with *Pseudomonas*, for example, showed the tremendous versatility of microorganisms in degrading complex organic compounds.

If it appears that in the first half of the twentieth century microbiologists had little interest to spare for basic ecological theories, it is equally true that during the same time period general ecologists treated the ecological processes of decomposition, detritus formation, and mineral nutrient cycling with equal disdain. These processes were taken for granted and, in contrast to primary production, were seldom scrutinized or quantified. Considering that nutrient regeneration (recycling) can be one of the key constraints of primary production, this neglect is somewhat surprising. But even if general ecologists had been interested in quantifying microbial cycling processes, there was little in their training to prepare them for this task. The microbiologists of that period could have offered little assistance.

The principle approach of general ecology was field observation; for microbiologists it was laboratory experimentation on pure cultures. Because of their small size and short generation time, microorganisms have potential advantages for studies in population dynamics, but the observational difficulties described earlier discouraged their use in such investigations. Notable exceptions are the classic experiments of Gause (1934) on populations of the ciliate protozoa *Paramecium caudatum* and *Didinium nasutum*, the latter preying on the former species. The above and similar experiments with *Schizosaccharomyces pombe* (yeast), which is preyed upon by *Paramecium bursaria* (ciliate protozoan), revealed the same out-of-phase cyclic oscillations that are characteristic for some predator-prey populations in nature. At that time, these ecologically oriented, mixed culture experiments elicited little response among microbiologists, while among zoologists

and ecologists they sparked a great deal of additional research and lively debate.

Communication between classical and microbial ecologists in the early twentieth century was minimal or nonexistent; the two disciplines had no common language or approach. Some microbiological methods were, however, developed in this period that allowed microbes to be observed in ways that were compatible with classical ecological approaches. Among the few techniques developed for the *in situ* observation of microorganisms in the first half of this century, the contact slide (Cholodny et al., 1930; Rossi et al., 1936) deserves mentioning. In this method, microscope slides buried in soil or sediment provide a surface for microbial growth that is not unlike that of soil particles. Careful removal and microscopic observation of these slides provides an opportunity to observe microorganisms as they grow and interact in their natural environment.

These early studies established the basis for later research in microbial ecology. Stimulated by a general interest in the environment and ecology, as well as by the availability of radiotracer, microanalytical and electron microscopic techniques, a rapid upswing in the pace and range of ecologically oriented microbiological research occurred in the early 1960s. Much of this research was prompted by the noticeable and troublesome presence in the environment of synthetic pollutants that were not, or not rapidly enough, degraded by microorganisms, and by eutrophication problems due to detergent fillers and fertilizer runoff. Additional concern arose about the effects of radiation and pollutants on microbial cycling activities in soil and natural waters. The biomagnification of DDT, PCBs, mercury, and other pollutants called attention to food web interrelations and to the key position of microbial forms as primary producers in aquatic food webs.

A great stimulus to microbial ecology was space exploration. The possibility of soft-landing instruments on remote planets prompted the development of methods and instrument packages for detection of microbial life. The testing of these methods and instruments, in turn, stimulated interest in the microbial communities of extremely harsh terrestrial environments, e.g., the dry valleys of Antarctica. The nitrogen fertilizer shortage that developed in wake of the energy crisis of the early 1970s resulted in renewed and intense interest in symbiotic and nonsymbiotic nitrogen fixation processes. The energy crisis also called for research on fuel generation from renewable resources such as organic wastes and plant biomass, with microorganisms as obvious candidates to effect such conversions. The progress in methodology and in our understanding of the structure and function of microbial ecosystems that has taken place in the last twenty-five years makes up a large portion of the material covered in this textbook.

Sources of Information for the Microbial Ecologist

Long before the time was ripe for microbial ecology to emerge as a distinct area of specialization, a considerable amount of fundamental knowledge on microbe-environment interactions accumulated in several microbial subdisciplines. As described earlier, the microbiology of soil, with its important agricultural implications, attracted the attention and talent of such distinguished investigators as Winogradski and Beijerinck. Plant pathology, with its related practical implications, also received early attention. In strong analogy to the progress in medical microbiology, the pure culture technique led rapidly to the isolation and characterization of various plant pathogenic fungi and bacteria. Beijerinck made a crucial contribution also to this field with his investigation of plant diseases caused by submicroscopic, filterable principles. Continuation of Beijerinck's unfinished work by others led to the startling discovery of a crystallizable plant pathogenic principle: the tobacco mosaic virus. In contrast to human disease, the sanitation-immunization approach has limited relevance to the control of plant pathogens in

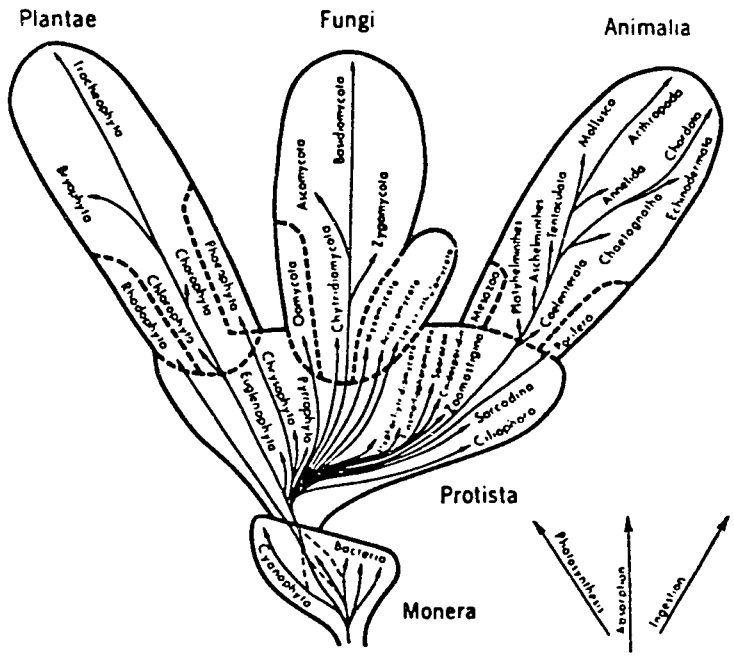


FIG. 2.1
...ker's classification ... is a five-kingdom ... based on three levels ...nization: prokaryotic ...om Monera), eukar- ...nicellular (kingdom ...a), and eukaryotic ...ellular and multinu- (kingdoms Fungi, An- ..., and Plantae).

...ng to Woese's theory, bacteria began to live ... urkaryotes and eventually these prokaryotic ...ria and their urkaryotic host cells became ...lly dependent. Over time, the prokaryotic ...ers in this mutually beneficial relationship ...oped into cellular organelles (mitochondria ...hloroplasts), giving rise to the eukaryotic

...y of Acellular ...oorganisms

... of the major classification systems deals ...the acellular microorganisms. Effectively, ...iruses, viroids, and prions are treated as nonliving entities. The reproductive capacity of acellular microorganisms being integrally linked to the host organisms in which they multiply, the viruses, viroids, and prions are normally considered along with their hosts.

Prions

Prions are specific infectious protein molecules that contain the information that codes for their own replication (Prusiner, 1982, 1984). They seem to be an exception to what appeared to be a universal characteristic of living systems—the storage of genetic information in nucleic acid molecules. All other organisms store their genetic information in nucleic acids, DNA (deoxyribonucleic acid) or less commonly RNA (ribonucleic acid). It is not yet clear how a protein can direct its own replication and thus we do not understand how prions replicate.

Prions were discovered during the search for the cause of scrapie, an infectious and usually fatal disease of sheep. Prions and the consequences of their reproduction within the cells of the organisms they infect may explain several diseases that until now have no known cause. Much more research is needed to reveal the importance of the discovery of prions.

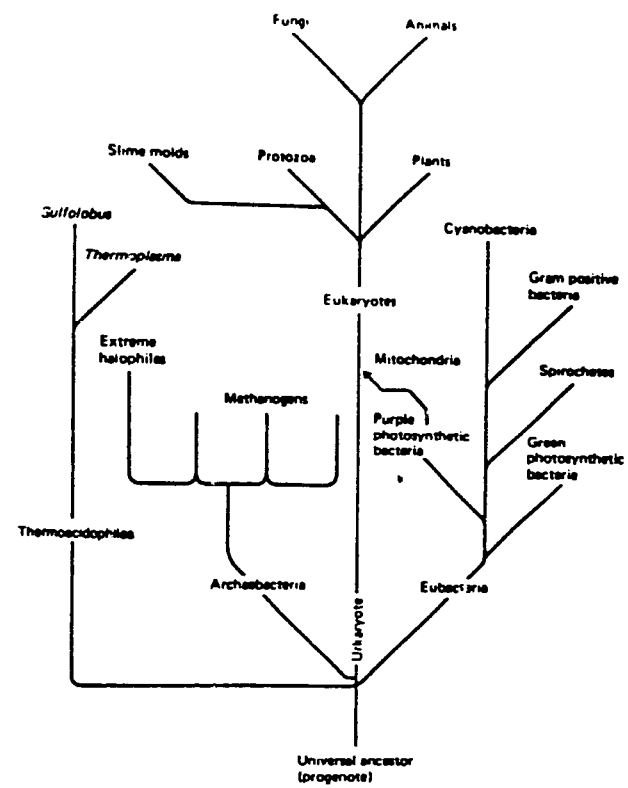


FIGURE 2.2
The three-kingdom classification system Woese proposed is based on the discovery that the archaebacteria are fundamentally different from all other organisms. This theory was developed by using the modern techniques of molecular biology, and in particular the examination of the macromolecules comprising key structures of the organism, especially the RNA of the ribosomes. Unlike previous classification systems, the use of analysis of conserved gene products permits a direct assessment of genetic and thus evolutionary relatedness.

Viroids

Whereas prions are protein molecules, viroids are composed exclusively of RNA (Diener, 1981). Viroids, like prions, are simply molecules that contain the information for their own reproduction; they have no other structures, and their

7

Measurement of Numbers, Biomass, and Activities in Microbial Ecosystems

Understanding the structure and functioning of ecosystems depends upon more than simply recognizing the interrelationships among populations; it requires quantitative information about numbers of organisms, biomass of populations, rates of activity, rates of growth and death, and cycling and transfer rates of materials within ecosystems. This quantitative approach, more than anything else, distinguishes ecologists from naturalists. In this chapter we will examine how microbial ecologists quantitatively assess the microbial components of ecosystems.

Numbers, biomass and activity represent distinct ecological parameters. Though normally correlated to each other, it is wrong to use them in an interchangeable manner. The nature of the ecological problem dictates which of the above parameters is the most appropriate one to measure. On occasion, technical problems force us to measure a less relevant parameter, such as numbers, and to calculate from these a more relevant parameter, such as biomass. Whenever this is done, it is important to assess critically whether such a projection is justified under the prevailing circumstances.

It stands to reason that a change in microbial numbers usually correlates to a similar change in biomass and in activity. Since such correlations appear to be so self-evident, one needs to be alert to situations when such correlations are either not proportional or entirely absent. Due to life cycles or nutrient availability, the cell size of microorganisms may change. In such situations, biomass may increase or decrease without a corresponding change in microbial numbers. Similarly, in the case of a sporulating fungus or actinomycete, numbers may increase dramatically with little or no change in biomass. Microbial activity will correlate with numbers and biomass only as long as environmental conditions remain constant. Any change in temperature, nutrient availability, or other environmental determinants will alter microbial activity without necessarily changing either numbers or biomass. Since unwarranted extrapolations abound in the literature, microbial ecologists need to evaluate those critically and on a case-by-case basis.

It is rarely feasible to subject a whole ecosystem to enumeration or measurement procedures. Instead, these procedures are performed

'presentative subsamples, and from these
urements projections are made to the whole
stem (Board and Lovelock, 1973). Sampling
ns and their statistical implications are dis-
d in more detail in the Appendix. It is im-
nt to understand that each determination
sts of three phases: sample collection, sample
ssing, and the actual measurement. The
er of sample collection and sample pro-
g can profoundly influence the outcome of
easurements and, therefore, all three pro-
es must be taken into consideration when
sults are interpreted.

he term representative means that the sample
reflect the diversity and density of organisms
entirety of the sampled environment. In
environments, the distribution of micro-
sms is not homogeneous, but patchy. Any
sample is, of necessity, minute as compared
whole environment being sampled and
re may easily over- or underestimate the
undance. This is particularly true because
ganisms live within microhabitats that are
ognized at the time of sample collection.
ource of error can be minimized by pro-
composite samples, prepared from in-
l ones collected by the use of a suitable
g grid. Confidence limits of extrapolations
mples to the entire environment should
stically determined.

le Collection

l approaches must be used for sampling
anisms from such diverse ecosystems
ts of animals, the surface layer of soils,
sediment of the deep sea. The lack of
cess to some environments requires the
mote control sampling devices. In some
tion procedures, sample processing and
nting procedures are coupled with sam-
he method of obtaining samples is de-
l by the physical and chemical properties
osystem being examined, by the expected abundance of microorganisms, and by the enumeration or measurement procedures to be performed. Some sampling approaches are compared in Table 7.1.

Sampling procedures must insure that numbers or activity of microorganisms are not altered, either positively or negatively, in a nonquantifiable manner during collection of the sample. Sampling procedures also must insure that samples are representative and are not contaminanted with foreign microorganisms—that is, sampling procedures must insure that the microorganisms come only from the ecosystem being examined.

Soil Samples

Soil microbiologists often do not use aseptic technique, but pragmatically rely on a shovel and pail because of the abundance of microorganisms in the soil relative to possible contaminants from air or nonsterile containers. When soil must be collected from a particular depth, a soil corer is used. A soil corer is a hand or motor-driven hollow tube with a sharp cutting edge and is used with or without a liner to hold the core of collected soil. Soil corers are designed to minimize compaction during sample recovery.

The sampling of microorganisms present in soil in relatively low numbers may require the use of attractants or baits. In some cases it is sufficient to provide a surface that will, by absorption, concentrate the naturally occurring dissolved nutrients. The buried slide technique of Cholodny-Rossi has been used extensively in the sampling and observation enumeration of microorganisms in soils and sediments (Cholodny, 1930; Rossi et al., 1936). In this technique a glass microscope slide is implanted into the soil or sediment (Figure 7.1). Assuming that the clean glass slide surface is nonselective and acts like the surface of mineral particles in soil, the types and proportions of the organisms that adhere to the slide can be considered representative of the soil community in general. A modern variation on the buried slide technique is the exposure of electron microscope grids into natural environments (Hirsch and Pankratz, 1970). The retrieved grids are observed with the electron microscope to reveal more microbial morphological detail than light microscopy can.

TABLE 7.1
Comparison of microbial sampling approaches in major natural environments

Environment	Access	Numbers	Sampling devices	Sample processing
Air	Direct	Low	Filters, Anderson samplers	Concentration on filters
Water	Direct or remote	High or low	Nets, containers or filters	Dilution or concentration
Sediment	Remote	High	Grabs or corers	Serial dilution
Soil	Direct	High	Shovels or corers	Serial dilution

Another variation of the buried slide technique is to replace glass slides with flattened glass capillaries, which are called pedoscopes when used in soil, and peloscopes when used in sediments (Aristovskaya and Parinkina, 1961; Perfilev and Gabe, 1969; Aristovskya, 1973) (Figure 7.2). The capillaries resemble sediment or soil pore spaces and microorganisms may enter them freely. The flattened optical surface of the capillaries facilitates microscopic observation and counting after retrieval. The capillaries may also be filled with nutrient solutions to attract microorganisms, in which case the technique resembles an enrichment culture and will, therefore, not reflect the original composition of the natural microbial community of the environment. Major achievements attributed to the use of capillary techniques in soils include: 1) the observation of microbial landscapes of silt deposits, such as the abundance of *Caulobacter*, algae and flagellate protozoa in wet soils, the development of *Metallogenium* indicative of iron-manganese deposition and the extensive growth of *Gallionella* when ferrous iron is present; 2) the enumeration of new genera that had remained unknown due to their unusual characteristics; and 3) the observation of life cycle stages in natural habitats (Perfilev and Gabe, 1969).

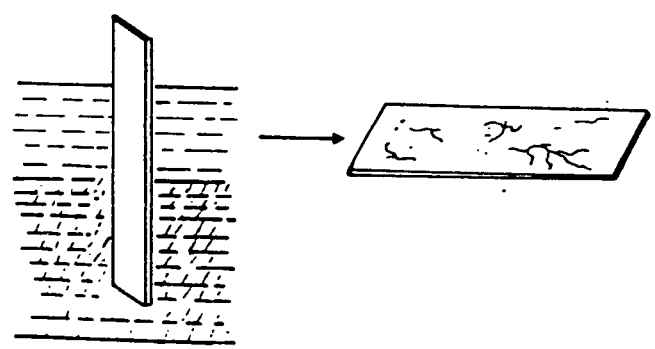


FIGURE 7.1
Schematic representation of buried slide method for collection and enumeration of microorganisms.

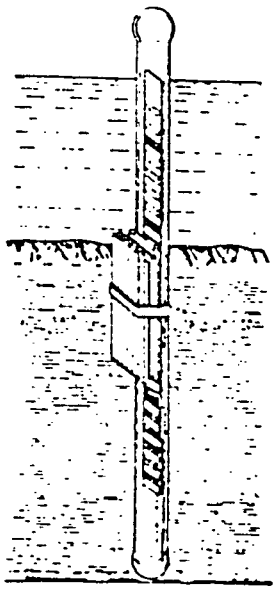


FIGURE 7.2
Pedoscope for *in situ* observation of microorganisms in sediment. (Pedoscope is a similar device for observation in soil.) (From Aristovskaya, 1973; reprinted by permission; Swedish National Research Council.)

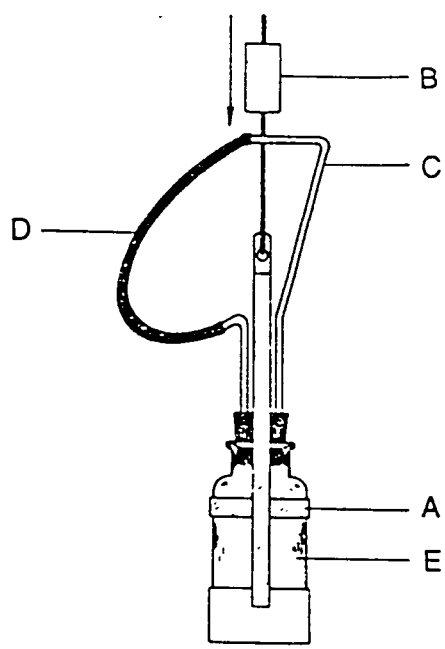
Water Samples

Sampling problems are typically greater in aquatic habitats than in soils because remote collection normally is required. Various sampling devices are used for collecting water and sediment samples; each has its advantages and limitations. Many of these devices are designed to ensure that the sample really comes from a particular location—from exactly 100 meter depth, for example.

Water samples collected for enumeration of algae and/or protozoa often are collected with Nansen and VanDorn sampling bottles but these devices are not suitable for bacteriological sampling because they cannot be sterilized. Planktonic algae and protozoa are often collected in nets towed by a boat, but many microflagellates will not survive this collection method. The planktonic organisms may be funneled into collection bottles where they are concentrated.

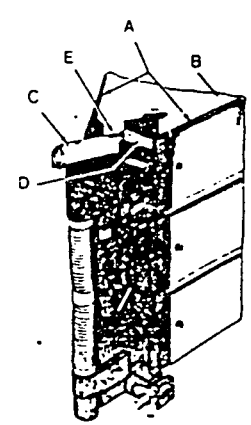
Water samplers for collection of bacterial populations generally consist of an evacuated chamber that can be opened at a given depth, allowing water to fill the chamber (Bordner and Winter, 1978). The J-Z sampler, for example, consists of an evacuated glass bottle or a compressed rubber bulb with a sealed glass tube inlet (ZoBell, 1941) (Figure 7.3). The device is lowered to the desired depth and a weighted messenger

FIGURE 7.3
J–Z sampler for collection of water samples at shallow depths: (A) bracket; (B) messenger; (C) glass tube to be broken by messenger (D) rubber inlet tube; (E) partially evacuated sample bottle.



is sent down the line to break the glass inlet tube, allowing the water to enter the flask or bulb. The Niskin sterile bag water sampler consists of an evacuated polyethylene bag with a sealed rubber tube inlet. The sampling bag is mounted on a spring-loaded holder, lowered to the desired depth, and a messenger is then lowered that releases a knife blade that cuts the rubber inlet hose, causing springs to spread the sampling bag, thus drawing in a water sample (Niskin, 1962) (Figure 7.4). Because the sampling devices are lowered through the water column to reach the desired depth, the collected sample may be contaminated with water from a different depth. Contamination may also originate from the sampling wire used to lower the device, and more elaborate sampling devices are needed to preclude such contamination (Jannasch and Maddux, 1967) (Figure 7.5). Specialized sampling devices also are required for collecting water samples from great depths because of decompression problems during recovery; devices have been designed to open at a specific depth,

FIGURE 7.4
Sterile Niskin butterfly water collecting bag: (A) spring loaded holder; (B) sterile plastic bag; (C) rubber inlet tube; (D, E) knife blade for opening inlet when triggered by messenger.



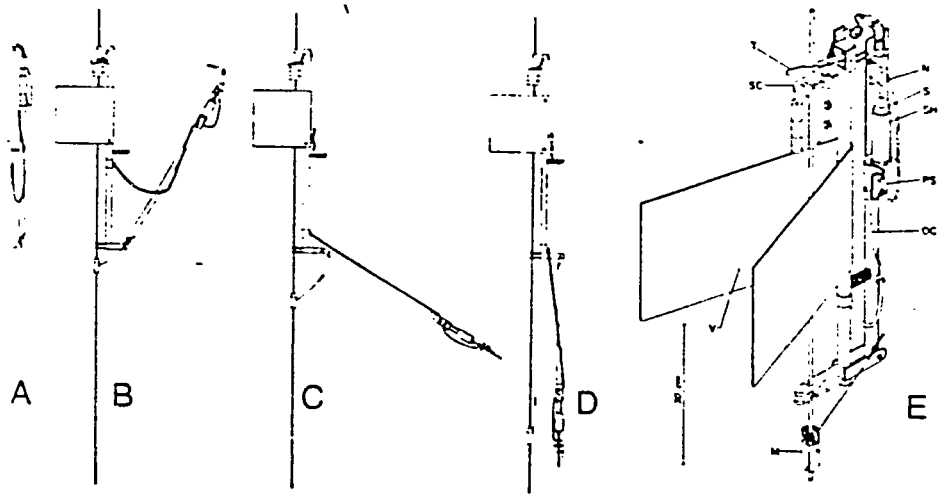
allowing a sample to enter, and to close again in order to maintain the appropriate pressure during recovery (Jannasch and Wirsen, 1977) (Figure 7.6).

Recovery of microorganisms from the air-water interface of aquatic habitats is by adsorption onto a suitable material (Crow et al., 1975). Sterile nuclepore filters, teflon, or cellophane may be used for this purpose. Densities of microorganisms on surfaces are often high.

Sediment Samples

For collecting sediment samples from marine or freshwater ecosystems there are a variety of grab samplers or corers. Grab samplers have two spring-loaded jaws that are triggered when the sampler reaches bottom or when a messenger is lowered. When triggered, the jaws of the grab sampler snap shut, dig into the sediment, and collect a sample. Most grab samplers do not prevent contamination of the sample with overlying water. Box corers, which are frequently used by benthic biologists but only rarely by marine microbiologists, recover sediment in a relatively undisturbed state (Tait and DeSanto, 1972). Core samples are also used for collection of sediments for microbiological analyses. Some caution must be used to prevent compression of the sample so that the vertical stratification of the microbial community can be preserved. Coring devices can have a sterile lining that aids in handling the sample and permits dividing the core sample into vertical sections.

In addition to these remote sampling devices that are deployed from the surface of the water, divers and submersibles are sometimes used for the collection of samples for microbiological analyses. These means of collection have the advantage of enabling the collectors to see what they are sampling and to make rational decisions about what to collect and also to describe the samples. Deep diving submersibles such as the Alvin, which has been used to investigate the deep ocean thermal vent regions, have been par-



:E 7.5
:bial water sampling device desinged to avoid contamination from the hydro-
:o wire: (A) vane maintains direction into current; (B) when triggered by a
:ger, the sampling arm swings out and sterile cover of sample tube pulls away;
:ringe plunger retracts to draw in sample; (D) sampling inlet closes; (E) detail of
:ing apparatus. (From Jannasch and Maddux, 1967; reprinted by permission;
:l of Marine Research)

:rly valuble because in addition to samples
: recovered for laboratory processing, some
:les can be collected, processed, left *in situ*
:cubation, and later recovered for analysis.

:amples

:ling microorganisms from the air requires
:rocessing be coupled with sample collection
:bial numbers in air are generally quite low
: is impractical to collect large volumes of
: later processing. Air samples are, therefore,
:ally collected by passing a measured volume
through a membrane filter (Gregory 1973).
:ore size of the membrane filter may be
: for the collection of different types of
:organisms. In the Anderson air sampler,
air is drawn through a graded series of grids of decreasing pore size (Andersen, 1958). An agar plate is placed under each grid to collect microorganisms and other airborne particles that, by their inertia, impact on the agar surface. Air velocity increases with decreasing grid openings, thus increasingly smaller particles land on successive agar plates.

Biological Samples

Retrieving microorganisms from plants and animals involves collection of a vital fluid, such as sap or blood; dissection to recover a particular tissue, such as liver; collection of excreted products, such as fecal matter; or sampling of a surface tissue. In cases where the surface is exposed,

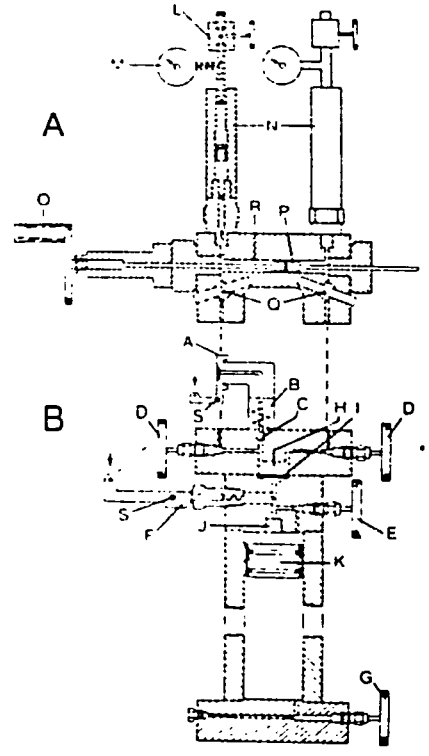


FIGURE 7.6
Sampling device for collection of undecompressed water samples from great depths: (A) transfer unit; (B) filter sampler, I) nuclepore filter, B) intake lid, K) piston for maintaining pressure and regulating flow rate through filter. For other details of apparatus and its operation see original reference. (From Jannasch and Wirsen, 1977; reprinted by permission; American Society for Microbiology.)

microorganisms may be scraped or washed from the tissue. As examples, microorganisms can be recovered from plant surfaces by washing with sterile solutions (Dickinson and Preece, 1976), from the oral cavity by scraping and washing tooth surfaces (Gibbons and van Houte, 1975), and from human skin by swabbing (Rosebury, 1962). Such nondestructive sampling requires that the microorganisms not be tightly bonded to the plant or animal, and concern must be given to the efficiency of such recovery methods.

In other cases the tissue or cells may be preserved together with the associated microorganisms. This is especially useful when examination of the anatomical relationship, or enumeration of microbial populations, is required. Such approaches are useful when considering associations between microbial populations, such as between phage and bacteria, as well as when examining associations between plants or animals and microbiota.

Sample Processing

Only rarely are naturally occurring microbial populations found in concentrations that are convenient for measurements or counting and therefore the microorganisms in the sample normally must be either concentrated or diluted. Samples with too many microorganisms may be brought to appropriate concentrations by serial dilutions (the successive diluting of the sample with an appropriate diluent), and samples with too few microorganisms can be concentrated by centrifugation or by passage through membrane filters. The conditions employed for processing affect the eventual numbers of microorganisms that are enumerated.

If counts are to reflect accurately the numbers of viable microorganisms present in the sample at the time of collection, then processing must be accomplished quickly because microorganisms reproduce rapidly in the collection vessels, yielding artificially elevated counts. This problem, known as the bottle effect, is particularly observed with seawater, which naturally lacks adsorptive surfaces; when placed into a glass contained nutrients are concentrated by adsorption on the wall, becoming more concentrated and available to microbes which are then able to reproduce

:atly increase their numbers. Conditions
processing samples can also kill some of
roorganisms present in the original sample,
ielding artificially depressed counts.
samples are to be diluted for viable count
tures, microorganisms must be evenly dis-
in the diluent. This is a difficult task,
ally for soil and sediment samples where
rganisms are usually bound to particles.
ciency of recovery of microorganisms is
dependent on the chemical composition
notic strength of the diluent, the time of
temperature, the chemical composition
persant if one is used, and the degree of
n (Jensen, 1968; Litchfield et al., 1975)
7.2 and 7.3). The optimal recovery process
rom sample to sample, precluding an ab-
tandardization of methodology.
amples must be concentrated by filtration,
iate filters must be selected. Filters with
hat are too large fail to collect the mi-
nisms, filters with pore sizes that are too
og easily and cannot filter sufficient vol-
n general, polycarbonate filters produced
lepore are considered superior to nitro-
e filters produced by Millipore because
latness of the surface and uniformity of

2
ixing time on viable counts of soil bacteria

ne r	Viable count (#/g) on soil extract agar incubated 12 days at 25°C. Soil 1	Soil 2
	7.4×10^6	2.9×10^6
	8.1×10^6	4.2×10^6
	$*8.7 \times 10^6$	3.9×10^6
	8.4×10^6	7.0×10^6
	7.1×10^6	$*7.1 \times 10^6$

*Optimal mixing time
n, 1968.

TABLE 7.3
Effect of chemical composition of diluent on viable counts of soil bacteria

Diluent	Viable count (#/g) on soil extract agar—8-day incubation at 25°C with 1-min. delay between preparation of dilutions and plating
Tap water	1.9×10^7
Pyrophosphate 0.1%	2.3×10^7
Ringer's solution	1.6×10^7
Winogradsky's solution	3.0×10^7

After Jensen, 1968

the pores. The particular chemical composition of the filter may also affect the viable counts of microorganisms (Green et al., 1975; Lin, 1976).

It is obvious that conditions not compatible with the physiological requirements of particular groups of microorganisms will lead to their underestimation becuase a loss of viablility. For example, enumeration of obligate anaerobes requires that diluents be sparged free of oxygen and that all processing be carried out under an oxygen-free atmosphere. The enumeration of obligate psychrophiles requires that the diluent and all glassware, such as pipettes, be prechilled to avoid killing these microorganisms.

When the enumeration procedure is not designed to discriminate between living and dead microorganisms, the samples may be preserved by adding formaldehyde or glutaraldehyde. Such preparations are suitable for direct microscopic observations.

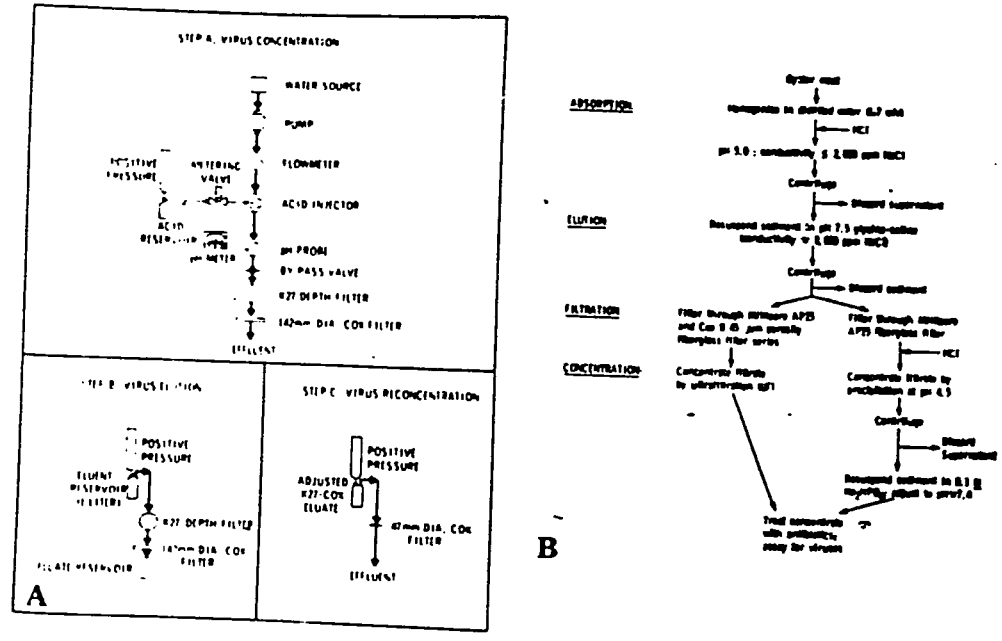
The recovery of viruses from environmental samples requires special processing to concentrate the viruses (Sobsey, 1982; Gerba 1983). Viruses can be concentrated from water by repetitive adsorption and elution processes; in one such procedure, after acidification, viruses in large volumes of water can be adsorbed onto epoxy,

fiberglass, or nitrocellulose filters (Sobsey et al., 1973). Adsorbed viruses can then be eluted with an alkaline solution. The sorption and elution process can be repeated, resulting in a many thousandfold concentration of the viruses. Viruses can also be eluted from particles such as occur in sewage sludge, using flocculating materials, and adsorptive filters that maintain the infectivity of viruses during these concentration procedures. Somewhat different procedures are required for recovery of viruses from animal or plant tissues (Sobsey et al., 1978) (Figure 7.7).

Determination of Microbial Numbers

In the case of a pure bacterial culture, the question "How many microorganisms are there?" is relatively simple to answer. When dealing with the mixed communities of environmental samples, however, the problem becomes exceedingly complex and, in most cases, defies a simple and absolute answer. Quantification is possible, but the numbers obtained need to be qualified by the technique used; enumeration techniques need to

FIGURE 7.7
An example of a viral recovery and concentration method that has been used for examination of shellfish. (From Sobsey, et al., 1978; reprinted by permission; American Society for Microbiology.)



ssen with care to assure that results are nt to the question being asked. In this r we will consider some of the complexities umerating microorganisms and some of the aches used for this purpose.

icroorganisms are extremely diverse and methods used to enumerate one group of rganisms may be inappropriate for the ation of another group. The methods used umerating viruses (Primrose et al., 1982), a (Herbert, 1982), fungi (Parkinson, 1982), Round, 1982), and protozoa (Finlay, 1982) ite different. Special techniques must be for enumerating specific physiological , such as psychrophilic bacteria or obli- anaerobic bacteria. Enumeration of such l groups requires special sampling and ing procedures. Precise differential criteria e used for enumerating an individual mi- l species such as *Escherichia coli*, which n used as an indicator of sewage contam- .

e enumeration process must begin by de- which microorganisms are to be enumer- Are all microorganisms to be enumerated only certain groups to be counted? Are nbers of microorganisms to be converted nates of biomass? What are the charac- s of the habitat being examined? There universal methods that can be applied to oorganisms and all habitats; the diversity oorganisms and their habitats requires the variety of methodological approaches. enumeration process can be broken down ee distinct phases: sampling, sample pro- , and the actual counting procedure. All of the process must be taken into con- n when interpreting the results.

re are two principal approaches used for ting microorganisms: direct observation, t count procedures and indirect or viable ocedures. Occasionally, numbers are also ed from procedures that measure specific ical constituents of microorganisms. it should be recognized that such procedures actually measure biomass. Each approach has its advantages and limitations (Atlas, 1982).

Direct Count Procedures

Microorganisms can be counted by direct microscopic observations (Jones and Mollison, 1948; Frederick, 1965; Gray, 1967; Gray et al., 1968; Harris et al., 1972; Daley and Hobbie, 1975). Direct count procedures yield the highest estimates of numbers of microorganisms and are occasionally used for the indirect calculation of biomass. There are, however, several major drawbacks to direct observational methods, including the inability to distinguish living from dead microorganisms, the underestimation of microorganisms in samples containing high amounts of background debris, and the inability to perform further studies on the observed microorganisms.

For relatively large microorganisms such as protozoa, algae, and fungi, direct counts can be conveniently accomplished using a counting chamber (Finlay et al., 1979). A variety of counting chambers that hold specified volumes, such as a haemocytometer or Petroff-Hauser chamber, can be used for counting cells (Parkinson et al., 1971).

A modified agar film technique of Jones and Mollison (1948) often is used for direct observation and enumeration of fungi (Skinner et al., 1952; Thomas et al., 1965; Parkinson, 1973). In this technique the sample is mixed with agar and pipetted onto glass slides, where it forms a thin film. The dried agar film can be stained with phenolic aniline blue, and the slides then can be viewed. Length of mycelia can be estimated by counting intersections with a superimposed grid. Alternately, the microscope image can be projected onto a screen and traced. The length can be determined by running a distance-measuring device (map measure) along the traced lines. If the average width of the mycelial filaments is also measured, the fungal biomass can be calculated (Tables 7.4 and 7.5). A problem with this technique is that unless adequate dispersion is

TABLE 7.4
Length of fungal mycelium in tundra soils

Country and location	Sites	Mean mycelium length (m/g oven dry soil)
Canada	Mesic meadow	1005
(Devon Is.)	Raised beach	199
United Kingdom	Blanket bog	4968
(Moor House)	Juncus Moor	1667
	Limestone grass	826
Antarctica	Hut Bank	6328
(Signy Is.)	Mountain moss	2783
	Grassland	288
	Old moraine	84
	New moraine	4
	Marble knolls	144
	Marble schist soils	44
Norway	Wet meadow	5000
(Hardangervidda)	Dry meadow	1000
	Birch wood	7000
Ireland	Blanket bog	995
Sweden	Mire	3260
United Kingdom	Wood	341
Canada	1900 m elevation	580
(Mt. Allen)	2500 m elevation	801
	2800 m elevation	50

After Dowding and Widden, 1974.

TABLE 7.5
Length and biomass of fungal mycelium in soil

	Length of hyphae m/g	Fungal biomass g/m²
Soil 1	174	0.31
Soil 2	619	2.69
Soil 3	457	1.39
Soil 4	1602	7.66

Miller, unpublished data.

'yphae remain hidden among the soil
nd can only be seen when soil particles
n (Skinner et al., 1952). This problem
ercome by establishing the optimum
ime and using a modern high speed
'er.

ear film technique for enumeration of
be combined with fluorescence mi-
using fluorescein diacetate as the stain
e the biomass of living fungi (Babuik
1970; Soderstrom, 1977). Only meta-
ctive cells are stained in this procedure
uorescence occurs only after enzymatic
f the stain molecule; thus use of fluo-
icetate stain in conjunction with a vital
does not distinguish living from dead
hae permits the estimation of both living
fungal biomass. A problem with this
is the high background fluorescence
s because esterases are released and
the dye.

orescense microscopy with stains such
orange (AODC method), 4'6-diamidino-
idole (DAPI), and fluorescein isothio-
ITC) are widely used for direct counting
(Strugger 1948; Babuik and Paul, 1970;
n and Meyer-Reil, 1974; Daley and
'75; Daley, 1979, Geesey and Costerton,
man, 1980, Porter and Feig, 1980).
fluorescing immersion oil is important
e background autofluorescence. Poly-
nuclepore filters are superior to cellulose
irect counting of bacteria because they
rm pore size and a flat surface that
he bacteria on top of the filter (Hobbie
7) (Table 7.6). The nuclepore filters
ned with irgalan black or other suitable
ite a black background against which
microorganisms can be counted (Jones
, 1975). When acridine orange is used
bacteria and other microorganisms
range and green. The green or orange
lates with the physiological state of
ganism but attempts to separate living
microorganisms by the color of flu-

orescence are often misleading and unsatisfactory. DAPI, which stains the DNA of bacterial cells and produces an intense blue fluorescence, has been found to be superior to acridine orange for visualizing small bacterial cells (Coleman, 1980).

Counts by direct epifluorescent microscopy are typically two orders of magnitude higher than counts obtained by cultural techniques (Table 7.7). Many small and unusually shaped bacteria are observable and countable with epifluorescence microscopy; but these forms are usually impossible to cultivate on laboratory media and it is not clear whether these are "degenerate" nonviable representatives of known bacteria or forms with as yet unknown physiological requirements. In some cases there is a high positive correlation between direct fluorescent microscopic counts and viable plate counts (Figure 7.8); in other cases the correlation is low (Table 7.8). The differences between direct and plate counts may simply reflect the selectivity of the media and incubation conditions used, the proportion of living and dead or injured bacteria, and the particular species in the sample.

The value of the direct count epifluorescent microscopy approach to enumeration is that it is applicable to a variety of habitats without the bias inherent in viable plate count procedures. It allows the estimation of numbers of microorganisms in marine, freshwater, and soil habitats despite the great differences in population sizes and physiological types that occur in these various habitats. Direct counts are often directly proportional to biomass, and thus can be used to estimate microbial biomass. Tedious size measurements must be made, however, to convert accurately the direct microscopic counts to biomass estimates.

Additionally if the numbers of dividing cells are counted it is possible to estimate *in situ* growth rates (Hagstrom et al., 1979). The frequency of dividing cells has been shown to correlate with other measures of microbial growth such as rates of RNA synthesis measured by incorporation of labelled adenine (Christian et al., 1982). This fre-

TABLE 7.6
Comparison of Nuclepore (polycarbonate) and Sartorius (cellulose nitrate) filters for direct counts

Site	Count (#/ml) Sartorius (0.5μm)	Nuclepore (0.2μm)
Estuary	8.6×10^4	1.7×10^6
Reservoir	4.0×10^5	7.2×10^5
Pond	1.7×10^6	4.0×10^6

After Hobbie et al., 1977.

quency of dividing cells (FDC) method assumes that the time between the initiation of cell constriction and cell separation is constant; this assumption is not always valid (Staley and Konopka, 1985). It is also difficult to recognize dividing cells using light microscopic methods.

The numbers of specific types of microorganisms can be estimated using fluorescent antibody techniques (Hill and Gray, 1967; Schmidt, 1973; Strayer and Tiedje, 1978; Fliermans et al., 1979; Bohlool and Schmidt, 1980). The fluorescent antibody technique is extremely specific for an individual microbial species and permits autecological studies, that is, studies of individual microorganisms in their natural environments (Bohlool and Schmidt, 1973) (Table 7.9). In the FITC method antibodies are conjugated with a fluorochrome, such as fluorescein isothiocyanate. Problems with the fluorescent antibody technique include nonspecific fluorescence of the background in some samples, the high degree of specificity of the antigen-antibody reaction that may preclude staining of different strains of the same species, and possible cross-reactions between different organisms. Fluorescent antibody techniques have been applied to studies on selected microbial species in their natural habitats, including ecologically important organisms (Eren and Pramer, 1966); monoclonal antibodies have been used to specifically detect methanogens in environmental samples (Conway de Macario et al., 1981, 1982), and such specific fluorescent dyes are applicable to enumerate many other specific populations.

Fluorescent staining methods can also be used in combination with other procedures to determine numbers of living organisms. By combining the AODC method with INT (2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride) staining, for example, it is possible to determine both total numbers of microorganisms and the numbers of microorganisms carrying out respiration (Zimmerman et al., 1978). The method is based on the fact that electron transport systems in respiring microorganisms will reduce INT to INT-formazan and therefore respiring bacteria will accumulate intracellular dark red spots. When

TABLE 7.7
Comparison of direct epifluorescent counts and viable plate counts

Sample	Soil: Direct count	Soil: Viable count	Marine water: Direct count	Marine water: Viable count
A	5.0×10^8	3.1×10^7	2.2×10^5	1.3×10^4
B	1.1×10^9	6.2×10^7	8.2×10^4	7.6×10^2
C	2.0×10^9	1.7×10^8	1.3×10^6	2.1×10^4

Atlas, unpublished data.

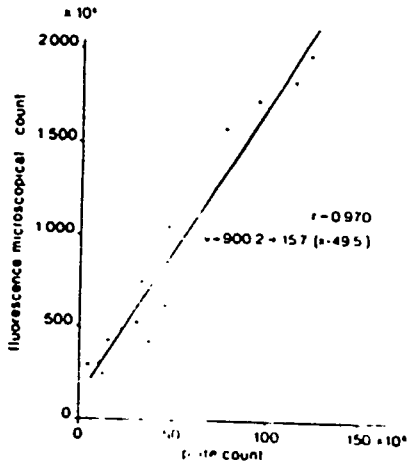


FIGURE 7.8
Correlation between direct microbial counts by fluorescence microscopy and viable plate counts for soil samples. (From Trüldenius, 1973; reprinted by permission of Swedish National Research Council.)

applied to Baltic Sea water samples, 6–12 percent of the bacteria were found to be active; in freshwater samples, 5–36 percent of the bacteria were active (Zimmerman et al., 1978). This method can be difficult to interpret depending on the size of the bacterial cells and INT grains that develop.

Numbers of actively growing bacteria can also be determined by incubation with nalidixic acid, an antibiotic that inhibits cell division (Kogure et al., 1979). Microscopic observation after fluorescent staining reveals elongated cells (actively growing microbes) and those of normal size and shape (dormant or dead microbes).

Another technique for determining bacterial numbers and the spectrum of actively metabolizing cells is autoradiography combined with direct microscopic observation (Brock and Brock, 1968; Waid et al., 1973; Ramsay, 1974; Fliermans and Schmidt, 1975; Hoppe, 1976; Faust and Correll, 1977; Meyer-Reil, 1978). In this method bacteria are incubated with a radiolabelled substrate, such as tritiated glucose, collected on a bacteriological filter, which is placed on a glass slide and coated with a photographic emulsion. The actively metabolizing bacteria are radioactive and can be identified by the dark silver grains surrounding

TABLE 7.8
Counts of bacteria (numbers/mL)

Area	Season/Year	Direct count	Viable count	
			4°C	20°C
Beaufort Sea				
Ice	Winter 1976	9.9×10^4	6.6×10^1	4.5×10^1
Water	Summer 1975	8.2×10^5	9.6×10^3	7.3×10^3
	Winter 1976	1.8×10^5	6.1×10^2	1.1×10^3
	Summer 1976	5.2×10^4	5.0×10^4	2.7×10^4
N.W. Gulf of Alaska				
Water	Summer 1975	3.0×10^5	1.0×10^5	5.2×10^5
N.E. Gulf of Alaska				
Water	Winter 1976	1.4×10^5	1.3×10^5	2.4×10^5

After Kaneko et al., 1978

TABLE 7.9
Quantification of *Rhizobium japonicum*, USDA 110, in the rhizosphere of soybean plants grown in the field

Sample (weeks after planting)	Number *R. japonicum* 110/g soil*	
	Uninoculated	110-inoculated
2	4.1×10^3	6.2×10^6
3	4.9×10^3	1.9×10^6
4	4.9×10^3	3.6×10^7
5	N.T.	6.4×10^5
6	N.T.	1.7×10^6

(N.T. = Not Tested)
* = Based on counts of 20 microscopic fields per sample.
After Bohlool and Schmidt, 1973.

the cell. Applying this method to nearshore water samples indicates that 2.3–56.2 percent of the total bacteria are metabolically active (Meyer-Reil, 1978) (Table 7.10).

Electron microscopy instead of light microscopy can be used for direct counting of microorganisms (Gray, 1967; Nikitin, 1973; Todd et al., 1973; Bowden, 1977; Larsson et al., 1978). Results of enumerations with scanning electron microscopy are comparable to those obtained by epifluorescent microscopy (Bowden, 1977) (Table 7.11). Caution must be observed in the use of electron microscopic techniques because of the possibility of producing artifacts when specimens are metal-coated to increase contrast for observation and when specimens are placed under high vacuum. As magnification increases, numerous viewing fields may have to be scanned before a microorganism is observed and, therefore, electron microscopic observation has been applied mainly to samples with naturally dense or artificially enriched microbial communities.

A particle counter, such as a Coulter counter, can also be used to estimate directly numbers of microorganisms (Kubitschek, 1969). The Coulter counter electronically measures the number of particles within a fixed size range. Se[illegible] timates of bacteria and protozoa can b[illegible] by setting appropriate size ranges. Th[illegible] with Coulter counter analysis is that s[illegible] living particles are counted together w[illegible] organisms and this difficulty has limited [illegible] fulness of this method. However recent [illegible] in image analysis permit the specific re[illegible] of microorganisms in complex samples. [illegible] be expected that cell sorters will see [illegible] usage in the enumeration microorgani[illegible]

Viable Count Procedures

There are two basic approaches to via[illegible] procedures: 1) the plate count techni[illegible] 2) the Most Probable Number (MPN) te[illegible] All viable count procedures require se[illegible] of microorganisms into individual repr[illegible] units. All viable count procedures are [illegible] for certain microorganisms; the degree [illegible] tivity varies with the particular viable co[illegible] cedure. This selectivity is a disadvanta[illegible] trying to estimate the total viable microb[illegible] mass within an ecosystem, but it perm[illegible] estimation of numbers of particular [illegible] microorganisms.

Plate Count Methods The agar plat[illegible] method, which has been widely used for [illegible] meration of viable microorganisms es[illegible] bacteria, has been severely criticized (P[illegible] 1969; Buck 1979). The problem lies in the [illegible] of the method and/or the misinterpreta[illegible] the results. All too often users of this te[illegible] fail to recognize that "total counts" ca[illegible] be achieved using this method.

Plate count procedures employ variou[illegible] and incubation conditions. Agar is mos[illegible] used as the solidifying agent because most [illegible] lack the enzymes necessary for depolym[illegible] agar. Dilutions of samples can be spread [illegible] top of the agar (surface spread method). [illegible] sample suspension can be mixed with th[illegible] just before the plates are poured (pou[illegible] method). One must consider whether the

7.10
ological variables measured in water samples taken from above sandy sedi-
t beaches of the Kiel Fjord and the Kiel Bight (4 to 13 July 1977)*

Colony-forming units/mL × 10^3 (plate counts)	Total no. of bacteria/mL × 10^6 (direct counts)	Total biomass (mg/mL × 10^{-3})	Number of active bacteria/mL × 10^6 (autoradiography)	Active bacteria /mL × total no. of bacteria)	Actual uptake rate of glucose (g/mL per h, × 10^{-3})
261	41.7	50.2	19.8	47.5	18.9
188	57.8	65.4	32.5	56.2	23.5
81.4	26.5	32.1	11.0	41.5	11.9
187	64.2	62.4	29.9	46.6	10.3
433	68.8	74.2	3.1	4.5	2.6
98.2	67.2	70.9	23.8	35.4	6.6
3.4	52.2	58.0	1.2	2.3	1.2
3.1	51.4	40.9	6.1	11.9	7.8
5.5	39.4	43.6	16.6	42.1	6.9
2.9	65.8	60.1	19.2	29.2	4.8
7.4	56.1	60.8	28.3	50.5	3.8
1.7	50.0	44.7	3.9	7.8	5.2

ark stations at the west side. A' to F' are corresponding stations at the east side of the Kiel Fjord and the Kiel
pectively. Stations A A' are located at the inner part, B/B' at the center part, and C/C' at the outer part of the
Stations D D', E E', and F F' are located at the Kiel Bight.
er-Reil, 1978

is can survive the plating procedure. Some
s are killed upon exposure to air in the
plate method; others cannot tolerate the
ture needed to maintain melted agar in
plate method. Because the agar used
ne media for bacteriological enumeration
tain organic contaminants, sometimes it is replaced with an alternative solidifying agent. When specific nutritional groups of microorganisms are to be enumerated, silica gel may be used for solidification. Because silica gel plates are more difficult to prepare than agar plates, they are used only when unavoidable.

The roll-tube method, used for enumeration

1
on of enumera-
epifluorescent and
electron micro-
estuary water

	Count (#/mL)		
	Scanning EM	Epifluorescence	
	0.2 μm Nuclepore	0.2 μm Nuclepore	0.2 μm Sartorius
Estuary 1	4.34×10^6	4.81×10^6	2.00×10^6
Estuary 2	3.30×10^6	2.92×10^6	0.78×10^6

After Bowden, 1977

of obligate anaerobes, is an extension of the pour plate method (Hungate, 1969; Hungate and Macy, 1973). The plates or tubes are incubated under specified conditions for a period of time to allow the bacteria to multiply and form macroscopic colonies after which the colonies are counted. It is assumed that each colony originated from a single bacterial cell. For this assumption to be valid it is necessary that the bacteria be widely dispersed in or on the agar. Plates with too many colonies cannot be accurately counted because one colony may represent more than one original bacterium. Plates with too few colonies also must be discarded from the counting procedure for statistical reasons.

The main considerations for doing plate count procedures are the composition of the medium, the incubation conditions, and the period of incubation. Media for the enumeration of heterotrophs not capable of nitrogen fixation must contain a usable source of carbon, nitrogen, and phosphorus and must also contain required cations and anions, such as iron, magnesium, sodium, calcium, chloride, and sulfate. It is not usually clear why a particular medium yields the highest numbers of microorganisms from a particular ecosystem. Clearly, nutritional requirements, including growth factors, must be met by the media. General purpose media have nutrient concentrations that are much higher than those found in the natural ecosystem being studied. Concentrations of nutrients in many of these media used for total enumerations are high enough to be toxic to at least some microorganisms.

The real advantage of viable enumeration procedures is that conditions can be adjusted so that only members of a defined group are enumerated. Plate count media may be designed to be selective or differential. Selective plate count procedures are designed to favor the growth of the desired group of microorganisms. Growth of other groups is precluded by media composition and/or incubation conditions. Differential media do not preclude the growth of other microorganisms, but permit detection of the desired group by some distinguishing characteristic.

Media can be designed for the selective enumeration of fungi. The viable plate count technique, though, is generally not the method of choice for enumerating fungi, because this technique favors enumeration of nonfilamentous fungi and spores (Menzies, 1965). Plate counts, however, are suitable for enumeration of yeasts. To prevent overgrowth of fungal colonies by bacteria, which are likely to be more numerous than fungi in the sample, bacterial inhibitors are added to the media. The dye rose bengal and the antibiotics streptomycin and neomycin are often added as bacterial inhibitors. A simple technique for suppression of bacteria involves the lowering of the pH of the medium to 4.5–5.5. Most fungi are unaffected by this pH, while most bacteria are suppressed.

Media designed to inhibit the growth of one group of organisms, while permitting growth of another group, are known as selective media. For example, Sabouraud dextrose agar is used to enumerate fungi based on its low pH and carbohydrate source of carbon. Incorporation of penicillin or methylene blue, which inhibit Gram positive organisms, into a medium is often used to select for Gram negative bacteria. Antibiotic resistant microorganisms can be enumerated on media with added antibiotics.

Differential media may be designed in a variety of ways. Reagents may be incorporated into the media that permit immediate visual differentiation of the desired bacteria, or reagents may be added after incubation to detect the desired bacteria. The key advantage of differential media is that the procedure permits one to distinguish between the microorganisms being enumerated and others present in the sample.

Eosin methylene blue (EMB) agar and MacConkey's agar are widely utilized media used in the determination of water quality. These media are both selective and differential. They select for the growth of Gram negative bacteria by incorporating an inhibitor of Gram positive bacteria. They differentiate bacteria capable of utilizing lactose by formation of characteristically colored colonies. On EMB agar coliform bacteria, which

am negative and utilize lactose, form a teristic colony with a green metallic sheen. ites of coliform counts determined in this r are often used as an indicator of water and for quality control in the food industry ., 1977). Less than ten coliforms/liter are ered safe for potable water supplies.

important new methodological approach detection of specific microorganisms in imental samples is the development of gene (Grunstein and Hogness, 1975; Hill et al., sayler et al., 1985). Currently the micro- ms must be cultured before they can be d with a gene probe, but the hope is that sitivity of this method will permit detection roorganisms in environmental samples the need for culturing. In this technique probe is prepared by isolating segment of information and labelling it with either otin. Bacterial cells growing on a viable medium are transfered to a suitable solid , such as a nitrocellulose filter, and lysed, e denatured DNA, which adsorps to the he filter paper with the attached DNA is ed with the gene probe. Hybridization oc- he base sequence of the lysed cells matches e sequence of the gene probe and the n of the hybrid can be detected by auto- phy for ^{32}P-labelled probes and by en- development for biotin-labelled probes. manner bacteria with specific genetic es can be specifically detected. DNA- bidization techniques should prove par- useful for the detection of specific geno- environmental samples when the specific ons are in relatively low numbers com- other populations.

Probable Number The most probable (MPN) method, an alternative to plate ethods for determination of viable or- uses statistical analyses and successive of the sample to reach a point of extinction , 1950; Alexander, 1965; Melchiorri- , 1972; Colwell, 1979). Replicate dilutions, three to ten replicates per dilution, are scored as positive or negative and the pattern of positive and negative scores are used in connection with appropriate statistical tables to obtain the most probable number of viable microorganisms. The MPN procedure gives a statistically based estimate of the number of microorganisms in the sample and when relatively few replicate tubes are used the confidence intervals are quite large. The MPN method for enumeration of bacteria has the advantage of permitting the use of liquid culture, avoiding the need to add a solidifying agent such as agar, with its possible contaminants, but it is more laborious and less precise than the plate count.

It is essential that criteria be established for differentiating positive from negative replicates. In many cases the MPN test procedures are designed so that increases in turbidity (growth) can be seen and scored as positive. In other procedures more elaborate tests, such as protein or chlorophyll determination, are required to score positive tubes. As with plate count procedures, MPN determinations can use selective and differential media.

A modified MPN method designed by Singh (1946) is often used for estimating numbers of protozoa. Multiple glass or polypropylene rings are embedded in sodium chloride agar in petri dishes, forming wells. The agar surface is inoculated with a heavy suspension of a bacterium, such as *Enterobacter aerogenes*. Dilutions of an environmental sample are inoculated into each of the wells and protozoa present in the sample dilutions feed on the provided bacterial paste, producing a readily recognizable clearing. Clearing of the bacteria within a ring is scored positive and the lack of clearing is scored negative.

An MPN method can be used for the enumeration of enteric viruses. In this method serial dilutions of the samples containing viruses are added to tubes of suitable host cells in tissue-cultures (Farrah et al., 1977). Following incubation, the tubes are examined for cytopathic effect (CPE), that is, the death of the infected cells. Quantitation can be achieved using MPN tables scoring tubes showing CPE as positive.

The numbers of viruses may also be quantitated as the $TCID_{50}$ (tissue culture infectious dose—50%), the lowest dilution of the virus suspension that caused CPE in 50% of the tubes.

As in the plate count procedures, media and incubation conditions can be adjusted in the MPN procedure to select for particular groups of microorganisms or to differentiate microorganisms with desired characteristics. Obviously, the combinations of incubation conditions and media that can be used for enumerating specific groups of bacteria are infinite. Each procedure must be carefully selected and tested to permit the correct interpretation of results.

Determination of Microbial Biomass

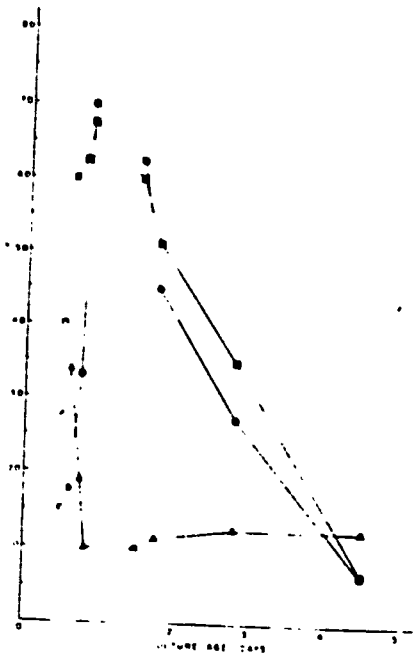
Biomass is an important ecological parameter because it measures the quantity of energy being stored in a particular segment of the biological community. Measurement of biomass is used in determining the standing crop of a population and the transfer of energy between trophic levels within an ecosystem. Biomass literally means "mass of living material" and can be expressed in units of weight (grams) which can be converted to units of energy (calories). Unfortunately, the direct measurements of microbial biomass, such as by filtration and dry weight, or centrifugation and packed cell volume measurements as practiced on pure cultures, are rarely applicable to environmental samples. These techniques tend to measure mineral and detritus particles and nonmicrobial biomass along with microorganisms and fail to discriminate between trophic levels, that is, between producers and consumers. Consequently, the determination of microbial biomass is often imprecise.

Biochemical Assays

The most practical approach to the determination of microbial biomass is to assay for specific biochemicals that indicate the presence of microorganisms. Ideally, all the microbial biomass to be determined should have the same quantity of the biochemical being assayed, so that there is a direct correlation between the amount of the biochemical measured and the biomass of microorganisms. Also the biochemical being assayed should be present only in the biomass to be determined. These two conditions are rarely, if ever, met, so that the results of quantifying a particular biochemical must be extrapolated with caution in order to estimate the biomass of microorganisms that are present.

ATP and Total Adenylate ATP is present in all microorganisms and can be measured with great sensitivity. Though dependent on physiological state, ATP concentrations are fairly uniform relative to cell carbon for many bacteria, algae, and protozoa (Figure 7.9). Because ATP is lost rapidly following the death of cells, measuring ATP concentrations can be used to estimate living biomass (Holm-Hansen, 1969; Paerl and Williams, 1976; Deming et al., 1979; Stevenson et al., 1979). ATP can be detected by the luciferin-luciferase assay in which reduced luciferin reacts with oxygen to form oxidized luciferin in the presence of luciferase enzyme, magnesium ions, and ATP. Light is emitted in this reaction and the amount of emitted light is directly proportional to the ATP concentration. ATP can also be quantitatively measured using high press liquid chromatography. The method used to extract the ATP has a marked effect on the sensitivity and reliability of the assay (Deming et al., 1979; Stevenson et al., 1979). The ATP must be extracted rapidly and in a manner that maintains the integrity of the ATP because ATP is easily converted to AMP. A variety of methods are used, including extraction with various organic solvents or hot buffers; the efficiency of a particular procedure depends upon the nature of the sample and the microbial community.

ATP measurements can be used to estimate microbial biomass. A factor of 250–286 is often used for conversion of ATP to cellular carbon for aquatic samples (Hamilton and Holm-Hansen,



1. 7.9
tion of ATP and viable count. In cultures older day, the ATP content per viable cell remains constant even during large changes in population rom Holm-Hansen, 1973; reprinted by permis- pyright 1973; University of South Carolina

Holm-Hansen and Booth, 1966; Holm-n, 1973; Paerl and Williams, 1976); for soil of 20 is used to convert ATP to biomass (Ausmus, 1973; Jenkinson and Oades, Oades and Jenkinson, 1979). There are some ies, however, in the accuracy of estimating al biomass based upon ATP measure- Some microorganisms alter their ATP trations radically when nutritional or logical conditions change. Also, in some ems such as soil, sediment, and nearshore are, ATP may be absorbed on particles (Bancroft et al., 1976; Jenkinson and Oades, 1979). The sorption of ATP interferes with its extraction and quantification. In addition, the presence of plant or animal cells, which also contain ATP, limits applicability of this method in some ecosystems.

Perhaps a better measure of microbial biomass, but one that is not universally accepted, is the total adenylate pool (A_T). The adenylate pool is insensitive to metabolic state: A_T = ATP + ADP + AMP. The total adenylate pool is determined in order to calculate energy charge, as described later in this chapter, and can conveniently be used simultaneously to estimate numbers and biomass of microorganisms. The advantage of using A_T is that it does not vary greatly during changes in metabolic activities of the organisms, whereas ATP is highly dependent on the physiological state of the organism. When determinations of the total adenylate pool are used in conjunction with ATP measurements, the energy charge, which is a measure of the physiological state of the organisms within the sample, can be calculated (Wiebe and Bancroft, 1975; Karl and Holm-Hansen, 1978). The energy charge (EC), which is independent of population size is calculated as EC = (ATP + 1/2 ADP)/(ATP + ADP + AMP). Actively growing cells have an EC ratio of 0.8–0.95; cells in stationary growth phase have an EC ratio of about 0.6 and senescent or resting cells have an EC ratio of less than 0.5.

Cell Wall Components Almost all bacteria contain muramic acid in their cell walls and the specific relationship between murein and bacteria makes quantitation of this cell-wall component useful for estimating bacterial biomass (Millar and Casida, 1970; Moriarty, 1975, 1977, 1978; King and White, 1977; White et al., 1979). The assay for muramic acid is based on the release of lactate from muramic acid and either enzymatic (Moriarty, 1977) or chemical analysis (King and White, 1977) to determine the concentration of lactate.

The conversion of muramic acid measurements to biomass assumes that all Gram positive bacteria have a ratio of 45 μg MA/mg C and that all Gram negative bacteria have 12 μg MA/mg C. In reality there is a gradient of concentrations of muramic acid in Gram positive and Gram negative bacteria. To accurately use this method, it is necessary to estimate the proportions of Gram negative and Gram positive bacteria in the sample; erroneous estimates of these proportions will yield inaccurate estimates of biomass.

Gram negative bacteria contain lipopolysaccharide as part of their cell wall complex. Lipopolysaccharide can be quantitated using Limulus amoebocyte lysate (LPS) method (Watson et al., 1977, Watson and Hobbie, 1979). This method uses an aqueous extract from the blood cells of horseshoe crabs (*Limulus polyphemus*) that reacts specifically with lipopolysaccharide to form a turbid solution; the degree of turbidity is directly proportional to the lipopolysaccharide concentration and can be quantitatively assayed. Because lipopolysaccharide occurs only in the cell walls of Gram negative bacteria and occurs in a relatively constant proportion, it is a useful method for estimating numbers of bacteria in ecosystems dominated by Gram negative forms. The LPS method is very sensitive, capable of detecting concentrations as low as 1000/mL and correlates well with counts obtained by other measures of biomass (Table 7.12 and Figure 7.10).

To estimate fungal biomass, chitin can be measured (Swift, 1973; Sharma et al., 1977; Willoughby, 1978) because chitin occurs in the cell walls of many fungi but not in plants or other soil microorganisms. Measurement of chitin can therefore be used to estimate fungal biomass in the presence of other microbial populations and can be used to determine fungal biomass associated with plants, including plant roots in soil. The presence of microarthropods, though, does interfere with such determinations.

Chlorophyll and Other Photosynthetic Pigments Assuming the absence of plants, it is possible to estimate numbers of photosynthetic algae and bacteria by measuring chlorophyll or other photosynthetic pigments (Edmondson, 1974; Cohen et al., 1977; Holm-Hansen and Riemann, 1978). Chlorophyll a, the dominant photosynthetic pigment in algae and cyanobacteria is a useful measure of the biomass of these photosynthetic microorgansisms even though there may not be a constant relationship between biomass and chlorophyll content (Banse, 1977). Estimates of the biomass of photosynthetic microorganisms based upon chlorophyll determinations have been found to correlate well with such estimates based upon ATP determinations (Paerl et al., 1976). Chlorophyll a can be extracted with solvents, such as acetone or methanol, and quantified by measuring absorbance with a spectrophotometer using a wavelength of 665 nm. This wavelength can be adjusted to the corresponding absorption maxima to measure bacterial chlorophylls; for example, 850 nm is used for determining the biomass of purple photosynthetic bacteria. It is thus possible to estimate the biomass of different photosynthetic microbial populations in the same sample by estimating various chlorophylls that absorb at different wavelengths (Stanier and Smith, 1960; Caldwell and Tiedje, 1975).

The chlorophyll concentration in the extract can also be determined by spectrofluorometry (Lorenzen, 1966; Loftus and Carpenter, 1971; Sharabi and Pramer, 1973; Caldwell, 1977). Chlorophyll a, for example, has an excitation wavelength of approximately 430 nm and a wavelength of maximum fluorescence of approximately 585 nm. The spectrofluorometric determination is more selective than the spectrophotometric method. The double selectivity of excitation and fluorescence maxima tend to reduce interference of nonspecific absorption that may affect spectrophotometric determinations (Sharabi and Pramer, 1973).

DNA Concentrations of DNA are maintained in relatively constant proportions within microorganisms and determination of DNA can be used

unts of cell
and muramic
lls of a Gram
Gram nega

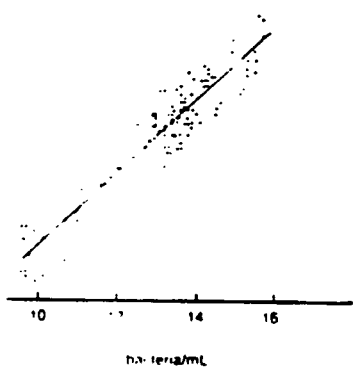
	Cell C mg	ATP μg	Muramic acid μg
Gram Positive			
Bacillus	9×10^{-2}	1.1×10^{1}	3×10^{6}
Gram Negative			
Pseudomonas	1×10^{-1}	0.5×10^{6}	0.5×10^{6}

After Moriarty, 1977

e of microbial biomass (Holm-Hansen Hobbie et al., 1972; Paul and Meyers, y and Olson, 1985). For environmental here sensitivity is critical for accurate minations, reaction with fluorescent s ethidium bromide or Hoechst 33258, ofluometry are generally employed. ification of the DNA is necessary in s to prevent interference and it is control for the presence of eukaryotic mates of biomass based upon DNA determinations and those by direct counts show significant correlations (McCoy and Olson, 1985).

Protein Protein is easily quantified (Lowry et al., 1951) and bacterial haeme proteins can be specifically detected by chemiluminescence (Oleniacz et al., 1968) but the use of protein measurements for estimating numbers of microorganisms is limited to situations where background protein levels are negligible. Also, different microorganisms contain different amounts of protein and thus, protein determinations are best used for estimation of microbial biomass when only a single population is present. Hence, total protein measurements have only limited applicability to environmental samples.

LPS and direct counts of bacteria (note am = 10^{-15} g). (From Watson, et al., 1977; permission: American Society for)



Physiological Approaches to Biomass Determination

Physiological approaches for estimating microbial biomass have been developed based upon respiratory activities. One such approach, which has been used to measure microbial biomass in soil, involves fumigating soil with chloroform and then measuring the CO_2 released from the mineralization of the microorganisms killed by the fumigation (Jenkinson, 1976; Jenkinson and Powlson, 1976; Anderson and Domsch, 1978a). In this procedure the soil microbes are killed by fumigation with $CHCl_3$ and subjected to mineralization by reinoculating the soil with a small amount of the original soil; nonchloroform-treated

soils incubated under the same conditions act as controls and the amount of microbial carbon is calculated from the difference between the CO_2-C evolved from fumigated and nonfumigated samples. Conversion factors of 0.4–0.5 are recommended based on determinations made with various radiolabelled bacterial and fungal cultures. Average mineralization of fungi is 44 percent and 33 percent for bacteria; assuming a bacterial to fungal biomass ratio of 1.3 for soil, the combined average mineralization should be 41 percent. A high correlation has been found between soil ATP and biomass determined by this fumigation method (Oades and Jenkinson, 1979) (Table 7.13).

Another physiological approach for estimating microbial biomass involves measurement of respiration rates after substrate addition (Anderson and Domsch, 1973, 1975, 1978b). The peak respiration rate measured during a short period is assumed to be proportional to the numbers of viable microorganisms in the samples. Microbial inhibitors can be used in the procedure to obtain separate estimates of bacterial and fungal biomass. Good correlation has been reported between this peak respiration rate method and the chloroform fumigation method.

Measurement of Microbial Metabolism

Recent advances in analytical methodology provide the sensitivity needed to measure natural rates of microbial metabolic activities but it remains difficult to make such rate measurements that reflect actual activities within natural ecosystems (Christian et al., 1982; van Es and Meyer-Reil, 1982; Staley and Konopka, 1985). The difficulty lies in the fact that most methods require manipulation of the system in ways that can alter

TABLE 7.13
Comparison of biomass estimated by soil fumigations and ATP in various soils

Soil no.	CO_2-C Evolved by unfumigated soil in 10 days ($\mu g\ g^{-1}$)	CO_2-C Evolved by fumigated soil in 10 days ($\mu g\ g^{-1}$)	Biomass C ($\mu g\ g^{-1}$)	ATP in soil ($\mu g\ g^{-1}$)
1	70	114	88	0.75
2	205	315	220	2.07
3	131	240	218	3.14
4	20	43	48	0.64
5	236	613	754	9.03
6	108	158	99	0.90
7	117	341	448	2.97
8	264	565	603	4.56
9	76	143	135	1.37
10	259	642	766	7.00
11	257	646	778	6.55
12	85	189	208	1.32
13	229	525	591	4.20

After Oades and Jenkinson, 1979.

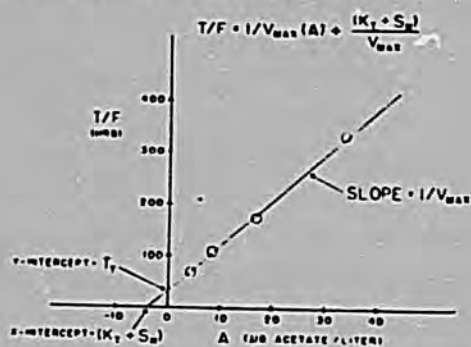
f microbial activity. Even minor manip- can greatly alter the microenvironmental ters that determine rates of microbial ac- as examples, simply enclosing a water produces a wall effect that can change s of microbial activity and removal of for activity determinations can change parameters, such as oxygen concentra- hereby changing the rates of microbial s being measured.

rophic Potential

proach to measuring natural rates of het- ic microbial activity is to measure uptake tracer levels of radiolabelled substrates mine the heterotrophic potential for the n of that substrate (Wright and Hobbie, 66; Hobbie and Crawford, 1969; Wright, 78). This approach assumes that micro- s take up solutes from solution according rder or saturation kinetics and that the uptake increase with increasing concen- of the substrate up to a maximal uptake ...). By plotting the rates of uptake of labelled substrates against their concen- V_{max}, can be calculated. The concentra- substrates used must be low and must nate their natural concentrations in the ent. In addition to measuring the max- ke potential, this method can be used te the turnover time for a given substrate nsport constant that reflects the affinity ven substrate. Natural turnover time is s the number of hours required for the heterotrophic population to take up espire a quantity of substrate equal in ation to the existing *in situ* concentration. procedure for determining heterotrophic consists of adding various concentrations abel d substrate to samples and incu- sa ples under conditions that simulate nv onm nt. After incubation the cells ter on a filter and the incorporated ity is counted by liquid scintillation. uptake was only measured by incor- poration into cells; however, this method neglected the amount of substrate that had been taken up and metabolized. Therefore, the method was modified so that $^{14}CO_2$ produced during respiration is trapped and this amount is added to the counts of ^{14}C that are incorporated into the cells (Hobbie and Crawford, 1969).

If one assumes that uptake by a heterogeneous heterotrophic population acts like a single enzyme because uptake by different bacteria is functionally equivalent the results should be comparable to Michaelis-Menten enzyme kinetic analyses. Using the linearized form of the Michaelis-Menten equation: $T/F = (K_t + S_n)/V_{max} + A/V_{max}$, where T/F in hours is time divided by the fraction of added substrate assimilated into the cells or converted into CO_2, A is the concentration of substrate added, $K_t + S_n$ is the transport constant plus the natural concentration of that substrate, and V_{max} is the maximal rate of substrate uptake, a plot of T/F versus A is linear (Figure 7.11). Using T/F as the "Y" axis and A as the "X" axis, the slope of the resulting line is equal to $1/V_{max}$; the "Y" intercept is equal to T_t (the

FIGURE 7.11
Measurement of heterotrophic potential showing modified Lineweaver-Burke plot of uptake kinetics: T_t = turnover time, V_{max} = maximal uptake velocity, $K_t + S_n$ = transport constant + natural substrate concentration, T/F = velocity, A = substrate concentration. (From Wright and Hobbie, 1966; reprinted by permission; copyright 1966, Ecological Society of America.)



turnover time) and the "X" intercept is equal to $K_t + S_n$. If the *in situ* concentration of the substrate is measured independently, then the transport constant K_t for that substrate can be determined. Conversely, if K_t can be determined independently, the equation may be used to calculate S_n, which is frequently too low to be detectable by direct chemical analysis.

The value of measuring V_{max} is that it gives a real estimate of *in situ* heterotrophic activities because the V_{max} value is a function of the existing population. It is sensitive to environmental changes, even when these changes do not affect population size. V_{max} is of great value when comparing seasonal and spatial differences of the metabolic activities of indigenous microorganisms.

The estimation of rates of microbial activity with this method is limited by the substrate(s) used. Using replicate samples, it is possible to determine the relative V_{max} values and turnover times for a variety of substrates. Studies of heterotrophic potentials have utilized acetate, glucose and other carbohydrates, glutamate and other amino acids, or mixed ^{14}C-labelled photosynthate produced by algae incubated with $^{14}CO_2$ in light.

There are numerous problems with the performance and interpretation of heterotrophic potential measurements that should be mentioned. The substrate concentrations added must be within the right range to give a straight line slope that allows for calculation of V_{max}, T_t, and $K_t + S_n$. This generally requires adding many substrate concentrations, some of which are not utilized in the calculation. Adding too high a concentration of substrate saturates the system and should yield a rate equal to V_{max}. Excessive concentrations of added substrate, however, may be inhibitory. A more refined mathematical treatment is necessary to account for competitive and noncompetitive inhibitory effects. At present, the measurement of heterotrophic potential does not account for competitive inhibition and assumes a unique transport system for each substrate being measured but this assumption is not valid as many substrates are carried by common transport enzymes.

The measurement of heterotrophic potential also assumes that all members of the microbial population are responding in the same way to variations in solute concentration. Again, this need not be true (Vaccaro and Jannasch, 1967). In some cases there is evidence for differential uptake rates by various bacterial populations and by bacterial versus algal populations. Lower concentrations of added substrate generally give satisfactory slopes for calculation of V_{max} for bacterial populations and higher concentrations of substrates give satisfactory slopes for calculation of V_{max} for algal populations. Although heterotrophic potential measurements correct for $^{14}CO_2$ production, no correction is made for production of other volatile compounds and this can cause a serious error when methane or volatile fatty acids are major metabolic products.

In addition to measurement of V_{max} and T_t, the percent respiration ($^{14}CO_2$ produced/$^{14}CO_2$ + $^{14}CO_2$ + ^{14}C assimilated) can be calculated. The percent respiration is indicative of the energy required for maintenance; the greater the percent respiration, the greater the metabolic energy being used for maintenance of the microbial population and the lower the percent respiration, the greater the proportion of metabolic energy being used for assimilation and growth.

The specificity of the heterotrophic potential measurement is both a problem and an advantage for the substrate being measured. The method gives an estimate for specific heterotrophic activity for a particular substrate; it does not measure overall heterotrophic activity. Measurement of heterotrophic potentials permits studies on the effects of factors, such as pollutants on microbial activities.

Growth Rate Based Upon Nucleotide Incorporation

Because DNA is synthesized in growing cells at a rate that is proportional to biomass, the rate of DNA synthesis reflects the growth rates of microorganisms (van Es and Meyer-Reil, 1982). Microbial growth rates have been determined in

ironmental samples by incubating samples with ated thymidine, using autoradiography of ples to determine rates of nucleotide incor- ation (Brock and Brock, 1968; Brock, 1969). s technique is only applicable to organisms t can be recognized microscopically and for ich the relation between growth rate and ac- mulation of radiolabel can be determined in- endently. Various other analyses have been d to determine the incorporation of radiola- led nucleotides into RNA and DNA (Karl, 9, 1980, 1981; Fuhrman and Azam, 1980; Karl l., 1981a, 1981b; Staley and Konopka, 1985). he concentrations of RNA and DNA are wn, the growth rates of the indigenous mi- bial community can be determined by this hod.

ductivity and Decomposition

Photosynthesis In addition to heterotrophic ential measurements, which assess secondary luctivity, rates of primary production (syn- is) can be made using radiotracer and other hods. Both heterotrophic and autotrophic as- lation of carbon dioxide can be measured g radiolabelled bicarbonate by incubating a ple containing the indigenous microbial com- nity with the radiolabelled substrate and then rmining the amount of $^{14}CO_2$ assimilated into cellular organic matter by filtering the cells counting the ^{14}C trapped on the filters (Hubel, Gorden, 1972). Washing the filters eliminates unincorporated ^{14}C radiolabelled bicarbonate. residual ^{14}C-containing organic compounds be oxidized with acid dichromate and the ased $^{14}CO_2$ trapped and quantitated.

In actual field measurements, photosynthetic heterotrophic-chemolithotrophic incorpora- of $^{14}CO_2$ are differentiated using light and bottle sets. In a typical procedure, both r (light) and opaque or covered (dark) bottles filled with water samples, radiolabelled bi- onate is added and the bottles are incubated *u* for several hours or an entire photoperiod. Incorporation results from the dark bottles are subtracted from incorporation results in the light bottles to obtain the net photosynthetic incor- poration. Unlabelled CO_2 (bicarbonate) in the samples is measured to calculate the actual specific $^{14}CO_2$ activity in the water samples and, with sufficient measurements, rates of productivity for daily or annual periods can be determined.

The time frame of $^{14}CO_2$ incorporation ex- periments is relevant to the interpretation of the results. Short experimental periods of one or two hours give values close to the gross primary pro- ductivity, because there is little chance for the incorporated ^{14}C to be mineralized again by res- piration. The values obtained for long exposure times will be closer to net primary productivity (photosynthesis minus respiration), because some of the incorporated ^{14}C will be mineralized under these conditions.

Respiration $^{14}CO_2$ release from labelled sub- strates can also be used to determine decom- position rates for specific substrates. The complete degradation of a compound to its mineral components in which the organic carbon of the compound is converted to carbon dioxide by respiration is called mineralization. Rates of de- composition of both synthetic and natural organic compounds can be determined with this method (Figure 7.12). Measurements of $^{14}CO_2$ release from synthetic organic chemicals, such as pesticides, are essential for evaluating the environmental safety of such compounds because, unless mi- neralized by microorganisms, they will accumulate in the environment. In performing such analyses it is essential to consider the position of the ^{14}C label because one portion of a molecule can be degraded while another portion remains intact (Bartha and Pramer, 1970).

Various other methods that do not employ radiolabelled substrates can also be used to mea- sure rates of microbial respiration. These methods normally measure either rates of oxygen con- sumption or rates of carbon dioxide production. As long as aerobic conditions are maintained,

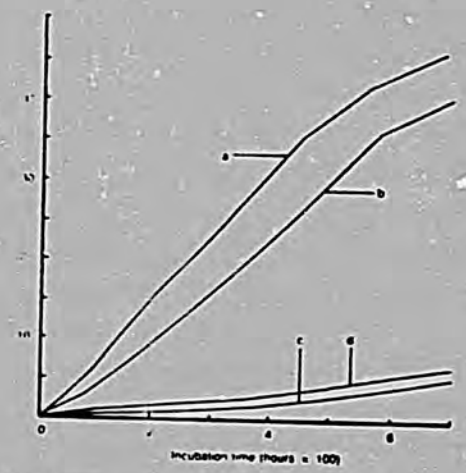


FIGURE 7.12
Decomposition of ^{14}C-labelled hemlock lignocellulose by soil microorganisms. The use of lignocellulose labelled specifically in different portions of the molecule permits identification of the origin of the CO_2.
(a) ^{14}C cellulose labelled lignocellulose soil #1.
(b) ^{14}C cellulose labelled lignocellulose soil #2.
(c) ^{14}C lignin labelled lignocellulose soil #1.
(d) ^{14}C lignin labelled lignocellulose soil #2.
(From Crawford, et al., 1977; reprinted by permission; American Society for Microbiology.)

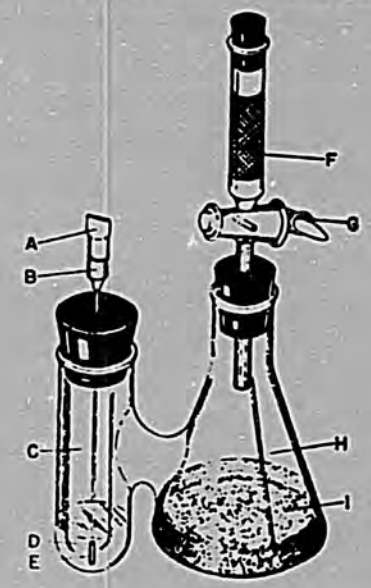
CO_2 evolution can be used as a measure of mi- crobial respiratory activity with reasonable ac- curacy (Pramer and Bartha, 1972). For long-term studies, rates of carbon dioxide production are often determined using specially designed enclosed flasks, such as the Biometer flask (Bartha and Pramer, 1965) (Figure 7.13) or using flow-through incubation systems, such as "gas trains," that pass a stream of CO_2-free air through the incu- bation flask and trap CO_2 from the effluent gas stream (Atlas and Bartha, 1972). The carbon dioxide that is trapped can be quantitated by titration of the trapping base solution with acid of known concentration to reach a point of neutrality.

Besides measuring rates of carbon dioxide evolution, rates of oxygen consumption can also be used as a measure of respiratory activity. Various oxygen electrodes are available for such measurements and newly developed microprobes can be used for *in situ* measurements (Revsbeck, 1983; Revsbeck et al., 1983).

Specific Enzyme Assays

A variety of enzyme assays also can be used for measuring the metabolic activities of indigenous microorganisms. Some, such as measurement of

FIGURE 7.13
The Biometer flask is a compact, commercially available enclosed unit used for measuring carbon dioxide production from soil. It has the advantage of being able to withdraw and replace CO_2-absorbing alkali without exposing the system to contamination by atmospheric CO_2. (A) rubber closure, (B) syringe needle, (C) side-arm, (D) alkali, (E) needle guard, (F) CO_2 absorbent (ascarite), (G) stopcock, (H) sample compartment, (I) (soil). (From Bartha and Pramer, 1965; reprinted by permission; copyright Williams and Wilkins, Baltimore.)



ase and phosphatase activities, assay ivities of a relatively large portion of al community; other assays, such as nt of cellulase, chitinase, and nitro- ivities, assay the metabolic functions important segments of the microbial (Table 7.14). Enzymes involved in ical cycling are of interest to microbial of particular importance are enzymes for carbon and nitrogen cycling. It is important that the microbial community not be altered during the assay procedure if the measurement of enzymatic activity is to reflect *in situ* activities accurately. Caution must be given to maintenance of *in situ* conditions, particularly with reference to temperature, moisture content, and E_h, and incubation periods must be short enough to preclude changes in numbers of microorganisms, which could alter the levels of enzymes present in the sample.

nzyme assays

Substrate	Description of assay
se Triphenyltetrazolium chloride	Dehydrogenases convert triphenyltetrazolium chloride to triphenyl-formazan, the triphenylformazan is extracted with methanol and quantitated spectrophotometrically.
p-Nitrophenol phosphate	Phosphatases convert the *p*-nitrophenol phosphate to *p*-nitrophenol which is extracted in aqueous solution and quantitated spectrophotometrically
Gelatin	Gelatin hydrolysis, as an example of proteolytic activity, can be measured by the determination of residual protein.
Starch	The amount of residual starch is quantitated spectrophotometrically by the intensity of the blue color resulting from its reaction with iodine.
Chitin	Production of reducing sugars is measured using anthrone reagent.
Cellulose	Production of reducing sugars is measured using anthrone reagent.
Carboxymethyl cellulose	Cellulases alter the viscosity of carboxymethyl cellulose which can be measured.
Acetylene	Nitrogenase, besides reducing dinitrogen gas ($N{\equiv}N$) to ammonia (NH_3), is also capable of reducing acetylene ($HC{\equiv}CH$) to ethylene (C_2H_4), the rate of formation of ethylene can be monitored using a gas chromatograph with flame ionization detector and the rate of nitrogen fixation can be calculated using an appropriate conversion factor.
Nitrate	Dissimilatory nitrate reductase can be assayed the disappearance of nitrate or by measuring the evolution of denitrification products, such as nitrogen gas and nitrous oxide, from samples using a gas chromatograph; denitrification can be blocked at the nitrous oxide level by the addition of acetylene, permitting a simpler assay procedure.

Summary

Numbers, biomass, and metabolic activity are the fundamental biotic parameters of microbial ecosystems. Much remains to be done to improve our accuracy and sensitivity in measuring these parameters. Further advances in microanalytical techniques are likely to improve these measurement procedures and reduce interferences. It is equally important to be aware of the true meaning and limitations of each measurement procedure. Knowing that a "total viable count" typically enumerates only about 1 percent of a microbial community places such data in the proper perspective. Although under some conditions numbers, biomass and metabolic activity show proportional correlations, under many realistic circumstances they do not. This dictates caution and critical thinking when one parameter is calculated or projected from another directly measured parameter.

REFERENCES & SUGGESTED READINGS

Alexander, M. 1965. Most-probable-number method for microbial populations. In C. A. Black (ed.) *Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties.* American Society of Agronomy, Madison, Wis., pp. 1467–1472.

American Public Health Association. 1975. *Standard Methods for the Examination of Water and Wastewater*, 14th Ed. American Public Health Association.

Andersen, A. A. 1958. A new sampler for the collection, sizing, and enumeration of the viable airborne bacteria. *Journal of Bacteriology* 76:471–484.

Anderson, J. P. E., and K. H. Domsch. 1973. Quantification of bacterial and fungal contributions to soil respiration. *Archiv fur Mikrobiologie* 93:113–127.

Anderson, J. P. E., and K. H. Domsch. 1975. Measurement of bacterial and fungal contributions to respiration of selected agricultural and forest soils. *Canadian Journal of Microbiology* 21:314–322.

Anderson, J. P. E., and K. H. Domsch. 1978a. Mineralization of bacteria and fungi in chloroform-fumigated soils. *Soil Biology and Biochemistry* 10:207–213.

Anderson, J. P. E., and K. H. Domsch. 1978b. A physiological method for the quantitative measurement of microbial biomass in soil. *Soil Biology and Biochemistry* 10:215–221.

Aristovskaya, T. V. 1973. The use of capillary techniques in ecological studies of microorganisms. *Bulletin from the Ecological Research Committee* (Stockholm) 17:47–52.

Aristovskaya, T. V., and O. M. Parinkina. 1961. New methods of studying soil microorganism associations. *Soviet Soil Science* 1:12–20.

Atlas, R. M. 1982. Enumeration and estimation of microbial biomass. In R. G. Burns and J. H. Slater (eds.), *Experimental Microbial Ecology.* Blackwell Scientific Publications, Oxford, England, pp. 84–104.

Atlas, R. M., and R. Bartha. 1972. Degradation and mineralization of petroleum by two bacteria isolated from coastal water. *Biotechnology and Bioengineering* 14:297–308.

Atlas, R. M., and J. S. Hubbard. 1974. Applicability of radiotracer methods of measuring $^{14}CO_2$ assimilation for determining microbial activity in soil including a new *in situ* method. *Microbial Ecology* 1:145–163.

Atlas, R. M., D. Pramer, and R. Bartha. 1978. Assessment of pesticide effects on nontarget soil microorganisms. *Soil Biology and Biochemistry* 10:231–239.

Ausmus, B. S. 1973. The use of the ATP assay in terrestrial decomposition studies. *Bulletin from the Ecological Research Committee* (Stockholm) 17:225–234.

Babuik, L. A., and E. A. Paul. 1970. The use of fluorescein isothiocyanate in the determination of the bacterial biomass in grassland soil. *Canadian Journal of Microbiology* 16:57–62.

W., T. J. Novitsky, H. L. Quinby, and ·s 1977. Determination of bacterial num- ·mass in the marine environment. *Ap- ·nvironmental Microbiology* 33:940–946.

(·, R. J. Bobbie, J. S. Herron, J. D. S. J. Morrison. 1979. Biochemical mea- ·of microbial mass and activity from envi- samples. In J. W. Costerton and R. R. ·s). *Native Aquatic Bacteria: Enumera- ·ry and Ecology*. ASTM Special Technical ·No. 695, American Society for Testing ·ials, Philadelphia, pp. 69–81.

J., and K. Bancroft. 1975. Use of the ·energy charge ratio to measure growth ·tural microbial communities. *Proceedings ·onal Academy of Science USA* 72:2112–

·v, L. G. 1976. Methods for studying mi- ·ms on decaying leaves and wood in fresh D. W. Lovelock and R. Davies (eds.). ·s for the Study of Mixed Populations* Press, London, pp. 31–50.

W. 1972. The coliform count. In R ·d.) *Water Pollution Microbiology*. John · Sons, New York, pp. 333–345.

: T. 1978. Measurement and significance ·bacteria of natural waters. *Applied and ·ental Microbiology* 36:297–305.

Wright, R. T. 1973. Some difficulties in using ^{14}C-organic solutes to measure heterotrophic bacterial activity. In L. H. Stevenson and R. R. Colwell (eds.). *Estuarine Microbial Ecology*. University of South Carolina Press, Columbia, pp. 199–217.

Wright, R. T., and J. E. Hobbie. 1965. The uptake of organic solutes in lake water. *Limnology and Oceanography* 10:23–28.

Wright, R. T., and J. E. Hobbie. 1966. Use of glucose and acetate by bacteria and algae in aquatic ecosystems. *Ecology* 47:447–464.

Yoshinari, T., and R. Knowles. 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. *Biochemical and Biophysical Research Communications* 69:705–710.

Zimmerman, R., R. Iturriaga, and J. Becker-Birck 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. *Applied and Environmental Microbiology* 36:926–935.

Zimmerman, R., and L.-A. Meyer-Reil 1974. A new method for fluorescence staining of bacterial populations on membrane filters. *Kieler wissenschaftliche Meeresforschungen* 30:24–27.

ZoBell, C. E. 1941. Apparatus for collecting water samples from different depths for bacteriological analysis. *Journal of Marine Research* 4:173–188.

8

Effects of Abiotic Factors on Microorganisms

Abiotic Limitations to Microbial Growth

Liebig's Law of the Minimum

Justus Liebig, a German agricultural chemist active during the middle of the nineteenth century, recognized that, like atoms in a molecule, elements in a living organism are present in distinct proportions (Liebig, 1840). According to Liebig's law, the total yield or biomass of any organism will be determined by the nutrient present in the lowest (minimum) concentration in relation to the requirements of that organism. In any given ecosystem there will be some limiting nutritional factor, and hence, for example, crop yield cannot be increased by the addition of excess phosphorus if the soil is suffering from a shortage of nitrogen.

Liebig's Law of the Minimum applies to microorganisms just as it applies to plants and animals. Increase in the concentration of a particular limiting nutrient allows the affected population to grow or reproduce until another factor becomes limiting. In a given ecosystem, the growth of one microbial population may be limited by concentrations of available phosphorus and adding nitrogen will not permit additional growth of that population. Within the same ecosystem, however, growth of another microbial population may be limited by concentrations of available nitrogen and the addition of nitrogen would permit further growth of those organisms.

Shelford's Law of Tolerance

The occurrence and abundance of organisms in an environment are determined not only by nutrients, but also by various physicochemical factors such as temperature, redox potential, pH, and many others. Shelford's Law of Tolerance describes how such abiotic parameters control the abundance of organisms in an ecosystem, and states that for survival and growth each organism requires a complex set of conditions (Shelford, 1913). For an organism to succeed in a given environment, each of these conditions must remain within the tolerance range of that organism and if any condition, such as temperature, exceeds the minimum or maximum tolerance of the organism, the organism will fail to thrive and will be eliminated. Consequently, psychrophilic microorganisms cannot grow in ecosystems with high temperatures; obligately an-

·croorganisms cannot survive conditions ·ygen tension; obligately halophilic mi- ·nnot grow in freshwater lakes; and so ·e tolerance ranges of microorganisms ·luctuations of chemical and physical ·an ecosystem do not determine which ·nisms are present but rather which mi- ·sms can be present on a sustained basis ·osystem.

·ility the presence and success of an ·or a group of organisms in an ecosystem ·both on nutrient requirements and on (Odum, 1971). The population levels ·ganisms are controlled in an ecosystem ·uantity and diversity of materials for ·y have a minimum requirement, by ·hysical factors, and by the tolerance ·the organisms themselves to these and ·ponents of the environment. Tolerance ·a given parameter are interactive with ·meters and thus, a microorganism which ·e able to survive at a particular tem- ·in an e· ·ystem with a particular hy- ·n concentration, may be able to survive ·me temperature in another ecosystem ·ferent hydrogen ion concentration. The ·e nature of environmental determinants ·es the task of microbial ecologists in ·recisely the controlling or limiting factors ·ecosystems. Nevertheless, from a be- ·vancy of physico-chemical conditions, ·ngle one can be identified that, by ex- ·he tolerance limit, excludes an organism ·ven environment.

·easures of environmental determinants ·average condition prevailing in a large ·d fail to consider the existence of mi- ·s within the samples where microor- ·ctually live. As an example, the oral ·often considered to be an aerobic en- ·t because of the continuous passage of ·h it but there are anaerobic micro- within the oral cavity where obligate ·s survive and grow. One must also con- ·fact that interactions among microbial populations can alter the environment in such a way as to allow for or prevent the growth of other microorganisms. For example, a microorganism may produce acidic metabolic products, preventing the survival in that ecosystem of microorganisms that cannot tolerate conditions of low pH, or conversely a microorganism may carry out a metabolic activity, such as nitrogen fixation, whose by-products are required for growth by another microorganism.

Another important consideration in examining limiting or tolerance factors is availability. A nutrient may be present in an ecosystem but in a form that is not available to microorganisms. A microbial ecologist must consider not only the presence of materials but also their availability to the microbes. Toxic compounds, for example, may be bonded to particles such as clay and be unavailable for interaction with microorganisms; microorganisms are protected from the direct effects of the toxic material in such situations and can therefore tolerate higher concentrations of the toxic substance. Reduced availability of required substances can prevent microbial growth. Water or nutrients, for example, may become bound and unavailable for microbial uptake. Lack of availability may also occur because the chemical is in the wrong form. For example, nitrogen gas is present in virtually all ecosystems, but this form of nitrogen is not usable by most microorganisms and, thus, in many ecosystems available nitrogen is a limiting factor; phosphate is unavailable in environments that have high divalent cation concentrations; iron in its oxidized (Fe^{3+}) form is insoluble and unavailable for microbial uptake.

Environmental Determinants

Having considered the underlying reasons for examining the physical and chemical properties of an ecosystem, we shall now consider some specific environmental determinants that control microbial growth and activity in an ecosystem.

The extremes of environmental conditions that microorganisms may have to tolerate include: high temperatures approaching that of boiling water, low temperatures approaching freezing, low acidic pH values, high alkaline pH values, high salt concentrations, low water availability, high irradiation levels, low concentrations of usable nutrients, and high concentrations of toxic compounds. Many microorganisms that inhabit extreme environments, including hot springs, salt lakes, and Antarctic desert soils, possess specialized adaptive physiological features that permit them to survive and function within the physicochemical constraints of these ecosystems (Alexander, 1976; Gould and Corry, 1980; Kushner, 1978, 1980). The membranes and enzymes of microorganisms inhabiting extreme environments often have distinct modifications that permit them to function under conditions that would inhibit active transport and metabolic activities in organisms lacking these adaptive features. Because relatively few microbes possess these adaptations, diversity in extreme environments generally is low.

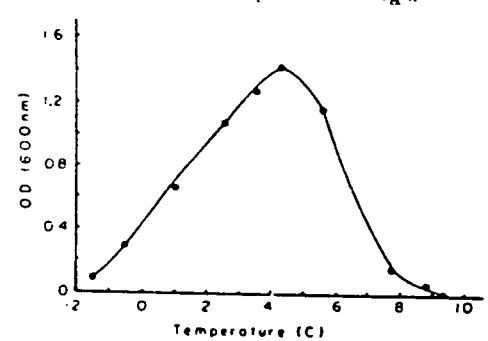
Temperature

All microorganisms have a characteristic optimal growth temperature at which they exhibit their highest growth and reproduction rate. Microorganisms also have minimal growth temperatures below which they are metabolically inactive and do not demonstrate growth and upper temperature limits beyond which they fail to grow. In most ecosystems, temperature fluctuates on a daily and seasonal basis. Measurement of temperature at the time of sample collection does not reflect the range of temperatures which the organism must tolerate, nor does it indicate whether that single temperature prevails during the entire time of microbial growth and activity. Considering the temperature fluctuations that occur in most habitats and the abilities of most microorganisms to tolerate some fluctuation around their optimal growth temperatures, the recording accuracy for temperature in most cases need only be to the degree centigrade.

Both the optimal growth temperature and the range of temperatures that a particular microorganism can tolerate determine whether that microorganism will survive and what role it will play in a given ecosystem. Some microorganisms can grow below 0°C; others can grow at almost 100°C. Microorganisms are classified as psychrophiles, mesophiles, and thermophiles, respectively, if their optimal growth temperatures are low (5–15°C), moderate (25–40°C), or high (40–60°C). A typical growth curve for a marine psychrophile (Figure 8.1) indicates that optimal growth occurs at 4°C, that the organism is capable of growth at −1.8°C (the freezing point of seawater) and that the bacterium will not grow at or above 10°C (Morita, 1975).

It is of interest to consider the adaptations that allow psychrophilic microorganisms to grow in temperature ranges that are prohibitive to most mesophilic organisms. In most mesophilic organisms, a control mechanism shuts off protein synthesis if temperatures fall to approximately 5°C or lower; psychrophilic organisms apparently

FIGURE 8.1
Growth attained by an Antarctic psychrophile during 80 hour incubation, measured by optical density, showing low optimal growth temperature and restricted range of temperature tolerance. (From Morita, 1975; reprinted by permission; American Society for Microbiology.)



to override these control mechanisms
Ingraham, 1978).

Growth at High Temperatures High
re adaptations of thermophilic micro-
include high proportions of saturated
membranes, which prevent melting at
eratures (Campbell and Pace, 1968;
, 1979; Brock, 1978). Many thermo-
oorganisms produce enzymes that are
denatured at high temperatures.
unusual amino acid sequences occur
proteins of thermophiles, stabilizing
eins at elevated temperatures (Zuber,
he thermophiles, when growing at tem-
near the maximal growth temperature,
mino acid and growth factor requirements
apparent at the optimal growth tem-
This temperature-dependent auxotrophy
for selective heat inactivation of certain
pathways The maximum heat tolerance
tive thermophilic cells coincides with
stability of their ribosomes When the
growth temperature is exceeded, the
melt and protein synthesis ceases.
branes of thermophilic microorganisms
major proportion of high molecular
d branched fatty acids that permit them
their semipermeable properties at high
ures. Thermophiles have relatively high
s of guanine and cytosine in their DNA
the melting point and add stability to
molecules of these organisms.
microorganisms in several areas of nat-
curring high temperatures, namely hot
and deep-sea hydrothermal vents, have
ensively studied. Bacterial growth at
ures up to 250°C by a natural population
from a hot deep-sea vent has been re-
Baross and Deming, 1983, Baross et al.,
at the experimental proof of this study
eing contested (Trent et al., 1984). Mi-
owth has been unquestionably measured
ratures up to 110°C in cultures of ex-
thermophilic bacteria isolated from shal-
low and deep marine hot vents where high hydrostatic pressure prevents boiling (Stetter, 1982; Jannasch and Mottl, 1985; Deming and Baross, 1986). The extremely thermophilic isolates obtained from the hot vents, most of which are archaebacteria, are all anaerobic, with growth ranges from 65° to 110°C and growth optima from 86° to 105°C. One of the thermophilic, methanogenic vent isolates differs from all other archaebacteria in having a unique macrocyclic glycerol diether instead of a tetraether as the polar membrane lipid, which may explain the retention of membrane fluidity at extremely high temperatures. The warm vents (6° to 23°C) contain between 10^4 and 10^9 cells per milliliter and significant numbers of bacteria (10^4/mL) can be observed in hot (greater than 300°C) vent water. Thermophilic carbon dioxide-, sulfate-, and sulfur-reducing bacteria that use H_2 as the source of electrons are the best candidates for possible microbial activities in hot zones where bottom seawater mixes below the surface with rising hydrothermal fluid.

Steam vents in areas of volcanic activity may have temperatures of 500°C, and many hot springs have temperatures near 100°C. Microorganisms living in hot springs obviously must be adapted to function at high temperatures as well as low concentrations of organic matter, oxygen, and, depending on the particular hot spring, either acid or alkaline pH values. Despite these extreme conditions, several microorganisms do possess the adaptive features necessary to live in hot spring habitats. Many of the organisms living in hot spring habitats are obligate thermophiles and are restricted to growth at high temperatures. These thermophilic microorganisms have adaptive features that allow them to carry out active metabolism at temperatures over 60°C.

Microorganisms occupy habitats of differing maximal temperatures along the temperature gradient from the source of the hot spring, bacteria being the most tolerant of the elevated temperatures (Brock, 1978). At temperatures above 75°C, only a few bacterial species can survive and grow; these species include members of the genera *Thermus* and *Sulfolobus*. *Pacinus stearothermophilus* is often the dominant bacterial species in temperature zones of 55–70°C, but many other microorganisms, including cyanobacteria and algae, also occur in such habitats Cyanobacteria occur as layers of growth within specific zones of thermal ponds, growing in higher temperature zones than algae, which are restricted to growth below 55°C.

Survival and Death of Microorganisms at High and Low Temperatures Inability to grow at a particular temperature does not necessarily lead to the death of a microorganism. The temperature tolerance of endospores, cysts, and other resting stages is well known, and even vegetative cells can survive for periods of time at temperatures outside their active growth range Survival is often greatest at temperatures below the organism's growth range and thermophiles are frequently isolated, for example, from Antarctic soils (Horowitz et al., 1972)

Although microorganisms may survive low temperatures, their growth rates and metabolic activities decline below the optimal temperature. In the microbial ecology of foods, this fact is the basis of preservation and increasing shelf life of products by freezing or refrigeration (Deroster, 1970). Lower growth rates and metabolic activities are exhibited by many microorganisms in natural soil and aquatic habitats during winter when temperatures are low. Temperatures above the growth range of the organism, however, frequently result in death of the organism Some microorganisms produce resistant resting stages, such as bacterial endospores and sclerotia of fungi, which permit survival at elevated temperatures at which vegetative cells of that organism would not survive. Even such resistant resting stages as the bacterial endospore have upper temperature limits for survival.

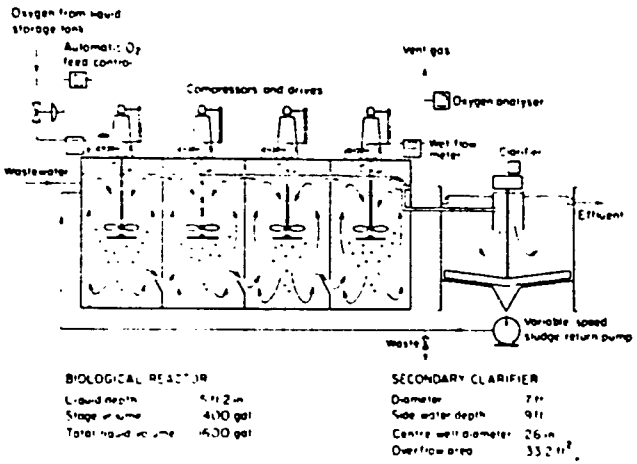
The fact that elevated temperatures kill microorganisms is used in the preservation of many food products and the heat sterilization of various items. Concern with food products generally is with the relatively resistant organisms that can cause spoilage and disease (Frazier and Westhof, 1978). The resistance of organisms to elevated temperatures is expressed as the thermal death time (TDT), the time required at a particular temperature to kill a specified number of organisms. Examples of thermal death times for several bacteria, including endospore formers, are listed in Table 8.1. The TDT shows the equivalent times and temperatures required to cause an identical reduction in numbers of viable microorganisms. These are used by the food industry to determine the appropriate processes for the pasteurization (reduction of numbers of viable microorganisms) or sterilization (complete killing of viable microorganisms) of a product.

The effects of temperature on microorganisms are interactive with other environmental parameters For example, moisture has a marked influence on the thermal death of microorganisms Moist heat is more effective in killing microorganisms than dry heat. Whereas 15–20 minutes at 120°C with steam is adequate to sterilize many materials, such as bacteriological media, dry heat at 160–180°C for three to four hours is often needed to achieve similar results. The pH also influences thermal killing; microorganisms tend to be more resistant to high temperatures at neutral pH than at acid or alkaline pH values.

TABLE 8.1
Approximate thermal death times for bacteria

Organisms	Time (min.)	Temperature (°C)
Escherichia coli	20–30	57
Staphylococcus aureus	19	60
Bacillus subtilis (spores)	50–20	100
Clostridium botulinum (spores)	100–330	100

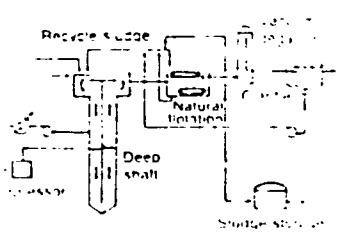
After Frazier and Westhoff, 1978.



wastewater
in developed
ale achieves
al oxidation
e using oxy-
air in its
Higher oxy-
ssures result
oxygen trans-
o Howell,
by permis-
The Institute
London.)

photosynthetic activity of algae, they shallow. Oxygenation is usually in- b consequent odor problems. Their is strongly influenced by

shaft process for wastewater treatment oxygen partial pressures and transfer increased hydrostatic pressure. The shaft deep, and is separated by a central di- tion is initially established by air injection half of the shaft. After circulation is es- injection is switched to the downcomer in the drawing. Air bubbles are retained period and the increasing hydrostatic tates O_2 dissolution. (From Howell, 1977, permission; copyright The Institute of Pe- don.)



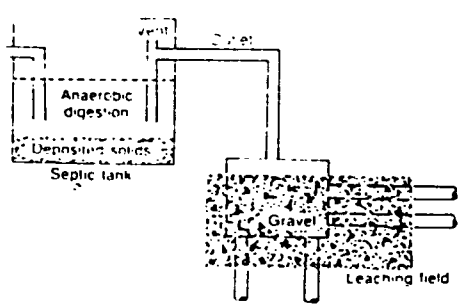
temperature fluctuations and their usefulness, therefore, is largely restricted to warmer climates.

Most frequently, treated sewage is discharged into surface waters, but in some arid areas sufficient surface water flow may not exist. In addition, it may be necessary to regenerate the groundwater removed from wells. In such areas, partially treated sewage is used for groundwater recharge. Typically, the sewage is subjected to primary settling, and is subsequently channeled through a series of holding ponds. The first holding pond tends to reaerate the oxygen-depleted water. After settling of most of the algal and bacterial biomass, the water is transferred to large, shallow infiltration ponds. From these infiltration ponds the water flows through natural sand and soil layers and slowly returns to the underground aquifer. Clogging of the infiltration ponds by undegraded microbial polysaccharides and by accumulation of reduced ferrous sulfide is a recurrent problem in groundwater recharge operations. The problem is countered by periodic rest periods for individual infiltration basins. These interruptions allow degradation of the excess polysaccharides and reaerate the sediment of the infiltration ponds with the concurrent oxidation of the clogging ferrous sulfide.

Anaerobic Treatments

Anaerobic wastewater treatment methods are generally slower, but save the energy necessary for forced aeration. Some anaerobic treatment systems can also salvage a part of the chemical energy content of the wastewater by generating methane, a useful fuel (Cillie et al., 1969; Toerien and Hatting, 1969; Zinder, 1984). The simplest anaerobic treatment system is the septic tank (Figure 12.18), popular in rural areas without sewer systems. The organics in the wastewater undergo limited anaerobic digestion. Residual solids settle to the bottom of the tank. The clarified effluent is distributed over a leaching field where dissolved organics undergo oxidative biodegradation. Septic tank treatment does not reliably destroy intestinal pathogens and it is important that the leaching field not be in the proximity of water wells. The Imhoff tank (Figure 12.19) is an improved septic tank design that maintains anaerobic conditions more strictly, produces some utilizable methane, and facilitates the settling of solids, but requires expert maintenance (Imhoff and Fair, 1956; Mitchell, 1974).

FIGURE 12.18
Diagram of a septic tank. (From Mitchell, 1974; reprinted by permission; copyright Prentice-Hall.)



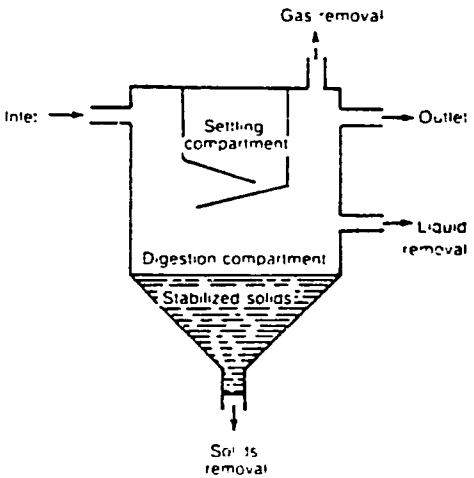


FIGURE 12.19
Diagram of an Imhoff tank. A separate settling compartment is baffled in a way so as to prevent gas bubbles from carrying solids back up into it. The flow passes along this baffled compartment perpendicularly to the plane of the drawing. Stabilized solids are removed from the bottom of the tank. Gases are vented and may be utilized as fuel. (From Mitchell, 1974; reprinted by permission; copyright Prentice-Hall.)

Large-scale anaerobic digestors (Figure 12.20) are used for further processing of sewage sludge produced by primary and secondary treatments. While anaerobic digestors can and have been used directly for the treatment of sewage, the economics of the situation favor aerobic processes for such relatively dilute wastes. Large scale anaerobic digestors, therefore, are used primarily for processing of settled sewage sludge and for treatment of some very high BOD industrial effluents.

Conventional anaerobic digestors are large fermentation tanks designed for continuous operation under anaerobic conditions. Provisions for mechanical mixing, heating, gas collection, sludge addition, and drawoff of stabilized sludge are incorporated. The digestor contains high

ect of Temperature on Microbial es In addition to affecting survival and , temperature influences the metabolic ac- of microorganisms. In general, higher atures that do not kill the microorganisms n higher metabolic activities. For example, ed O_2 consumption occurs as temperature es (Figure 8.2). The increased respiratory reflects the underlying influence of tem- e on enzymes up to the temperature where denaturation occurs (Hargrave, 1969). e change in enzyme activity caused by a se in temperature is known as the 10- temperature quotient or Q_{10} value. In gen- zymes have Q_{10} values near 2, that is, an e in temperature of 10°C within the tol- range of the enzyme results in a doubling vity. Examples of some Q_{10} values for mi- enzymes are shown in Table 8.2. In ad- to Q_{10} values for individual enzymes, the t must consider the effects of temperature rates of metabolic activities of the com- of microbial populations. Populations of

8.2
ship of temperature and bacterial respiration in ments. Increased O_2 consumption occurs at emperatures. (From Hargrave, 1969; reprinted ission; Journal of the Fisheries Research Board da.)

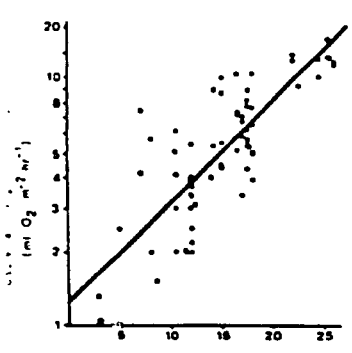


TABLE 8.2
Q_{10} values for some enzymes

Catalase	2.2(10–20C)
Maltase	1.9(10–20C)
Maltase	1.4(20–30C)
Succinic oxidase	2.0(30–40C)
Urease	1.8(20–30C)

$$Q_{10} = \frac{\text{activity at temp T + 10C}}{\text{activity at temp T}}$$

sulfate reducers in salt marsh sediment, for example, have been reported to have a Q_{10} of 3.5 for the reduction of sulfate (Figure 8.3) (Abdollahi and Nedwell, 1979).

Radiation

The spectrum of electromagnetic radiation is continuous from extremely energetic short wavelength gamma rays to long wavelength low energy radio waves. Portions of this continuous spectrum are defined as ultraviolet, visible, infrared radiation, and so forth. The entire global ecosystem is driven by the radiant energy of the sun; photosynthetic interaction with visible light radiation is of profound importance for terrestrial life. The other portions of the electromagnetic spectrum also are ecologically important and have marked effects on life.

Ionizing Radiation Radiations are designated as ionizing radiations if their interaction with matter produces unstable ions and free radicals that interact with living matter in a destructive manner. Gamma rays and x-rays range in wavelength from 10^{-11} cm to 10^{-6} cm, with gamma rays at the shorter, more energetic end of the range. They are highly penetrating and their energy levels are destructive to microorganisms. Low level irradiation may cause mutations and high exposure doses destroy both nucleic acids and enzymes and kill microorganisms. Exposure levels

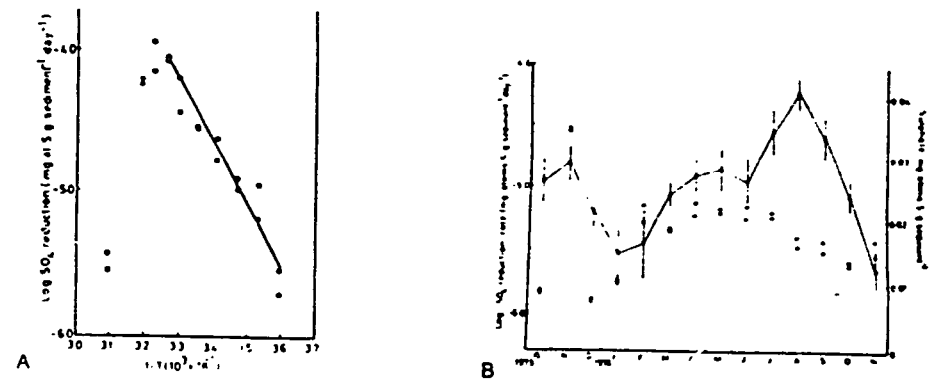


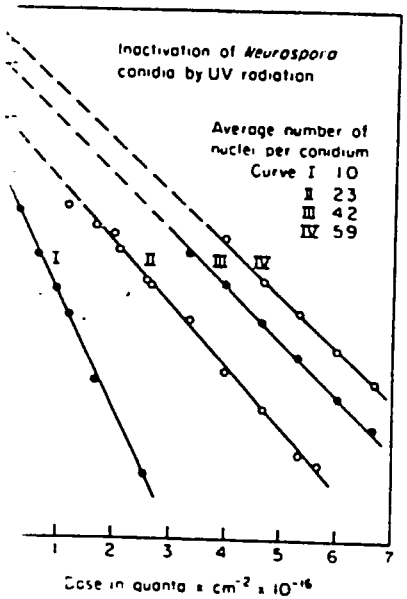
FIGURE 8.3
(A) Arrhenius plot of sulfate reduction in sediment showing linear decrease in activity with decreasing temperature; (B) the seasonal *in situ* rates of sulfate reduction calculated from the above Arrhenius plot and actual field temperatures. Open circles indicate calculated rates of sulfate reduction. Closed circles indicate actual *in situ* concentrations of sulfate. High rates of sulfate reduction occurred during summer and fall and showed a negative correlation with actual sulfate concentrations. (From Abdollahi and Nedwell, 1979; reprinted by permission; copyright Springer-Verlag.)

from cosmic and natural radioactive sources are low, nevertheless low level radiations may have contributed to the spontaneous mutations which form the basis for natural selection over geologic ages.

The resistance of microorganisms to gamma radiation varies. As is true with other environmental extremes, microorganisms tend to be more tolerant to ionizing radiation than macroorganisms. As a practical consequence, it is even necessary to add biocides to the cooling waters of nuclear reactors because the high ionizing radiation levels fail to prevent microbial proliferation.

Bacterial endospores are highly resistant to gamma radiation; it takes 0.3–0.4 Mrads to effect a 90 percent kill, whereas one-tenth of this dose effects the same percentage kill in most vegetative bacteria. Vegetative cells of *Micrococcus radiodurans*, though, display very high radiation resistance, tolerating as much as 1 Mrad without a reduction in viable count (Anderson et al., 1956). Extremely efficient DNA repair mechanisms, rather than any unusual protective substances, are responsible for the radiation resistance of this bacterium.

Ultraviolet Radiation Solar radiation includes ultraviolet light radiation, visible light radiation, and infrared radiation. Ultraviolet (UV) radiation (1-320 nm) is too energetic for photosynthetic use and is destructive to microorganisms (Figure 8.4) (Norman, 1954). The most strongly germicidal wavelength (260 nm) coincides with the absorption maximum of DNA, suggesting that damage to DNA is the principal mechanism of the germicidal effect. UV-induced dimerization of thymine bases in DNA strands causes faulty transcription, resulting in defective enzymes. UV-damaged DNA can be repaired enzymatically by excision of the damaged portions, but this process requires time. The survival of the UV-damaged cells is enhanced by conditions that are adverse to rapid growth; optimal growth conditions allow for rapid expression of the UV damage with resulting cell



...ation of *Neurospora* conidia by uv radiation. ...was at 254 nm. Conidia with higher numbers ...were less susceptible to uv inactivation proba... ...se uv acts by interference with DNA replica... ...he presence of redundant DNA information ...that demage. (From Norman, 1954; reprinted ...sion; copyright Wistar Institute of Biology.)

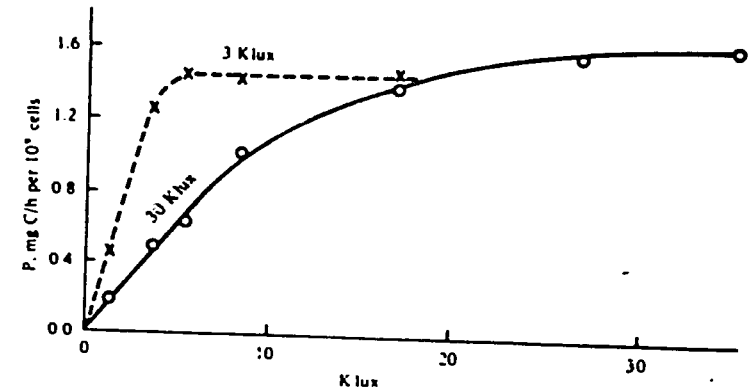
...isible light also has a reactivation effect ...amaged cells (Rupert, 1964; Halldal and ...972). Part of the effect may be photo... ...blue light tends to break the UV-induced ...dimers. In addition, visible light triggers ...pair mechanism that is, paradoxically, ...ted by the more damaging UV irradia... ...ature, microorganisms are exposed to ...iation only in combination with high ...ht intensities, which makes this adap... ...derstandable.

...e Light Radiation The visible light ...is a relatively narrow band in the total electromagnetic spectrum. It ranges from 320 nm (violet) to the far red end of the spectrum of 800 nm. The quantity of light in a given habitat depends upon the obstructions the light beam has to penetrate. Light intensity decreases by the square of the distance from the source, primarily because of absorption and scattering. The sum of absorption and scattering processes, called extinction, is high in ecosystems with many suspended particles. The term turbidity, which refers to the cloudiness or number of suspended particles in a water body, often is used to describe the resistance of a water body to light penetration.

The intensity of light reaching a particular habitat influences rates of photosynthesis (Halldal, 1980). In the absence of light, photosynthesis ceases. Up to some optimal light intensity, rates of photosynthetic activity increase (Figure 8.5) (Steeman-Nielson et al., 1962). As with many other environmental factors, the response of an organism to changes in light intensity will depend on the light intensity to which the organism is adapted. This is clearly shown in Figure 8.5 by the different response curves of algae that were adapted to 3 Klux and those that had been growing at 30 Klux. In most cases, organisms can tolerate a wider range of an environmental factor, such as light, if the change is gradual, allowing the organism more time to adapt, than if the change occurs abruptly. Interestingly, some of the cyanobacteria can alternate between oxygen-evolving photosynthesis at high light intensities and anoxygenic photosynthesis, using hydrogen sulfide as electron donor, at low light intensities (Cohen et al., 1975).

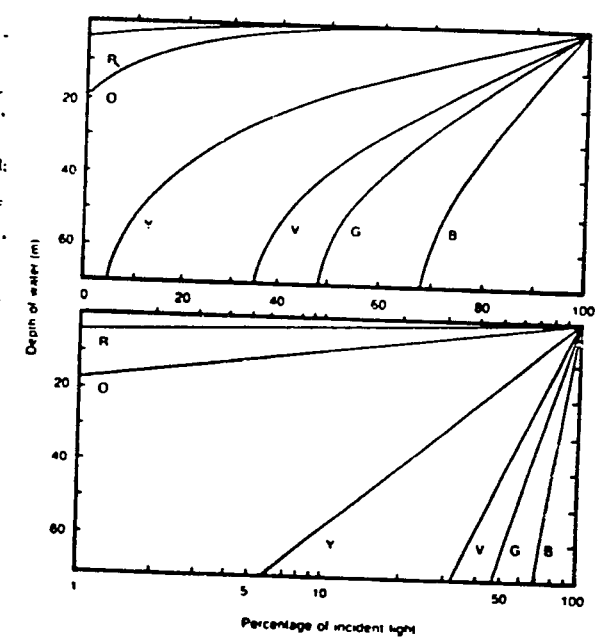
The quality, that is, the color as well as the intensity of light, influences rates of photosynthesis. Not all wavelengths of light penetrate equally well (Figure 8.6). Energy of shorter wavelengths penetrates further than longer energy wavelengths. Blue light, for example, can penetrate further into aquatic habitats than red light, which is one factor that leads to a spatial separation of algae and bacteria with different pigments that can absorb light energy of differing wavelengths.

FIGURE 8.5
The rate of photosynthesis (P) per unit cell as a function of light intensity for *Chlorella vulgaris* adapted to high (30 Klux) and low (3 Klux) light intensities. The graph shows higher rates of photosynthesis at low light intensities by low light adapted cells. Maximal rates of photosynthesis (saturation) occur at 5 Klux for low light adapted cells and at 20–25 Klux for high light intensity adapted cells. (From Steeman-Nielson, et al., 1962; reprinted by permission. Scandinavian Society of Plant Physiology.)



Some microorganisms exhibit phototactic behavior; they move toward or away from a light source (Carlile, 1980; Konopka, 1984). This behavior leads to vertical migrations of motile microorganisms that adjust their depth in the water column to that of optimal light intensity. Buoyancy regulation is ecologically important to planktonic cyanobacteria that inhabit thermally straitifed

FIGURE 8.6
Penetration of light of varying wavelengths into a water column, generally showing that shorter wavelengths of visible light (e.g., blue) penetrate to greater depths than longer wavelengths (e.g., red); R = red; O = orange; Y = yellow; V = violet; G = green; B = blue; upper graph arithmetic scale; lower graph on semi log scale. (From Wetzel, 1975; reprinted by permission; copyright W. B. Saunders Co.)

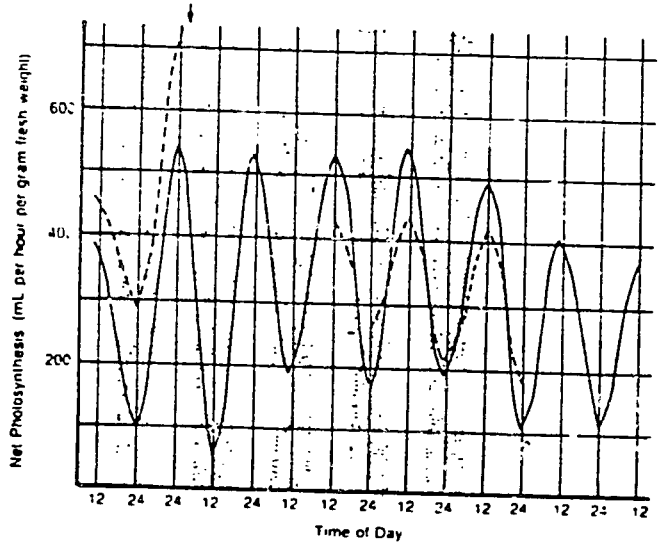


Konopka, 1984). In these lakes, the depths
ch light energy and inorganic nutrients are
bundant, are very different. Gas-vacuolate
sms which can migrate between these
may have a competitive advantage over
lankton, which are incapable of vertical
ion.

oyancy in planktonic cyanobacteria is reg-
in response to the levels of light energy
organic nutrients (such as nitrogen and
orus). These factors may alter buoyancy
u of their effects upon the rates of energy
ion and utilization. When growth rate is
limited, the light intensity at which neutral
cy occurs is hypothesized to be directly
to the concentration of limiting nutrient.
can mediate this response by either raising
ot pressure to collapse some existing gas
or increasing cellular amounts of ballast
les such as polysaccharide (Konopka,

It also appears to be a key stimulus in the establishment of circadian rhythms in some eukaryotic microorganisms (Figure 8.7) (Sweeney and Haxo, 1961). Thus, light affects not only the growth and survival but also the behavior of microorganisms.

In addition to considering light energy as a limiting factor for primary production, solar radiation may be considered as a stress factor. Solar radiation contains radiation in the ultraviolet spectrum, which can cause cell death or mutation. Even excessive intensity of visible light is detrimental because it causes photooxidative changes. Surface ecosystems, such as the tops of leaves, surfaces of oceans, rocks, soils, and animals, airborne particles and droplets in clouds receive high amounts of ultraviolet radiation. Microorganisms on snow, ice, and in brines with crystalline salts receive unusually high UV and visible light exposures because of the reflectivity of their environment. Microorganisms in these environments are selected in part for their tolerance to ultraviolet radiation. Pigmentation for



8.7
rhythms in Ace-
. Light and dark
re shown by shad-
ficity of photo-
continues in con-
(After Sweeney
. 1961; reprinted
ner by permission.
1961 by the
Association for
vement of

shielding the sensitive DNA and efficient repair mechanisms constitute adaptations that provide such tolerance.

Pressure

Atmospheric Pressure Atmospheric pressure is the pressure exerted by the weight of the air column which at the surface of the earth, (sea level), is approximately 1 atmosphere (760 mm mercury). In general, microorganisms are not affected by changes in atmospheric pressure. At extremely low atmospheric pressures, such as in the upper atmosphere or in an artificial vacuum, however, water may evaporate and oxygen becomes limiting, rendering microorganisms metabolically inactive (Gregory, 1973).

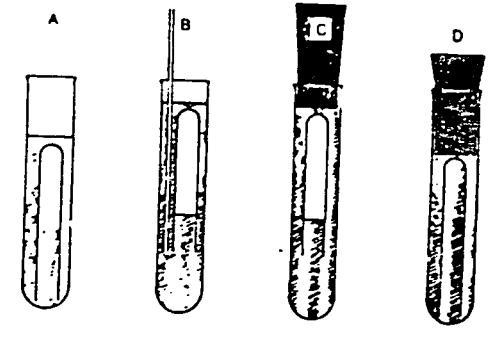
Hydrostatic Pressure Whereas atmospheric pressure reflects the weight of the air column, hydrostatic pressure is the pressure exerted by the weight of a water column. Hydrostatic pressure increases by approximately 1 atmosphere for every 10 meters of depth. Because the atmospheric pressure at sea level is 1 atmosphere, the pressure at 10 m is approximately 2 atmospheres. The precise pressure at a given depth depends on the weight of the water and hence pressure is greater at a given depth in saline water than at the same depth in freshwater.

The hydrostatic pressure can be calculated by measuring the density and the depth of the water. In routine hydrographic measurements, readings from shielded and unshielded reversing thermometers are compared; hydrostatic pressure compresses the mercury in the unshielded instrument, resulting in a lower reading at a given temperature. Using suitable conversion tables, the difference between the readings indicates the hydrostatic pressure at the point of thermometer reversion.

To study the growth and metabolism of microorganisms under high hydrostatic pressure simple culture tubes in cylinders can be pressurized by a hydraulic press (Figure 8.8) (ZoBell and Hittle, 1967). Precautions must be taken to avoid hyperbaric oxygenation and compression heat. Sudden decompression may rupture microbial cell membranes by releasing gas bubbles, but steady hydrostatic pressure in the range of 1 to 400 atmospheres has little or no effect on the growth and metabolism of most microorganisms. Higher hydrostatic pressures tend to inhibit or stop the growth of terrestrial and shallow-water organisms, but not that of barotolerant deep-sea organisms, which may be exposed to pressures as high as 1000 atmospheres.

In response to pressures, organisms are either sensitive (that is, they grow optimally at pressures about 1 atmosphere and growth rates decline as pressure increases), barotolerant (that is, they tolerate elevated pressures up to given limits without a decline in growth), or barophilic (that

FIGURE 8.8
Culture tube for use in pressure cylinders for prevention of hyperbaric oxygenation (A). A measured amount of oxygen is introduced into an oxygen-free solution prior to pressurization (B). A rubber stopper acts as piston (C), which is forced into tube by external hydrostatic pressure (D) created by a hydraulic press. (From ZoBell and Hittle, 1967; reprinted by permission; National Research Council Canada.)



ow optimally at elevated pressures and ompletely inhibited or even killed at ic pressure). The existence of obligate has been a question of prolonged con- Sampling and isolation of deep-sea bac- out loss of *in situ* pressure is required to determine the existence of de- n-sensitive strains (Yayanos and Dietz, ly reports by ZoBell and coworkers 964) about barophilic deep-sea micro- could not be readily confirmed by other s. Barotolerant organisms were found bligate barophiles, probably because amples underwent decompression and temperature changes during retrieval. S, the deep-sea research submersible e Woods Hole Oceanographic Institute to a mission in 1500 m deep water. escaped but their lunches, including wiches, sank with the submersible. Alvin was raised ten months later, the unspoiled condition of the food called ion to microbial metabolic rates under static pressure and prompted a series ations by Holger Jannasch and asso- the *in situ* activities of deep-sea mi- ns (Jannasch and Wirsen, 1977; Jan- ; Jannasch and Nelson, 1984). Various media, some containing radiolabelled rces, were incubated *in situ* on the oor and in refrigerated incubators at essures and temperatures in the lab- hese tests concluded that low tem- -4°C, only moderately limit the activity microorganisms, but that the com- low temperature and high hydrostatic poses a severe limitation on microbial pically, microbial metabolism at deep- es was an order of magnitude or more the activity of the same deep-sea or- 1 atmosphere and identical tem-

t became possible to retrieve and in- sea samples without decompression, the majority of isolates recovered using an apparatus that prevented decompression showed highly bartolerant characteristics, while the remainder (4 out of 15) exhibited barophilic characteristics (Jannasch et al., 1982). The limited evidence to date indicates that the deep-sea microbial community is only moderately barotolerant rather than barophilic, and its activity is severely constrained, though not halted, by the prevailing high hydrostatic pressures.

Considerable difficulty has been experienced in trying to maintain barophilic bacteria in the laboratory. Most of the enrichment cultures from the deep sea lose their viability after two or three transfers to new media, although some barophiles have survived in sediment samples for several months when stored at *in situ* pressures and temperatures. Even at refrigeration temperatures, deep-sea barophiles die off much more rapidly at 1 atmosphere than when compressed to *in situ* pressures. Despite the technical difficulties some barophilic bacteria have been cultured. A clearly barophilic deep-sea *Spirillum* has been isolated by Yayanos and co-workers that grows at least fifteen times faster at pressures between 300 and 600 atmospheres than at 1 atmosphere (Yayanos et al., 1979). At least some of the barophilic bacteria thus far studied have survived decompression during recovery from the deep sea, so controlled temporary decompression may have no lethal or irreversibly damaging effects.

An explanation for the distribution of more or less temperature-and pressure-adapted bacteria in the deep sea might be found in accumulating data on the considerable particle flux from surface waters to the deep sea and observations of bacterial attachments to these articles, such as organic detritus and fecal pellets. Most of the deep-sea bacteria in the sediments and water column may thus represent relatively recent arrivals from areas of low or moderate pressure.

High hydrostatic pressure appears to inhibit microbial synthesis of RNA, DNA, and protein, membrane transport functions, and rates of activity of various enzymes (Baross et al., 1972; Hardon and Albright, 1974). Enzyme proteins, in order to be active, must be in proper tertiary configuration. It has been suggested that high hydrostatic pressures distort this configuration, leading to low enzyme activity. In the case of a barotolerant organism, the distortion would be minimal. It appears that most enzymes actually are capable of normal functioning over the wide range of pressures found in most habitats and hydrostatic pressure should not preclude enzymatic activities even in the deep sea. Theoretically, an obligately barophilic microorganism might have its enzyme in proper tertiary configuration only at elevated hydrostatic pressure and would not function well at lower pressures (Figure 8.9) (Kim and ZoBell, 1972).

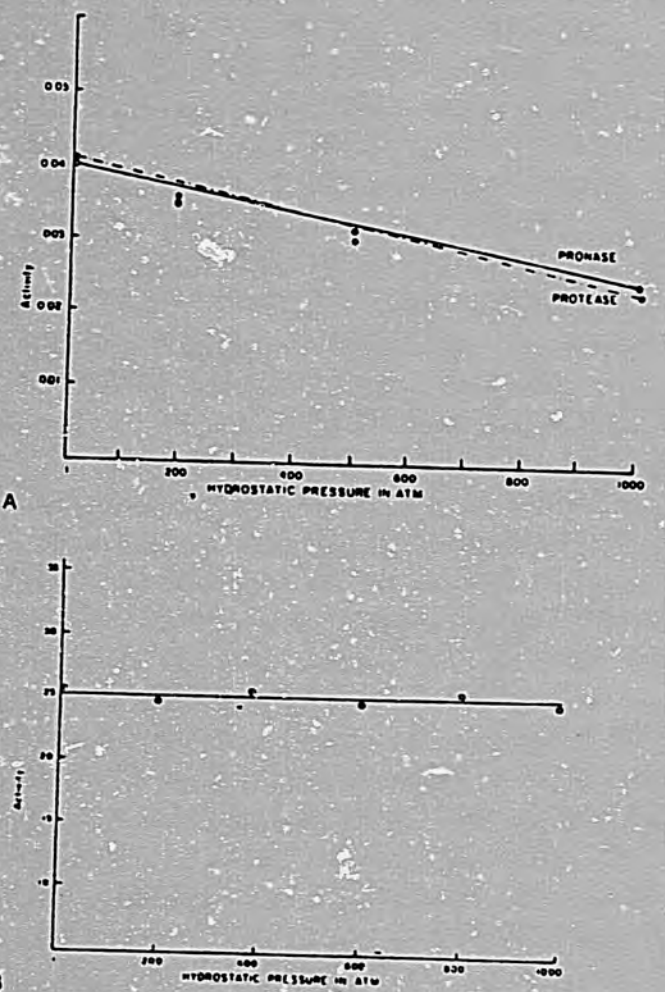
Osmotic Pressure Whereas high hydrostatic pressure is restricted to special habitats, all microorganisms must cope with osmotic pressure, which results from differences in solute concentrations on opposite sides of a semipermeable membrane. In order to equilibrate and equalize solute concentrations, water molecules attempt to move across the membrane in the direction of the higher solute concentration. In some hypotonic habitats, water molecules attempt to move into microbial cells, which could expand and rupture the cells; in hypertonic habitats, water molecules attempt to move out of the cells, which could dehydrate and shrivel the cells.

Microorganisms have a variety of adaptive mechanisms that permit them to tolerate osmotic pressure within certain ranges. The rigid cell walls of bacteria and other microorganisms protect these organisms from osmotic shock by preventing the pressure exerted by incoming water molecules from expanding and bursting the cells. The contractile vacuoles of some protozoa serve a similar function by pumping out excess incoming water.

Few microorganisms can tolerate the high osmotic pressures characteristic of saline brines, as occur in some salt marshes and land-locked lakes, or high sugar concentrations such as occur in tree sap. On the other hand, numerous microorganisms can withstand the low osmotic pressure of distilled water. The term osmophile is used to describe organisms that can tolerate high sugar concentrations and the term halophile, to describe salt tolerant or salt-requiring microorganisms. Some osmophilic yeasts are capable of growth at greater than 65% wt/wt sugar concentration ($a_w \approx 0.94$) (Brown, 1976).

Salinity

In addition to affecting osmotic pressure, high salt concentrations tend to denature proteins; that is, they disrupt the tertiary protein structure, which is essential for enzymatic activity. High concentrations of salt dehydrate cells and denature enzymes. High salinity habitats include salt lakes, which occur in arid regions where evaporation exceeds freshwater inflow or where a lake is fed by a salt spring. Relatively few organisms can grow in highly saline waters, and often the biota of salt lakes are restricted to a few halophilic and salt tolerant bacterial species (Kushner, 1968; 1980; Post, 1977). Obligate halophiles possess specialized enzymes that are in their active configuration only at high salt concentrations (Figure 8.10) (Larsen, 1962). Halophilic microorganisms have high internal concentrations of potassium chloride, and the enzymes of such microorganisms must have a greater tolerance to salt than enzymes from microorganisms that are not salt tolerant. In many cases high concentrations of salt are required by halophiles to maintain their enzymatic activities (Larsen, 1967). Many extreme halophiles have unusual membranes, such as the purple bilayer membranes of *Halobacterium*. The cell wall of *Halobacterium*, an archaebacterium, lacks murein and appears to be stabilized by sodium ions. The ribosomes of *Halobacterium* require high concentrations of potassium for stability. It is these adaptive features that permit halophiles to live in the saturated brine environments of



In less extreme cases many marine quire sodium ions and NaCl concen- near 3 percent are necessary for the ese bacteria. The true marine bacteria appear specifically to require sodium for all membrane transport systems whereas sodium-dependent transport in terrestrial bacteria has so far been shown to occur only with specific me-

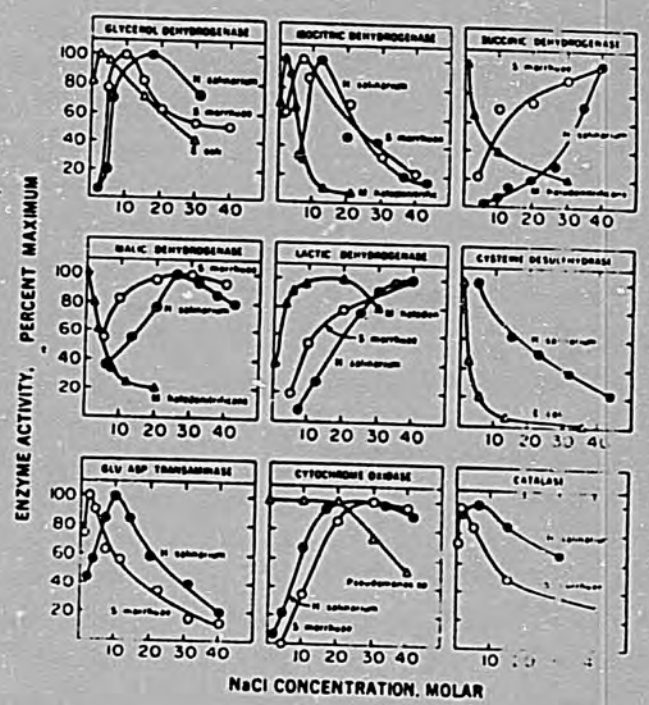


FIGURE 8.10
Effects of sodium chloride concentrations on various enzymes of halophilic and nonhalophilic bacteria. Some of the enzymes of halophilic bacteria require high salt concentrations for maximal activity (e.g., succinic dehydrogenase of *Halobacterium salinarium*). Enzyme activities of nonhalophilic organisms decrease at high salt concentrations (e.g., glycerol dehydrogenase of *E. coli*). (From Larsen, 1962; reprinted by permission; copyright Academic Press.)

tabolites, which often are not essential for growth (MacLeod, 1985).

Water Activity

In some environments it is important to consider the water activity (a_w) rather than the total amount of water (Brown, 1976; Griffin, 1981; Reid, 1980). The water activity is an index of the water actually available for microbial use. The water activity depends on the number of moles of water, the number of moles of solute, and on the activity coefficients for water and the particular solute. Water activity can be decreased not only by solutes (osmotic forces), but also by absorption to solid surfaces (matric forces). By definition, a_w of free distilled water is 1.0. Osmotic and matric forces usually lower a_w to some fraction of this value. Most microorganisms require a_w values above 0.96 for active metabolism but some filamentous fungi and lichens are capable of growth at a_w values as low as 0.60 (Table 8.3). Such microorganisms are described as xerotolerant.

Water activity in soils is traditionally measured in relation to water-holding capacity (WHC) of the soil. A measured amount of dry soil is allowed to soak up water. After excess water is allowed to drain in a humid chamber, the weight increase of the soil sample is recorded. This value is 100% of the water-holding capacity. Optimal conditions for activity of aerobic soil microorganisms occur between 60% and 80% WHC. The

er activities growth of isms

Water Activity (A_w)	Bacteria	Fungi
1.00	*Caulobacter* *Spirillum*	
0.90	*Lactobacillus* *Bacillus*	*Fusarium* *Mucor*
0.85	*Staphylococcus*	*Debaryomyces*
0.80		*Penicillium*
0.75	*Halobacterium*	*Aspergillus*
0.60		*Saccharomyces rouxii* *Actinomyces bisporus*

ontent of soil can be determined the wet and dry weights of the soil ple is weighed and then placed in lly for 12–14 hours at 105°C, after ht is again measured. The difference een the initial (wet weight) and ht) readings represents the loss of ater content is conveniently ex- atio of dry weight to wet weight. may also be expressed as percent aterholding capacity.

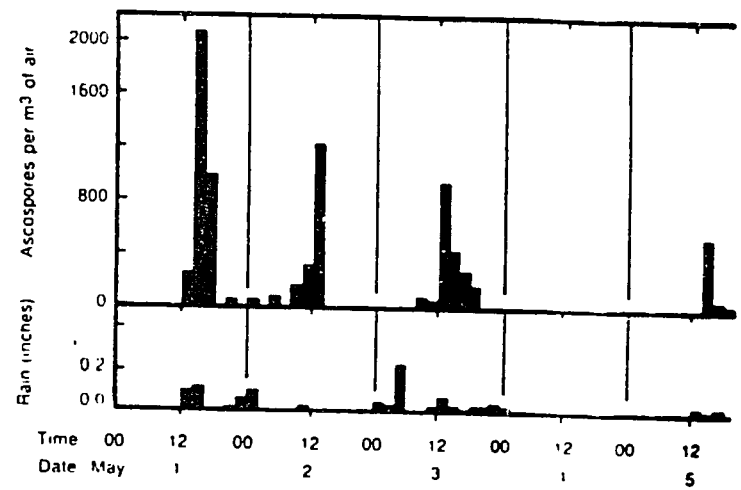
uations it is sometimes more con- rmine water content on a volume lone by measuring the bulk density A known volume of soil is collected, d and the weight determined. Soil ater content will have a low weight e following drying, assuming that were filled with water and not

activities in the soil fluctuate with ent. During periods of drought mi- ies may be severely limited. The of soils not only affects microbial s, but also indirectly by affecting t gases; in water-logged soils dif- limited, generally resulting in an- aerobic conditions that may result in a shift from aerobic to anaerobic metabolism.

The water content in the atmosphere is generally low and limiting for microbial growth (Gregory, 1973). In some regions, such as in tropical areas, the humidity in the air is sufficient to allow microbial growth. In such humid areas microorganisms may grow on exposed surfaces using water from the air. Lichens tolerate desiccation and grow on receiving only intermittent precipitation. A number of fungi and lichens grow on exposed plant surfaces. Various fungi grow on canvas and leather in humid regions. In addition to influencing survival in the atmosphere, water as rainfall causes a flux of microorganisms into and out of the atmosphere. Rainfall washes some organisms out of the atmosphere, others are released to the atmosphere when raindrops trigger their release (Figure 8.11) (Hirst et al., 1955).

Desert soils, by definition, receive less than 25 cm of rainfall per year. Many deserts are extremely dry and are subject to extreme diurnal variations in temperature because of the lack of moisture in the atmosphere overlying these areas to moderate the loss of heat. Microorganisms living in dry desert soils must be able to tolerate long periods of desiccation (Stains, 1984).

FIGURE 8.11
Concentration of ascospores of *Venturia inaequalis* in air related to rainfall. High numbers of ascospores were found shortly following the rainfall. (From Hirst, et al., 1955; reprinted by permission; Administration A—Fish, Food, and Plant Pathology—of England and the Controller of Her Britannic Majesty's Stationery Office.)



In the dry valleys of Antarctica microorganisms must also tolerate very low temperatures and, during part of the year, high irradiation levels (Horowitz et al., 1972). In such environments many microorganisms have developed adaptations that allow them to survive in a dormant state during unfavorable conditions and to grow actively only during those brief periods of time when conditions are favorable, such as after a rainstorm. Many of the bacteria and fungi living in desert soils form spores that allow them to persist, if necessary, for decades between growth periods. When there is adequate moisture, the spores germinate and for a brief period the organisms can actively grow and reproduce.

Rock-inhabiting (lithobiontic) microorganisms colonize rock surfaces, or the rock fabric adjacent to the surface, in a wide variety of climates and environments. Some microorganisms grow on the rock surface. Conspicuous among these are the lichens, which often form brighly colored growths. These organisms are particularly resistant to desiccation.

Some microorganisms, called endoliths, also grow within the rock matrix (Friedmann and Ocampo, 1976; Friedmann, 1982; Friedmann and Ocampo-Friedmann, 1984). The morphology of endolithic colonization in cold and hot deserts is remarkably similar. The uppermost 1 to 3 mm of the rock are free from microorganisms. Colonies are present in the next few millimeters, where the microorganisms adhere to or grow between the rock crystals. Endolithic microorganisms in hot deserts and in Antarctica share a common survival strategy: they are capable of switching their metabolic activities on and off in response to rapid changes in environmental conditions.

There are, however, major differences between the endolithic microorganisms of hot and cold deserts (Friedmann and Ocampo-Friedmann, 1984). In hot deserts, all endolithic samples examined contained only procaryotes, which form a narrow green zone a few millimeters below and parallel to the rock crust; the photosynthetic primary producers are cyanobacteria, nearly always members of the genus *Chroococcidiopsis*. In hot deserts, a favorable combination of available water and moderate temperature to occur in early

after dew fall. Later in the day, the ure rises and the relative humidity drops ly. The combination of temperature in- a hydrated state and the concomitant water imposes a severe environmental at eucaryotic organisms do not seem to This is probably the reason that, in hot endolithic microbial communities are d exclusively of bacteria

endolithic microbial communities in polar how a higher level of complexity than hot deserts. In most cases, polar en- communities contain a number of or- including eukaryotes, that are dominant. and algae together form a symbiotic sociation in which the fungi and algae regated bands within the rock matrix ery different from the usual integrated chen organization The coherent, pro- seudo-tissue termed plectenchyma is Instead, loose filaments and cell clusters ween the rock crystals In endolithic he rock matrix prevents the development lso eliminates the need for a coherent ma. The activity of the cryptoendolithic mmunity results in the mobilization or pounds in the rock as well as in a char- exfoliative weathering pattern

at

ement of air and water aids the passive of microorganisms Of equal importance le of movement in importing and dis- nutrients for microbial growth and in metabolic by-products The movement ls in and out of ecosystems is controlled s such as solubility diffusion viscosity, ravity, porosity, and the flow charac- of the ecosystem.

ecosystems are characterized by ex- low, such as rivers, or by turbulence, eans Other ecosystems such as ponds, ally quiescent or static, such as soil. Ecosystems with extensive flow or turbulence have greater mixing capacity. Materials are moved into and out of such ecosystems in part by the movement or mixing of the ecosystem.

Diffusion Even in ecosystems which lack extensive mixing, materials may move by diffusion. Diffusion results in a spreading out of a substance from its source, or a lowering of concentration. Diffusion of materials through ecosystems depends in part on temperature. The molecular weight of the solute and the viscosity (flow characteristics) of the solvent determine in part the ability of a solution to diffuse through an ecosystem. Mixing is often augmented by differences in specific gravity and by thermal convection. Gaseous molecules, for example, with low specific gravities, tend to rise, generally moving upward through aquatic ecosystems. In the atmosphere, diffusion of gases is augmented by convection along temperature gradients with warmer gases rising and cooler gases descending through the atmosphere. The movement of gases along thermal gradients results in turbulence and mixing

In terrestrial ecosystems diffusion of materials is a function of porosity Porosity refers to the number of pores in a soil or sediment particle matrix. The pores are sometimes referred to as interstitial spaces. These pores may be filled with liquid or gases. Diffusion of materials occurs through the pores and exchange rates between material in the interstitial spaces and external sources affects diffusion rates and the availability of materials essential for microbial growth and activity.

Adsorption Movement of materials through an ecosystem may be restricted by surface adsorption and tension Surface tension may restrict movement of materials into an ecosystem, for example, from the atmosphere into an aquatic ecosystem, resulting in the accumulation of nutrients and pollutants. Adsorption may bind materials to particles, decreasing the availability of those materials within the ecosystem. If the substance is an essential nutrient this can decrease productivity, but if the substance is toxic, adsorption can increase microbial growth. Because of adsorption, even excessive levels of microbial inhibitors fail to suppress completely microbial activity in soil.

In environments with very dilute nutrient concentrations, the local enrichment of nutrients on adsorptive surfaces increases their microbial utilization. This phenomenon appears to aid the survival of copiotrophic microorganisms in oligotrophic environments (Kjelleberg, 1984). The concentration of organic and inorganic nutrients by adsorption onto particles accounts in part for the preferential growth of many microorganisms on surfaces. The adsorptive properties of an ecosystem depend on available particle surface areas, charged binding sites, hydrophilic binding sites, and other factors, such as pH. In some cases, adsorption is essential for microbial reproduction. For example, many microorganisms only grow or reproduce on surfaces such as leaves, skin, teeth, etc. Some bacterial viruses must adsorb onto specific receptor sites located on pili before penetrating into bacterial cells where they reproduce.

The mechanisms of adherence of microorganisms to a variety of biotic and abiotic surfaces have been intensively examined in recent studies (Marshall, 1976; Fletcher and Marshall, 1982; Costerton, 1984; Dazzo, 1984, Fletcher and McEldowney, 1984). Bacteria growing in a wide variety of environmental and pathogenic situations have been shown to be surrounded by extracellular glycocalyx structures that mediate the attachment process (Costerton, 1984); in some cases proteins known as lectins mediate the specific attachment processes (Dazzo, 1984). Charge interactions also bring about adsorption Adhesion holds the bacterial cell in a suitable habitat, positioning the cell within the surface zone, which is rich in nutrients, because organic molecules are attracted to these interfaces. Development of a f[illegible], anionic, exopolysaccharide matrix (glycocal[illegible]) acts as an ion-exchange resin, attracting and co[illegible]centrating charged nutrients. With cell growth, this matrix burgeons until a thick, coherent biofilm is formed. This sessile biofilm mode of growth may protect adherent cells from surfactants, bacteriophages, phagocytic amoebae, chemical biocides, antiseptics, antibiotics, disinfectants, antibodies, and phagocytic leukocytes.

Hydrogen Ion Concentration

Microorganisms generally cannot tolerate extreme pH values Under highly alkaline or acidic conditions, some microbial cell components may be hydrolyzed or enzymes may be denatured. There are, however, some acidophilic bacteria that tolerate or even require extremely acidic conditions for growth The optimal pH values and the tolerance of ranges of pH for various bacteria are shown in Table 8.4

Amino acids are zwitterions, having both basic and acidic portions The pH affects the dissociation of these functional groups on protein molecules; in order to perform enzymatic activity, enzymes must be in a particular state of dissociation Certain pH values will be optimal for activities of specific enzymes Optimal pH values may depend on other factors, such as salt concentrations (Figure 8.12) (Lieberman and Lanyi, 1972).

The pH of an environment affects microorganisms and microbial enzymes directly and also influences the dissociation of many molecules that indirectly influence microorganisms. The pH determines in part the solubility of CO_2 influencing the rates of photosynthesis, the availability of required nutrients, such as ammonium and phosphate, which limit microbial growth rates in many ecosystems; and the mobility of heavy metals, such as copper, which are toxic to microorganisms.

. optimum, and
pH in multiplira-
ious bacteria

Organism	pH		
	Minimum	Optimum	Maximum
Escherichia coli	4.4	6.0–7.0	9.0
Proteus vulgaris	4.4	6.0–7.0	8.4
Enterobacter aerogenes	4.4	6.0–7.0	9.0
Pseudomonas aeruginosa	5.6	6.6–7.0	8.0
Erwinia carotovora	5.6	7.1	9.3
Clostridium sporogenes	5.0–5.8	6.0–7.6	8.5–9.0
Nitrosomonas spp.	7.0–7.6	8.0–8.8	9.5
Nitrobacter spp.	6.6	7.6–8.6	10.0
Thiobacillus thiooxidans	1.0	2.0–2.8	4.0–6.0
Lactobacillus acidophilus	4.0–4.6	5.8–6.6	6.8

After Doetsch and Cook, 1973

Potential

zymatic reactions are oxidation-reduction in which one compound is oxidized and compound is reduced. The ability of an to carry out oxidation-reduction re- an organism depends on the oxidation- state of the environment. In a solution, ation of oxidized to reduced components the oxidation-reduction potential or tential (E_h). Some microorganisms can only in oxidizing environments, while n exist only in reducing environments 13) (Baas-Becking and Wood, 1955; ner, 1974). A positive E_h value favors a negative E_h is indicative of a reducing ent. Some essential nutrient elements, ron and manganese, are soluble at low tentials but precipitate in oxidizing ents.

redox potential is greatly influenced by nce or absence of molecular oxygen. ents in equilibrium with atmospheric ve E_h around +800 mV; environments d oxygen tensions have lower redox Heterotrophic activity keeps the redox of aerobic natural waters at 400–500 mV, lower than expected for water fully equilibrated with the atmosphere. Below the surface of water-logged soil and sediment, E_h values are usually negative (Figure 8.14). Low redox potentials may be caused by extensive growth of heterotrophic microorganisms that scavenge all available oxygen. Such is often the case in highly polluted ecosystems where microorganisms utilize the available oxygen for decomposition processes. E_h of sediments rich in organic matter can be as low as −450 mV. At these low E_h values, sulfate reduction yielding H_2S, and CO_2 reduction yielding CH_4, can readily occur. It should be pointed out that it is difficult to measure E_h in natural habitats, and that heterogeneity is great; the development and use of microelectrodes, however, now makes it possible to measure redox potentials on a scale relevant to microbial microhabitats (Revsbech and Jørgensen, 1986). Because of the dynamics at interfaces one can have a low redox in the presence of oxygen; diurnal events of the latter are common.

Magnetic Force

The effects of magnetism upon microorganisms has been generally ignored as most bacteria appear

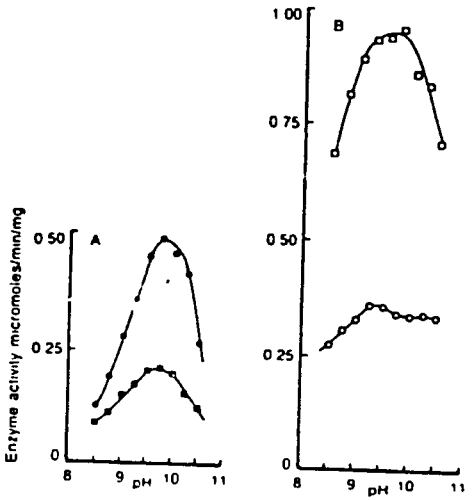


FIGURE 8.12
The pH dependence of threonine deaminase (shown as micromoles product formed per minute per milligram protein) from *Halobacterium cutirubrum* assayed at 0.05 and 4.0 M NaCl concentrations in the absence of ADP (A) and presence of ADP (B). In the absence of ADP, an increase of the NaCl concentration caused a narrowing of the pH optimum; in the presence of ADP, when the cooperative substrate kinetics were no longer observed, the pH response of the enzyme broadened with increased salt concentration. (From Lieberman and Lanyi, 1972; reprinted from Biochemistry by permission; American Chemical Society.)

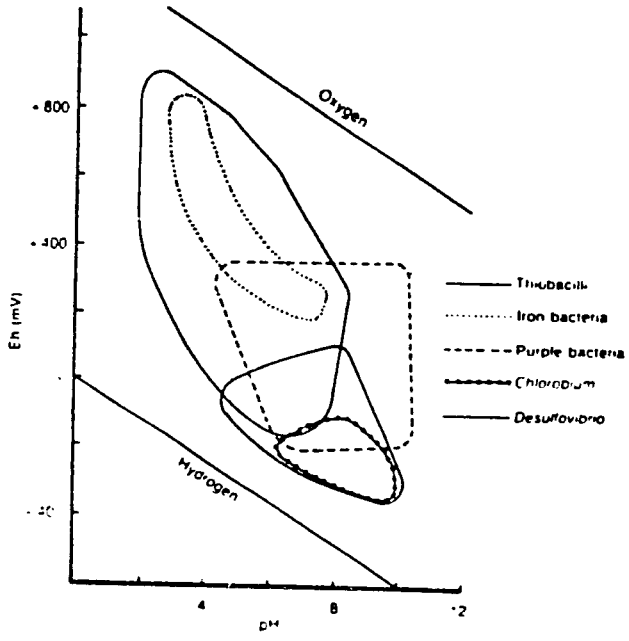
to be totally unaffected by changes in magnetic fields. Some bacteria, however, exhibit magnetotaxis, motility directed by a geomagnetic field (Blakemore, 1975, 1982; Moench and Konetzka, 1978). Magnetotactic bacteria contain dense inclusion bodies, which may impart a magnetic moment upon the cell (Figure 8.15). Some of these magnetotactic bacteria contain approximately 4 percent iron by weight. These inclusions, called magnetosones, may allow the cells to orient themselves, and in a magnetic field, allow them to move actively in a particular direction. The adaptive value of magnetotactic behavior is yet unclear. Because the magnetotactic microorganisms that have been described are microaerophilic or anaerobic, it has been suggested that magnetotactic orientation may help these motile organisms to locate deeper, more reduced organic sediments. The investigation of this and other aspects of magnetotactic behavior has been hampered by the difficulty in growing pure culture of these organisms and most observations have been made only on mixed enrichments.

Organic Compounds

Organic matter in an ecosystem is frequently the limiting factor for growth of heterotrophic microorganisms. Specific organic substrates often favor the growth of particular populations with specific catabolic activities. Some microorganisms require relatively high levels of organic matter, whereas others grow only at relatively low concentrations.

In aquatic ecosystems, the chemical oxygen demand (COD) is often used as an index of the total organic carbon in the sample. The COD is determined by measuring the amount of oxidizing reagent consumed during oxidation of organic matter with dichromate or permanganate. The biological oxygen demand (BOD) yields an estimate of the readily useable concentrations of organic matter. The BOD is determined by allowing microorganisms to consume oxygen in solution during oxidation of the organic matter present in the sample. A high BOD indicates the abundance of organic substrates that can be used by microorganisms aerobically. When the BOD is high, however, microorganisms often use much of the available oxygen for the degradation of organic matter, creating conditions of oxygen depletion and resulting in the death of higher organisms, such as fish, that require O_2 for survival.

Most natural ecosystems are not characterized by high concentrations of usable organic matter; rather, microorganisms in many ecosystems, such as the oceans, live at very low concentrations of nutrients. Periods of starvation are probably experienced by most free-living bacteria. Starvation survival is important in microbial ecology



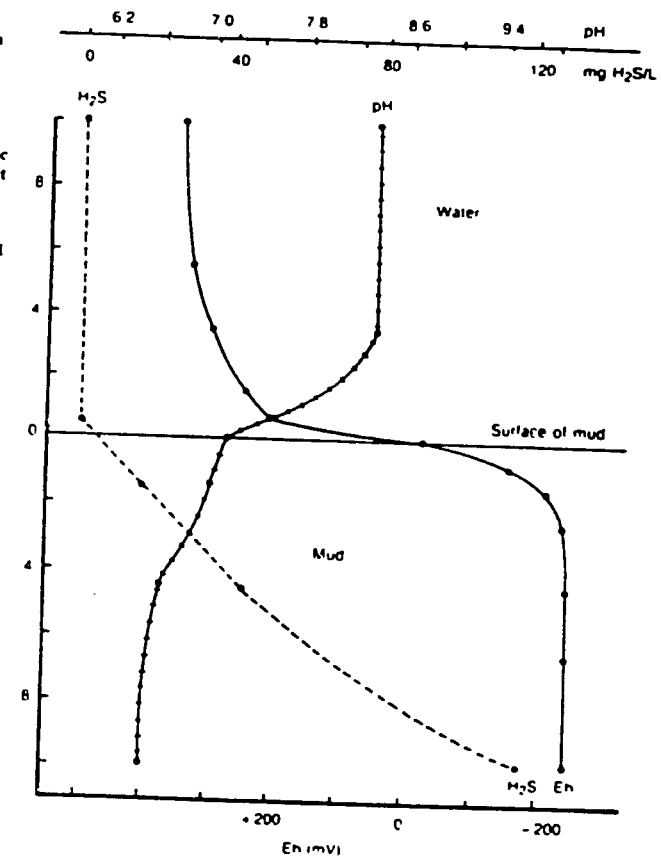
8.13
and E_h tolerance
of various bacteria
in several cycling
s. The thiobacilli
bacteria are ex-
olerant to highly
d aerobic condi-
ich are characteris-
r natural habitat.
um and *Desulfo-*
r restricted to an-
nd neutral to
lkaline pH zones.
interaction of pH
signified by the
ydrogen and oxy-
tials. (Reprinted
nheimer, 1974;
Haas Beckling and
55, reprinted by
on John Wiley and
and VEB Gustav
erlag.)

it is a mechanism that perpetuates the permitting the genome to express itself environmental conditions become fa- (Kjelleberg, 1984).

e is growing evidence of the existence ia especially suited for the exploitation utrient flux habitats, and that they can ished from organisms especially suited nutrient flux environments (Poindexter, tton, 1985). As opposed to copiotrophs growing on high nutrient concentrations), atural distribution implies that nutrient e favors their survival and is an im- actor in their competitiveness, the oli- (low-nutrient) bacteria possess phys- properties that permit them to efficiently vailable limited nutrient resources. Sub- take characteristics of oligotrophs must be suitable for acquisition of growth substrates against steep gradients between the cell and its surroundings and acquired nutrients must be so managed that the cell can maintain its integrity and growth. To achieve a high uptake capacity relative to nutrient-consuming processes, oligotrophs ideally should be small spheres, slender rods or envelope-appendaged bacteria with high affinities (low K_m) for uptake for a variety of utilizable substrates. Indeed many oligotrophs, such as *Caulobacter*, form appendages and are more efficient at transporting substrates into their cells than at excreting byproducts.

Organic compounds can be potential nutrients, but some of them act as inhibitors or poisons. Various metabolic products, such as carboxylic acids, alcohols, and phenolics, are toxic to microbial populations, especially when they ac-

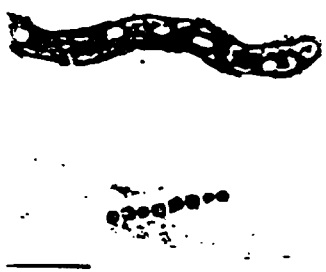
FIGURE 8.14
E_h, pH and H_2S values in a lake. A sulfur-rich lake shore habitat showing reduced pH and E_h below surface of mud. H_2S concentrations were low in water with high E_h (aerobic zone) and high in sediment with low E_h (anaerobic zone). (Reprinted from Rheinheimer, 1974, based on Suckow, 1966; reprinted by permission John Wiley and Sons Ltd. and VEB Gustav Fischer Verlag.)



cumulate in some anaerobic environments where they are not readily metabolized to less toxic degradation products. Some microorganisms specifically excrete allelopathic substances (allelochemics), which are chemicals (other than food substances) produced by one species that affect growth, health, behavior, or population dynamics of other species (Whittaker and Feeny, 1971); production of antibiotics and bacteriocins are prime examples of allelochemics.

Inorganic Compounds

Many inorganic compounds are essential nutrients for microorganisms (Stotzky and Norman, 1964). There are also many inorganic substances that



rographs of magnetotactic bacteria show-
dation and regular arrangement of iron-rich
(Courtesy of R. Blakemore, University of
hire.)

io microorganisms (Ehrlich, 1978). It
necessary to consider the chemical
organic compounds when considering
ounds as microbial nutrients or inhib-
anic compounds of importance in ex-
ecology of microorganisms include
oxygen, carbon dioxide, carbon mon-
rogen, nitrogen, nitrous oxide, and hy-
ide; the elemental form of sulfur, the
monium, ferrous iron, ferric iron,
calcium, silicon, sodium, potassium,
alt, copper, manganese, molybdenum,
nickel, zinc, mercury, cadmium, and
the anions phosphate, carbonate, bi-
sulfide, sulfate, nitrite, nitrate, chloride,
romide, fluoride, silicate, selenite, and

n and Its Reactive Forms Oxygen ex-
ral forms with varying degrees of tox-
lovitch, 1977). Molecular oxygen nor-
ts as O_2 in the atmosphere. Singlet
^{1}) has a higher energy and is more
icroorganisms. It can be formed both
lly and chemically. Peroxidase enzyme systems can form singlet oxygen from hydrogen peroxide. The reactivity of singlet oxygen is a key factor in some biological interactions, such as the ability of blood phagocytes to kill invading microorganisms. Singlet oxygen also forms in the upper atmosphere photochemically and reacts with normal molecular oxygen to produce ozone. Ozone (O_3)is a strong oxidizing agent that kills microorganisms. Ozone is sometimes used as a sterilizing agent, for example, in some water treatment operations.

Another highly reactive oxygen species is the superoxide anion. Superoxide anions are generated by reactions of oxygen with many biochemicals such as flavins, quinones, and thiols. The superoxide anion can destroy lipids and other biochemical components of the cell. It is relatively long-lived and may explain the oxygen sensitivity of some obligate anaerobes. Various obligate anaerobes are not killed by O_2 directly, but are prevented from growing by the presence of oxygen, apparently because of the production and lack of effective removal of superoxide anions (Morris, 1975). The superoxide anion can be converted to hydrogen peroxide plus O_2 by the action of the enzyme superoxide dismutase. Peroxide anions, as in hydrogen peroxide, are also quite toxic to microorganisms. Hydrogen peroxide can be decomposed by catalase enzymes to $H_2O + O_2$ or by peroxidase enzymes to H_2O with the coupled oxidation of a broad range of electron donors.

Carbon Dioxide and Monoxide Carbon dioxide is required for both autotrophic and heterotrophic metabolism. CO_2 may be available in solution as carbonate or bicarbonate. Carbon dioxide consumption is often used as a measure of productivity. Production of CO_2 is a useful measure of the mineralization of organic matter.

Carbon monoxide is another form of inorganic carbon; it strongly inhibits microbial respiration pathways. Carbon monoxide binds with iron-containing proteins and molecules such as cytochromes, blocking electron transport. Some microorganisms can metabolize CO, but most are inhibited by carbon monoxide.

Nitrogen For most microorganisms, nitrogen gas is an inert form of nitrogen. Only a restricted group of nitrogen-fixing bacteria are able to convert gaseous nitrogen into combined forms of nitrogen. The most important inorganic nitrogen-containing nutrients are ammonium, nitrate, and nitrite ions. Nitrogen is required for protein formation. In addition to being required for protein synthesis, some chemolithotrophic microorganisms oxidize ammonium or nitrite for generating energy. Some microorganisms use nitrate as the terminal electron acceptor in respiration pathways, resulting in denitrification, which returns nitrogen gas to the atmosphere. The fact that ammonium is a cation and nitrate and nitrite are anions results in differential leaching of these latter nitrogen-containing ions from environments such as soil.

Phosphorus Phosphate is required for microbial generation of ATP and for nucleic acid and membrane synthesis. High concentrations of phosphate, however, may be inhibitory to microbial growth. Concentrations of available nitrogen and phosphorus often limit both productivity and decomposition in aquatic habitats. Additions of useable nitrogen and phosphorus are often used to increase productivity in aquaculture practices. The elimination of the phosphate limitation is sometimes undesirable, as it can lead to prolific algal or cyanobacterial growth. This growth has occurred following addition of phosphates, in the form of household detergents, to lakes where productivity was phosphate-limited. On the other hand, controlled addition of nitrogen and phosphorus can enhance decomposition of polluting organic compounds. For example, the extent of petroleum biodegradation in seawater has been found to be limited by both nitrogen and phosphorus (Figure 8.16); this limitation can be overcome by application of oleophilic nitrogen and phosphorus fertilizer (Atlas and Bartha, 1972, Atlas, 1977).

Sulfur Inorganic sulfur is required by microorganisms for formation of proteins and other

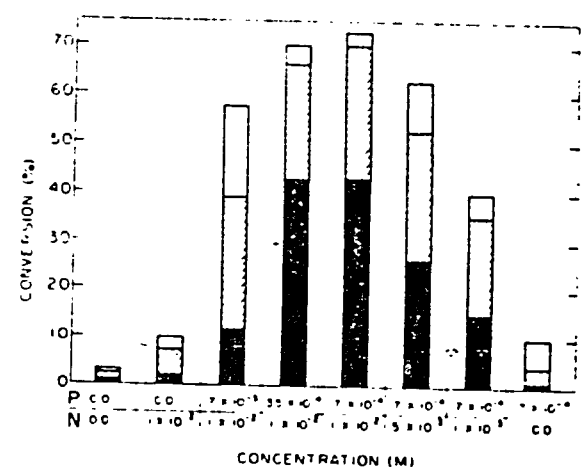


FIGURE 8.16
Limiting effects of nitrogen and phosphorus on the biodegradation of petroleum in seawater. Solid portions of histogram indicates mineralization of oil measured by CO_2 evolution. Total height of histogram indicates the biodegradation of oil as measured by weight loss. Height of solid + cross-hatched portion indicates biodegradation measured by gas chromatography. Natural levels of available N and P in seawater limit extent of biodegradation of oil spills. (From Atlas and Bartha, 1972; reprinted from *Biotechnology and Bioengineering* by permission; John Wiley and Sons.)

cular components. Some oxidized forms of t, such as sulfur dioxide, are toxic to mi- ganisms and may even be used as disinfecting ts. Hydrogen sulfide is used by some pho- otrophic bacteria as an electron donor. Hy- en sulfide is also toxic to many enzyme sys- that involve heavy metal atoms. Numerous organisms produce hydrogen sulfide as a bolic product of protein metabolism.

Other Elements Iron is required in cyto- mes and other iron proteins. The oxidation of iron depends on the pH and the redox ntial. Ferrous and ferric ions demonstrate rent degrees of solubility, which affects their ability for biological uptake.

hloride ions are essential for maintenance embrane function. Chlorate and hypochlorite are strong oxidizing agents and are toxic to organisms. Hypochlorite is often used as infectant for drinking water and as a final in sewage treatment and enters ecosystems, as rivers, as part of the sewage effluent. urements of residual chlorine are made on effluents to determine the concentrations e toxic compounds.

ilicon is required by diatoms, silicoflagellates, adiolaria for building cell wall structures. xample of the depletion of silicate during ns of diatoms is shown in Figure 8.17.

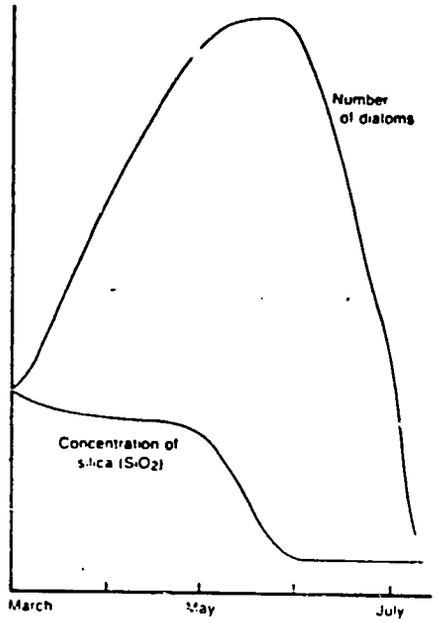


FIGURE 8.17
A graph illustrating that the amount of silica available in a lake can be the limiting factor in the growth of diatoms. Note silica depletion following diatom bloom and subsequent crash in the diatom population. Concentrations of other nutrients were measured but none showed the type of correlation shown in the graph. (From Jensen and Salisbury, 1972; reprinted by permission; copyright Wadsworth Publishing Co., Belmont, Calif.)

mmary

are a large number of abiotic physical and ical factors that influence the growth, sur- and metabolic activity of microorganisms. iety of physical measurements and analytical dures are needed in order to characterize abiotic factors that govern the microbial in- ents in a habitat. In some instances, one or factors can be identified that regulate the th and activities of individual microbial pop- ns in a given ecosystem. Often the effect e abiotic factor on a microbial population is influenced by other abiotic factors. In some cases, microorganisms are limited in their activities by the availability of some essential nutrient; in other cases, some environmental factor may exceed the maximum or minimum tolerance of a particular microbial population.

Factors that have major influences on microorganisms include: electromagnetic radiation, which in the form of visible light is the principal energy source for the global ecosystem, but which as high-energy ionizing radiation is destructive for life; temperature, which determines in many cases rates of growth and activity as well as the particular microorganisms that occur within an ecosystem and what functions they perform; pressure due to atmospheric, hydrostatic, and osmotic forces, which effects the survival of organisms; redox potential, which effects what forms of microbial metabolism may occur; pH, which influences survival and nutrient supply; availability of liquid water, which is essential for all active life processes; and the concentrations and chemical forms of organic and inorganic compounds, which may be essential nutrients or may act as inhibitors and poisons.

REFERENCES & SUGGESTED READINGS

Abdollahi, H., and D. B. Nedwell. 1979. Seasonal temperature as a factor influencing bacterial sulfate reduction in a saltmarsh sediment. *Microbial Ecology* 5:73–79.

Alexander, M. 1976. Natural selection and the ecology of microbial adaptation in a biosphere. In M. R. Heinrich (ed.). *Extreme Environments. Mechanisms of Microbial Adaptation.* Academic Press, New York. pp. 3–25.

Anderson, A. W., H. D. Hordan, R. F. Cain, G. Parrish, and D. Duggan. 1956. Studies on a radioresistant *Micrococcus*. I. Isolation, morphology, cultural characteristics and resistance to gamma irradiation. *Food Technology* 10:575–578.

Atlas, R. M. 1977. Stimulated petroleum biodegradation. *Critical Reviews in Microbiology* 5:371–386.

Atlas, R. M., and R. Bartha. 1972. Degradation and mineralization of petroleum in seawater: limitation by nitrogen and phosphorus. *Biotechnology and Bioengineering* 14:309–318.

Baas-Becking, L. M. G., and E. J. F. Wood. 1955. Biological processes in the estuarine environment. *Koninklijke Nederlandse Akademie Van Wetenschappen Proceedings B* 58:160–181.

Baross, J. A., F. J. Hanus, and R. Y. Morita. 1972. Effects of hydrostatic pressure on uracil uptake, ribonucleic acid synthesis, and growth of three obligately psychrophilic marine vibrios, *Vibrio alginolyticus* and *Escherichia coli*. In R. R. Colwell and R. Y. Morita (eds.). *Effects of the Ocean Environment on Microbial Activities.* University Park Press, Baltimore. pp. 180–222.

Baross, J. A., and J. W. Deming. 1983. Growth of "black smoker" bacteria at temperatures of at least 250°C. *Nature* (London) 303:423–426.

Baross, J. A., J. W. Deming, and R. R. Becker. 1984. Evidence for microbial growth in high-pressure, high-temperature environments. In M. J. Klug and C. A. Reddy (eds.). *Current Perspectives in Microbial Ecology.* American Society for Microbiology, Washington, D.C. pp. 186–195.

Black, C. A. (ed.). 1965. Methods of Soil Analysis: Part 2—Chemicals and microbiological properties. *American Society of Agronomy*, Madison, Wis.

Blakemore, R. 1975. Magnetotactic bacteria. *Science* 190:377–379.

Blakemore, R. P. 1982. Magnetotactic bacteria. *Annual Review of Microbiology* 36:217–238.

Brock, T. D. 1978. *Thermophilic Microorganisms and Life at High Temperatures.* Springer-Verlag, New York.

Brown, A. D. 1976. Microbial water stress. *Bacteriological Reviews* 40:803–846.

Button, D. K. 1985. Kinetics of nutrient limited transport and microbial growth. *Microbiological Reviews* 49:270–297.

Campbell, L. L., and B. Pace. 1968. Physiology of growth at high temperatures. *Journal of Applied Bacteriology* 31:24–35.

Carlile, M. J. 1980. Positioning mechanisms—the role of motility, taxis and tropism in the life of microorganisms. In D. C. Ellwood, M. J. Latham, J. N. Hedger, and J. M. Lynch (eds.). *Contemporary Microbial Ecology.* Academic Press, London. pp. 55–74.

Castenholz, R. W. 1979. Evolution and ecology of thermophilic microorganisms. In M. Shilo (ed.). *Strategies of Microbial Life in Extreme Environments.* Verlag Chemie, Weinheim, New York. pp. 373–392.

ery large, slowly cycled reservoir for 8 × 10^{15} metric tons) is N_2 gas of the (79 percent). Large, but essentially reservoirs of nitrogen are present in × 10^{15} metric tons) and sedimentary metric tons) rock as bound, nonex- ammonia (Blackburn, 1983). Physi- and biological weathering releases om these reservoirs so slowly that it le influence on yearly cycling models. deposits of more readily available itrogen are rare. Availability of com- en is an important limiting factor for duction in many ecosystems. The only mulations of nitrate occur on some e Chilean coast; these nitrate deposits from the decomposition of guano seabirds. The dry climate of these prevented the leaching of nitrate 76, Delwiche, 1970).

ganic nitrogen salts, ammonium, ni- trate, are highly water-soluble and are distributed in dilute aqueous oughout the ecosphere; they form ctively cycled reservoirs. Living and matter also provide relatively small, d reservoirs of nitrogen. In temperate dulized soil organic matter, humus, tantial and relatively stable nitrogen he nitrogen of humus becomes avail- ke by living organisms only through neralization, a process measured in centuries. In tropical climates, the and humidity favor the rapid direct n of organic matter, limiting the ac- d litter and humus.

nimals, and most microorganisms bined forms of nitrogen for incor- cellular biomass (Brown and John- ut the ability to fix atmospheric ni- ricted to a limited number of bacteria ic associations. While many habitats lants for a supply of organic carbon sed as source of energy, all habitats r on the bacterial fixation of atmospheric nitrogen or on human intervention through the distribution of nitrogen fertilizers synthesized by the Haber-Bosch process, which was invented shortly before the First World War. Plants could not continue their photosynthetic metabolism without the availability of fixed forms of nitrogen provided by microorganisms or by synthetic fertilizer.

The biogeochemical cycling of the element nitrogen is highly dependent on the activities of microorganisms. A generalized scheme for the biogeochemical cycling of nitrogen is shown in Figure 11.1. A more detailed representation of nitrogen flow through the biosphere, indicating the magnitude of some critical transfer rates, is shown in Figure 11.2. Recent quantitative estimates of global biological fixation (Burns and Hardy, 1975; Soderlund and Svensson, 1976) were upwardly revised from earlier ones (Dewiche, 1970). Fixation on land, which includes considerable amounts from agriculturally managed legume crops, amounts to 135 million metric tons per annum (mta). This greatly exceeds N-fixation in the much larger marine environment (40 million mta). Anthropogenic nitrogen inputs in the form of synthetic fertilizer (30 million mta), combustion (19 million mta), and nitrogen fixation by leguminous and other crops (44 million mta) approach the total of nitrogen fixation in grasslands (45 million mta), forests (40 million mta), other terrestrial areas (10 million mta), and the marine environment (40 million mta). Synthetic nitrogen fertilizer input is projected to rise further and may reach 100 million mta by the year 2000. Volcanic activity, ionizing radiation, and electrical discharges supply additional combined nitrogen to the atmosphere; when washed down with precipitation, this combined nitrogen becomes available to the biosphere, but abiotically fixed nitrogen is estimated to be only 10–20 percent of the biological fixation. Microorganisms are also responsible for the return of molecular nitrogen to the atmosphere through denitrification and for the transformations that affect the mobility and accessibility of fixed nitrogen to the inhabitants

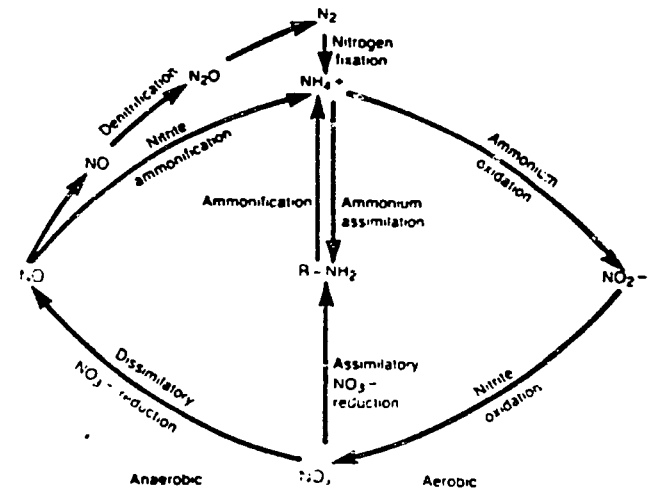


FIGURE 11.1
The nitrogen cycle showing chemical forms and key processes in biogeochemical cycling of nitrogen. The left portion of the cycle represents anaerobic, the right portion aerobic processes. The critical steps of nitrogen fixation, nitrification, and denitrification are all mediated by bacteria. R-NH_2 represents amino groups in cell protein.

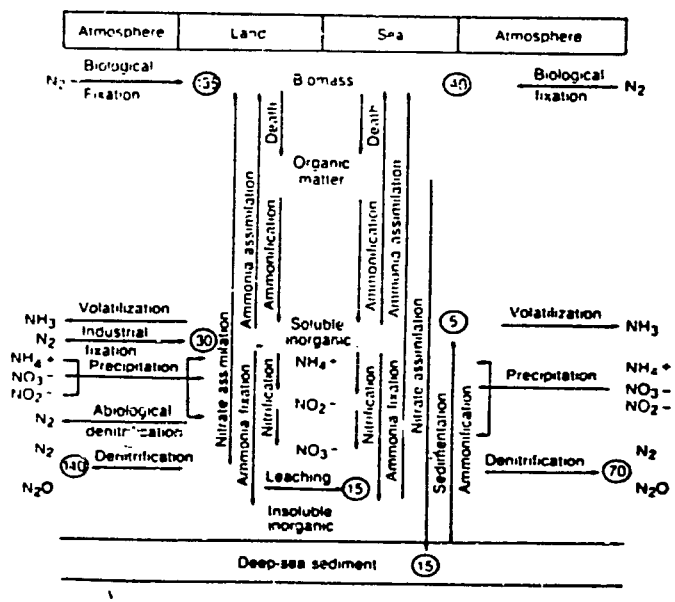


FIGURE 11.2
General scheme for transfer of nitrogen between the atmosphere, marine, and terrestrial environments indicating relative transfer rates (numbers are millions of metric tons/yr) and interconversions within the terrestrial and aquatic reservoirs. (From Burns and Hardy, 1975; reprinted by permission; copyright Springer-Verlag.)

ıosphere allows the gas chromato-
ırement of the denitrification process
a single product, N_2O. Measurement
tion with time under these conditions
estimate of denitrification rates. The
plicable to pure cultures as well as
ntal samples and *in situ* measure-
s limited in sensitivity by the low
bility in many habitats.
steps within the nitrogen cycle are
pression. For example, ammonium
inhibit the fixation of atmospheric
ification occurs readily in neutral
soils, but is inhibited in anaerobic
idic soils. Assimilatory nitrate re-
ity is repressed by ammonia; dis-
trate reductase is repressed by

various environmental conditions
nitrogen cycling processes, there
ation of cycling processes. Fixation
curs in both surface and subsurface
ixation occurs exclusively in aerobic
itrification predominates in water-
nd in anaerobic aquatic sediments.
of nitrogen within a given habitat
easonal fluctuations; during spring
ns of cyanobacteria, for example,
en fixation in aquatic habitats usually
ecting population fluctuations and
needed energy and mineral nutrients
molecular nitrogen.

Cycle

active element with stable valence
2 to +6 (Table 11.2) and is among
abundant elements in the crust of
an average concentration of 520
becomes a limiting nutrient (Ehrlich,
en, 1983). At least some elemental
s and some sulfide ores appear to
ic origin (Ivanov, 1968). Eruptive
anic activity continues to introduce

additional sulfur into the ecosphere at relatively low rates. With the notable exceptions of ferric and calcium sulfates, most sulfate salts are readily soluble in water. Sulfate is the second most abundant anion in seawater and the SO_4^{2-} of the marine environment represents a very large, slowly cycled sulfur reservoir. Living and dead organic matter comprise a smaller, more rapidly cycled sulfur reservoir. Largely inert sulfur reservoirs are metal sulfides in rock, elemental sulfur deposits, and fossil fuels. Human activities, including strip mining and the burning of fossil fuels, have mobilized a part of these inert sulfur reservoirs with destructive pollution consequences. Some of these consequences will be discussed later in this chapter.

Plants, algae, and many heterotrophic microorganisms assimilate sulfur in the form of sulfate. For incorporation into cysteine, methionine, and coenzymes in the form of sulfhydryl (-SH) groups, sulfate needs to be reduced to the sulfide level by assimilatory sulfate reduction. A direct uptake as sulfide is not feasible for most microorganisms because of the very high toxicity of H_2S. In assimilatory sulfate reduction, toxicity is avoided by immediately reacting the reduced sulfur with an acceptor—for example, serine—to yield cysteine.

Sulfur is released as mercaptans and H_2S from the reduced organosulfur state during decomposition of dead organic matter (Bremner and Steele, 1978). Analogous to ammonification, this process is referred to as desulfuration. Volatile

TABLE 11.2
Oxidation states of sulfur in various compounds

Form	Example	Oxidation state
S^{2-}	sulfides, mercaptans	−2
S^0	elemental sulfur	0
S_2O_4	hyposulfite	+2
SO_3^{2-}	sulfite	+4
SO_4^{2-}	sulfate	+6

mercaptans and H_2S are the offensive odor components of rotten eggs and cabbage. The action of cysteine desulfhydrase can be described as follows (equation 6):

$$(6)\quad HSCH_2 \cdot CHNH_2 \cdot COOH + H_2O \xrightarrow[\text{desulfhydrase}]{\text{cysteine-}} HOCH_2 \cdot CHNH_2 \cdot COOH + H_2S$$

Hydrogen sulfide is a highly reactive compound and may be oxidized chemically or photochemically by atmospheric oxygen. Alternatively, it may be subject to microbial oxidation under aerobic conditions or it may be phototrophically oxidized under anaerobic conditions. Under anaerobic conditions, sulfate as well as elemental sulfur may serve as electron acceptors, while organic substrates are oxidized. The microbial cycling of sulfur is summarized in Figure 11.4.

Oxidative Sulfur Transformations

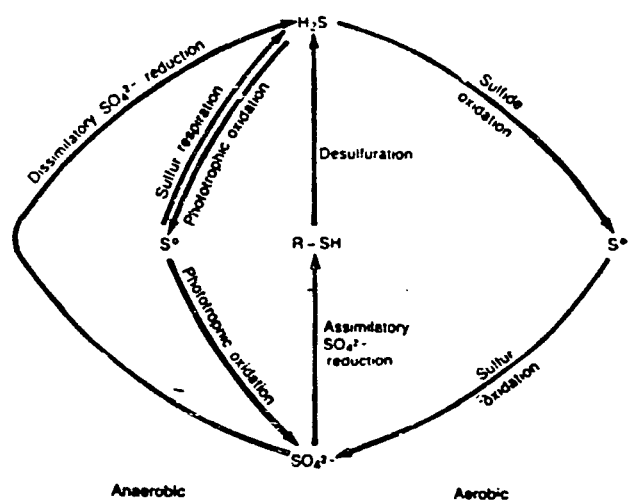
In the presence of oxygen, reduced sulfur compounds are capable of supporting chemolithotrophic microbial metabolism. *Beggiatoa*, *Thiovulum*, *Thiothrix*, and the more recently described thermophilic *Thermothrix* (Caldwell et al., 1976), are filamentous, microaerophilic bacteria capable of oxidizing H_2S according to equation 7.

$$(7)\quad H_2S + 1/2\,O_2 \rightarrow S^0 + H_2O \quad (\Delta G = -50.1 \text{ kcal/mole})$$

Sulfur globules are deposited within the cells. In the absence of H_2S these sulfur globules are slowly oxidized further to sulfate. These typical gradient organisms position themselves on the interface of an anaerobic environment, the sediment, and the partially oxygenated water in contact with the sediment (Jorgensen and Revsbeck, 1983).

The large size and observable sulfur globules of *Beggiatoa* allowed Winogradsky to develop the concept of microbial chemolithotrophy almost a hundred years ago. Although it was clear that *Beggiatoa* derived energy from H_2S and S^0 oxidation, until very recently it was impossible to grow *Beggiatoa* in pure culture under strictly

FIGURE 11.4
Representation of the sulfur cycle showing biogeochemical transformations of oxidized and reduced forms of sulfur. R-SH represents sulfhydryl groups of cell protein. Note the similarities of the sulfur cycle to the nitrogen cycle (Figure 11.1).



As the sum of the above reactions
metalic iron is rapidly converted
droxide and ferrous sulfide. The
are:

$$+ 2H_2O \rightarrow Fe(OH)_2 + H_2$$

$$H_2 + CaSO_4 \rightarrow H_2S + Ca(OH)_2 + 2H_2O$$

$$H_2S + Fe^{2+} \rightarrow FeS + H_2$$

$$Fe^0 + CaSO_4 + 4H_2O \rightarrow FeS + 3Fe(OH)_2 + Ca(OH)_2$$

horus Cycle

not an abundant component of the
osgrove, 1977; Ehrlich, 1981). Its
further restricted by its tendency
in the presence of divalent metals
and ferric (Fe^{3+}) ions at neutral
H. Large, slowly cycled reservoirs
occur in marine and other aquatic
mall, actively cycled reservoirs of
e dissolved phosphate in soils and
hosphate in living and dead organic
gely inert reservoir has been phos-
ch as apatite ($3Ca_3[PO_4]_2 \cdot Ca[FeCl]_2$).
voir is being increasingly tapped by
industry. Much of this phosphate
lost to marine sediments, raising
ut future availability of an important
primary phosphates, such as
ly one of the three PO_4^{3-} valences
a metal. Primary phosphates have
olubilities. Secondary and tertiary
ave only one or no hydrogen atoms
cule, respectively, and their water
e progressively lower. In the man-
hosphate fertilizer the tertiary phos-
ate is converted by acid treatment
and primary "superphosphate."

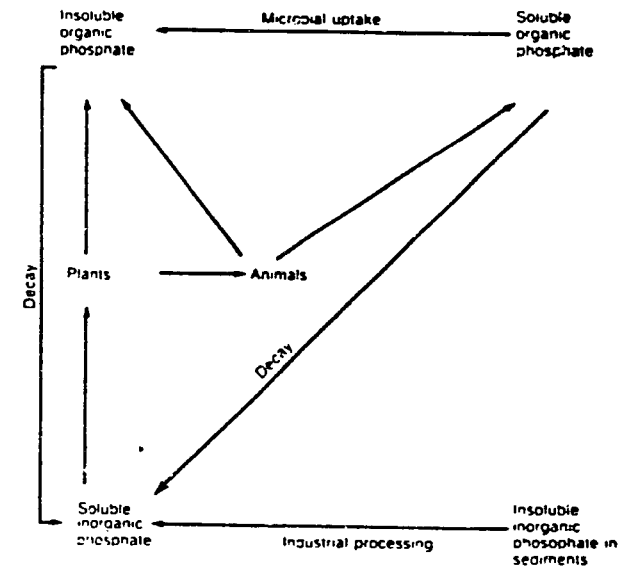
Phosphorus is an essential element in all living systems. Within biological systems, the most abundant forms of phosphorus are phosphate esters. Most of the phosphorus in cells is in nucleic acids. Phosphate diester bonds form the links within nucleic acid molecules. Phosphate also forms an essential portion of the ATP molecule. The hydrolysis of phosphate from ATP to ADP forms the basis for most energy transfer reactions within biological systems. Phospholipids, which contain hydrophilic phosphate groups, are essential components of cell membranes.

The microbial cycling of phosphorus for the most part does not alter the oxidation state of phosphorus (Stewart and McKercher, 1982). Most phosphorus transformations mediated by microorganisms can be viewed as transfers of inorganic to organic phosphate or as transfers of phosphate from insoluble, immobilized forms to soluble or mobile compounds (Figure 11.7).

Although phosphate is normally not reduced by microorganisms, it appears that some soil and sediment microorganisms may be capable of utilizing phosphate as a terminal electron acceptor under appropriate environmental conditions. Reliable and recent documentation for this transformation is not available. Phosphate is likely to serve as a terminal electron acceptor in the absence of sulfate, nitrate, and oxygen. The final product of phosphate reduction would be phosphine (PH_3). Phosphines are volatile and spontaneously ignite on contact with oxygen, producing a green glow. The production of phosphines is sometimes observed near burial sites and swamps where there is extensive decomposition of organic matter. Phosphines can also ignite the methane produced in similar environments, giving rise to "ghostly" light phenomena. A microbial involvement in phosphine production is likely but requires experimental confirmation.

Within many habitats phosphates are combined with calcium, rendering them insoluble and unavailable to plants and many microorganisms. A number of heterotrophic microorganisms are

FIGURE 11.7
The phosphorus cycle showing various transfers, none of which alters the oxidation state of phosphate.



capable of solubilizing phosphates from such sources. Phosphate is assimilated by these microorganisms, which also solubilize a large proportion of the insoluble inorganic phosphate, releasing it for use by other organisms. The mechanism of phosphate solubilization is normally by production of organic acids. Some chemolithotrophic microorganisms, such as *Nitrosomonas* and *Thiobacillus*, mobilize inorganic phosphates by producing nitric and sulfuric acids, respectively (Tiessen and Stewart, 1985).

Within soils, phosphate also exists as insoluble iron, magnesium, and aluminum salts. The mobilization of insoluble ferric phosphates may occur when microorganisms reduce ferric ions to ferrous ions under anaerobic conditions. Flooding of soils enhances release of phosphate by this mechanism.

Soluble forms of inorganic phosphates can be readily taken up by plants and microorganisms and assimilated into organic phosphates. As an example, inorganic phosphate reacts with ADP to generate ATP when coupled with a sufficiently exothermic reaction. Inorganic phosphate also reacts with carbohydrates such as glucose at the initiation of glycolysis.

The reverse process, mineralization of organic phosphates, also occurs; this process is catalyzed by phosphatase enzymes. Many microorganisms produce phosphatase enzymes. Some bacteria and fungi produce phytase, which releases soluble inorganic phosphates from inositol hexaphosphate (phytic acid) (equation 24).

$$\text{Inositol hexaphosphate} + 6H_2O \xrightarrow{\text{phytase}} \text{inositol} + 6\,PO_4^{3-}$$

Microbial activities may also immobilize phosphorus, rendering it inaccessible to the biological community. The assimilation of phosphorus into

l constituents, such as membranes, osphate from the available pool. In ons, microrganisms aid plants by osphates; in others they also compete or available phosphate resources. vity in many habitats is phosphate- uatic environments, phosphate con- xhibit seasonal fluctuations that are ith algal and cyanobacterial blooms. ation of phosphorus, especially in ats, greatly limits primary produc- atic habitats, phosphorus may exist particulate form. These forms exhibit eactivity and availability to the bi- munity. Phosphate concentrations habitats are closely correlated with n. Phosphate-limited lakes normally hic. The addition of phosphates from h as detergents fillers causes these me eutrophic (Chapra and Robertson,

Cycle

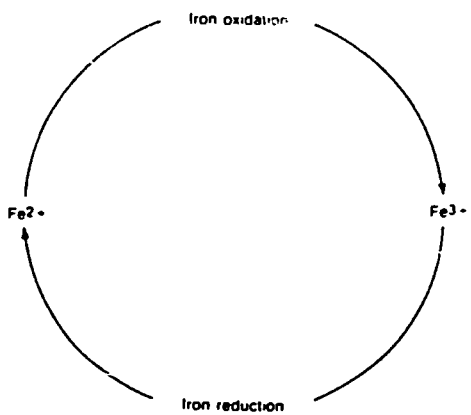
urth most abundant element in the earth, but only a small portion of vailable for biogeochemical cycling S1; Nealson, 1983). The cycling of largely of oxidation-reduction re- reduce ferric iron to ferrous iron and us iron to ferric iron (Figure 11.8). ion-reduction reactions are important nic iron-containing compounds and iron compounds.

Fe^{3+}) and ferrous (Fe^{2+}) ions have nt solubility properties. Ferric iron n alkaline environments as ferric Ferric iron may be reduced under nditions to the more soluble ferrous some anaerobic conditions, however, S may be evolved to precipitate iron lfide. Flooding of soil, which creates nditions, favors the accumulation of ferrous iron. In aerobic habitats, such as well-drained soil, most of the iron exists in the ferric state.

In organic compounds iron is often attached to organic ligands by chelation. Chelated iron can undergo oxidation-reduction transformations and these are utilized in electron transport processes. Cytochromes of electron transport chains contain iron that undergoes oxidation-reduction transformations during the transfer of electrons.

The low solubility of ferric iron necessitates relatively elaborate uptake and transport mechanisms for this otherwise abundant element. Several groups of bacteria utilize siderophores, special iron chelating agents, to facilitate the solubilization and uptake of iron. The excretion of siderophores is induced by iron deficiency. To date, two groups of siderophores have been studied extensively. Phenol-catechol derivatives are synthesized by enterobacteria and bear such common names as enterochelin and enterobactin. Streptomycetes and some other bacterial groups synthesize derivatives of hydroxamic acids referred to as ferrioxamines. In each case the ferric iron is chelated by multiple hydroxyl or carbonyl groups as the ferric iron is enclosed in the molecular cage of the chelator. Siderophores have molecular weights between 500 and 1,000 and are not transported back through the cell membrane, but rather pass on the iron molecule to siderophore receptor proteins in the membrane. The siderophore receptor shuttles the iron molecule through the membrane and releases the siderophore to chelate additional molecules of ferric iron (Lewin, 1984).

FIGURE 11.8
The iron cycle showing interconversion of ferrous and ferric iron.



Under alkaline to neutral conditions, ferrous iron is inherently unstable in the presence of O_2 and is oxidized spontaneously to ferric iron. Under such conditions microorganisms have little chance to extract energy from the oxidation process. Under acidic reaction conditions in oxygenated environments, however, ferrous iron is relatively stable and under such conditions acidophiles such as *Thiobacillus ferrooxidans* and some strains of *Sulfolobus acidocaldarius* are capable of the chemolithotrophic oxidation of ferrous iron.

$$(25)\quad Fe^{2+} + H^+ + \tfrac{1}{4} O_2 \rightarrow Fe^{3+} + \tfrac{1}{2} H_2O \quad (\Delta G = -6.5 \text{ kcal/mole})$$

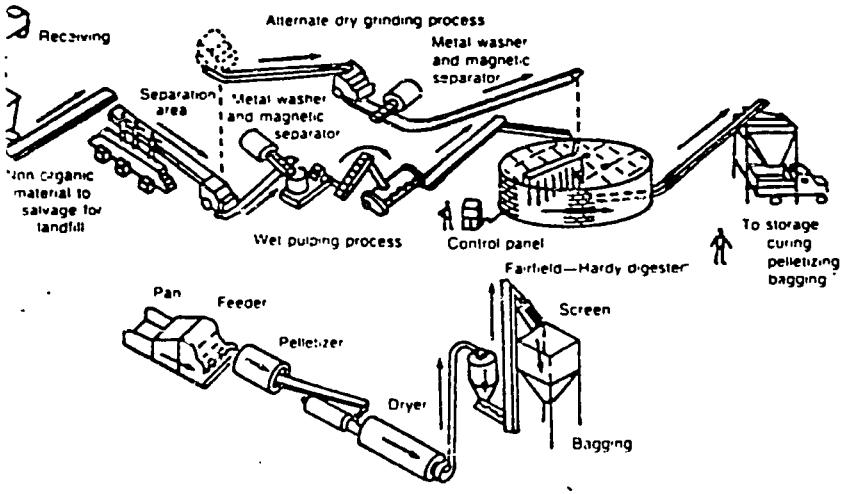
Alternatively, the same microorganisms can also oxidize reduced sulfur compounds. Non-acidophilic iron bacteria, such as the prosthecate *Hyphomicrobium*, *Pedomicrobium*, and *Planctomyces*, the filamentous *Sphaerotilus* and *Leptothrix*, were at times described as being chemolithotrophs or at least as deriving energy from ferrous iron oxidation. In addition, a number of additional "iron bacteria" such as *Gallionella*, *Metallogenium*, *Seliberia*, *Ochrobium*, *Siderocapsa*, *Naumanniella*, and *Siderococcus* were described from enrichments but were never obtained as stable axenic cultures (Jones, 1986). Although these organisms appear to catalyze ferrous iron oxidation to some extent and become encrusted with the ferric precipitate, the chemolithotrophic or energy-yielding nature of the process has never been demonstrated convincingly. Cell walls may simply act as catalytic surfaces for ferric iron precipitation or the cells may use ferrous iron oxidation as a sink for harmful excess oxygen.

The activity of iron-oxidizing bacteria can lead to substantial iron deposits. Typically, groundwater seeping through sand formations dissolves ferrous salts. Underground, a lack of oxygen usually prevents iron oxidation. When groundwater seeps to the surface, usually in a swampy area, iron oxidizers convert the ferrous (Fe^{2+}) ions to ferric (Fe^{3+}), which precipitates as ferric hydroxide and forms bog-iron deposits. Such easily accessible surface deposits were mined extensively and smelted in the early industrial age.

Since oxygenic photosynthesis changed the atmosphere of our planet to an oxidizing one, most iron in the biosphere is kept in the oxidized (ferric) state. Where limited oxygen diffusion and vigorous heterotrophic microbial activity create anaerobic conditions, as occurs in the hypolimnion of stratified lakes, waterlogged soils, and aquatic sediments, ferric iron may act as an electron sink and be reduced to Fe^{2+}. A large and heterogeneous group of heterotrophic bacteria appear to be involved in iron reduction including *Bacillus*, *Pseudomonas*, *Proteus*, *Alcaligenes*, clostridia, and enterobacteria.

The mechanism of iron reduction remains largely unexplored. The facts that nitrate inhibits Fe^{3+} reduction, and nitrate reductase negative mutants lose their ability to reduce Fe^{3+}, link iron reduction to the nitrate reductase system at least in some microorganisms (Nealson, 1983). Some iron reduction may occur non-enzymatically when reduced products of microbial metabolism, such as formate or H_2S, react chemically with Fe^{3+}. In soils, iron reduction is linked to a condition called gleying. Anoxic conditions due to water-logging or high clay content give rise to the formation of reduced ferrous iron, which gives the soil a greenish-grey color and a sticky consistency. The predominant iron reducers within gleyed soils appear to be *Bacillus* and *Pseudomonas* species.

Compost plant flow diagram



·ucess of composting. (From Pavoni et al., 1973; reprinted by permission; · Nostrand Reinhold Co.)

·ra, *Thermoactinomyces*, and *Clos-*
·*rmocellum*. Important fungi in the
composting process are *Geotrichum*
Aspergillus fumigatus, *Mucor pusillus*,
·*n thermophile*, *Thermoascus auran-*
·*orula thermophila* (Finstein and Mor-

·imal composting, several conditions
Adequate moisture (50–60 percent
·nt) must be present, but excess mois-
·rcent or above) should be avoided,
·rferes with aeration and lowers self-
·use of its large heat capacity. Carbon-
·tios should not be greater than
1. Lower nitrogen content does not
formation of a sufficient microbial bi-
·cessive nitrogen (C:N = 25:1, or nar-
·s to volatilization of ammonia, causing
·ms, and lowers the fertilizer value of
·: compost.

The economic viability of composting is often mistakenly judged only by the sale value of the generated compost. The primary purpose of composting, however, is to dispose of noxious wastes in an environmentally acceptable manner. Any costs recovered from the sale of compost reduce the cost of waste disposal operation, but should not be expected to render the waste disposal operation self-supporting. Landfill operations may have lower direct costs than com-
ng, but the long-range environmental costs in terms of groundwater contamination favor the composting process. Because of their water pollution potential, raw or anaerobically digested sewage sludges and other unstable organic wastes are acceptable by federal and state regulations only in a few designated landfill operations. In addition, compost is useful in soil improvement in certain situations.

Compost is a good soil conditioner and supplies some plant nutrients, but cannot compete with synthetic fertilizers in agricultural production. If sewage sludge was a major component of the compost mixture, the finished compost may contain relatively high concentrations of potentially toxic heavy metals, such as cadmium, chromium, and thallium. Little is known about the behavior of these metals in agricultural soils and, because of the possibility that some of these heavy metals may contaminate agricultural products, the large-scale use of sewage sludge-derived compost in agriculture does not seem wise at this time. Compost finds application in parks and gardens for ornamental plants, and in land reclamation, such as for strip mining reclamation and highway beautification projects. Compared to alternative disposal methods, composting has considerable environmental advantages and is likely to be practiced in the future on an increased scale.

Treatment of Liquid Wastes

Liquid wastes are produced by everyday human activities (domestic sewage) and by various agricultural and industrial operations. Following natural drainage patterns or sewers, liquid waste discharges enter natural bodies of surface water, such as groundwater, rivers, lakes, and oceans. At much slower rates, they may also percolate to the groundwater table, especially if this is high or if fissures are present in the unsaturated (vadose) soil layer. These same water bodies are used by people in alternative ways, such as sources of drinking water, household, industrial, and irrigation water, for fish and shellfish production, swimming and other recreational activities. Therefore, it is crucial to maintain the quality of these natural waters to the best of our ability. Water quality is a broad concept. Its maintenance means that natural waters cannot be overloaded with organic or inorganic nutrients, toxic, noxious, or esthetically unacceptable substances. They should not become vehicles of disease transmission from fecal contamination nor should their oxygenation, temperature, salinity, turbidity, or pH be altered significantly.

Natural waters have an inherent self-purification capacity. Organic nutrients are utilized and mineralized by heterotrophic aquatic microorganisms. Ammonia is nitrified and, along with other inorganic nutrients, is utilized and immobilized by algae and higher aquatic plants. Allochthonous populations of enteric and other pathogens are reduced in numbers and eventually eliminated by competition and predation pressures exerted by the autochthonous aquatic populations. Thus, sufficiently low amounts of raw sewage can be accepted by natural waters without significant deterioration of water quality. Dense human populations, community living patterns, and large-scale agricultural and industrial activities typically produce liquid wastes on a scale that overwhelms the homeostasis of aquatic communities and causes unacceptable deterioration of water quality (Dart and Stretton, 1977; LaRiviere, 1977).

Historically, the first human concern with water quality was prompted by destructive epidemics of cholera, typhoid fever, dysentery, and other diseases spread by waterborne pathogens. Waste treatment, protection of drinking water sources, and disinfection of drinking water and sewage, gradually introduced in the early years of this centrury, largely eliminated the spread of waterborne epidemics in developed countries; concern remained, however, for the health, environmental, and esthetic problems caused by an overload of organic nutrients in sewage-polluted waters. Even as treatment methods were developed that alleviated most of the above problems, total sewage volume increased further, and the inorganic nutrient content of sewage started to command additional attention because of "cultural eutrophication" problems caused by the excessive mineral nutrient enrichment of natural waters (National Academy of Sciences, 1969; Keeny et al., 1971). Man-made synthetic organic molecules, not readily subject to microbial degradation during

atment, also cause additional contem-
cerns.

al Oxygen Demand (BOD)

orary liquid waste treatment, the usual
erations is the reduction of the bio-
xygen demand (BOD) associated with
and dissolved organics, occasionally
the removal of inorganic nutrients
trant organics prior to the discharge
ent (Hawkes, 1963; Mitchell, 1974).
asure of oxygen consumption required
obial oxidation of readily degradable
l ammonia in sewage. The 5-day BOD
he oxygen consumed by a properly
ge sample during 5 days of incubation
sewage sample is diluted in oxygen-
ater and enclosed without air space
ottle. After incubation, the decrease
l oxygen is measured, usually by an
trode. Since some organics are not
lized by microorganisms and since
omass is also formed, BOD is lower
mical oxygen demand (COD) mea-
strong chemical oxidants.
its solubility is low, dissolved oxygen
aters seldom exceeds 8 mg/L, and
eterotrophic microbial activity, it is
rably lower than that. Replenishment
oxygen from the atmosphere (reaer-
by photosynthetic O_2 evolution can
bly slower than the utilization rate
heterotrophic microorganisms in
of abundant organic substrates.
, the principal impact of sewage
natural waters is the lowering or
l their dissolved oxygen (D.O.) con-
xygen is exhausted, self-purification
w drastically. Fermentation products
ction of secondary electron sinks,
te and sulfate, give rise to noxious
, and colors; the water becomes an-
ptic. Oxygen deprivation kills ob-
bic organisms including some mi-

crobial forms, fishes, and invertebrate animals. The decomposition of these dead organisms constitutes an additional oxygen demand. Turbidity and toxic metabolic products, such as H_2S, also interfere with photosynthetic oxygen regeneration, thus further slowing the recovery process.

Input of a single dose of sewage into a lentic (stagnant non-flowing) environment causes a septic period of oxygen depletion with an associated reduction in biological diversity (Hynes, 1960). After a prolonged septic period, oxygen diffusion eventually reaerates the system and allows the mineralization of the accumulated fermentation products. The mineral nutrients liberated from the degraded organic matter may then give rise to an algal bloom. If there is no further disturbance, secondary succession will eventually restore the aquatic community to its former state. When a lotic (flowing river) environment receives a steady input of raw sewage, the above described events may be observed as steady state conditions at various distances downstream from the sewage outfall. The relative changes in some environmental parameters and populations in a river receiving sewage are illustrated in Figure 12.7. Depending on the rate of sewage discharge, flow rate, water temperature, and other environmental factors, water quality may return close to the original state at distances from a few up to several dozen kilometers downstream from the sewage outfall (Hynes, 1960; Warren, 1971).

Septic conditions in natural waters, whether they occur temporarily or as a steady state, are clearly undesirable. To avoid these conditions, sewage treatment aims to reduce the BOD of the sewage prior to discharge. Typically, this is achieved in several steps, each step bringing about a more complete reduction of the original BOD. These steps are referred to as primary, secondary, and tertiary sewage treatment.

Sewage enters the treatment plant through screens, traps and skimming devices that remove larger objects, grit, floating scum and grease. Primary sewage treatment removes only suspended solids. This removal is achieved in settling

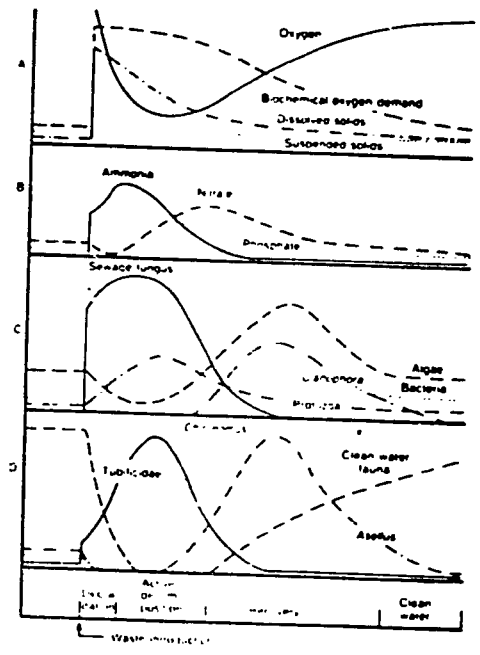


FIGURE 12.7
Diagrammatic presentation of the effects of an organic effluent on a river and the changes as one passes downstream from the outfall. (A) and (B) physical and chemical changes; (C) changes in microorganisms; and (D) changes in invertebrate animals. Sewage fungus is predominantly *Sphaerotilus natans*. (From Warren 1971; after Hynes, 1960; reprinted by permission; W. B. Saunders Co.)

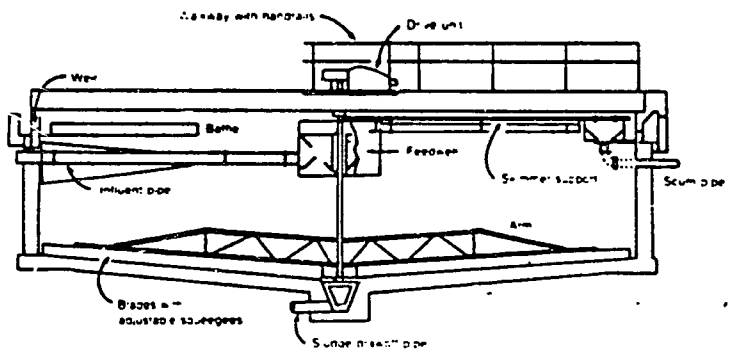
tanks or basins (Figure 12.8) where the solids are drawn off from the bottom. They may be subjected to anaerobic digestion and/or composting prior to final disposal in landfills or as soil conditioner. It should be pointed out that only a low percentage of the suspended or dissolved organic material is actually mineralized during liquid waste treatment. Most of the organic material is removed by settling, and in a way the disposal problem is merely displaced to the solid waste area rather than solved by complete recycling. Nevertheless, the fragility of aquatic ecosystems, owing to their low dissolved oxygen content, renders this displacement essential (Warren, 1971).

The liquid portion of the sewage containing dissolved organic matter is subjected to further treatment or is discharged after primary treatment only. Liquid wastes vary in composition, and if they contain mainly solids and little dissolved organic matter, primary treatment may remove 70–80 percent of the BOD and may be considered adequate. For typical domestic sewage (Table 12.1), however, primary treatment removes only 30–40 percent of the BOD, and secondary treatment is necessary for acceptable BOD reduction (Schroeder, 1977).

In secondary sewage treatment, a smaller portion of the dissolved organic matter is mineralized and a larger portion is converted from a dissolved state to removable solids. The combination of primary and secondary treatments reduces the original BOD of the sewage by 80–90 percent. The secondary sewage treatment step relies on microbial activity. The treatment may be aerobic or anaerobic and may be conducted in a large variety of devices. In some of these, the microorganisms are associated with surface films, in others they are homogeneously suspended. A correctly designed and operated secondary treatment unit should produce effluents with BOD and/or suspended solids less than 20 mg/l.

Aerobic Treatments

A simple and relatively inexpensive film-flow type aerobic sewage treatment installation is the trickling filter (Figure 12.9). The sewage is distributed by a boom-type sprinkler revolving over a bed of porous material. It slowly percolates through this porous bed and the effluent is collected at the bottom. The porous material of the filter bed becomes coated by dense, slimy bacterial growth. *Zooglea ramigera* and similar bacteria play a



g tank for
ment of sew-
larifier cross
nd large de-
ed out from
fore entering
nk. (From
77; reprinted
; copyright
)

s of typical municipal wastewater

	Concentration, mg/L
	700
	500
	300
	200
l	200
	50
	150
chemical oxy-	300
al (BOD)	
carbon (TOC)	200
gen demand	400
N)	40
	15
onia	25
	0
	0
as P)	10
	3
	7
	100

ler, 1977.

principal role in generating the slime matrix (Figure 12.10), which accommodates a heterogeneous microbial community including bacteria, fungi, protozoa, nematodes, and rotifers (Mack et al., 1975; Hawkes, 1977). This microbial community absorbs and mineralizes the dissolved organic nutrients in the sewage, thus reducing the BOD of the effluent (Fig. 12.11). Aeration is provided passively by the porous nature of the bed. Insects, mainly fly larvae, consume the excess microbial biomass generated, but constitute a nuisance to nearby residential areas. They can be controlled

FIGURE 12.9
Trickling filter for the secondary treatment of sewage. An enlarged section shows construction details. (From Warren, 1971; reprinted by permission; copyright W. B. Saunders Co.)

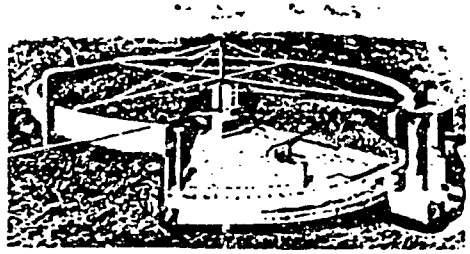


FIGURE 12.10
Zoogloea ramigera, a sewage bacterium that produces large amounts of extracellular polysaccharide. It plays an important role in surface slime and floc formation during sewage treatment. (Courtesy of P. Dugan, Ohio State University.)

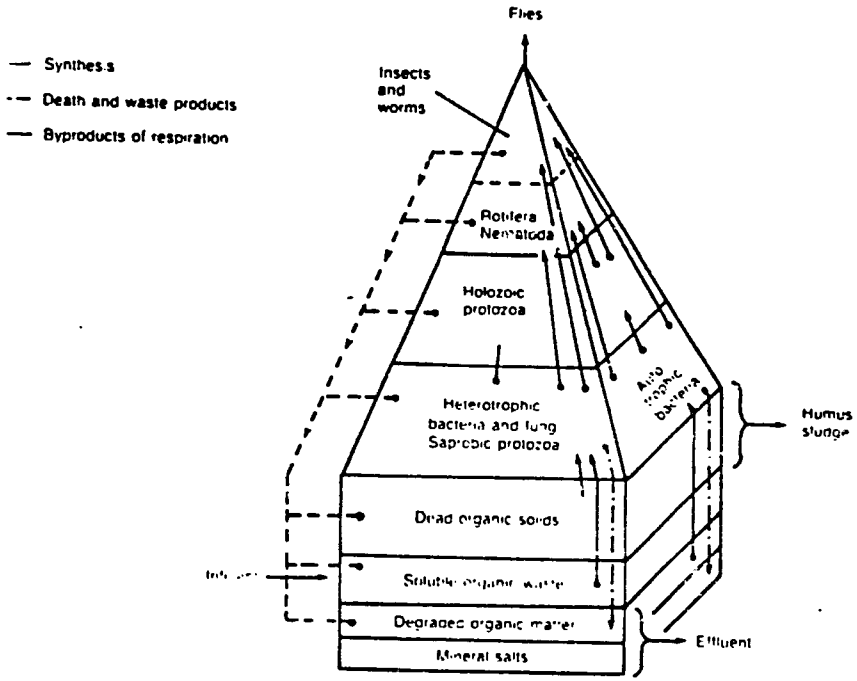
by the continuous rather than intermittent operation of the trickling filters because the fly larvae are suppressed by continuous spreading and successfully feed on the exposed microbial biomass only when the sprinkling is shut off.

The effluent from trickling filters is further clarified by allowing sloughed-off biomass to settle prior to discharge. The sewage may be passed through two or more trickling filters or can be recirculated several times through the same filter. A drawback of this otherwise simple and inexpensive treatment system is that a nutrient overload may lead to excess microbial slime, reducing aeration and percolation rates and necessitating a renewal of the trickling filter bed. Cold winter temperatures strongly reduce the effectiveness of these outdoor treatment facilities.

A more advanced aerobic film-flow type treatment system is the so-called rotating biological contactor or biodisc system. Closely spaced discs, usually manufactured from plastic material (Figure 12.12) are rotated in a trough containing the sewage effluent. The partially submerged discs become coated with a microbial slime similar to that described in case of the trickling filters. The continuous rotation keeps the slime well aerated and in contact with the sewage. The thickness of the microbial slime layer in all film-flow processes is governed by the diffusion of nutrients through the film. When the film grows to a thickness that prevents nutrients from reaching the innermost microbial cells, these will die, autolyze, and cause the detachment of the slime layer. The sloughed-off microbial biomass can be easily removed by settling (Howell, 1977; Schroeder, 1977). Biodisc systems require less space than trickling filters, are more efficient and stable in operation, and produce no aerosols, but they require a higher initial investment. They have been successfully used in treatment of both domestic and industrial sewage effluents.

A popular aerobic suspension type of liquid waste treatment system is the activated sludge process (Figs. 12.13 and 12.14). After primary settling, the sewage, containing dissolved organic compounds, is introduced into an aeration tank. Aeration is provided by air injection and/or mechanical stirring. Microbial activity is maintained at very high levels by reintroduction of most of the settled activated sludge, hence the name of the process. During the holding period in the aeration tank, vigorous development of heterotrophic microorganisms takes place.

The heterogeneous nature of the substrate allows the development of diverse heterotrophic bacterial populations. Gram negative rods predominate with coliforms (*Escherichia*), *Enterobacter*, pseudomonads, *Achromobacter*, *Flavobacterium*, and *Zoogloea* being most frequently isolated. *Micrococcus*, *Arthrobacter*, various coryneforms and mycobacteria also occur, along with *Sphaerotilus*, and other large filamentous bacteria. Filamentous fungi and yeasts normally occur in low numbers and play a subordinate role in the activated sludge process. The protozoa are mainly represented by the ciliates. Along with rotifers, they are important predators of bacteria. The bacteria occur individually in free suspension and also aggregate as floc. The floc consists pre-



2.11
ways of energy and materials transfer during treatment of dissolved organic means of trickling filters. Respiration at each level leads to mineralization of aterial and to release of heat energy. (After Hawkes, 1963; reprinted by per- ergamon Press Ltd.)

tly of microbial biomass cemented by slimes, such as produced by *Zoogleea* and similar organisms. Most of the ciliate , such as *Vorticella*, are of the attached ling type; they adhere to the floc but lominantly on the bacteria in suspension. is too large to be ingested by the ciliates rs, and thus may be considered a defense sm against predation. In the raw sewage, d bacteria predominate, but during the me in the aeration tank the numbers of d bacteria decrease. At the same time, sociated with floc greatly increase in numbers (Casida, 1968; Pike and Curds, 1971). The diversity and density of bacteria in activated sewage sludge renders this material a popular inoculum for various enrichment cultures. Some typical total and viable bacterial counts at various stages of sewage treatment are shown in Table 12.2.

During the holding period in the aeration tank, a portion of the dissolved organic substrates is mineralized. Another portion is converted to microbial biomass. In the advanced stage of aeration, most of the microbial biomass is associated with floc that can be removed from suspension by

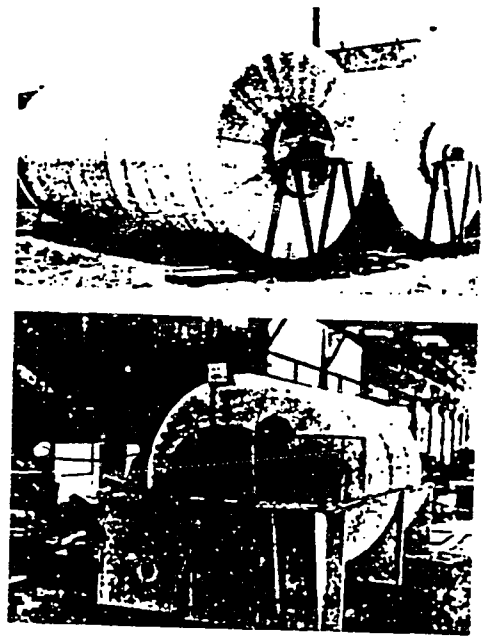
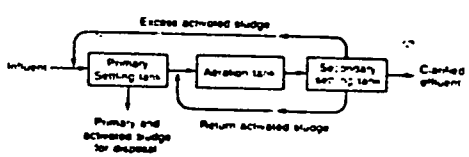


FIGURE 12.12
Two views of rotating biological contactor or "biodisc unit." (From Schroeder, 1977; reprinted by permission; copyright McGraw-Hill.)

settling. The settling characteristics of the sewage sludge floc are critical for their efficient removal. Poor settling characteristics are associated with

FIGURE 12.13
Flow diagram of an activated sludge secondary sewage treatment system. A portion of the sludge is recycled as inoculum for the incoming sewage. (After Imhoff and Fair, 1956; reprinted by permission; John Wiley and Sons.)

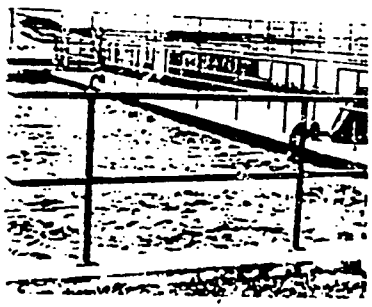


the "bulking" of the sewage sludge, a problem caused by proliferation of filamentous bacteria, such as *Sphaerotilus*, *Beggiatoa*, *Thiothrix*, and *Bacillus*, and also filamentous fungi, such as *Geotrichum*, *Cephalosporium*, *Cladosporium*, and *Pencillium*. The causes for bulking are not always understood, but this condition is frequently associated with high C:N and C:P ratios and/or low dissolved oxygen concentrations.

A portion of the settled sewage sludge is recycled for inoculation of the incoming raw sewage; the excess sludge requires additional treatment by anaerobic digestion and/or composting, or has to be disposed of in landfills. The practice of ocean dumping is no longer considered acceptable. Combined with primary settling, the activated sludge process tends to reduce the BOD of the effluent to 5–15 percent of the raw sewage.

The treatment also drastically reduces the numbers of intestinal pathogens in the sewage, even prior to final disinfection by chlorination. This reduction is accomplished by the combined effects of competition, adsorption, predation, and settling. Predation by ciliates, rotifers, and *Bdellovibrio* is probably indiscriminate and affects pathogens as well as nonpathogenic heterotrophs. Pathogens, however, tend to grow poorly or not at all under the conditions prevailing in the aeration tank, while the nonpathogenic heterotrophs proliferate vigorously. Thus, nonpathogenic heterotrophs compensate for their removal by predation, while the pathogens are decimated continuously. Settling of the floc removes additional pathogens. Typically, numbers of *Salmonella*, *Shigella*, and *Escherichia coli* are 90–99 percent lower in the effluent of the activated sludge treatment process than in the incoming raw sewage. Enteroviruses are removed to a similar degree; here the main removal mechanism appears to be adsorption of the virus particles to the settling sewage sludge floc.

The activated sludge process is essentially a continuous culture process, suitable for mathematical and computer modeling (Pike and Curds, 1971). In a steady-state condition, the growth of



14
sin of an activated sludge sewage treatment
n Casida, 1968; reprinted by permission;
hn Wiley and Sons.)

bacteria, those associated with floc, qual to the sludge wastage, those re- Figure 12.15 the steady-state popu- microorganisms are modeled at various d sludge wastage rates. Model (A) as- presence of attached ciliates, a con- t most closely resembles the real-life Simulation (B) assumes only free- ciliates; simulation (C) assumes the ciliates. The role of the attached ciliates ling the dispersed bacteria is clearly hus, the presence of the attached ciliates is critical for removal of the portion of the BOD consisting of dispersed bacteria that would be otherwise discharged with the effluent. In fact, the types and numbers of protozoa associated with the activated sludge floc can be used as an indicator of sludge condition, and thus treatment performance. The sludge is in poor condition when few ciliates and many flagellates are observed; ciliates predominate in "good" sludge. The role of the ciliates was also experimentally demonstrated in benchscale treatment units inoculated with controlled bacterial and protozoan populations as shown in Table 12.3.

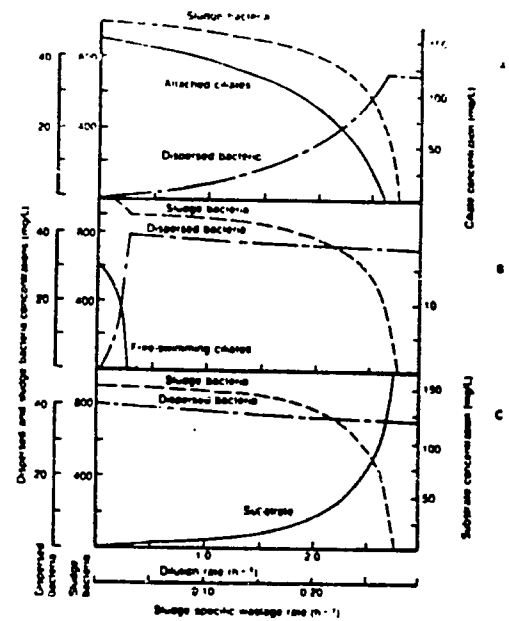
The activated sludge treatment system is an efficient and flexible one that is able to withstand considerable variations in sewage flow rate and concentration. It is widely used for the treatment of domestic sewage and industrial effluents. Some interesting variations of the process were developed for treatment of industrial wastes, and are shown in Figures 12.16 and 12.17. Both modifications are designed to improve the oxygen transfer rate and thus achieve a higher BOD reduction rate per unit volume of treatment system. The "unox" process (Figure 12.16) developed by Union Carbide uses oxygen instead of air for aeration. Closed tanks and a specially designed stirring system prevent the wastage of oxygen. The deep shaft process of ICI (Figure 12.17) uses

total and viable
samples from
ges of sewage
ed in the sus-
mass

	Bacterial count				
	In samples (number/mL)		In biomass (number/g)		Percent of bacteria viable
	Total	Viable	Total	Viable	
Settled sewage	6.8×10^{7}	1.4×10^{7}	3.2×10^{12}	6.6×10^{10}	2.0
Activated sludge mixed liquor	6.6×10^{7}	5.6×10^{7}	1.4×10^{12}	1.2×10^{10}	0.85
Filter slimes	6.2×10^{10}	1.5×10^{9}	1.3×10^{12}	3.2×10^{10}	2.5
Secondary effluents	5.2×10^{7}	5.7×10^{5}	4.3×10^{12}	4.7×10^{10}	1.1
Tertiary effluents	3.4×10^{7}	4.1×10^{4}	3.4×10^{12}	4.1×10^{9}	0.12

After Pike and Curds, 1971.

FIGURE 12.15
Steady-state populations of microorganisms at various dilution and sludge wastage rates by computer modeling. Model (A) assumes the presence of attached ciliates; model (B) the presence of free-swimming ciliates; and model (C) assumes the absence of ciliates. Sludge wastage rate is kept at 1/10th of the dilution rate. Substrate concentration curve shown in (C) is identical for (B) and (A), but is omitted from these plots. The simulations clearly show the strong effect of the attached ciliates on the dispersed bacteria. The reduction of the latter helps to achieve a low BOD discharge. (From Pike and Curds, 1971; reprinted by permission; copyright Academic Press, London.)



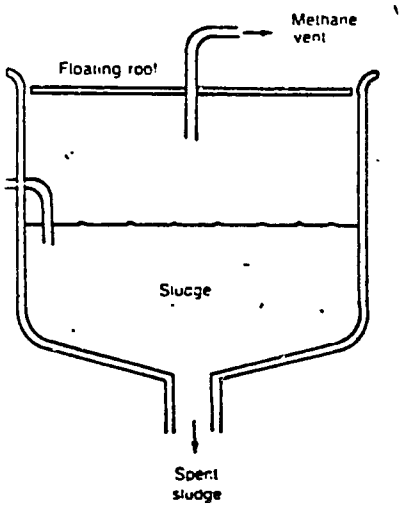
air injection and achieves high oxygen dissolution by increasing hydrostatic pressure and an ingenious circulation pattern (Howell, 1977).

Oxidation ditches and lagoons are low-cost substitutes for the previously described treatment systems. They tend to be inefficient and require large holding capacities and long retention times. As oxygenation is usually achieved by diffusion

TABLE 12.3
Effect of ciliated protozoa on the effluent quality of bench scale, activated sludge plants

Effluent analysis	Without ciliates	With ciliates
BOD (mg/L)	53–70	7–24
COD (mg/L)	198–250	124–142
Permanganate value (mg/L)	83–106	52–70
Organic nitrogen (mg/L)	14–21	7–10
Suspended solids (mg/L)	86–118	26–34
Optical density at 620 nm	0.95–1.42	0.23–0.34
Viable bacteria count (millions/mL)	106–160	1–9

After Pike and Curds, 1971.



:.20
»plified diagram of an anaerobic digestor.
chell, 1974; reprinted by permission; copy-
nce-Hall.)

of suspended organic matter; values be-
and 100 g/l are considered favorable.
lerable part of this suspended material
al biomass, viable counts of which can
h as 10^9–10^{10} bacteria per ml. Anaerobic
e typically two to three orders of magnitude higher than aerobic ones. Fungi and protozoa are present in low numbers only and are not considered to play a significant role in anaerobic digestion.

The anaerobic digestion of wastes can be considered as a two-step process even though it really is a coupled sequence of microbiological interactions. First, complex organic materials including microbial biomass are depolymerized and converted to fatty acids, CO_2, and H_2. A large variety of nonmethanogenic obligately or facultatively anaerobic bacteria participate in these processes. In the next step, methane is generated either by the direct reduction of methyl groups to methane, or by the reduction of CO_2 to methane by molecular hydrogen or by other reduced fermentation products, such as fatty acid, methanol, or even carbon monoxide (Balch et al., 1979). Some of the methanogenic bacteria and the mechanisms of their methane production are listed in Table 12.4.

The operation of anaerobic digestors requires the close control of several parameters, such as retention time, temperature, pH, and C:N and C:P ratios. The reactors are usually heated to 35–37°C for optimal performance. pH has to be controlled to stay within the 6.0–8.0 values, with pH 7.0 being optimal. Extremes of pH, and the influx of heavy metals or other toxic materials can easily upset the operation of the anaerobic

4
of biogenic
and some repre-
methanogenic

Source of CH_4-carbon	Electron donor	Methanogenic bacteria
CO_2	H_2	*Methanobacterium bryantii* *Methanobacterium thermoautotrophicum* *Methanomicrobium mobile*
CO_2	H_2, formate	*Methanococcus vannielii* *Methanobrevibacter ruminantium*
CO_2	H_2, CO, formate	*Methanobacterium formicicum*
CO_2	H_2	
methanol, methylamine, di- and trimethylamine, acetate		*Methanosarcina barkeri*

digestor. In a "stuck" or "sour" digestor methane production is interrupted and fatty acids and other fermentation products accumulate. It is a difficult task to restore a stuck digestor to normal operation. It is usually necessary to clean out the reactor and charge it with large volumes of anaerobic sludge or appropriate methanogenic bacteria from an operational unit.

A number of advanced process designs were developed recently for anaerobic sewage treatment (Switzenbaum, 1983). The relatively high cost of anaerobic digestors has previously limited them to processing very high BOD liquids, primarily sewage sludge. Some advanced designs make anaerobic digestors more efficient and economical in handling more dilute wastes, such as primary-treated sewage. The anaerobic activated sludge process closely parallels its aerobic counterpart: settled biomass is continuously recycled to maximize the rate of digestion per reactor volume. Other designs involve sludge beds, anaerobic filters or anaerobic expanded fluidized beds. Each design attempts to assure that the dilute sewage passes through a very dense stationary anaerobic biomass. This passage minimizes reactor volume and fluid retention time while it maximizes the efficiency of the digestion process.

A normally operating anaerobic digestor yields a reduced volume of sludge. This material still causes odor and water pollution problems and can be directly disposed of only at a limited number of landfill sites. Aerobic composting further consolidates the sludge and renders it suitable for disposal in any landfill site or for use as a soil conditioner. The gas produced consists of methane, CO_2, small amounts of other gases, such as N_2, H_2S, and occasionally traces of H_2. This gas is either burned directly in the treatment plant to drive the pumps and maintain the temperature of the digestor at the desired level or, after purification, may be added to municipal gas distribution systems (Finstein and Morris, 1979).

Anaerobic digestors are relatively expensive to construct and require expert maintenance, though some of the cost is offset by the production of the methane gas. The use of anaerobic lagoons as an inexpensive alternative is seldom feasible because of the noxious odors associated with such an operation. A special case in which the anaerobic lagoon performs in a relatively acceptable fashion is in treatment of meat packing plant effluents. Fatty materials and animal stomach contents form a thick, insulating scum layer on the surface of such anaerobic lagoons that substantially reduces the odor problem and renders such facilities acceptable if they are a sufficient distance from residential areas.

Tertiary Treatments and Disinfection

The previously discussed aerobic and anaerobic biological liquid waste treatment processes are designed to reduce the BOD represented by dissolved, biodegradable organic substrates; all are classified as secondary liquid waste treatment. Tertiary treatment is any practice beyond secondary. Tertiary liquid waste treatments are aimed at the removal of nonbiodegradable organic pollutants and mineral nutrients, especially nitrogen and phosphorus salts. The removal of nonbiodegradable organic pollutants, such as chlorophenols, polychlorinated biphenyls, and other synthetic pollutants (see Chapter 13) is necessary because of the potential toxicity of these compounds. Activated carbon filters are used in removal of such materials from secondary-treated industrial effluents. Previous secondary treatment is necessary in order to avoid overloading this expensive treatment stage with biodegradable materials that can be removed in more economic ways.

Concern about nitrogen salts and phosphate in sewage effluents is relatively recent and is connected with the phenomenon of cultural eutrophication of natural waters (Keeney et al., 1971; Godzing, 1972). Eutrophication, the process of becoming rich in nutrients, is a natural sequence in the geological history of lakes and ponds. Initially deep, cold, nutrient poor (oligotrophic) waters become shallower, warmer and more eu-

silt and organic matter accumulates. .. the lake is filled in. Cultural eutro- frequently referred to only as eutro- with the same intended meaning, is the human activities and causes dramatic lake character within years or decades geological ages. Sudden nutrient en- hrough sewage discharge or agricultural gers explosive algal blooms. Owing to own or unknown causes, such as mutual exhaustion of micronutrients, toxic or disease, the algal population usually The decomposition of the dead algal by heterotrophic microorganisms ex- e dissolved oxygen in the water, pre- extensive fish kills and septic conditions. utrophication does not go to the above algal mats, turbidity, discoloration, and he fish population from valuable species olerant but less valued forms, represent le eutrophication changes.

phication is a complex phenomenon that derstood in the context of Leibig's Law nimum. Many natural oligotrophic lakes ed by phosphorus or by nitrogen, but rient limitation possibilities, such as dis- $)_2$ or sulfur, may also exist. If the sewage supplies large amounts of the inorganic which happens to be limiting in the re- ater body, a eutrophication response is occur. If this is the case, and the sewage cannot be stopped or diverted, the ob- ecy is to remove the causative inorganic from the sewage discharge.

phosphate filler of many detergent for- s, which is added to precipitate divalent h as Ca^{++} and Mg^{++} in hard water, is quently blamed in eutrophication cases. Washington, located adjacent to Seattle ounding communities, the pattern of eu- tion and the improvement following sew- rsion from Lake Washington to Puget correlated best with phosphate concen- (Edmondson, 1970). Work on Wisconsin dicated that phosphate concentrations exceeding 10μg P/l at the beginning of the growing season are likely to result in destructive algal blooms. In this and many other cases there is little doubt that phosphate has been the main offender, but in the case of nitrogen-limited waters a treatment to remove phosphate alone is clearly useless. In practice, if eutrophication of the receiving waters is a concern, an effort is made to remove both nitrogen and phosphorus from the effluent (Godzing, 1972).

Phosphate is commonly removed by precipitation as calcium or iron phosphate. This can be accomplished as an integral part of primary or secondary settling, or in a separate facility with recycling of the precipitating agent. Nitrogen, present mainly as ammonia, can be removed by "stripping," that is, by volatilization as NH_3 at a high pH. Some of the ammonia eliminated from the sewage in this manner, however, may return to the watershed in the form of precipitation and still cause eutrophication problems. Another alternative for ammonia nitrogen removal is breakpoint chlorination. Addition of hypochlorous acid in 1:1 molar ratio results in the formation of monochloramine (Equation 1):

$$(1)\quad HOCl + NH_3 \rightarrow NH_2Cl + H_2O$$

Further addition of hypochlorous acid results in the formation of dichloramine (Equation 2):

$$(2)\quad HOCl + NH_2Cl \rightarrow NHCl_2 + H_2O$$

Breakpoint chlorination occurs at the approximate molar ration of two moles of chlorine to one mole of ammonia (7.6 mg Cl to 1 mg ammonium-N) and results in the near-quantitative conversion of ammonia to molecular nitrogen according to the simplified reaction (Equation 3):

$$(3)\quad 2NHCl_2 + H_2O \rightarrow N_2 + HOCl + 3H^+ + 3Cl^-$$

As chlorination of the sewage effluent is commonly practiced for disinfection purposes, chlorination to this "breakpoint" can accomplish the removal of ammonium nitrogen in the same process. The removal of ammonium nitrogen also lowers the BOD of the effluent, since the nitrification process would consume oxygen dissolved in the receiving water.

Biological processes for removal of nitrogen and, to a lesser degree, phosphorus have been proposed and some of these are gaining acceptance. If the primary concern about ammonium nitrogen is its BOD, this problem can be solved by in-plant nitrification. Vigorous and prolonged aeration is necessary to convert ammonium nitrogen to nitrate by biological nitrification. After conversion to nitrate, it is also possible to remove most of the nitrogen from effluent by denitrification. To achieve this result, aeration is discontinued and organic matter added. The anaerobic oxidation of organic matter converts the nitrate to gaseous denitrification products (see Chapter 11). Methanol has been used as an additional carbon source, but cost considerations favor the use of the dissolved carbon substrates of the raw sewage itself to drive the denitrification process. This usage can be accomplished by intermittent aeration or by an admixture of raw sewage to extensively aerated and thus nitrified sewage. Bacterial uptake and storage of phosphorus in the form of polyphosphates may be encouraged by treatment process modifications. Other schemes involve the uptake of nitrogen and phosphorus into algal biomass in shallow holding ponds. Problems with harvesting and utilizing the algal biomass in animal feed, for example, need to be solved before this approach can be practiced on a large scale. Higher aquatic plants, such as water hyacinths and cattails, also have potential for the removal of nitrogen and phosphorus from sewage effluents and might prove to be easier to harvest if appropriate uses for such biomass could be found. One should be aware, however, of the practical difficulty of devoting large land areas to such operations near urban centers.

A highly advanced tertiary treatment system is currently operating in South Tahoe, California and the description of this system (Figure 12.21) serves well to illustrate the integration of various previously mentioned approaches (Slechta and Culp, 1967). This advanced treatment system became necessary to prevent the eutrophic deterioration of scenic Lake Tahoe, an oligotrophic crater lake. After conventional primary and secondary treatments, phosphate is precipitated by liming and ammonia is removed by stripping the high pH effluent. This step is accomplished in a stripping tower at elevated temperature and with vigorous aeration. After ammonia stripping, the pH is adjusted to neutrality. Following additional settling and clarification, aided by polyelectrolyte addition, nonbiodegradable organics are removed by filtration through activated carbon. The effluent is disinfected by chlorination. The lime sludge from the phosphate precipitation step is recycled by conversion to CaO in a recalcinating furnace. The quality of the treated effluent is very high (Table 12.5).

Disinfection

The final step in sewage treatment is disinfection, designed to kill enteropathogenic bacteria or viruses that were not eliminated during the previous steps of sewage treatment. Disinfection is commonly accomplished by chlorination, using either chlorine gas (Cl_2) or hypochlorite ($Ca[OCl]_2$ or NaOCl). Chlorine gas reacts with water to yield hypochlorous and hydrochloric acids (Equation 4):

$$(4)\quad Cl_2 + H_2O \rightarrow HOCl + HCl$$

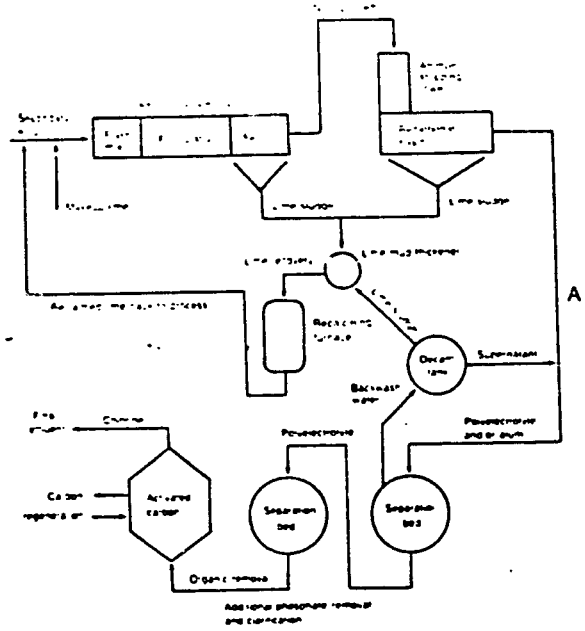
Hypochlorite also reacts with water to form hypochlorous acid (Equation 5):

$$(5)\quad Ca(OCl)_2 + 2H_2O \rightarrow 2HOCl + Ca(OH)_2$$

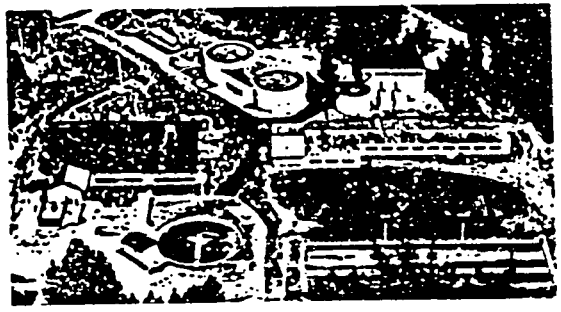
or (Equation 6)

$$(6)\quad NaOCl + H_2O \rightarrow HOCl + NaOH.$$

The hypochlorous acid or the hypochlorite (ClO^-) ion is the actual disinfectant. The total concen-



A



B

21
n of the South
d.) advanced ter-
water treatment
osphate, nitro-
ecalcitrant or-
ounds are re-
photograph of
Tahoe sewage
system. (A from
d Culp, 1967; re-
permission;
lution Control
. B from Warren,
nted by permis-
. Saunders Co.)

of hypochlorous acid and hypochlorite is
ated as free residual chlorine. Hypochlorite
trong oxidant and this is the basis of its
cterial action. As an oxidant, it also reacts
residual dissolved or suspended organic
r, ammonia, reduced iron, manganese and sulfur compounds. The oxidation of such compounds competes for HOCl and reduces its disinfectant action, making it necessary to chlorinate to a point that these reactions are satisfied with several mg/L excess free residual chlorine remaining in solution. This process requires high

TABLE 12.5
Quality of purified reclaimed water after tertiary treatment at South Tahoe, CA

Parameter	Wastewater (mg/L)	Reclaimed water (mg/L)
BOD	200–400	1
COD	400–600	3.25
Total organic carbon	–	1–7.5
Phosphate	25–30	0.2–1.0
Organic nitrogen	10–15	0.3–2.0
Ammonia	25–35	0.3–1.5
Nitrate and nitrite	0	0

After Mitchell, 1974.

amounts of hypochlorite, resulting in high salt concentrations in the effluent. Therefore, it is desirable to remove nitrogen and other contaminants by alternate means and use chlorination for disinfection only.

A disadvantage of disinfection by chlorination is that more resistant types of organic molecules, including some lipids and hydrocarbons, are not oxidized completely but become, instead, partially chlorinated. Chlorinated hydrocarbons tend to be toxic and are difficult to mineralize (see Chapter 13). Since alternative means of disinfection are more expensive, chlorination remains the principal means of sewage disinfection.

Treatment and Safety of Water Supplies

Closely related to the safe disposal of liquid wastes is the problem of safe and healthful water for drinking and other uses. Increasing population densities and urbanization during the eighteenth and nineteenth centuries were initially not accompanied by adequate sanitation practices. This situation created conditions for devastating epidemics caused by enteropathogenic microorganisms such as *Vibrio cholerae* (cholera), *Salmonella typhi* (typhoid fever), various other *Salmonella* and *Shigella* strains (causing gastrointestinal infections of varying severity), and *Entamoeba histolytica*, the causative agent of amoebic dysentery. The common feature of these diseases is that the infectious organisms are shed in the feces of sick individuals and clinically asymptomatic carriers. Fecal contamination, through untreated or inadequately treated sewage effluents entering lakes, rivers, or groundwaters that in turn serve as municipal water supplies, creates conditions for rapid dissemination of the pathogens. The primary route of infection is ingestion with drinking water, but additional infection possibilities arise by washing fruits, vegetables, and eating utensils with contaminated water. The obvious remedy to this situation is to disrupt the transmission of enteropathogenic organisms through fecal contamination of water supplies. This is done to a certain degree during sewage treatment, but the adequate treatment of all sewage discharges is by no means assured. A major sanitation effort, therefore, is necessary to treat and safely distribute public water supplies. Such sanitation practices have led to the virtual elimination of waterborne infections in developed countries, but such infections continue to be major causes of sickness and death in undeveloped regions (Sobsey and Olson, 1983).

Public water supplies originate from groundwater through wells or from surface waters such as rivers, lakes, or reservoirs. In the case of

ater, disinfection is often all that is re- Surface waters and some groundwaters be treated by settling and filtration. Dis- errous iron and divalent manganese are l prior to settling by addition of perman- Filtration is carried out through gravel sand beds. Microbial films on the surface ed particles enhance the effectiveness of ation. The filter beds have to be back- frequently to prevent clogging.

nfection of municipal water supplies has ditionally accomplished by chlorination, ibed for the disinfection of sewage dis- (Equations 4–6). This treatment is rel- inexpensive and the free residual chlorine of the treated water represents a built- y factor against pathogens surviving the treatment period or against recontami- The disadvantage of chlorination is the of trace amounts of trihalomethane compounds. The fact that THMs were in virtually all municipal water supplies d chlorination for disinfection linked the on of these contaminants to the chlori- process. Since some of the THMs are ed carcinogens, the U. S. Environmental ion Agency established in 1979 a maximal unit in drinking water of 100 μg/L. To thin this limit using traditional chlorine tion, organic compounds were removed e water meticulously by sand filtration er techniques. This method is often both ical and too expensive. Fortunately, it nd that disinfection by monochloramine tive but produces much lower amounts ls. As an example, traditional chlorination River water produced 160 μg THM/L hloramine treatment produced THM levels ntly below 20 μg/L. Monochloramine may rated right in the water to be disinfected ng ammonium prior to or simultaneously lorine or hypochlorite. Once hypochlorous formed according to equations 4–6, it with the added ammonia according to n 1, generating monochloramine. The use of chloramines as drinking water disinfectants is the least expensive way to reduce THM formation and this practice is spreading rapidly (Wolfe et al., 1984).

Ozone (O_3) has been used for water disinfection with good results both in Europe and in the United States. Ozone treatment kills pathogens reliably and does not result in the synthesis of undesirable THM contaminants. Since ozone is an unstable gas, however, water treated with ozone does not have any residual antimicrobial activity and is prone to chance recontamination. Ozone has to be generated from air on site in ozone reactors, using electrical corona discharge. Since only about 10 percent of the electricity is actually generating ozone and the rest is lost as heat, disinfection by ozone is considerably more expensive than by chlorination (Mitchell, 1974).

Water Quality Testing

The public health importance of clean drinking water requires an objective test methodology to evaluate the effectiveness of treatment procedures and to establish drinking water safety standards (Bonde, 1977; Hoadley and Putka, 1977). The detection of actual enteropathogens, such as *Salmonella* or *Shigella*, in routine monitoring studies would be a difficult and uncertain undertaking. Instead, the bacteriological tests of drinking water simply establish the fact and the degree of fecal contamination of the water sample by demonstrating the presence of so-called indicator organisms. The most frequently used indicator organism for fecal contamination is the normally nonpathogenic *Escherichia coli*. Positive tests for *E. coli* do not prove the presence of enteropathogenic organisms, but establish the possibility of such a presence. Because *E. coli* is numerous and easier to grow than the enteropathogens, the test has a built-in safety factor for detecting potentially dangerous fecal contamination.

Ideally an indicator organism should be present whenever the pathogens concerned are present, should be present only when there is a real

danger of pathogens being present, must occur in greater numbers than the pathogens to provide a safety margin, must survive in the environment as long as the potential pathogens, and must be easy to detect with a high reliability of correctly identifying the indicator organism regardless of what other organisms are present in the sample. *E. coli* meets many of these criteria but there are limitations to its use as an indicator organism and various other species have been proposed as additional or replacement indicators of water safety. There probably is no universal indicator organism for determining water quality. Under different conditions different populations may be better indicators than others (Wolf, 1972).

The classic test for the detection of fecal contamination involves, in the first step, inoculation of lactose broth with undiluted or appropriately diluted water samples. Gas formation detected in small inverted test tubes (Durham tubes) gives positive presumptive evidence of contamination by fecal coliforms; this is the presumptive test. Gas formation in lactose broth at elevated incubation temperature is a characteristic of fecal *Salmonella*, *Shigella*, and *E. coli* strains, but also of the nonfecal coliform *Enterobacter aerogenes*. In this second stage, the confirmed test, positive lactose broth cultures are streaked out on a lactose-peptone agar medium containing sodium sulfite and basic fuchsin (m-Endo agar). Fecal coliform colonies on this medium acquire a characteristically greenish metallic sheen while *Enterobacter* forms reddish colonies, and nonlactose fermenters form colorless colonies. Subculture in lactose broth at 35°C should yield gas formation and completes a positive test for fecal coliforms; this is the completed test.

It is possible to simplify this three-stage test. In the Eijkman test, suitable dilutions are incubated in lactose broth at 44.5°C. At this temperature fecal coliforms still grow, but nonfecal coliforms are inhibited. Gas formation in lactose broth at 44.5°C constitutes a one-step positive test, but precise temperature control is mandatory, since temperatures only a few degrees higher

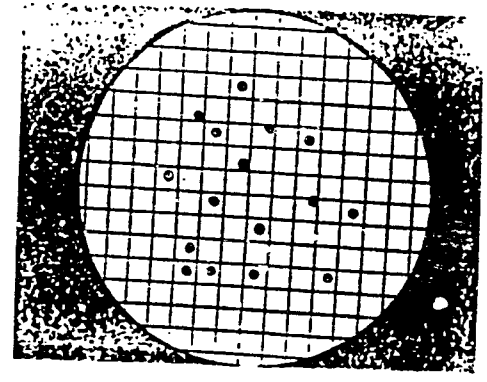


FIGURE 12.22
Filter used in filtration of fecally contaminated water showing typical colonies of *Escherichia coli* after incubation on Endo-agar.

inhibit or kill fecal coliforms. Another modern technique is filtration of known volumes of diluted or undiluted water samples through 0.45 μm pore size millipore filters and incubating these filters directly on Endo medium. Colonies of fecal coliforms show up with a characteristic metallic sheen and can be counted (Figure 12.22). The dried filters can be filed for a permanent record.

The standards for tolerable limits for fecal contamination (Table 12.6) are somewhat arbitrary, but have large built-in safety factors and

TABLE 12.6
U.S. water standards for coliform contamination

Water use	Maximum permissible coliform count per 100 mL
Municipal drinking water	1
Waters used for shellfishing	70
Recreational waters	1000

After Mitchell, 1974.

by long experience: they vary with
ter use. The most stringent standard
municipal water supplies used by
Somewhat higher coliform counts
ally be tolerated in a private well
one family, since such a well could
source of a widespread epidemic.
water standard does not absolutely
ossibility of ingesting enteropath-
nking water, but seems to reduce
to a statistically tolerable limit.
afety factors are twofold. Entero-
likely to be present in much lower
fecal coliforms and in addition, a
infective bacteria are usually unable
dy defenses. A minimum infectious
eral hundred to several thousand
ecessary for various diseases to
tual infection. Drinking water sup-
the 1/100 mL coliform standard
en demonstrated to be the source
bacterial infections (Olson and

orm counts are also used to es-
ty of water for shellfish harvesting
l uses. Shellfish tend to concentrate
ther particles through their filter-
y. Some shellfish are consumed
an become a source of infection
pathogens. This is reflected by
ringent standard for waters in use
Clinical evidence for infection
enic coliforms through recrea-
aters, for wading or swimming is
ut, as a precaution, beaches are
hen fecal coliform counts exceed
standard of 1,000/100 mL. Some
ds require that disinfected sewage
exceed the above limit.
nonpathogenic and pathogenic
in natural waters was discussed
ntext of allochthonous organisms
interactions. In general, fecal
of competitors at the low substrate

concentrations that prevail in natural waters and so tend to be eliminated by competition and predation. Low water temperatures, sediment adsorption, and anoxic conditions are factors that occasionally contribute to the prolonged survival of fecal coliforms in natural waters.

Enteroviruses and the cysts of some pathogenic protozoa are somewhat more resistant to disinfection by chlorine, chloramine, or ozone than bacteria and, occasionally, active virus particles or live cysts are recovered from water treated to meet fecal coliform standards (Sobsey and Olson, 1983; Olson and Nagy, 1984). Thus, the possibility exists that water treated according to accepted standards may occasionally become a source of viral or protozoan infection. As many as 100 different viral types have been demonstrated to be shed in human feces, but practical concern has been mainly with the causative viruses of infectious hepatitis, polio, and viral gastroenteritis. There is evidence that infectious hepatitis was, on occasion, spread by water supplies, though a much more prevalent way of infection is the consumption of raw shellfish from fecally contaminated waters. Spread of polio infection through water supplies and/or recreational use of beaches has been suspected in some cases, but conclusive proof is lacking (Shuval and Katznelson, 1972; Vaughan et al., 1979). The situation with viral gastroenteritis is similar. It seems fair to say that while the possibility of the occasional spread of viral infection through drinking water adequately treated by bacteriological standards cannot be excluded, there is no supporting evidence for actual epidemics caused by such water. Similarly, it is unclear whether properly disinfected water has ever spread protozoan pathogens such as the flagellate *Giardia lamblia* or the amoeba *Negleria fowleri* (Sobsey and Olson, 1983). There is ample evidence, of course, for destructive epidemics by enteroviruses and protozoa caused by untreated drinking water in various underdeveloped countries.

The considerable uncertainty about the haz-

ards posed by enteroviruses in water supplies and recreational waters is due to the difficulty of detecting and enumerating viruses in such waters. For detection, viruses have to be grown in suitable tissue culture cell lines and/or detected by immunological procedures. Virus concentrations are generally low, 0.1–1 particle per mL treated sewage effluent and as low as 0.1–1 per L in receiving natural waters. Therefore, the first problem is to concentrate the viruses sufficiently for detection and enumeration. Table 12.7 lists and compares some of these methods.

The oldest procedure is the gauze pad method. Gauze pads are suspended in water for about 24 hours. They concentrate viruses by absorption. Water expressed from the retrieved gauze pad contains higher numbers of viruses than directly collected water samples. Sample incorporation relies on concentrated media that allow the introduction of relatively large (10–50 mL volume) water samples.

Ultracentrifugation concentrates viruses by sedimentation. It requires expensive instrumentation and is time-consuming. Filters do not actually filter out viruses, but retain them by adsorption. Subsequently, viruses are eluted from the filter, or the filter is dissolved. Hydro-extraction is actually a dialysis process. Water is extracted through the dialysis tubing by concentrated polyethylene glycol. Virus particles remain with other large molecules in the tubing and are concentrated. Phase separation involves partitioning between two liquid phases. Electrophoresis takes advantage of the negative charge on virus particles. Adsorption to and elution from various particulate materials has also been used with varying success.

Considerable research effort is currently di-

TABLE 12.7
A comparison of various methods for detecting viruses in water

	Sample volume, liters	Concentration factor	Percent recovery efficiency
Gauze pad method	?	10–50	0.5–5
Sample incorporation method	0.06–0.15	10–20	95
Ultracentrifugation	1–10	30–?	20–100
Membrane filter adsorption	10–100	1000	5–38
Soluble ultrafilters	10	1000	35–100
Hydro extraction	1	100	24–64
Phase separation (two steps)	5	1000	35–100
Electrophoresis	0.3	100	100
Adsorption to particulate materials:			
Calcium phosphate	3.0	1000	50–100
Colbalt chloride	1	1000	100
Aluminum hydroxide	3.8–19	1000	50–100
Ion exchange resins	0.1	50–100	20–50
Passive haemagglutination	?	?	80–100
Insoluble polyelectrolytes	1000	1,000–100,000	35–80

After Shuval and Katznelson, 1972.

ating the duration and mechanisms survival in natural waters. Gen- difficult at this time, but it appears ses are eliminated from natural lowly than fecal coliforms. Ad- diment particles appears to offer rotection against inactivation and, cumstances, may promote the dis- viruses (Smith et al., 1978). Chang- ntal conditions, such as pH and cause the desorption of the still particles. Recent surveys sporad- d human enteroviruses in both d saltwater environments but the eroviruses showed little correlation er of fecal coliforms. Whether this rential survival or to nonidentical sources is not clear at this time. , cultivation, and enumeration from s clearly needs further scientific e standards and routine monitoring be established.

Summary

Practical applications of microbial ecology are evident in the practices of biodeterioration control, and soil water, and waste management. Spoilage of food and perishable materials is prevented by sterilization and aseptic containment. Alternatively, unfavorable physicochemical conditions are used to retard biodeterioration. Agricultural soils are managed to encourage aerobic and discourage anaerobic cycling processes. In addition, fertilizer technology and soil pH control take into account and often utilize microbial cycling activities. The composting of solid waste and the biological treatment of sewage are complex microbial processes that recycle and/or stabilize organic wastes and protect the environment. Disinfective treatment of municipal water supplies guards against the spread of water-borne epidemics. The effectiveness of such disinfective treatment is monitored through counts of indicator organisms.

ES ESTED INGS

. E. Fox, L. J. Magrum, C. R. S. Wolfe. 1979. Methanogens: re- unique biological group. *Bacterio-* 43:260–296.

77. Bacterial indicators of water *ces in Aquatic Microbiology* 1:273–

Ir. 1968. *Industrial Microbiology*. Sons, New York.

Mitchell. 1976. Control of marine ical repellents. In J. M. Sharpley dan (eds.). *Proceedings of the Third odegradation Symposium*. Applied ers, Essex, England, pp. 515–521.

1 R. Henzen, G. J. Stander, and 969. The application of the process tion. IV. Anaerobic digestion. 3:623–643.

L. Matsuuchi, and B. Armbruster. 1976. Secretion of adhesive polymers and attachment of marine bacteria to surfaces. In J. M. Sharpley and A. M. Kaplan (eds.). *Proceedings of the Third International Biodegradation Symposium*. Applied Science Publishers, Essex, England, pp. 433–442.

Dart, R. K., and R. J. Stretton. 1977. *Microbiological Aspects of Pollution Control*. Elsevier Publishing Company, Amsterdam.

Desrosier, N. W. 1970. *The Technology of Food Preservation*, 3rd ed. The AVI Publishing Co., Westport, Conn.

Di Salvo, L. H., and G. W. Daniels. 1975. Observations on estuarine microfouling using the scanning electron microscope. *Microbial Ecology* 2:234–240.

Edmondson, W. T. 1970. Phosphorus, nitrogen and algae in Lake Washington after diversion of sewage. *Science* 169 690–691.

Epstein, E., G. B. Wilson, W. D. Burge, D. C.

Mullen, and N. K. Enkiri. 1976. A forced aeration system for composting wastewater sludge. *Journal Water Pollution Control Federation* 48:655–694.

Finstein, M. S., F. C. Miller, P. F. Strom, S. T. MacGregor, and K. M. Psarianos. 1983. Composting ecosystem management for waste treatment. *Biological Technology* 1:347–353.

Finstein, M. S., and M. L. Morris. 1975. Microbiology of municipal solid waste composting. *Advances in Applied Microbiology* 19:113–151.

Finstein, M. S., and M. L. Morris. 1979. Anaerobic digestion and composting: microbiological alternatives for sewage sludge treatment. *American Society for Microbiology News* 45:43–48.

Flint, K. P. 1982. Microbial ecology of domestic wastes. In R. G. Burns and J. H. Slater (eds.). *Experimental Microbial Ecology*. Blackwell Scientific Publications, Oxford, England.

Frazier, W. D., and D. C. Westhoff. 1978. *Food Microbiology*. McGraw-Hill Book Co., New York.

Gebhardt, M. R., T. C. Daniel, E. E. Schweizer, and R. R. Allmaras. 1985. Conservation tillage. *Science* 230:625–630.

Godzing, T. J. 1972. The role of nitrogen in eutrophication processes. In R. Mitchell (ed.). *Water Pollution Microbiology*. Wiley-Interscience, New York, pp. 43–68.

Hartenstein, R. 1981. Sludge decomposition and stabilization. *Science* 212:743–749.

Hawkes, H. A. 1963. *The Ecology of Waste Water Treatment*. Pergamon Press Ltd., Oxford, England.

Hawkes, H. A. 1977. The ecology of activated sludge. In K. W. A. Chater and H. J. Somerville (eds.). *The Oil Industry and Microbial Ecosystems*. Institute of Petroleum, London, pp. 217–233.

Hoadley, A. W., and B. J. Putka (eds.). 1977. *Bacterial Indicators/Health Hazards Associated with Water*. ASTM Technical Publication 635, American Society for Testing Materials, Philadelphia.

Howell, J. A. 1977. Alternative approaches to activated sludge and trickling filters. In K. W. A. Chater and J. H. Somerville (eds.). *The Oil Industry and Microbial Ecosystems*. Institute of Petroleum, London, pp. 199–216.

Hurst, A., and D. L. Collins-Thompson. 1979. Food as bacterial habitat. *Advances in Microbial Ecology* 3:79–134.

Hynes, H. B. N. 1960. *The Biology of Polluted Waters*. Liverpool University Press, Liverpool, England.

Imhoff, K., and G. M. Fair. 1956. *Sewage Treatment*, 2nd ed. John Wiley and Sons, New York.

Keeny, D. R., R. A. Herbert, and A. J. Holding. 1971. Microbiological aspects of the pollution of fresh waters with inorganic nutrients. In G. Sykes and F. A. Skinner (eds.). *Microbial Aspects of Pollution*. Academic Press, London, pp. 181–200.

LaRiviere, J. W. M. 1977. Microbial ecology of liquid waste treatment. *Advances in Microbial Ecology* 1:215–259.

Mack, W. N., J. P. Mack, and O. Ackerson. 1975. Microbial film development in a trickling filter. *Microbial Ecology* 2:215–226.

Mitchell, R. 1974. *Introduction to Environmental Microbiology*. Prentice-Hall, Englewood Cliffs, N.J.

National Academy of Sciences. 1969. *Eutrophication: Causes, Consequences, Correctives*. National Academy of Sciences, Washington, D.C.

Olson, B. H., and L. A. Nagy. 1984. Microbiology of potable water. *Advances in Applied Microbiology* 30:73–132.

Pavoni, J. L., J. E. Heer, Jr., and D. J. Hagerty. 1975. *Handbook of Solid Waste Disposal, Materials, and Energy Recovery*. Van Nostrand Reinhold Co., New York.

Phillips, R. E., R. L. Blevins, G. W. Thomas, W. W. Frye, and S. H. Phillips. 1980. No-tillage agriculture. *Science* 208:1108–1113.

Pike, E. B., and C. R. Curds. 1971. The microbial ecology of the activated sludge process. In G. Sykes and F. A. Skinner (eds.) *Microbial Aspects of Pollution*. Academic Press, London, pp. 123–147.

Pye, V. I., and R. Patrick. 1983. Ground water contamination in the United States. *Science* 221:713–718.

Schroeder, E. D. 1977. *Water and Wastewater Treatment*. McGraw-Hill Book Co., New York.

Shuval, H. J., and E. Katznelson. 1972. The detection of enteric viruses in the water environment. In R. Mitchell (ed.). *Water Pollution Microbiology*. Wiley-Interscience, New York, pp. 347–361.

Sieburth, J. M. 1975. *Microbial Seascapes*. University Park Press, Baltimore.

Slechta, A. F., and G. L. Culp. 1967. Water reclamation studies at the South Tahoe public utility district. *Journal of Water Pollution Control Federal* 39:787–814.

Smith, E. M., C. P. Gerba, and J. L. Melnick. 1978. Role of sediment in persistence of enterovi-

ie estuarine environment. *Applied and ental Microbiology* 35:685–689.

l. D., and B. H. Olson. 1983. Microbial water-borne disease. In P. S. Berger and an (eds.). *Assessment of Microbiology lity Standards of Drinking Water*. Envi- l Protection Agency. Washington, D.C.,

ium, M. S. 1983. Anaerobic treatment of r. recent developments. *American Society iology News* 49:532–536.

D. F., and W. H. J. Hatting. 1969. Anaer- tion. I. The microbiology of anaerobic *Water Research* 3:385–416.

artment of Health, Education and Welfare. *itary Landfill Facts*. SW 41s. Government ffice. Washington D.C.

J. M., E. F. Landry, M. Z. Thomas, le, and W. F. Penello. 1979. Survey of human enterovirus occurrence in fresh and marine surface waters on Long Island. *Applied and Environmental Microbiology* 38:290–296.

Waksman, S. A. 1952. *Soil Microbiology*. John Wiley and Sons. New York.

Warren, C. E. 1971. *Biology and Water Pollution Control*. W. B. Saunders, Philadelphia.

Wolf, H. W. 1972. The coliform count as a measure of water quality. In R. Mitchell (ed.). *Water Pollution Microbiology* Wiley-Interscience, New York, pp. 333–345.

Wolfe, R. L., N. R. Ward, and B. H. Olson. 1984. Inorganic chloramines as drinking water disinfectants: a review. *Journal of the American Water Works Association*. 76:74–88.

Zinder, S. H. 1984. Microbiology of anaerobic conversion of organic wastes to methane:recent developments. *ASM News* 50:294–298.

13

Microorganisms and Some Novel Pollution Problems

During the second half of the twentieth century, pollution problems have become more acute than ever before. Pollution and its control have ceased to be the narrow concerns of only sanitary engineers; they have become slogans for mass movements and the focal topic of noisy scientific, public, and legislative debates. The current environmental predicament of our planet has two recognizable components: conventional pollution that now occurs on a vastly increased scale and novel pollution problems that were largely unknown before the Second World War. The population explosion, combined with an international trend towards urbanization, has increased the intensity of conventional pollution to a level where the old sanitation principle "the solution to pollution is dilution" is clearly an inadequate approach. To stave off unacceptable deterioration of the environment, industrialized societies are being forced to pay heavily for the abatement of sewage, solid waste, and air pollution. The scientific background of these conventional pollution problems is well understood and effective control is largely a question of technology and finances. In contrast, many of the novel pollution problems, caused by chemicals to which the environment had not been exposed previously, have caught scientists and laymen alike off guard. Of necessity, regulatory decisions of far-reaching economic and environmental consequences are being made on the basis of scant or contested evidence.

The majority of the novel pollution phenomena involve man-made organic chemicals, such as pesticides, plastics, and other synthetics that persist, degrade too slowly, or are converted to even less desirable residues. Acid mine drainage, oil, and heavy metal pollution do not involve synthetics, but human activities are responsible for introducing these materials at problem levels into the ecosphere. The principles that govern the distribution and fate of these chemical pollutants in the ecosphere show similarities regardless of their origin. Quite often, novel pollution problems have global as well as local implications, whereas the impact of conventional pollution is typically felt only within definable geographic boundaries, such as within a watershed.

In response to some of the above-mentioned novel pollution phenomena, efforts are being made to develop genetically engineered microorganisms that will aid in the abatement of these problems. The use of recombinant DNA technology is being

nable microbial evolution to keep
advances of synthetic organic
his approach to environmental
own potential problems, however,
uses are being extensively debated
al., 1985).

and Biomagnification
tic Molecules

ble environmental conditions, all
ic compounds are degraded. While
never been put to a rigorous and
st, it appears self-evident from the
do not observe a large-scale accu-
ny natural organic substance. If any
ound produced in the ecosphere
tly resistant to recycling, huge de-
material would have accumulated
ic geological ages. This is clearly
substantial organic deposits, such
accumulate only under conditions
rse to biodegradation.

r (1965) formalized this general un-
s the "principle of microbial infal-
ssing the empirical observation that
ganic compound is totally resistant
ation provided that environmental
favorable. This fact should hardly
. The evolution of various biopoly-
w and gradual, allowing time for a
tion of microbial catabolism appro-
ery newly available substrate. The
this evolution was measured in bil-
s. In contrast, the explosive devel-
nthetic organic chemistry during the
has led to the large-scale production
ing variety of synthetic organic
hat, either by design or by accident,
ound up in the environment. Once
ate varied. Most of the synthetic
nd natural counterparts or were suf-
ar to naturally occurring compounds
t to microbial metabolism (Dagley,

1975). Others were xenobiotic, that is foreign to biological systems, and had molecular structures and chemical bond sequences not recognized by existing degradative enzymes. They were resistant or were metabolized incompletely with the result that some man-made organic compounds accumulated in the environment. Human ingenuity outpaced microbial evolution, and the fallibility of the presumed "biological incinerators" was exposed.

One can visualize numerous reasons why a xenobiotic organic compound should prove recalcitrant (totally resistant) to biodegradation (Alexander, 1981). Unusual substitutions, such as with chlorine and other halogens, unusual bonds or bond sequences, such as tertiary and quaternary carbon atoms, highly condensed aromatic rings, excessive molecular size (in the case of polyethylene and other plastics), are some of the more common reasons for recalcitrance. Other more subtle reasons may be the failure of the compound to induce the synthesis of degrading enzymes, even though it may be susceptible to their action, a failure of the compound to enter the microbial cell for lack of suitable permeases, unavailability of the compound due to insolubility or adsorption phenomena, an excessive toxicity of the parent compound or its metabolic products. On occasion, microbial transformations are synthetic rather than degradative and result in residues that are more stable than the parent compound.

Unfortunately, the term degradation has been used to describe transformations of every type, including those that yield products more complex than the starting material, as well as those responsible for the complete oxidation of organic compounds to CO_2, H_2O, NO_3^-, and other inorganic components. This ambiguous terminology has proven confusing to legislators and interested laypeople alike, who, for example, would hear one expert testify that the insecticide DDT is degradable, since it is converted in part to DDD, DDE, and other closely related compounds. (Figure 13.1) (Wedemeyer, 1956). Another expert would insist that DDT is persistent, because the

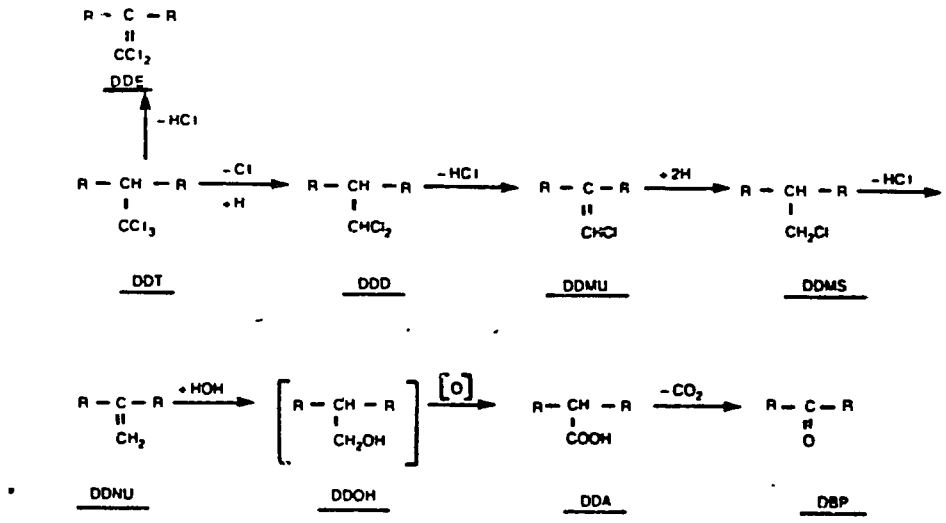


FIGURE 13.1
Transformations of the insecticide DDT by *Enterobacter aerogenes*. All transformations are restricted to the $-CCl_3$ moiety. Anaerobic conditions favor dechlorination steps. The final product DBP is still recalcitrant and biologically active. "R" represents a *p*-chlorophenyl moiety. Abbreviations: DDT = 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane, DDE = 1,1-dichloro-2,2,-bis(*p*-chlorophenyl) ethylene, DDD = 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane, DDMU = 1-chloro-2,2-bis(*p*-chlorophenyl) ethylene, DDMS = 1-chloro-2,2-bis(*p*-chlorophenyl) ethane, DDNU = unsym-bis(*p*-chlorophenyl) ethylene, DDOH = 2,2-bis(*p*-chlorophenyl) ethanol, DDA = 2,2-bis(*p*-chlorophenyl) acetate, DBP = 4,4'-dichlorobenzophenone. (Based on Wedemeyer, 1976; reprinted by permission; American Society for Microbiology.)

basic carbon skeleton remains unaltered. To preclude misunderstanding, the term mineralization has been proposed for describing the ultimate recycling of an organic molecule to its mineral constituents (ultimate biodegradation) and is used here in this sense.

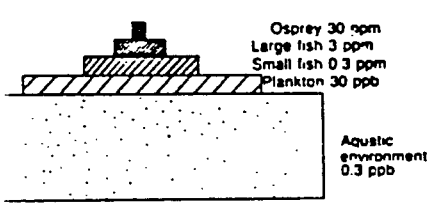
What happens if a persistent organic compound is introduced into the ecosphere? Particle size and solubility characteristics, among other factors, will influence its mode and rate of travel, but chances are good that it will be distributed to places far removed from its original application site. The fallout from atmospheric nuclear tests has dramatized the capacity of atmospheric forces to distribute chemicals globally from point sources (Hanson, 1967). The experience with chlorinated hydrocarbon insecticides has been similar: they have been detected in remote and even Arctic regions, thousands of miles removed from the nearest possible application site (Cade et al., 1971). It is important, therefore, to keep in mind that it is insufficient to assess the effect of a persistent organic chemical on the environment to which it is directly applied. It may be distributed and it may elicit unexpected detrimental effects in a more fragile ecosystem far removed from the original application site.

The production and release of persistent or-

hemicals into the environment have a rel- short history. Their distribution, however, spread (National Research Council, 1972; and Burns, 1975). Distribution tends to organo-chlorines in environments that were directly exposed often are present in the its per billion (ppb) range. Why should w concentrations still cause concern? They because of a phenomenon called "biological ication," or "biomagnification" for short. subject to this phenomenon, the pollutant be both persistent and lipophilic (Gosset 1983). Because of their lipophilic character, nute dissolved amounts are partitioned from rounding water into the lipids of both pro- tic and eukaryotic microorganisms (Figure Concentrations in their cells as compared surrounding medium may increase by one e orders of magnitude. The microorganisms gested by the members of the next higher c level. Only 10–15 percent of the biomass sferred to the higher trophic level, the rest sipated in respiration. The persistent lipo- pollutant, however, is neither degraded nor ted to a significant extent and so is preserved ically without loss in the smaller biomass e second trophic level. Consequently, its ntration increases by almost an order of magnitude. The same thing occurs at the successively higher trophic levels. The top trophic level, composed of birds of prey, mammalian carnivores, and large predatory fish, may carry a body burden of the environmental pollutant that exceeds the environmental concentration by a factor of 10^4–10^6.

At such levels, if the pollutant is a biologically active substance, such as a pesticide, it may cause death or serious debilitation of the affected organism. DDT and other chlorinated hydrocarbons were implicated in the death or reproductive failure of various birds of prey (Cade et al., 1971). In the case of local application even a relatively short food chain such as the sprayed vegetation → earthworm → robin can be destructive to the bird. We humans derive our food from various trophic levels and are in a less exposed position than a top level carnivore. Nevertheless, at the time of unrestricted DDT use, the average American, with no occupational exposure, carried a body burden of 4–6 ppm DDT and its derivatives. Although this amount was not considered acutely dangerous, the trend for an increasing contamination of the higher trophic levels of the ecosphere became sufficiently clear, and led to the ban on the use of DDT in the United States and most other developed countries for all but emergency situations.

E 13.2
gnification of DDT. Minute concentrations of dis- DDT move by physical partitioning into plank- urther concentration of the pollutant takes place gh the shrinkage of biomass between successive c levels. DDT concentrations are idealized and, ypical, they do not represent actual analytical



Recalcitrant Halocarbons

Numerous xenobiotic pollutants, such as DDT, are halocarbons which have proven recalcitrant to microbial attack. The carbon-halogen bond is a highly stable one. Cleavage of this bond is not exothermic, but rather, requires a substantial energy input; it is an endothermic reaction. As a consequence, halocarbons are chemically and biologically stable. Stability is desirable in pesticides, but the same property causes persistence and is undesirable as an environmental contaminant. There are only limited photochemical and biochemical mechanisms in the biosphere for their detoxification and recycling. Furthermore, the

energetics of their degradation are such that there is little prospect for microorganisms ever to evolve a capability for utilization of extensively halogenated carbon compounds as growth substrates.

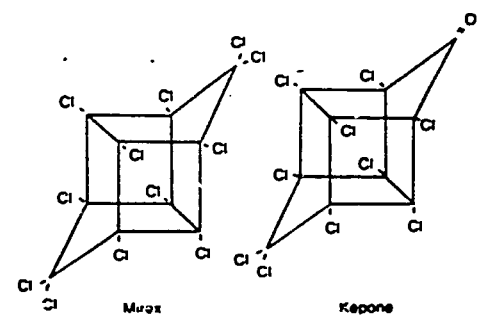
Important groups of halocarbons include halocarbon propellants, solvents and refrigerants, certain organochlorine insecticides, polychlorinated or polybrominated biphenyls and triphenyls, chlorodibenzodioxins and chlorodibenzofurans.

Halocarbon Propellants and Solvents Halocarbon propellants and solvents are C_1-C_2 alkanes in which all or nearly all hydrogen atoms are replaced by fluorine-chlorine combinations. They serve as solvents and aerosol propellants in spray cans for such things as cosmetics, paint, and insecticides, and as the working fluid in the condensor units of air conditioners and refrigerators. Most commonly used are CCl_3F and CCl_2F_2 freons, designated by the codes F-11 and F-12, respectively. Spilled halocarbon propellants and solvents have caused serious groundwater contamination problems. Being inert and quite volatile, some of these components upon release rise to the stratosphere and by photochemical interaction are believed to deplete the protective ozone layer of the earth (Molina and Rowland, 1974; National Research Council, 1976). A partial destruction of the ozone layer would result in increased UV radiation on the surface of the earth, causing an increased incidence of skin cancer and mutagenesis. While the rate and extent of ozone layer destruction by halocarbons is a matter of controversy, legislation in the United States has been enacted to phase out halocarbons as aerosol propellants. At this time, there is no suitable substitute for halocarbons as refrigerants and their use as coolants will continue regardless of their recalcitrance.

The less extensively halogenated C_1 - C_2 alkanes are subject to microbial utilization at slow rates. Dichloromethane was shown to serve as the only carbon source to a pseudomonad and dichloroethane served as substrate to microbial consortia (Stucki et al., 1981). The fully chlorinated but unsaturated tetrachloroethylene ($Cl_2C=CCl_2$) was subject to stepwise dechlorination and partial conversion to CO_2 by a methanogenic bacterial consortium growing on acetate in an anaerobic reactor (Vogel and McCarty, 1985). Dehalogenation of organic compounds is thermodynamically favored under anaerobic conditions. Aerobically, Fogel et al., (1986) demonstrated extensive degradation of trichloroethylene (TCE) and other halogenated C_2 compounds by a methane utilizing microbial consortium. It was proposed that the low specificity of methane monooxygenase allows the conversion of TCE to TCE epoxide which subsequently spontaneously hydrolyzes to products utilizable by microorganisms. *Methylococcus capsulatus* was reported to convert chloro- and bromomethane to form aldehyde dichloromethane to CO and trichloromethane to CO_2 while growing methane (Dalton and Stirling, 1982). Thus, methanotrophic bacteria show some promise for bioremediation of halocarbon-polluted aquifers.

Pesticides Mirex ($C_{10}Cl_{12}$) and Kepone ($C_{10}Cl_{10}O$) are very extensively chlorinated insecticidal compounds (Figure 13.3). Mirex has been used in the United States in the fire ant

FIGURE 13.3
Structural formulae of the insecticides Mirex and Kepone. Extensive chlorination renders these compounds extremely resistant to biodegradation.

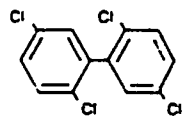


:ram. Kepone was manufactured ·ı export and as a precursor for other ·epone came to national attention in ·tates when it poisoned a substantial ·orkers in the manufacturing plant . Virginia. The subsequent investi- ·led extensive environmental con- ·ound the plant. The Kepone incident the indefinite closing for fishing of ·ated section of the James River. No ·ineralization was demonstrated for ·e or Mirex, although both compounds ·o limited dechlorination by anaerobic ·nsortia (Orndorff and Colwell, 1980) ·radation (National Research Council,

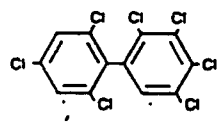
·ch less extensively chlorinated pes- ·s subject to some biochemical trans- ·oth *in vitro* and in the environment, ·e carbon skeleton persists in nature ·ely long periods. Pfaender and Alex- · succeeded in demonstrating the *in* ·lization of DDT ring-carbon by se- ·sing cell-free extracts of a hydrogen- ·cterium, whole cells of *Arthrobacter*, co-substrates, and a regimen of al- ·naerobic and aerobic conditions. ·s, they came to the conclusion that · DDT mineralization in the environ- ·not occur or does so at exceedingly ·ther than the unfavorable energetics ·lorination steps, no satisfactory ex- ·uld be found for this fact.

·ated Biphenyls (PCBs)

·ated biphenyls (PCBs) are mixtures · with one to ten chlorine atoms per ·gure 13.4). They are oily fluids with · points, great chemical resistance, ·l conductivity, and high light-breaking ·use of these properties, they have ·s plasticizers in polyvinyl polymers, ·coolants in transformers, and as heat



2,2',5,5'-Tetrachlorobiphenyl



2,2',3',4,4',5',6-Heptachlorobiphenyl

FIGURE 13.4
Polychlorinated biphenyls (PCBs). The above two compounds are only two examples of 210 different chloro-substitution possibilities. Resistance to biodegradation increases with the number of chloro-substitutions.

exchange fluids in general. Minor uses involve inks, paints, sealants, insulators, flame retardants, and, as microbiologists should know, microscope immersion oils. The chemical inertness of PCBs is paralleled by their great biological stability, which increases with the degree of their chlorination.

The structure of PCBs is similar in some ways to that of DDT and so is their behavior in the environment in regard to persistence and potential for biological magnification. Whereas most of the enumerated uses of PCBs would not seem to be conducive to widespread environmental contamination, the fact is that PCB residues have been detected in a large percentage of random environmental samples and they accumulate in animals of higher trophic levels (Gustafson, 1970, National Research Council, 1979).

PCB concentrations over 1 ppm have been detected in one-third of the sampled United States population with no known occupational exposure. In some freshwater habitats older individuals of predatory fish, such as trout and salmon, ac-

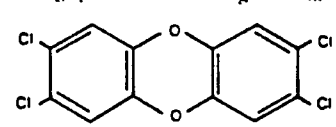
cumulate 20–30 ppm of PCBs and predatory as well as fish-eating birds were found to contain, on occasion, several hundred ppm of PCBs. In 1968, PCB-contaminated cooking oil, caused by a leaky heat exchanger, poisoned nearly 1000 people in Japan; the main symptoms were liver damage and a severe skin condition called chloracne. In this case, the victims had consumed several grams of the substance (Fujiwara, 1975). Low PCB-intakes from general environmental contamination are not known to harm humans or most other mammals, but along with chlorinated hydrocarbon insecticides, PCBs were implicated in egg-shell thinning and reproductive failure of some predatory and fish-eating birds. Reproductive failure in cultured mink was caused by the approximately 5 ppm PCB content of their fish-based feed and some species of fish, shrimp, and algae were affected by PCB concentrations in the low ppb range. Besides their acute effects, PCBs, and some other chlorinated hydrocarbons including DDT, are under suspicion of being potential human carcinogens, although this point is still controversial.

The assessment of environmental contamination by PCBs is still rudimentary. The various degrees of chlorination and the high number of isomers renders the positive identification and quantitative measurement of PCBs a difficult analytical task and, in the past, PCB residues were frequently confused with those of chlorinated hydrocarbon insecticides. It is possible that photochemical conversion of DDT to PCBs contributes to the widespread occurrence of these environmental contaminants (Maugh, 1973).

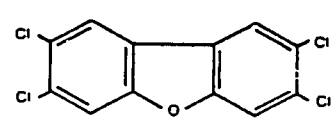
Biphenyl is able to serve some microorganisms as their only source of carbon and energy, and mono-, di-, tri-, and tetrachlorobiphenyls are, to some degree, subject to biodegradation as evidenced by formation of hydroxylated metabolites (Ahmed and Focht, 1973; Massé et al., 1984; Safe, 1984). In the more extensively chlorinated biphenyls, the substituents prevent ring hydroxylation and these highly chlorinated analogs cause the most concern with respect to persistence and environmental contamination. Voluntary curbs by manufacturers coupled with government regulations have removed PCBs from food-processing equipment and food-packaging material. Disposable items using PCBs as plasticizers have been phased out in an effort to reduce the amount of contaminating PCBs and, as of 1978, the use and discharge of PCBs in the United States came under a complete government ban. Continuing sources of PCB pollution, however, are previously contaminated sediments, landfills, and older electric transformers.

A frequent contaminant of PCBs and of the herbicide 2,4,5-T, to be discussed later in this chapter, are 2,3,7,8-tetrachlorodibenzodioxin (TCDD), related chlorodibenzo-dioxins and chlorodibenzofurans (Figure 13.5). Some of these contaminants arise during manufacture, others are formed through thermal degradation of the chlorophenols and chlorobiphenyls. TCDD and related compounds are characterized not only by high recalcitrance, but also by an unusually high degree of acute and chronic toxicity. Contamination as low as in the parts per billion (ppb) range is considered hazardous to human health. Human settlements that became contaminated

FIGURE 13.5
A: 2,3,7,8-tetrachloro-dibenzo-dioxin (TCDD), B: 2,3,7,8-tetrachloro-dibenzofuran, contaminants and/or thermal degradation products of PCBs and certain phenoxyalkanoic acid herbicides. These and related chlorodibenzo-dioxins and chlorodibenzofurans are extremely toxic and highly resistant to biodegradation.



2,3,7,8-TCDD

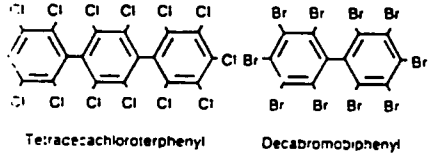


2,3,7,8-TCDF

TCDD and related substances as occurred nes Beach (United States) through spreading ntaminated waste oil and in Soveso (Italy) ed by the explosion of a chemical reactor, to the evacuation and indefinite closing of ffected areas (Tucker et al., 1983). Recently, pus et al. (1985) reported oxidation of TCDD some other chlorinated hydrocarbon pollu- by the lignin-degrading basidomycete *Pha- haete chrysosporium*. The amounts of $^{14}CO_2$ ed from the radiolabeled test substrates ed only between 1–4 percent and may not been in excess of the radiochemical im- nes of the test substances. Therefore, the egradability of TCDD continues to be tful.

Polybrominated biphenyls (PBBs), which are l as flame retardants, and polychlorinated henyls (PCTs), which have three attached yl rings and are employed as plasticizers ure 13.6), have pollution potentials similar CBs. To date, they have been manufactured much smaller scale than PCBs. Their behavior nvironmental contaminants received little at- ion until an unfortunate incident in 1976, when chemical plant that manufactured both PBB e retardants and cattle feed additives, large unts of cattle feed were inadvertently mixed PBBs and were fed to dairy cows on various s in Michigan. The incident necessitated the ruction of numerous dairy herds and caused t economic losses. Although no proven human fatalities were attributed to this occurrence, large numbers of people were contaminated with PBBs through the dairy products before the cause could be traced and eliminated. While the incident was clearly a case of human error, it has served as a forewarning of the long residence time and high biomagnification potential of PBBs as environmental pollutants.

RE 13.6
ples of polychlorinated terphenyls and polybromi- l biphenyls. Commercial mixtures contain numer- partially halogenated homologues. The extensive genation of these compounds result in high resis- to biodegradation.



Synthetic Polymers

Synthetic polymers are easily molded into complex shapes, have high chemical resistance, and are more or less elastic. Some can be formed into fibers or thin transparent films. These properties have made them popular in the manufacture of garments, durable and disposable goods, and packaging materials. Disposable goods and packaging material made out of various plastic materials have the largest enivronmental impact. Over 90 percent of the plastic material in municipal garbage consists of polyethylene, polyvinyl chloride and polystyrene, in roughly equal proportions (Figure 13.7).

Typically, these materials have molecular weights ranging from several thousands to 150,000 and appear to resist biodegradation indefinitely. Older reports on plastic biodegradation involve, on closer scrutiny, only the degradation of the plasticizers, the additives designed to render the plastic pliable. Frequently, plasticizers are esters of long-chain fatty acids and alcohols. Their biodegradation renders the material brittle, but the polymer structure is not affected. The contention that the basic polymer structures are not subject to biodegradation was based originally on relatively crude weight loss measurements, but recently has been confirmed by highly sensitive measurements using ^{14}C-labelled polymers. Resistance to biodegradation seems to be associated here with excessive molecular size. Biodegradation of long chain C_{30} and higher *n*-alkanes declines with increasing molecular weight and *n*-alkanes in excess of a molecular weight of 550–600 become refractory to biodegradation. If the

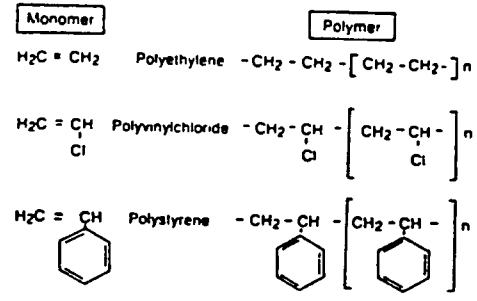


FIGURE 13.7
Chemical structure of some plastic materials of importance in environmental contamination. Left: monomeric starting material; right: polymer chain with repeating unit shown in brackets. Excessive molecular weigh; and a lack of depolymerizing exoenzymes make these polymers virtually nonbiodegradable. Monomers or short polymer chain fragments of the same materials tend to be biodegradable.

molecular size of polyethylene is reduced, by pyrolysis for example, to a molecular weight under 500, the fragments are susceptible to biodegradation.

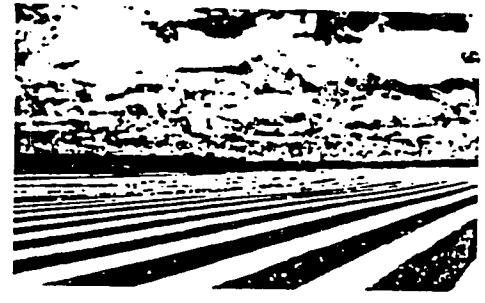
Plastic in the environment has been regarded as more of an aesthetic nuisance than a hazard, since the material is biologically quite inert. Reports about large numbers of floating plastic spherules in the ocean and their ingestion by small fish with consequent intestinal blockage, however, indicate that even inert materials are not necessarily innocuous (Carpenter, 1972).

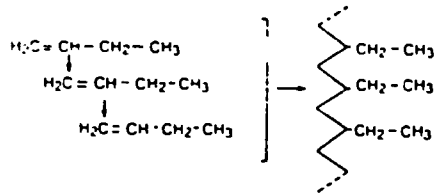
To date, development of biodegradable synthetic plastics has not progressed much beyond the experimental stage. Some polymers with ester linkages appear to be promising, such as the caprolactone polyester. A new agricultural practice involving plastics, the so-called film-mulching technique (Figure 13.8) lends special urgency to the development of biodegradable plastic film material. The practice involves the covering of fields with plastic film in order to control weeds and conserve moisture. It is impractical to collect or reuse the film at the end of the growing season and a large accumulation of plastic fragments in the field is equally undesirable. Since polyethylene, polybutene, and other polyolefins are susceptible to photochemical degradation, it is possible to devise materials that sustain sufficient photochemical damage during a growing season to become susceptible to subsequent microbial degradation (Figure 13.9) (Reich and Bartha, 1977). The resistance of the material to various irradiation levels can be adjusted by appropriate additions of antioxidants. Tests have shown that the native polymer was resistant to biodegradation, but the irradiated material became susceptible to it.

Alkyl Benzyl Sulfonates

Alkyl benzyl sulfonates (ABS) are the major components of anionic detergents. Like other surface active agents, the molecule has a polar (sulfonate) and a nonpolar (alkyl) end. Emulsification of fatty substances, and hence cleaning, occurs when ABS molecules form a monolayer around lipophilic droplets or particles. ABS molecules point with their nonpolar end towards the lipophilic substance and with the sulfonate end towards the surrounding water. Compared to

FIGURE 13.8
Photograph of film-mulched agricultural field. (Courtesy of B. L. Pollack; New Jersey Agricultural Experiment Station.)





IGURE 13.9
hemical structure of a polybutene film-mulch polymer hich is degradable by a combined photodegradation-iodegradation process. Left: monomeric precursor; ight: polybutene polymer. Photochemical reactions reak the polymer chain at the susceptible tertiary carbon allowing a subsequent biodegradative attack on the esulting chain fragments. (From Reich and Bartha, 977; reprinted by permission; copyright Williams and Vilkins.)

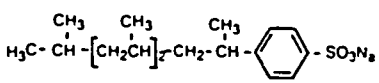
onventional soaps, nonlinear ABS (Figure 13.10) s resistant to biodegradation and causes extensive oaming of rivers that receive ABS-containing vastes. Nonlinear ABS is easier to manufacture and has slightly superior detergent properties, ut the methyl branching of the alkyl chain interferes with biodegradation, since the tertiary arbon atoms block the normal β-oxidation sequence. The problem was largely alleviated when he detergent industry switched to linear ABS hat was free from this blockage and was consequently more easily biodegraded. The ABS story s a remarkable one, since it was one of the first nstances when a synthetic molecule was redesigned to remove obstacles to biodegradation while essentially preserving the other useful characteristics of the compound (Brenner, 1969).

Oil Pollution

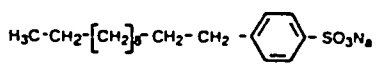
Pollution problems described in the preceding sections dealt with synthetic organic compounds unfamiliar to the biosphere and its recycling mechanisms. Petroleum is, in one sense, a natural product, resulting from the anaerobic conversion of biomass under high temperature and pressure. It has always entered the biosphere by natural seepage, but at rates much slower than the forced recovery of petroleum by drilling, currently estimated to be about two billion metric tons per year. Petroleum, or at least most of its components, is subject to biodegradation, but at relatively slow rates. The production, transportation, refining, and ultimate disposal are introducing, by conservative estimate, 3.2 million metric tons of oil annually into the oceans (National Research Council, 1985). This load is, of course, heavily concentrated around off-shore production sites, major shipping routes, and refineries and, therefore, frequently exceeds the self-purification capacity of the receiving waters. Oil floating on water is technically difficult to contain and collect. It is destructive to birds and various forms of marine life and, when driven ashore, causes heavy economic and esthetic damage. Cleanup costs are high, often amounting to 10–15 dollars per gallon of spilled oil. Prevailing weather conditions often completely thwart the containment and

IGURE 13.10
hemical structures of ome nonlinear and linear lkyl benzyl sulfonates ABS). The methyl branches n the former group of ompounds interfere with apid biodegradation and ause foaming problems in ontaminated water. Linear ABS compounds are free of his problem.

Nonlinear alkylbenzylsulfonate



Linear alkylbenzylsulfonate



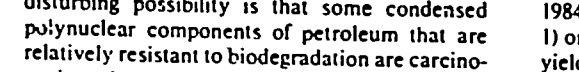
cleanup of oil slicks. Dispersal of the oil by detergents or sinking the oil with chalk or siliconized sand are essentially cosmetic measures. They remove the oil from the surface but increase the exposure of marine life to the pollutant and are, therefore, best avoided.

In addition to killing birds, fish, shellfish, and other invertebrates, oil pollution appears also to have more subtle effects on marine life. Dissolved aromatic components of petroleum disrupt, even at a low ppb concentration, the chemoreception of some marine organisms. Since feeding and mating responses largely depend on chemoreception, such disruption can lead to elimination of many species from the polluted area even when the pollutant concentration is far below the lethal level as defined in the conventional sense. Another disturbing possibility is that some condensed polynuclear components of petroleum that are relatively resistant to biodegradation are carcinogenic and may move up marine food chains and taint fish or shellfish.

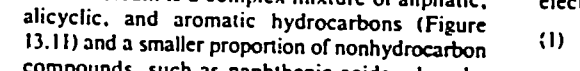
Petroleum is a complex mixture of aliphatic, alicyclic, and aromatic hydrocarbons (Figure 13.11) and a smaller proportion of nonhydrocarbon compounds, such as naphthenic acids, phenols, thiols, heterocyclic nitrogen, and sulfur compounds, as well as metalloporphyrins. There are several hundred individual components in every crude oil and the composition of each crude oil varies with its origin. Most xenobiotic pollutants can be regarded as substituted or modified hydrocarbons and, therefore, it is useful to review here briefly the biodegradation pathways of various hydrocarbon compounds.

Susceptibility to biodegradation varies with the type and size of the hydrocarbon molecule (Atlas, 1984). *n*-Alkanes of intermediate chain length (C_{10}-C_{24}) are degraded most rapidly. Short chain alkanes are toxic to many microorganisms, but they generally evaporate from oil slicks rapidly. Very long chain alkanes become increasingly resistant to biodegradation. As the chain length increases and the alkanes exceed a molecular weight of 500–600, they cease to serve as carbon sources. Branching, in general, reduces the rate of biodegradation since tertiary and quaternary carbon atoms interfere with degradation mechanisms or block degradation altogether. Aromatic compounds, especially of the condensed polynuclear type, are degraded more slowly than alkanes. Alicyclic compounds are frequently unable to serve as sole carbon sources for microbial growth unless they have a sufficiently long aliphatic side chain, but they can be degraded via co-metabolism by two or more cooperating microbial strains with complementary metabolic capabilities (Atlas, 1981).

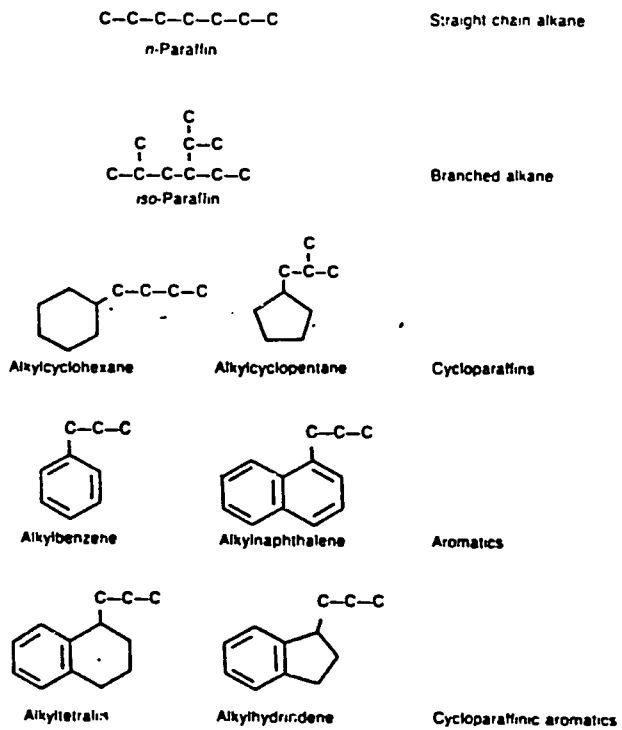
The initial attack on alkanes occurs by enzymes that have a strict requirement for molecular oxygen, that is, monooxygenases (mixed function oxidases), or dioxygenases (Singer and Finnerty, 1984; Britton, 1984). In the first case (equation 1) one atom of O_2 is incorporated into the alkane yielding a primary alcohol. The other is reduced to H_2O, with the reduced form of nicotinamide dinucleotide phosphate ($NADPH_2$) serving as electron donor:



In the second case (equation 2), both atoms of O_2 are transferred to the alkane, yielding a labile hydroperoxide intermediate that is subsequently reduced by $NADPH_2$ to an alcohol and H_2O (equation 3).



Most frequently, the initial attack is directed at the terminal methyl group, forming a primary alcohol that, in turn, is further oxidized to an aldehyde and fatty acid. Occasionally, both terminal methyl groups are oxidized in this manner, resulting in the formation of a dicarboxylic acid. This variation, described as diterminal or ω-oxidation, is one of several ways to bypass a



·URE 13.11
·ic structures of hydro-
·bons in crude oil. For
·ity, only the carbon
·letons are shown (the
·drogen atoms are omit-
·d). Homologues, isomers,
·d combinations result in
·ndreds of individual hy-
·rocarbon compounds in
·ude oil samples.

lock to β-oxidation due to branching of the car-
·on chain.

Once a fatty acid is formed, further catabolism
·curs by the β-oxidation sequence. The long-
·hain fatty acid is converted to its acyl coenzyme
·A form (equation 4) and is acted upon by a series
·f enzymes with the result that an acetyl CoA
·roup is cleaved off and the fatty acid is shortened
·y a two-carbon unit (equations 4-9).

·) $R\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COOH$

$\downarrow + CoA$

·) $R\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}CoA$

$\downarrow -2H$

(6) $R\text{-}CH_2\text{-}CH{=}CH\text{-}CO\text{-}CoA$

$\downarrow \quad +H_2O$

(7) $R\text{-}CH_2\text{-}CH(OH)\text{-}CH_2\text{-}CO\text{-}CoA$

$\downarrow \quad -2H$

(8) $R\text{-}CH_2\text{-}C(=O)\text{-}CH_2\text{-}CO\text{-}CoA$

$\downarrow \quad +CoA$

(9) $R\text{-}CH_2\text{-}CO\text{-}CoA + CH_3\text{-}CO\text{-}CoA$

This sequence is then repeated. The acetyl CoA units are converted to CO_2 through the tricarboxylic acid cycle. The β-oxidation sequence does not necessarily require the presence of molecular oxygen and after the initial oxygenation fatty acid biodegradation may proceed under anaerobic conditions.

A direct dehydrogenation of an intact hydrocarbon (equation 10) leading through a 1-alkene, (equation 11) alcohol, (equation 12) and aldehyde (equation 13) to a fatty acid (equation 14) has been suggested as a potential anoxic biodegradation mechanism:

(10) $R\text{-}CH_2\text{-}CH_3$

$\downarrow -2H$

(11) $R\text{-}CH{=}CH_2$

$\downarrow +H_2O$

(12) $R\text{-}CH(OH)\text{-}CH_3$

$\downarrow -2H$

(13) $R\text{-}CH_2\text{-}CHO$

$\downarrow \quad -2H, + H_2O$

(14) $R\text{-}CH_2\text{-}COOH$

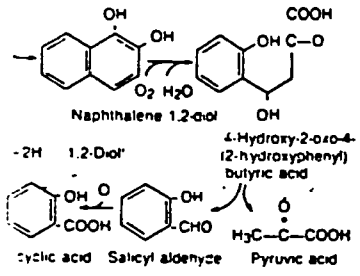
This sequence would allow the anaerobic metabolism of an intact hydrocarbon in the presence of an electron acceptor such as nitrate. The evidence for such a sequence at this time is very tenuous and significant metabolism of intact hydrocarbons in a strictly anaerobic environment is yet to be demonstrated. Geologic and environmental experience indicate that in the absence of molecular oxygen hydrocarbons persist.

Some microorganisms attack alkanes subterminally, that is, oxygen is inserted on a carbon atom within the chain instead at its end (Figure 13.12). In this manner, a secondary alcohol is formed first, which then by further oxidation is metabolized to a ketone and finally to an ester. The ester bond is cleaved yielding a primary alcohol and a fatty acid. The sum of the carbon atoms in the two fragments is equal to that of the parent hydrocarbon. The alcohol fragment is oxidized through the aldehyde to the fatty acid analog and both fragments are metabolized further by the β-oxidation sequence (Singer and Finnerty, 1984; Britton, 1984)).

Alicyclic hydrocarbons having no terminal methyl groups are biodegraded by a mechanism similar to the subterminal oxidation shown in Figure 13.12. In the case of cyclohexane (Figure 13.13), hydroxylation by a mixed function oxidase leads to an alicyclic alcohol. Dehydrogenation leads to the ketone. Further oxidation forms a lactone, which is subsequently hydrolyzed. The hydroxyl group is oxidized, in sequence, to an aldehyde and carboxyl group. The resulting dicarboxylic acid is further metabolized by β-oxidation. Microorganisms have been found that can grow on cyclohexane (Trower at al., 1985), and hence, must be capable of carrying out the whole degradation sequence. More frequently, however, the organisms capable of converting cyclohexane to cyclohexanone are unable to lactonize and open the ring and vice versa. Consequently, commensalism and co-metabolism play an important role in the biodegradation of alicyclic hydrocarbons (Perry, 1984; Trudgill, 1984).

Aromatic hydrocarbons are oxidized in one or several steps to catechol (Figure 13.14). The dihydroxylated aromatic ring is opened by oxidative "ortho cleavage" resulting in *cis, cis*-muconic acid. This is metabolized further to β-ketoadipic acid, which is oxidatively cleaved to the common tricarboxylic cycle intermediates, succinic acid, and acetyl-CoA. Alternatively, the catechol ring may be opened by meta cleavage, adjacent to rather than between the hydroxyl groups, yielding 2-hydroxy-*cis, cis*-muconic semialdehyde. Further metabolism leads to formic acid, pyruvic acid, and acetaldehyde. Condensed aromatic ring structures, if degradable, are also attacked by dihydroxylation and opening of one of the rings (Figure 13.15). The opened ring is degraded to pyruvic acid and CO_2 and a second ring is attacked in the same fashion. Many condensed polynuclear aromatic compounds, how-

abolism of a condensed aromatic ring plified) as shown for naphthalene. The re- al is metabolized further by *ortho* as shown in Figure 13.14.



se factors are difficult to influence. tion of oil slicks with hydrocarbon- icroorganisms and application to the n and phosphorus in oil-soluble form r" are being explored as means of polluting oil by stimulated biodegra- s, 1981, Bartha, 1986).

short run, petroleum and petroleum ntaining asphaltic components are not antitatively. The residues, along with ion products formed from reactions al degradation intermediates with each tar globules. The tar, a partially oxy- h-molecular weight material, is quite further degradation and floating tar encountered in the marine environ- asing quantities (Butler et al., 1973). al oil spills on land are generally ntain and clean up than on water. me and other low-viscosity distillation y seep into subsoils and persist there he prevailing anoxic conditions. They ollute the groundwater and pose an and in nearby basements and sewers. destructive to vegetation not only ontact toxicity, but because hydro- egradation in the soil renders plant anoxic. The lack of oxygen and accompanying H_2S evolution kills the roots of most plants, including those of large, well-established trees (Bossert and Bartha, 1984).

Natural gas seepages from underground pipe systems or from former landfills have a similar deleterious effect on vegetation. Here, the anoxic conditions are created by a combination of the physical displacement of soil air by natural gas and by the activity of *Methylomonas* and other bacteria capable of oxidizing gaseous alkanes. Destruction of ornamental and shade trees occurred on a large scale in some communities that replaced coal-derived generator gas with natural gas. The former is generated by blowing a mixture of steam and air through hot coals, giving rise to a combustible gas mixture containing hydrogen, carbon monoxide, nitrogen, and various impurities. This gas mixture had a high moisture content. When natural gas, which consisted mainly of methane, ethane and propane and was quite dry, was distributed through the same old municipal pipe networks, pipe joint gaskets dried out, shrank, and became leaky. The underground gas seepage caused widespread tree-kills through anoxic soil conditions, though the direct toxicity of methane and other gaseous alkanes to plants is quite low (Hoeks, 1972).

Oil sludges, mixtures of oil, water, and solids, are generated in oil refining operations and pose a disposal problem. A relatively low-cost disposal method involves spreading this waste on land in so-called land-treatment operations. Properly managed by liming, fertilization with nitrogen and phosphorus, and tillage for aeration, the hydrocarbon components are degraded by soil microorganisms in the upper aerobic soil layer, thus reducing the chance of any water pollution through infiltration or runoff (Bartha and Bossert, 1984).

Pesticides

Monocultures are inherently unstable ecosystems and, of necessity, a large part of the agricultural production effort goes towards the control of competing weeds, destructive insects, and other pests. The cost of manual labor and the increasing scale of agricultural operations in developed countries both have worked in favor of chemical pest control. By 1975, 1170 pesticides were registered for use in the United States—including 425 herbicides, 410 fungicides, and 335 insecticides. The total annual production of synthetic organic pesticides in the same year was 725 million kg. In terms of effectiveness, economy, and quality control, pesticides have been an unqualified success; the dangers inherent in our ignorance of the ultimate fate and unintended side effects of pesticides were recognized only gradually. Rachel Carson's controversial "Silent Spring" (1962) played a large role in alerting legislators and the general public to the proven and potential dangers of unwise pesticide use. In the subsequent highly emotional debate, sweeping endorsements or condemnations of pesticides were made by groups with conflicting interests and philosophies. In reality, a sudden discontinuation of pesticide use would surely precipitate an economic and public health crisis, while uncontrolled pesticide use would, in the long term, cause irreversible environmental damage. A responsible pesticide policy clearly demands careful risk-benefit analysis for each compound and its various uses.

Most of the currently used organic pesticides are subject to extensive mineralization within the time span of one growing season or less. Biochemical processes, alone or in combination with purely chemical reactions, are responsible for their disappearance. Synthetic pesticides show a bewildering variety of chemical structures, yet most can be traced to relatively simple hydrocarbon skeletons that bear a variety of substituent groups such as halogens, aminos, nitros, hydroxyls, and others. Aliphatic carbon chains are degraded by the β-oxidation sequence. The resulting C_2-fragments are further metabolized via the tricarboxylic acid cycle. Aromatic ring structures are metabolized by dihydroxylation and ring cleavage. Prior to these transformations, chemical groups attached to the aromatic ring are completely or partially removed. Substituent groups uncommon in natural compounds such as halogens, nitro- and sulfonate groups, if situated so as to impede the oxygenations, will frequently cause recalcitrance, whereas methyl, methoxy, carboxyl and carbonyl groups can often be removed metabolically from blocking positions. If the dihydroxylation can occur on a substituted aromatic compound, the previously described degradation pathways will be followed with minor modification according to the position and nature of the substituents. Saturated ring systems, such as cyclohexane and decalin, are more refractory than their aromatic analogs. They rarely support growth of any single microorganism, but some are degraded in the presence of other substrates and by the cooperative effort of several microbial strains. This phenomenon is observed also with other difficult-to-degrade compounds and relates to the concept of co-metabolism (Horvath, 1972).

"Co-metabolism" describes the phenomenon that occurs when a compound is transformed by a microorganism, yet the organism is unable to grow on the compound and does not derive energy, carbon, or any other nutrient from the transformation. Co-metabolic transformation occurs when an enzyme of a microorganism, growing on compound A, recognizes as substrate compound B and transforms it to a product. The transformation is limited, because the next enzyme of the organism that should attack in sequence has a higher specificity and does not recognize the product of B as a substrate. In the case of a pure culture, co-metabolism is a dead-end transformation without benefit to the organism. In a mixed culture or in the environment proper, however, such an initial co-metabolic transformation may pave the way for subsequent attack by another organism. In this manner, synergistic transformations of co-metabolic and also of the substrate-utilization type may eventually lead to the recycling of relatively recalcitrant compounds that do not support the growth of any selected pure al culture.

anic molecules to be degraded become

MICROBES IN THE ENVIRONMENT

Role in biogeochemical cycling. Microbes function as the recyclers of many different materials in the environment. They are essential to the carbon, nitrogen, oxygen, phosphorus, sulfur, iron, and hydrogen cycles in the environment.

As disease agents. There are numerous microorganisms which cause diseases in man and animals. These organisms are transmitted through the environment to man by the oral and respiratory routes. In order to do this, the microbe must exist in the environment for a period of time. Microbes may also be spread by contact between individuals.

As pollution indicators. Because of the large number of microbes which cause disease, it would be difficult to test a water for the presence of each type. This would require a large amount of time and expense. For this reason, a substitute test is used which looks for the presence of a pollution indicator. In the case of water pollution by fecal material, the coliform bacteria are used as the pollution indicator. These bacteria are not normally pathogens (disease producers), but occur in the fecal material of humans and animals in large numbers. Pathogens usually appear in an intermittent manner which makes them harder to detect. To be a good pollution indicator, a microbe must:

1. Be present in fecal matter in much larger numbers than the pathogen.
2. Have the same or longer die off rate as the pathogen.
3. Live at least as long as the pathogen in the water or other environment.
4. Be easy to work with in the laboratory.
5. Be easy to identify and test for.
6. Be harmless to lab workers.
7. Be absent in nonpolluted environments.
8. Have the same resistance (or somewhat greater) that the pathogen to disinfectants such as chlorine.

Bioremediation

MICROBIAL TREATMENT OF WASTEWATER
(See handout)

TREATMENT AND SAFETY OF WATER SUPPLIES

6. Flagellum - allows cell to move

METABOLISM

Microbes may use different methods to obtain energy. These are based on the material which is used as the final electron acceptor during the production of energy. Microorganisms obtain their energy from either chemical reactions or from light. The organisms are categorized according to the method used. A phototroph obtains energy from light and a chemotroph obtains energy by using chemical reactions. The chemotroph is the most important microbe in causing disease and in the cleanup of water pollution by organic chemicals. These organism use different types of metabolic reactions based on the electron acceptor used.

Energy is produced in an organism which uses aerobic metabolism (RESPIRATION) by many different biochemical reactions. These reactions usually involve biochemical cycles such as the Embden Meyerhoff Pathway and the Kreb's cycle. These cycles strip electron and hydrogens from organic compounds. The electrons and hydrogens are processed by the respiratory chain and the electron transport system. The electron eventually are passed to a final electron acceptor. In respiration or aerobic metabolism this is molecular oxygen. Cells which use this process must be provided with oxygen.

A second method of metabolism may be used by some microbes. This process is named fermentation and will take place in the absence of oxygen. The microbes use an organic compound as the final electron acceptor. An example of this method is the production of beer or wine.

An intermediate process may be used by other microbes. This process is named anaerobic respiration and allows the microbe to produce almost as much energy as respiration but proceeds in the absence of oxygen. The final electron acceptor may be sulfate, nitrate or some organic compounds.

MEASUREMENT OF MICROBES IN THE ENVIRONMENT
(See handout)

EFFECT OF ENVIRONMENTAL FACTORS ON MICROBES
(See handout)

CALIBRATION OF THE DISSOLVED OXYGEN METER/PREPARATION OF BLANK

NOTE: METER SHOULD BE CALIBRATED DAILY

1. Check BOD probe to be sure that there are no bubbles under the membrane.
2. Turn dissolved oxygen meter to the first position, "red line". The meter needle should move to the red line on the meter scale. If the needle does not go to the red line use the red line knob to adjust. If you cannot reach the red line by adjusting with the red line knob, the batteries must be replaced.
3. Turn meter knob to the zero position. Adust with the zero knob if necessary.
4. Collect about 950 ml of dilution water in a 1L beaker.
5. Carefully (SLOWLY) pour dilution water into each of three BOD bottles while bottle is tilted about 45 degrees.
6. Place BOD probe into one bottle, turn on the stirring bar, and and turn the knob to the 0-10 mg/l oxygen scale. Titrate the other two BOD dilution water bottles using the Winkler method.
7. Adjust the dissolved oxygen meter reading obtained with the BOD probe to the average value obtained by the Winkler method.
8. Remove probe from bottle and rinse with distilled water. Store probe in a BOD bottle which has about 50 ml of distiled water in it. Put glass stopper back in the dilution water BOD bottle used with the BOD probe. Add water for the water seal, put plastic overcap on and place bottle in the BOD incubator at 20 C. This bottle will be a dilution water blank to determine if the dilution water is suitable for the test.
9. Probe is now standardized and ready to process all samples.

CARE OF BOD PROBE

1. Check probe before each use to make sure that there are no bubbles under the membrane. If you see bubbles you must replace the membrane.
2. Store probe when not in use by placing the neck of the probe into a BOD bottle that has about 50 ml of distilled water in it. This is to keep the membrane moist and reduce the possibility of loss of the BOD probe fluid.
3. The membrane should be replaced on the average of 2-4 weeks.

WINKLER TITRATION FOR DISSOLVED OXYGEN (STD. METHODS 4500-O C, EPA METHOD 360.2)

1. Add 2 ml of alkali-azide-iodide reagent to bottle by allowing reagent to run slowly down the side of the oneck of the bottle.

2. Add 2 ml of manganous sulfate reagent slowly as in #1.
3. Stopper the bottle being careful not to trap any air bubbles, invert the bottle at least five times to mix reagent. At this point there should be a brown precipitate forming.
4. Allow bottle to stand until the precipitate has settled 2/3 of the way down the bottle. Invert the bottle again (at least five times) and allow to settle once more.
5. After the precipitate has [illegible] the bottle, restopper and add 2 ml of concentrated sulfuric acid. The precipitate will begin to dissolve. Invert the bottle another five times to totally dissolve the precipitate. An additional portion of acid may be added to fully dissolve the precipitate if necessary.
6. Titrate the brown colored liquid with sodium thiosulfate solution to a pale straw color and add two ml of starch indicator solution. Continue the titration until the blue color just disappears (it may become blue again after the solution stands for a period of time).
7. Oxygen concentration in mg/l is equivalent to the ml of thiosulfate titrant solution which is used. be careful to use the correct normality of the titrant to match the amount of sample used. The EPA method is easiest because you use the entire bottlr for titration. In this case you use a thiosulfate titrant of 0.0375 N. With the Standard Methods technique use 200 ml of sample from BOD bottle and a thiosulfate solution of 0.025 N.

PRECAUTIONS

1. Make sure BOD probe has no bubbles under the membrane.
2. Make a dilution blank each time you change dilution water or make up a new batch of dilution water.
3. Incubate all BOD bottles in the dark at 20 C.
4. Sample pH should be 6.5-7.5.
5. Sample should contain no residual chlorine.
6. Sample should contain no material toxic to bacteria.
7. Sample should be shaken well before being placed into BOD bottles. This will provide a homogeneous sample as well as getting rid of supersaturated oxygen levels.
8. Allow samples to come to 20 C before processing by the BOD test.
9. At the end of the incubation period use only those samples which give at least a change in dissolved oxygen of 2 mg/l and have a residual of at least 1 mg/l.
10. Attempt to prepare dilutions that overlap. In this way you may be able to have more than one bottle which meets the criteria in #9. This will give you duplicate analyses.

11. Duplicate at least one sample in every sample series. If the sample series is greater than 10 samples, duplicate one sample in every 10.

12. Dilutions may be prepared by placing a portion of the sample in the BOD bottle and adding dilution water. A graduated cylinder of appropriate size may be used to measure sample volumes from 11-300 ml. Use a 10 ml pipet for sample volumes of [illegible]

13. Dilution water blanks should change by less than 0.5 mg/l oxygen over the five day incubation period.

OXYGEN, DISSOLVED

Method 360.1 (Membrane Electrode)

STORET NO. 00299

1. Scope and Application
   1.1 The probe method for dissolved oxygen is recommended for those samples containing materials which interfere with the modified Winkler procedure such as sulfite, thiosulfate, polythionate, mercaptans, free chlorine or hypochlorite, organic substances readily hydrolyzed in alkaline solutions, free iodine, intense color or turbidity and biological flocs.
   1.2 The probe method is recommended as a substitute for the modified Winkler procedure in monitoring of streams, lakes, outfalls, etc., where it is desired to obtain a continuous record of the dissolved oxygen content of the water under observation.
   1.3 The probe method may be used as a substitute for the modified Winkler procedure in BOD determinations where it is desired to perform nondestructive DO measurements on a sample.
   1.4 The probe method may be used under any circumstances as a substitute for the modified Winkler procedure provided that the probe itself is standardized against the Winkler method on samples free of interfering materials.
   1.5 The electronic readout meter for the output from dissolved oxygen probes is normally calibrated in convenient scale (0 to 10, 0 to 15, 0 to 20 mg/l for example) with a sensitivity of approximately 0.05 mg/liter.
2. Summary of Method
   2.1 The most common instrumental probes for determination of dissolved oxygen in water are dependent upon electrochemical reactions. Under steady-state conditions, the current or potential can be correlated with DO concentrations. Interfacial dynamics at the probe-sample interface are a factor in probe response and a significant degree of interfacial turbulence is necessary. For precision performance, turbulence should be constant.
3. Sample Handling and Preservation
   3.1 See 4.1, 4.2, 4.3, 4.4 under Modified Winkler Method (360.2).
4. Interferences
   4.1 Dissolved organic materials are not known to interfere in the output from dissolved oxygen probes.
   4.2 Dissolved inorganic salts are a factor in the performance of dissolved oxygen probe.
      4.2.1 Probes with membranes respond to partial pressure of oxygen which in turn is a function of dissolved inorganic salts. Conversion factors for seawater and brackish waters may be calculated from dissolved oxygen saturation versus salinity data. Conversion factors for specific inorganic salts may be developed experimentally.

Approved for NPDES
Issued 1971

615
-97

Broad variations in the kinds and concentrations of salts in samples can make the use of a membrane probe difficult.

4.3 Reactive compounds can interfere with the output or the performance of dissolved oxygen probes.

4.3.1 Reactive gases which pass through the membrane probes may interfere. For example, chlorine will depolarize the cathode and cause a high probe-output. Long-term exposures to chlorine will coat the anode with the chloride of the anode metal and eventually desensitize the probe. Alkaline samples in which free chlorine does not exist will not interfere. Hydrogen sulfide will interfere with membrane probes if the applied potential is greater than the half-wave potential of the sulfide ion. If the applied potential is less than the half-wave potential, an interfering reaction will not occur, but coating of the anode with the sulfide of the anode metal can take place.

4.4 Dissolved oxygen probes are temperature sensitive, and temperature compensation is normally provided by the manufacturer. Membrane probes have a temperature coefficient of 4 to 6 percent/°C dependent upon the membrane employed.

5. Apparatus

5.1 No specific probe or accessory is especially recommended as superior. However, probes which have been evaluated or are in use and found to be reliable are the Weston & Stack DO Analyzer Model 30, the Yellow Springs Instrument (YSI) Model 54, and the Beckman Fieldlab Oxygen Analyzer.

6. Calibration

Follow manufacturer instructions.

7. Procedure

Follow manufacturer instructions.

8. Calculation

Follow manufacturer instructions.

9. Precision and Accuracy

Manufacturer's specification claim 0.1 mg/l repeatability with ±1% accuracy.

Bibliography

1. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 450, Method 422F (1975).

6/6

OXYGEN, DISSOLVED

Method 360.2 (Modified Winkler, Full-Bottle Technique)

STORET NO. 00300

1. Scope and Application
   1.1 This method is applicable for use with most wastewaters and streams that contain nitrate nitrogen and not more than 1 mg/l of ferrous iron. Other reducing or oxidizing materials should be absent. If 1 ml of fluoride solution is added before acidifying the sample and there is no delay in titration, the method is also applicable in the presence of 100–200 mg/l ferric iron.
   1.2 The Dissolved Oxygen (DO) Probe technique gives comparable results on all samples types.
   1.3 The azide modification is not applicable under the following conditions: (a) samples containing sulfite, thiosulfate, polythionate, appreciable quantities of free chlorine or hypochlorite; (b) samples high in suspended solids; (c) samples containing organic substances which are readily oxidized in a highly alkaline solution, or which are oxidized by free iodine in an acid solution; (d) untreated domestic sewage; (e) biological flocs; and (f) where sample color interferes with endpoint detection. In instances where the azide modification is not applicable, the DO probe should be used.
2. Summary of Method
   2.1 The sample is treated with manganous sulfate, potassium hydroxide, and potassium iodide (the latter two reagents combined in one solution) and finally sulfuric acid. The initial precipitate of manganous hydroxide, $Mn(OH)_2$, combines with the dissolved oxygen in the sample to form a brown precipitate, manganic hydroxide, $MnO(OH)_2$. Upon acidification, the manganic hydroxide forms manganic sulfate which acts as an oxidizing agent to release free iodine from the potassium iodide. The iodine, which is stoichiometrically equivalent to the dissolved oxygen in the sample is then titrated with sodium thiosulfate or phenylarsine oxide (PAO).
3. Interferences
   3.1 There are a number of interferences to the dissolved oxygen test, including oxidizing and reducing agents, nitrate ion, ferrous iron, and organic matter.
   3.2 Various modifications of the original Winkler procedure for dissolved oxygen have been developed to compensate for or eliminate interferences. The Alsterberg modification is commonly used to successfully eliminate the nitrite interference, the Rideal-Stewart modification is designed to eliminate ferrous iron interference, and the Theriault procedure is used to compensate for high concentration of organic materials.
   3.3 Most of the common interferences in the Winkler procedure may be overcome by use of the dissolved oxygen probe.

Approved for NPDES
Issued 1971

399

617

4. Sample Handling and Preservation

4.1 Where possible, collect the sample in a 300 ml BOD incubation bottle. Special precautions are required to avoid entrainment or solution of atmospheric oxygen or loss of dissolved oxygen.

4.2 Where samples are collected from shallow depths (less than 5 feet), use of an APHA-type sampler is recommended. Use of a Kemmerer type sampler is recommended for samples collected from depths of greater than 5 feet.

4.3 When a Kemmerer sampler is used, the BOD sample bottle should be filled to overflowing. (overflow for approximately 10 seconds). Outlet tube of Kemmerer should be inserted to bottom of BOD bottle. Care must be taken to prevent turbulence and the formation of bubbles when filling bottle.

4.4 At time of sampling, the sample temperature should be recorded as precisely as required.

4.5 Do not delay the determination of dissolved oxygen in samples having an appreciable iodine demand or containing ferrous iron. If samples must be preserved either method (4.5.1) or (4.5.2) below, may be employed.

4.5.1 Add 2 ml of manganous sulfate solution (6.1) and then 2 ml of alkaline iodide-azide solution (6.2) to the sample contained in the BOD bottle. Both reagents must be added well below the surface of the liquid. Stopper the bottle immediately and mix the contents thoroughly. The sample should be stored at the temperature of the collection water, or water sealed and kept at a temperature of 10 to 20°C, in the dark. Complete the procedure by adding 2 ml H_2SO_4 (see 7.1) at time of analysis.

4.5.2 Add 0.7 ml of conc. H_2SO_4 (6.3) and 1 ml sodium azide solution (2 g NaN_3 in 100 ml distilled water) to sample in the BOD bottle. Store sample as in (4.5.1). Complete the procedure using 2 ml of manganous sulfate solution (6.1), 3 ml alkaline iodide-azide solution (6.2), and 2 ml of conc. H_2SO_4 (6.3) at time of analysis.

4.6 If either preservation technique is employed, complete the analysis within 4–8 hours after sampling.

5. Apparatus

5.1 Sample bottles-300 ml ±3 ml capacity BOD incubation bottles with tapered ground glass pointed stoppers and flared mouths.

5.2 Pipets-with elongated tips capable of delivering 2.0 ml ±0.10 ml of reagent.

6. Reagents

6.1 Manganous sulfate solution: Dissolve 480 g manganous sulfate ($MnSO_4 \cdot 4H_2O$ in distilled water and dilute to 1 liter.

6.1.1 Alternatively, use 400 g of $MnSO_4 \cdot 2H_2O$ or 364 g of $MnSO_4 \cdot H_2O$ per liter. When uncertainty exists regarding the water of crystallization, a solution of equivalent strength may be obtained by adjusting the specific gravity of the solution to 1.270 at 20°C.

6.2 Alkaline iodide-azide solution: Dissolve 500 g of sodium hydroxide (NaOH) or 700 g of potassium hydroxide (KOH) and 135 g of sodium iodide (NaI) or 150 g of potassium iodide (KI) in distilled water and dilute to 1 liter. To this solution add 10 g of solution azide (NaN_3) dissolved in 40 ml of distilled water.

400 618

6.3 Sulfuric acid: concentrated.

6.4 Starch solution: Prepare an emulsion of 10 g soluble starch in a mortar or beaker with a small quantity of distilled water. Pour this emulsion into 1 liter of boiling water, allow to boil a few minutes, and let settle overnight. Use the clear supernate. This solution may be preserved by the addition of 5 ml per liter of chloroform and storage in a 10°C refrigerator.

6.4.1 Dry, powdered starch indicators such as "thyodene" may be used in place of starch solution.

6.5 Potassium fluoride solution: Dissolve 40 g $KF \cdot 2H_2O$ in distilled water and dilute to 100 ml.

6.6 Sodium thiosulfate, stock solution, 0.75 N: Dissolve 186.15 g $Na_2S_2O_3 \cdot 5H_2O$ in boiled and cooled distilled water and dilute to 1 liter. Preserve by adding 5 ml chloroform.

6.7 Sodium thiosulfate standard titrant, 0.0375 N: Prepare by diluting 50.0 ml of stock solution to 1 liter. Preserve by adding 5 ml of chloroform. Standard sodium thiosulfate, exactly 0.0375 N is equivalent to 0.300 mg of DO per 1.00 ml. Standardize with 0.0375 N potassium biiodate.

6.8 Potassium biiodate standard, 0.0375 N: For stock solution, dissolve 4.873 g of potassium biiodate, previously dried 2 hours at 103°C, in 1000 ml of distilled water. To prepare working standard, dilute 250 ml to 1000 ml for 0.0375 N biiodate solution.

6.9 Standardization of 0.0375 N sodium thiosulfate: Dissolve approximately 2 g (± 1.0 g) KI in 100 to 150 ml distilled water; add 10 ml of 10% H_2SO_4 followed by 20.0 ml standard potassium biiodate (6.8). Place in dark for 5 minutes, dilute to 300 ml, and titrate with the standard sodium thiosulfate (6.7) to a pale straw color. Add 1–2 ml starch solution and continue the titration drop by drop until the blue color disappears. Run in duplicate. Duplicate determinations should agree within ±0.05 ml.

6.10 As an alternative to the sodium thiosulfate, phenylarsine oxide (PAO) may be used. This is available, already standardized, from commercial sources.

7. Procedure

7.1 To the sample collected in the BOD incubation bottle, add 2 ml of the manganous sulfate solution (6.1) followed by 2 ml of the alkaline iodide-azide solution (6.2), well below the surface of the liquid; stopper with care to exclude air bubbles, and mix well by inverting the bottle several times. When the precipitate settles, leaving a clear supernatant above the manganese hydroxide floc, shake again. When settling has produced at least 200 ml of clear supernatant, carefully remove the stopper and immediately add 2 ml of conc. H_2SO_4 (6.3) (sulfamic acid packets, 3 g may be substituted for H_2SO_4)[5] by allowing the acid to run down the neck of the bottle, re-stopper, and mix by gentle inversion until the iodine is uniformly distributed throughout the bottle. Complete the analysis within 45 minutes.

7.2 Transfer the entire bottle contents by inversion into a 500 ml wide mouth flask and titrate with 0.0375 N thiosulfate solution (6.7) (0.0375 N phenyarsine oxide (PAO) may be substituted as titrant) to pale straw color. Add 1–2 ml of starch solution (6.4) or 0.1 g of powdered indicator and continue to titrate to the first disappearance of the blue color.

401

619

7.3 If ferric iron is present (100 to 200 mg/l), add 1.0 ml of KF (6.5) solution before acidification.

7.4 Occasionally, a dark brown or black precipitate persists in the bottle after acidication. This precipitate will dissolve if the solution is kept for a few minutes longer than usual or, if particularly persistent, a few more drops of H_2SO_4 will effect dissolution.

8. Calculation

8.1 Each ml of 0.0375N sodium thiosulfate (or PAO) titrant is equivalent to 1 mg DO when the entire bottle contents are titrated.

8.2 If the results are desired in milliliters of oxygen gas per liter at 0°C and 760 mm pressure multiply mg/l DO by 0.698.

8.3 To express the results as percent saturation at 760 mm atmospheric pressure, the solubility data in Table 422:1 (Whipple & Whipple, p 446–447, Standard Methods, 14th Edition) may be used. Equations for correcting the solubilities to barometric pressures other than mean sea level are given below the table.

8.4 The solubility of DO in distilled water at any barometric pressure, p (mm Hg), temperature, T°C, and saturated vapor pressure, u (mm Hg), for the given T, may be calculated between the temperature of 0° and 30°C by:

$$\text{ml/l DO} = \frac{(P - u) \times 0.678}{35 + T}$$

and between 30° and 50°C by:

$$\text{ml/l DO} = \frac{(P - u) \times 0.827}{49 + T}$$

9. Precision and Accuracy

9.1 Exact data are unavailable on the precision and accuracy of this technique; however, reproducibility is approximately 0.2 mg/l of DO at the 7.5 mg/l level due to equipment tolerances and uncompensated displacement errors.

Bibliography

1. Kroner, R. C., Longbottom, J. E., Gorman, R.A., "A Comparison of Various Reagents Proposed for Use in the Winkler Procedure for Dissolved Oxygen", PHS Water Pollution Surveillance System Applications and Development, Report #12, Water Quality Section, Basic Data Branch, July 1964.
2. Annual Book of ASTM Standards, Part 31, "Water", Standard D1589–60, Method A, p 373 (1975).
3. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 443, method 422 B (1975).

402

620

5010 INTRODUCTION

5010 A. General Discussion

Analyses for organic matter in water and wastewater can be classified into two general types of measurements: those that quantify an aggregate amount of organic matter comprising organic constituents with a common characteristic and those that quantify individual organic compounds. The latter can be found in Part 6000. The former, described here in Part 5000, have been grouped into four categories: oxygen-demanding substances, organically bound elements, classes of compounds, and formation potentials.

Methods for total organic carbon and chemical oxygen demand are used to assess the total amount of organics present. Gross fractions of the organic matter can be identified analytically, as in the measurement of BOD, which is an index of the biodegradable organics present, oil and grease, which represents material extractable from a sample by a nonpolar solvent, or total organic halide (TOX), which measures organically bound halogens. Trihalomethane formation potential is an aggregate measure of the total concentration of trihalomethanes formed upon chlorination of a water sample.

Analyses of organics are made to assess the concentration and general composition of organic matter in raw water supplies, wastewaters, treated effluents, and receiving waters, and to determine the efficiency of treatment processes.

5010 B. Sample Collection and Preservation

The sampling, field treatment, preservation, and storage of samples taken for organic matter analysis are covered in detail in the individual introductions to the methods. If possible, analyze samples immediately because preservatives often interfere with the tests. Otherwise, store at a low temperature (4°C) immediately after collection to preserve most samples. Use chemical preservatives only when they are shown not to interfere with the examinations to be made (see Section 1060). Never use preservatives for samples to be analyzed for BOD. When preservatives are used, add them to the sample bottle initially so that all portions are preserved as soon as collected. No single method of preservation is entirely satisfactory; choose the preservative with due regard to the determinations that are to be made. All methods of preservation may be inadequate when applied to samples containing significant amounts of suspended matter.

62

5020 QUALITY CONTROL

1000 contains important informa-
:evant to analyses included in Part
ive particular attention to Sections
(Quality Control), 1060 (Collection
eservation of Samples), 1080 (Re-
irade Water), and 1090 (Safety), all
h are critical for many of the Part
ethods.

special precautions when analyses
formed by independent laboratories.
e use of independent laboratories
s the same quality assurance pro-
observed for in-house analyses:
e samples, samples with known ad-
and blanks.

ration of samples with known additions may not be feasible for certain analyses. In such cases, consider using a mixture, in varying ratios, of several samples. Use the reported concentrations in the samples and the proportions in which they were mixed to calculate the expected concentration in the mixture. Examine laboratory performance using externally prepared standards and check samples (see Section 1020B).

Type I reagent water (Section 1080) should give satisfactory results for most of the analyses in Part 5000, but additional purification steps may be needed for certain methods, such as total organic halide (TOX) and trihalomethane formation potential (THMFP).

5210 BIOCHEMICAL OXYGEN DEMAND*

5210 A. Introduction

eral Discussion

iochemical oxygen demand (BOD)
ination is an empirical test in which
dized laboratory procedures are
determine the relative oxygen re-
ents of wastewaters, effluents, and
l waters. The test has its widest ap-
n in measuring waste loadings to
nt plants and in evaluating the
emoval efficiency of such treatment
s. The test measures the oxygen uti-
uring a specified incubation period
biochemical degradation of organic
l (carbonaceous demand) and the
used to oxidize inorganic material
sulfides and ferrous iron. It also
easure the oxygen used to oxidize
l forms of nitrogen (nitrogenous demand) unless their oxidation is prevented by an inhibitor. The seeding and dilution procedures provide an estimate of the BOD at pH 6.5 to 7.5.

Although only the 5-d BOD (BOD_5) is described here, many variations of oxygen demand measurements exist. These include using shorter and longer incubation periods, tests to determine rates of oxygen uptake, and continuous oxygen-uptake measurements by respirometric techniques. Alternative seeding, dilution, and incubation conditions can be chosen to mimic receiving-water conditions, thereby providing an estimate of the environmental effects of wastewaters and effluents.

2. Carbonaceous Versus Nitrogenous BOD

Oxidation of reduced forms of nitrogen, mediated by microorganisms, exerts nitrogenous demand. Nitrogenous demand historically has been considered an interference in the determination of BOD, as clearly evidenced by the inclusion of ammonia in the dilution water. The interference from nitrogenous demand can now be prevented by an inhibitory chemical.[1] If an inhibiting chemical is not used, the oxygen demand measured is the sum of carbonaceous and nitrogenous demands.

Measurements that include nitrogenous demand generally are not useful for assessing the oxygen demand associated with organic material. Nitrogenous demand can be estimated directly from ammonia nitrogen (Section 4500-NH_3); and carbonaceous demand can be estimated by subtracting the theoretical equivalent of the reduced nitrogen oxidation from uninhibited test results. However, this method is cumbersome and is subject to considerable error. Chemical inhibition of nitrogenous demand provides a more direct and more reliable measure of carbonaceous demand.

The extent of oxidation of nitrogenous compounds during the 5-d incubation period depends on the presence of microorganisms capable of carrying out this oxidation. Such organisms usually are not present in raw sewage or primary effluent in sufficient numbers to oxidize significant quantities of reduced nitrogen forms in the 5-d BOD test. Many biological treatment plant effluents contain significant numbers of nitrifying organisms. Because oxidation of nitrogenous compounds can occur in such samples, inhibition of nitrification as directed in ¶ B.4e6) is recommended for samples of secondary effluent, for samples seeded with secondary effluent, and for samples of polluted waters.

Report results as $CBOD_5$ when inhibiting the nitrogenous oxygen demand. When nitrification is not inhibited, report results as BOD_5.

3. Dilution Requirements

The BOD concentration in most wastewaters exceeds the concentration of dissolved oxygen (DO) available in an air-saturated sample. Therefore, it is necessary to dilute the sample before incubation to bring the oxygen demand and supply into appropriate balance. Because bacterial growth requires nutrients such as nitrogen, phosphorus, and trace metals, these are added to the dilution water, which is buffered to ensure that the pH of the incubated sample remains in a range suitable for bacterial growth. Complete stabilization of a sample may require a period of incubation too long for practical purposes; therefore, 5 d has been accepted as the standard incubation period.

If the dilution water is of poor quality, effectively, dilution water will appear as sample BOD. This effect will be amplified by the dilution factor. A positive bias will result. The method included below contains both a dilution-water check and a dilution-water blank. Seeded dilution waters are checked further for acceptable quality by measuring their consumption of oxygen from a known organic mixture, usually glucose and glutamic acid.

The source of dilution water is not restricted and may be distilled, tap, or receiving-stream water free of biodegradable organics and bioinhibitory substances such as chlorine or heavy metals. Distilled water may contain ammonia or volatile organics; deionized waters often are contaminated with soluble organics leached from the resin bed. Use of copper-lined stills or copper fittings attached to distilled water lines may produce water containing excessive amounts of copper (see Section 3500-Cu).

4. Reference

1. YOUNG, J.C. 1973. Chemical methods for nitrification control. *J. Water Pollut. Control Fed.* 45:637.

d by Standard Methods Committee, 1988.

·phy

E.J., P.D. McNamee & C.T. But-
. 1931. Selection of dilution water
oxygen demand tests. *Pub. Health*
084.

M.S. Nichols. 1937. Influence of
us and nitrogen on biochemical ox-
and. *Sewage Works J.* 9:34.

Ruchhoft, C.C. 1941. Report on the cooperative study of dilution waters made for the Standard Methods Committee of the Federation of Sewage Works Associations. *Sewage Works J.* 13:669.

Mohlman, F.W., E. Hurwitz, G.R. Barnett & H.K. Ramer. 1950. Experience with modified methods for BOD. *Sewage Ind. Wastes* 22:31.

5210 B. 5-Day BOD Test

Discussion

le: The method consists of fill-
ple, to overflowing, an airtight
specified size and incubating
ified temperature for 5 d. Dis-
en is measured initially and
ion, and the BOD is computed
rence between initial and final
the initial DO is determined
after the dilution is made, all
ke, including that occurring
rst 15 min, is included in the
rement.

g and storage: Samples for
is may degrade significantly
e between collection and anal-
g in low BOD values. Mini-
ion of BOD by analyzing
ptly or by cooling it to near-
perature during storage. How-
low temperature, keep holding
nimum. Warm chilled samples
re analysis.

amples—If analysis is begun
of collection, cold storage is
If analysis is not started
f sample collection, keep sam-
ow 4°C from the time of col-
in analysis within 6 h of
hen this is not possible because
site is distant from the lab-
e at or below 4°C and report
mperature of storage with the
case start analysis more than 24 h after grab sample collection. When samples are to be used for regulatory purposes make every effort to deliver samples for analysis within 6 h of collection.

2) Composite samples—Keep samples at or below 4°C during compositing. Limit compositing period to 24 h. Use the same criteria as for storage of grab samples, starting the measurement of holding time from end of compositing period. State storage time and conditions as part of the results.

2. Apparatus

a. Incubation bottles, 250- to 300-mL capacity. Clean bottles with a detergent, rinse thoroughly, and drain before use. As a precaution against drawing air into the dilution bottle during incubation, use a water-seal. Obtain satisfactory water seals by inverting bottles in a water bath or by adding water to the flared mouth of special BOD bottles. Place a paper or plastic cup or foil cap over flar d mouth of bottle to reduce evaporation of the water seal during incubation.

b. Air incubator or water bath, thermostatically controlled at 20 ± 1°C. Exclude all light to prevent possibility of photosynthetic production of DO.

3. Reagents

a. Phosphate buffer solution: Dissolve 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $Na_2HPO_4 \cdot 7H_2O$, and 1.7 g NH_4Cl in about 500 mL distilled water and dilute to 1 L. The pH should be 7.2 without further adjustment. Discard reagent (or any of the following reagents) if there is any sign of biological growth in the stock bottle.

b. Magnesium sulfate solution: Dissolve 22.5 g $MgSO_4 \cdot 7H_2O$ in distilled water and dilute to 1 L.

c. Calcium chloride solution: Dissolve 27.5 g $CaCl_2$ in distilled water and dilute to 1 L.

d. Ferric chloride solution: Dissolve 0.25 g $FeCl_3 \cdot 6H_2O$ in distilled water and dilute to 1 L.

e. Acid and alkali solutions, 1N, for neutralization of caustic or acidic waste samples.

1) Acid—Slowly and while stirring, add 28 mL conc sulfuric acid to distilled water. Dilute to 1 L.

2) Alkali—Dissolve 40 g sodium hydroxide in distilled water. Dilute to 1 L.

f. Sodium sulfite solution: Dissolve 1.575 g Na_2SO_3 in 1000 mL distilled water. This solution is not stable; prepare daily.

g. Nitrification inhibitor, 2-chloro-6-(trichloro methyl) pyridine.*

h. Glucose-glutamic acid solution: Dry reagent-grade glucose and reagent-grade glutamic acid at 103°C for 1 h. Add 150 mg glucose and 150 mg glutamic acid to distilled water and dilute to 1 L. Prepare fresh immediately before use.

i. Ammonium chloride solution: Dissolve 1.15 g NH_4Cl in about 500 mL distilled water, adjust pH to 7.2 with NaOH solution, and dilute to 1 L. Solution contains 0.3 mg N/mL.

4. Procedure

a. Preparation of dilution water: Place desired volume of water in a suitable bottle and add 1 mL each of phosphate buffer, $MgSO_4$, $CaCl_2$, and $FeCl_3$ solutions/L of water. Seed dilution water, if desired, as described in ¶ 4*d*. Test and store dilution water as described in ¶s 4*b* and *c* so that water of assured quality always is on hand.

Before use bring dilution water temperature to 20°C. Saturate with DO by shaking in a partially filled bottle or by aerating with organic-free filtered air. Alternatively, store in cotton-plugged bottles long enough for water to become saturated with DO. Protect water quality by using clean glassware, tubing, and bottles.

b. Dilution water check: Use this procedure as a rough check on quality of dilution water.

If the oxygen depletion of a candidate water exceeds 0.2 mg/L obtain a satisfactory water by improving purification or from another source. Alternatively, if nitrification inhibition is used, store the dilution water, seeded as prescribed below, in a darkened room at room temperature until the oxygen uptake is sufficiently reduced to meet the dilution-water check criteria. Check quality of stored dilution water on use, but do not add seed to dilution water stored for quality improvement. Storage is not recommended when BODs are to be determined without nitrification inhibition because nitrifying organisms may develop during storage. Check stored dilution water to determine whether sufficient ammonia remains after storage. If not, add ammonium chloride solution to provide a total of 0.45 mg ammonia/L as nitrogen. If dilution water has not been stored for quality improvement, add sufficient seeding material to produce a DO uptake of 0.05 to 0.1 mg/L in 5 d at 20°C. Incubate a BOD bottle full of dilution water for 5 d at 20°C. Determine initial and final DO as in ¶s 4*g* and *j*. The DO uptake in 5 d at 20°C should not be more than 0.2 mg/L and preferably not more than 0.1 mg/L.

c. Glucose-glutamic acid check: Because the BOD test is a bioassay its results can be influenced greatly by the presence of toxicants or by use of a poor seeding ma-

*Nitrification Inhibitor 2579-24 (2.2% TCMP), Hach Co., or equivalent.

illed waters frequently are con- with copper; some sewage seeds ely inactive. Low results always d with such seeds and waters. y check dilution water quality, veness, and analytical technique : BOD measurements on pure ompounds and samples with litions. In general, for BOD de- ns not requiring an adapted seed, ire of 150 mg glucose/L and 150 ic acid/L as a "standard" check lucose has an exceptionally high le oxidation rate but when it is lutamic acid, the oxidation rate d and is similar to that obtained y municipal wastes. Alterna- particular wastewater contains ble major constituent that con- the BOD, use this compound in e glucose-glutamic acid.

ne the 5-d 20°C BOD of a 2% the glucose-glutamic acid stan- solution using the techniques ¶s 4*d-j*. Evaluate data as de- ¶ 6, Precision and Bias.

ng:

source—It is necessary to have opulation of microorganisms ca- xidizing the biodegradable or- ter in the sample. Domestic , unchlorinated or otherwise- d effluents from biological ment plants, and surface waters wastewater discharges contain microbial populations. Some not contain a sufficient micro- ation (for example, some un- dustrial wastes, disinfected h-temperature wastes, or wastes ne pH values). For such wastes lution water by adding a pop- microorganisms. The preferred nt from a biological treatment essing the waste. Where this is le, use supernatant from do- tewater after settling at room for at least 1 h but no longer than 36 h. When effluent from a biological treatment process is used, inhibition of nitrification is recommended.

Some samples may contain materials not degraded at normal rates by the microorganisms in settled domestic wastewater. Seed such samples with an adapted microbial population obtained from the undisinfected effluent of a biological process treating the waste. In the absence of such a facility, obtain seed from the receiving water below (preferably 3 to 8 km) the point of discharge. When such seed sources also are not available, develop an adapted seed in the laboratory by continuously aerating a sample of settled domestic wastewater and adding small daily increments of waste. Optionally use a soil suspension or activated sludge, or a commercial seed preparation to obtain the initial microbial population. Determine the existence of a satisfactory population by testing the performance of the seed in BOD tests on the sample. BOD values that increase with time of adaptation to a steady high value indicate successful seed adaptation.

2) Seed control—Determine BOD of the seeding material as for any other sample. This is the *seed control*. From the value of the seed control and a knowledge of the seeding material dilution (in the dilution water) determine seed DO uptake. Ideally, make dilutions of seed such that the largest quantity results in at least 50% DO depletion. A plot of DO depletion, in milligrams per liter, versus milliters seed should present a straight line for which the slope indicates DO depletion per milliliter of seed. The DO-axis intercept is oxygen depletion caused by the dilution water and should be less than 0.1 mg/L (¶ 4*h*). To determine a sample DO uptake subtract seed DO uptake from total DO uptake. The DO uptake of seeded dilution water should be between 0.6 and 1.0 mg/L.

Techniques for adding seeding material to dilution water are described for two sample dilution methods (¶ 4*f*).

e. Sample pretreatment:

1) Samples containing caustic alkalinity or acidity— Neutralize samples to pH 6.5 to 7.5 with a solution of sulfuric acid (H_2SO_4) or sodium hydroxide (NaOH) of such strength that the quantity of reagent does not dilute the sample by more than 0.5%. The pH of seeded dilution water should not be affected by the lowest sample dilution.

2) Samples containing residual chlorine compounds—If possible, avoid samples containing residual chlorine by sampling ahead of chlorination processes. If the sample has been chlorinated but no detectable chlorine residual is present, seed the dilution water. If residual chlorine is present, dechlorinate sample and seed the dilution water (¶ 4*f*). Do not test chlorinated/dechlorinated samples without seeding the dilution water. In some samples chlorine will dissipate within 1 to 2 h of standing in the light. This often occurs during sample transport and handling. For samples in which chlorine residual does not dissipate in a reasonably short time, destroy chlorine residual by adding Na_2SO_3 solution. Determine required volume of Na_2SO_3 solution on a 100- to 1000-mL portion of neutralized sample by adding 10 mL of 1 + 1 acetic acid or 1 + 50 H_2SO_4, 10 mL potassium iodide (KI) solution (10 g/100 mL), and titrating with Na_2SO_3 solution to the starch-iodine end point for residual. Add to neutralized sample the relative volume of Na_2SO_3 solution determined by the above test, mix, and after 10 to 20 min check sample for residual chlorine. (NOTE: Excess Na_2SO_3 exerts an oxygen demand and reacts slowly with certain organic chloramine compounds that may be present in chlorinated samples).

3) Samples containing other toxic substances—Certain industrial wastes, for example, plating wastes, contain toxic metals. Such samples often require special study and treatment.

4) Samples supersaturated with DO—Samples containing more than 9 mg DO/L at 20°C may be encountered in cold waters or in water where photosynthesis occurs. To prevent loss of oxygen during incubation of such samples, reduce DO to saturation at 20°C by bringing sample to about 20°C in partially filled bottle while agitating by vigorous shaking or by aerating with clean, filtered compressed air.

5) Sample temperature adjustment—Bring samples to 20 ± 1°C before making dilutions.

6) Nitrification inhibition—If nitrification inhibition is desired add 3 mg 2-chloro-6-(trichloro methyl) pyridine (TCMP) to each 300-mL bottle before capping or add sufficient amounts to the dilution water to make a final concentration of 10 mg/L. (NOTE: Pure TCMP may dissolve slowly and can float on top of the sample. Some commercial formulations dissolve more readily but are not 100% TCMP; adjust dosage accordingly.) Samples that may require nitrification inhibition include, but are not limited to, biologically treated effluents, samples seeded with biologically treated effluents, and river waters. Note the use of nitrogen inhibition in reporting results.

f. Dilution technique: Dilutions that result in a residual DO of at least 1 mg/L and a DO uptake of at least 2 mg/L after 5 d incubation produce the most reliable results. Make several dilutions of prepared sample to obtain DO uptake in this range. Experience with a particular sample will permit use of a smaller number of dilutions. A more rapid analysis, such as COD, may be correlated approximately with BOD and serve as a guide in selecting dilutions. In the absence of prior knowledge, use the following dilutions: 0.0 to 1.0% for strong industrial wastes, 1 to 5% for raw and settled wastewater, 5 to 25% for biologically treated effluent, and 25 to 100% for polluted river waters.

Prepare dilutions either in graduated cylinders and then transfer to BOD bottles or

· directly in BOD bottles. Either di-
method can be combined with any
asurement technique. The number
es to be prepared for each dilution
on the DO technique and the num-
eplicates desired.

using graduated cylinders to pre-
lutions, and when seeding is nec-
dd seed either directly to dilution
to individual cylinders before di-
Seeding of individual cylinders
declining ratio of seed to sample
sing dilutions are made. When di-
re prepared directly in BOD bot-
when seeding is necessary, add seed
to dilution water or directly to the
ttles.

lutions prepared in graduated cyl-
If the azide modification of the ti-
iodometric method (Section 4500-
used, carefully siphon dilution
eded if necessary, into a 1- to 2-
ty graduated cylinder. Fill cylin-
full without entraining air. Add
uantity of carefully mixed sample
to appropriate level with dilution
x well with a plunger-type mixing
entraining air. Siphon mixed di-
to two BOD bottles. Determine
on one of these bottles. Stopper
d bottle tightly, water-seal, and
or 5 d at 20°C. If the membrane
method is used for DO measure-
hon dilution mixture into one
le. Determine initial DO on this
replace any displaced contents
le dilution to fill the bottle. Stop-
water-seal, and incubate for 5

tions prepared directly in BOD
sing a wide-tip volumetric pipet,
sired sample volume to individ-
ottles of known capacity. Add
amounts of seed material to the
BOD bottles or to the dilution
bottles with enough dilution
ed if necessary, so that insertion
will displace all air, leaving no bubbles. For dilutions greater than 1:100 make a primary dilution in a graduated cylinder before making final dilution in the bottle. When using titrimetric iodometric methods for DO measurement, prepare two bottles at each dilution. Determine initial DO on one bottle. Stopper second bottle tightly, water-seal, and incubate for 5 d at 20°C. If the membrane electrode method is used for DO measurement, prepare only one BOD bottle for each dilution. Determine initial DO on this bottle and replace any displaced contents with dilution water to fill the bottle. Stopper tightly, water-seal, and incubate for 5 d at 20°C. Rinse DO electrode between determinations to prevent cross-contamination of samples.

g. Determination of initial DO: If the sample contains materials that react rapidly with DO, determine initial DO immediately after filling BOD bottle with diluted sample. If rapid initial DO uptake is insignificant, the time period between preparing dilution and measuring initial DO is not critical.

Use the azide modification of the iodometric method (Section 4500-O.C) or the membrane electrode method (Section 4500-O.G) to determine initial DO on all sample dilutions, dilution water blanks, and where appropriate, seed controls.

h. Dilution water blank: Use a dilution water blank as a rough check on quality of unseeded dilution water and cleanliness of incubation bottles. Together with each batch of samples incubate a bottle of unseeded dilution water. Determine initial and final DO as in ¶s 4*g* and *j*. The DO uptake should not be more than 0.2 mg/L and preferably not more than 0.1 mg/L.

i. Incubation: Incubate at 20°C ± 1°C BOD bottles containing desired dilutions, seed controls, dilution water blanks, and glucose-glutamic acid checks. Water-seal bottles as described in ¶ 4*f*.

j. Determination of final DO: After 5 d

incubation determine DO in sample dilutions, blanks, and checks as in ¶ 4*g*.

5. Calculation

When dilution water is not seeded:

$$\text{BOD}_5,\ \text{mg/L} = \frac{D_1 - D_2}{P}$$

When dilution water is seeded:

$$\text{BOD}_5,\ \text{mg/L} = \frac{(D_1 - D_2) - (B_1 - B_2)f}{P}$$

where:

D_1 = DO of diluted sample immediately after preparation, mg/L,
D_2 = DO of diluted sample after 5 d incubation at 20°C, mg/L,
P = decimal volumetric fraction of sample used,
B_1 = DO of seed control before incubation, mg/L (¶ 4*d*),
B_2 = DO of seed control after incubation mg/L (¶ 4*d*), and
f = ratio of seed in diluted sample to seed in seed control = (% seed in diluted sample)/(% seed in seed control).

If seed material is added directly to sample or to seed control bottles:

f = (volume of seed in diluted sample)/(volume of seed in seed control)

Report results as CBOD$_5$ if nitrification is inhibited.

If more than one sample dilution meets the criteria of a residual DO of at least 1 mg/L and a DO depletion of at least 2 mg/L and there is no evidence of toxicity at higher sample concentrations or the existence of an obvious anomaly, average results in the acceptable range.

In these calculations, do not make corrections for DO uptake by the dilution water blank during incubation. This correction is unnecessary if dilution water meets the blank criteria stipulated above. If the dilution water does not meet these criteria, proper corrections are difficult and results become questionable.

6. Precision and Bias

There is no measurement for establishing bias of the BOD procedure. The glucose-glutamic acid check prescribed in ¶ 4*c* is intended to be a reference point for evaluation of dilution water quality, seed effectiveness, and analytical technique. Single-laboratory tests using a 300-mg/L mixed glucose-glutamic acid solution provided the following results:[1]

Number of months: 14
Number of triplicates: 421
Average monthly recovery: 204 mg/L
Average monthly standard deviation: 10.4 mg/L

In a series of interlaboratory studies,[3] each involving 2 to 112 laboratories (and as many analysts and seed sources), 5-d BOD measurements were made on synthetic water samples containing a 1:1 mixture of glucose and glutamic acid in the total concentration range of 3.3 to 231 mg/L. The regression equations for mean value, $\overline{X}$, and standard deviation, S, from these studies were:

$\overline{X}$ = 0.658 (added level, mg/L) + 0.280 mg/L
S = 0.100 (added level, mg/L) + 0.547 mg/L

For the 300-mg/L mixed primary standard, the average 5-d BOD would be 198 mg/L with a standard deviation of 30.5 mg/L.

a. Control limits: Because of many factors affecting BOD tests in multilaboratory studies and the resulting extreme variability in test results, one standard deviation, as determined by interlaboratory tests, is recommended as a control limit for individual laboratories. Alternatively, for each laboratory, establish its control limits by performing a minimum of 25 glucose-glutamic acid checks (¶ 4*c*) over a period of several weeks or months and calculating

and standard deviation. Use the standard deviations as the con- for future glucose-glutamic acid ompare calculated control limits ngle-laboratory tests presented to interlaboratory results. If con- are outside the range of 198 ± valuate the control limits and in- source of the problem. If mea- D for a glucose-glutamic acid utside the accepted control limit ect tests made with that seed and vater.

king range and detection limit: ing range is equal to the difference he maximum initial DO (7 to 9 minimum DO residual of 1 mg/ ied by the dilution factor. A lower limit of 2 mg/L is established by rement for a minimum DO de- f 2 mg/L.

ences

., W. & J. RESNICK. 1987. Personal ication with J. C. Young. Water Pol- ontrol Plant, Davenport, Iowa.

2. U.S. ENVIRONMENTAL PROTECTION AGENCY, OFFICE OF RESEARCH AND DEVELOPMENT. 1986. Method-by-Method Statistics from Water Pollution (WP) Laboratory Performance Evaluation Studies. Quality Assurance Branch, Environmental Monitoring and Support Lab., Cincinnati, Ohio.

8. Bibliography

SAWYER, C.N. & L. BRADNEY. 1946. Modernization of the BOD test for determining the efficiency of the sewage treatment process. *Sewage Works J.* 18:1113.

RUCHHOFT, C.C., O.R. PLACAK, J. KACHMAR & C.E. CALBERT. 1948. Variations in BOD velocity constant of sewage dilutions. *Ind. Eng. Chem.* 40:1290.

ABBOTT, W.E. 1948. The bacteriostatic effects of methylene blue on the BOD test. *Water Sewage Works* 95:424.

SAWYER, C.N., P. CALLEJAS, M. MOORE & A.Q.Y. TOM. 1950. Primary standards for BOD work. *Sewage Ind. Wastes* 22:26.

YOUNG, J.C., G.N. MCDERMOTT & D. JENKINS. 1981. Alterations in the BOD procedure for the 15th edition of Standard Methods for the Examination of Water and Wastewater. *J. Water Pollut. Control Fed.* 53:1253.

5220 CHEMICAL OXYGEN DEMAND (COD)*

5220 A. Introduction

emical oxygen demand (COD) is measure of the oxygen equivalent ganic matter content of a sample sceptible to oxidation by a strong oxidant. For samples from a spe- ce, COD can be related empiri- BOD, organic carbon, or organic The test is useful for monitoring and control after correlation has been established. The dichromate reflux method is preferred over procedures using other oxidants because of superior oxidizing ability, applicability to a wide variety of samples, and ease of manipulation. Oxidation of most organic compounds is 95 to 100% of the theoretical value. Pyridine and related compounds resist oxidation and vol-

by Standard Methods Committee, 1985.

atile organic compounds are oxidized only to the extent that they remain in contact with the oxidant. Ammonia, present either in the waste or liberated from nitrogen-containing organic matter, is not oxidized in the absence of significant concentration of free chloride ions.

1. Selection of Method

The open reflux method (B) is suitable for a wide range of wastes where a large sample size is preferred. The closed reflux methods (C and D) are more economical in the use of metallic salt reagents, but require homogenization of samples containing suspended solids to obtain reproducible results. Ampules and culture tubes with premeasured reagents are available commercially. Follow instructions furnished by the manufacturer.

Determine COD values of > 50 mg O_2/L by using procedures 5220B.4*a*, C.4, or D.4. Use procedure 5220B.4*b* to determine, with lesser accuracy, COD values from 5 to 50 mg O_2/L.

2. Interferences and Limitations

Volatile straight-chain aliphatic compounds are not oxidized to any appreciable extent. This failure occurs partly because volatile organics are present in the vapor space and do not come in contact with the oxidizing liquid. Straight-chain aliphatic compounds are oxidized more effectively when silver sulfate (Ag_2SO_4) is added as a catalyst. However, Ag_2SO_4 reacts with chloride, bromide, and iodide to produce precipitates that are oxidized only partially. The difficulties caused by the presence of the halides can be overcome largely, though not completely, by complexing with mercuric sulfate ($HgSO_4$) before the refluxing procedure. Although 1 g $HgSO_4$ is specified for 50 mL sample, a lesser amount may be used where sample chloride concentration is known to be less than 2000 mg/L, as long as a 10:1 ratio of $HgSO_4$:Cl^- is maintained. Do not use the test for samples containing more than 2000 mg Cl^-/L. Techniques designed to measure COD in saline waters are available.[1,2]

Nitrite (NO_2^-) exerts a COD of 1.1 mg O_2/mg NO_2^--N. Because concentrations of NO_2^- in waters rarely exceed 1 or 2 mg NO_2^--N/L, the interference is considered insignificant and usually is ignored. To eliminate a significant interference due to NO_2^-, add 10 mg sulfamic acid for each mg NO_2^--N present in the sample volume used; add the same amount of sulfamic acid to the reflux vessel containing the distilled water blank.

Reduced inorganic species such as ferrous iron, sulfide, manganous manganese, etc., are oxidized quantitatively under the test conditions. For samples containing significant levels of these species, stoichiometric oxidation can be assumed from known initial concentration of the interfering species and corrections can be made to the COD value obtained.

3. Sampling and Storage

Preferably collect samples in glass bottles. Test unstable samples without delay. If delay before analysis is unavoidable, preserve sample by acidification to pH ≤2 using conc H_2SO_4. Blend samples containing settleable solids with a homogenizer to permit representative sampling. Make preliminary dilutions for wastes containing a high COD to reduce the error inherent in measuring small sample volumes.

4. References

1. BURNS, E.R. & C. MARSHALL. 1965. Correction for chloride interference in the chemical oxygen demand test. *J. Water Pollut. Control Fed.* 37:1716.
2. BAUMANN, F.I. 1974. Dichromate reflux chemical oxygen demand: A proposed method for chloride correction in highly saline waters. *Anal. Chem.* 46:1336.

5220 B. Open Reflux Method

General Discussion

Principle: Most types of organic mat- are oxidized by a boiling mixture of omic and sulfuric acids. A sample is xed in strongly acid solution with a wn excess of potassium dichromate Cr_2O_7). After digestion, the remaining educed $K_2Cr_2O_7$ is titrated with ferrous monium sulfate to determine the unt of $K_2Cr_2O_7$ consumed and the oxable organic matter is calculated in ns of oxygen equivalent. Keep ratios of ent weights, volumes, and strengths stant when sample volumes other than mL are used. The standard 2-h reflux may be reduced if it has been shown a shorter period yields the same re-

Apparatus

Reflux apparatus, consisting of 500- or -mL erlenmeyer flasks with ground- s 24/40 neck* and 300-mm jacket Lie- West, or equivalent condenser† with 40 ground-glass joint, and a hot plate ng sufficient power to produce at least W/cm² of heating surface, or equiva-

Reagents

Standard potassium dichromate solu- 0.0417M: Dissolve 12.259 g $K_2Cr_2O_7$, ary standard grade, previously dried 03°C for 2 h, in distilled water and te to 1000 mL.

Sulfuric acid reagent: Add Ag_2SO_4, ent or technical grade, crystals or pow- to conc H_2SO_4 at the rate of 5.5 g SO_4/kg H_2SO_4. Let stand 1 to 2 d to lve Ag_2SO_4.

Ferroin indicator solution: Dissolve 5 g 1,10-phenanthroline monohydrate 695 mg $FeSO_4 \cdot 7H_2O$ in distilled water dilute to 100 mL. This indicator solution may be purchased already prepared.‡

d. Standard ferrous ammonium sulfate (FAS) titrant, approximately 0.25M: Dissolve 98 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in distilled water. Add 20 mL conc H_2SO_4, cool, and dilute to 1000 mL. Standardize this solution daily against standard $K_2Cr_2O_7$ solution as follows:

Dilute 10.0 mL standard $K_2Cr_2O_7$ to about 100 mL. Add 30 mL conc H_2SO_4 and cool. Titrate with FAS titrant using 0.10 to 0.15 mL (2 to 3 drops) ferroin indicator.

Molarity of FAS solution

$$= \frac{\text{Volume } 0.0417M\ K_2Cr_2O_7 \text{ solution titrated, mL}}{\text{Volume FAS used in titration, mL}} \times 0.25$$

e. Mercuric sulfate, $HgSO_4$, crystals or powder.

f. Sulfamic acid: Required only if the interference of nitrites is to be eliminated (see 5220A.2 above).

g. Potassium hydrogen phthalate (KHP) standard: Lightly crush and then dry potassium hydrogen phthalate ($HOOCC_6H_4COOK$) to constant weight at 120°C. Dissolve 425 mg in distilled water and dilute to 1000 mL. KHP has a theoretical COD[1] of 1.176 mg O_2/mg and this solution has a theoretical COD of 500 µg O_2/mL. This solution is stable when refrigerated for up to 3 months in the absence of visible biological growth.

4. Procedure

a. Treatment of samples with COD of > 50 mg O_2/L: Place 50.0 mL sample (for samples with COD of > 900 mg O_2/L, use smaller sample portion diluted to 50.0 mL) in a 500-mL refluxing flask. Add 1 g $HgSO_4$, several glass beads, and very slowly add 5.0 mL sulfuric acid reagent, with mixing to dissolve $HgSO_4$. Cool while mixing to avoid possible loss of volatile materials. Add 25.0 mL 0.0417M $K_2Cr_2O_7$ solution and mix. Attach flask to condenser and turn on cooling water. Add remaining sulfuric acid reagent (70 mL) through open end of condenser. Continue swirling and mixing while adding the sulfuric acid reagent. CAUTION: *Mix reflux mixture thoroughly before applying heat to prevent local heating of flask bottom and a possible blowout of flask contents.*

Cover open end of condenser with a small beaker to prevent foreign material from entering refluxing mixture and reflux for 2 h. Cool and wash down condenser with distilled water. Disconnect reflux condenser and dilute mixture to about twice its volume with distilled water. Cool to room temperature and titrate excess $K_2Cr_2O_7$ with FAS, using 0.10 to 0.15 mL (2 to 3 drops) ferroin indicator. Although the quantity of ferroin indicator is not critical, use the same volume for all titrations. Take as the end point of the titration the first sharp color change from blue-green to reddish brown. The blue-green may reappear. In the same manner, reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of sample.

b. Alternate procedure for low-COD samples: Follow procedure of ¶ 4a, with two exceptions: (i) use standard 0.00417M $K_2Cr_2O_7$, and (ii) titrate with 0.025M FAS. Exercise extreme care with this procedure because even a trace of organic matter on the glassware or from the atmosphere may cause gross errors. If a further increase in sensitivity is required, concentrate a larger volume of sample before digesting under reflux as follows: Add all reagents to a sample larger than 50 mL and reduce total volume to 150 mL by boiling in the refluxing flask open to the atmosphere without the condenser attached. Compute amount of $HgSO_4$ to be added (before concentration) on the basis of a weight ratio of 10:1, $HgSO_4$:Cl^-, using the amount Cl^- present in the original volume of sa ple. Carry a blank reagent through t same procedure. This technique has the a vantage of concentrating the sample wit out significant losses of easily digest volatile materials. Hard-to-digest volati materials such as volatile acids are lost, b an improvement is gained over ordina evaporative concentration methods.

c. Determination of standard solutio Evaluate the technique and quality of r agents by conducting the test on a standa potassium hydrogen phthalate solution.

5. Calculation

$$\text{COD as mg } O_2/\text{L} = \frac{(A - B) \times M \times 8000}{\text{mL sample}}$$

where:

A = mL FAS used for blank,
B = mL FAS used for sample, and
M = molarity of FAS.

6. Precision and Bias

A set of synthetic samples containin potassium hydrogen phthalate and NaC was tested by 74 laboratories. At a COI of 200 mg O_2/L in the absence of chlorid the standard deviation was ±13 mg/ (coefficient of variation, 6.5%). At COI of 160 mg O_2/L and 100 mg Cl^-/L, th standard deviation was ±14 mg/L (coef ficient of variation, 10.8%).

7. Reference

1. PITWELL, L.R. 1983. Standard COD. *Chem. Brit.* 19:907.

8. Bibliography

MOORE, W.A., R.C. KRONER & C.C. RUCHHOFT 1949. Dichromate reflux method for deter mination of oxygen consumed. *Anal. Chem.* 21:953.

MEDALIA, A.I. 1951. Test for traces of organi matter in water. *Anal. Chem.* 23:1318.

MOORE, W.A., F.J. LUDZACK & C.C. RUCHHOFT

ng 5000 or equivalent.
ng 2360, 91548, or equivalent.

‡GFS Chemical Co., Columbus, Ohio.

Determination of oxygen-consumed of organic wastes. *Anal. Chem.* 7.

DOBBS, R.A. & R.T. WILLIAMS. 1963. Elimination of chloride interference in the chemical oxygen demand test. *Anal. Chem.* 35:1064.

5220 C. Closed Reflux, Titrimetric Method

al Discussion

iple: See 5220B.1*a*.

rferences and limitations: See Volatile organic compounds are pletely oxidized in the closed sys- se of longer contact with the ox- ore each use inspect culture-tube breaks in the TFE liner. Select be size for the degree of sensitiv- l. Use the 25- × 150-mm tube s with low COD content because olume sample can be treated.

ilus

ction vessels: Preferably use bo- culture tubes, 16- × 100-mm, 20- n, or 25- × 150-mm, with TFE- w caps. Alternatively, use boro- ipules, 10-mL capacity, 19- to 20-

ng block, cast aluminum, 45 to ep, with holes sized for close fit tubes or ampules.

heater or oven, to operate at 150 OTE: Severe damage of most cul- closures from oven digestion in- potential source of contamina- increases the probability of leak- n oven for culture-tube digestion it has been determined that 2 h at 150°C will not damage the

le sealer: Use only a mechanical nsure strong, consistent seals.

ts

ard potassium dichromate diges- n, 0.0167M: Add to about 500 ed water 4.913 g $K_2Cr_2O_7$, primary standard grade, previously dried at 103°C for 2 h, 167 mL conc H_2SO_4, and 33.3 g $HgSO_4$. Dissolve, cool to room temperature, and dilute to 1000 mL.

b. Sulfuric acid reagent: See Section 5220B.3*b*.

c. Ferroin indicator solution: See Section 5220B.3*c*.

d. Standard ferrous ammonium sulfate titrant (FAS), approximately 0.10*M:* Dissolve 39.2 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in distilled water. Add 20 mL conc H_2SO_4, cool, and dilute to 1000 mL. Standardize solution daily against standard $K_2Cr_2O_7$ digestion solution as follows:

Add reagents according to Table 5220:I to a culture tube containing the correct volume of distilled water substituted for sample. Cool tube to room temperature and add 0.05 to 0.10 mL (1 to 2 drops) ferroin indicator and titrate with FAS titrant.

Molarity of FAS solution

$$= \frac{\text{Volume } 0.0167M\ K_2Cr_2O_7 \text{ solution titrated, mL}}{\text{Volume FAS used in titration, mL}} \times 0.10$$

e. Sulfuric acid: See Section 5220B.3*f*.

f. Potassium hydrogen phthalate standard: See Section 5220B.3*g*.

4. Procedure

Wash culture tubes and caps with 20% H_2SO_4 before first use to prevent contamination. Refer to Table 5220:I for proper sample and reagent volumes. Place sample in culture tube or ampule and add digestion solution. Carefully run sulfuric acid reagent down inside of vessel so an acid layer

TABLE 5220:I. SAMPLE AND REAGENT QUANTITIES FOR VARIOUS DIGESTION VESSELS

Digestion Vessel	Sample *mL*	Digestion Solution *mL*	Sulfuric Acid Reagent *mL*	Total Final Volume *mL*
Culture tubes:				
16 × 100 mm	2.5	1.5	3.5	7.5
20 × 150 mm	5.0	3.0	7.0	15.0
25 × 150 mm	10.0	6.0	14.0	30.0
Standard 10-mL ampules	2.5	1.5	3.5	7.5

is formed under the sample-digestion solution layer. Tightly cap tubes or seal ampules, and invert each several times to mix completely. CAUTION: *Wear face shield and protect hands from heat produced when contents of vessels are mixed. Mix thoroughly before applying heat to prevent local heating of vessel bottom and possible explosive reaction.*

Place tubes or ampules in block digester or oven preheated to 150°C and reflux for 2 h. Cool to room temperature and place vessels in test tube rack. Remove culture tube caps and add small TFE-covered magnetic stirring bar. If ampules are used, transfer contents to a larger container for titrating. Add 0.05 to 0.10 mL (1 to 2 drops) ferroin indicator and stir rapidly on magnetic stirrer while titrating with 0.10*M* FAS. The end point is a sharp color change from blue-green to reddish brown, although the blue-green may reappear within minutes. In the same manner reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of the sample.

5. Calculation

$$\text{COD as mg } O_2/L = \frac{(A - B) \times M \times 8000}{\text{mL sample}}$$

where:

A = mL FAS used for blank,
B = mL FAS used for sample, and
M = molarity of FAS.

6. Precision and Bias

Sixty synthetic samples containing potassium hydrogen phthalate and NaCl were tested by six laboratories. At an average COD of 195 mg O_2/L in the absence of chloride, the standard deviation was ±11 mg O_2/L (coefficient of variation, 5.6%). At an average COD of 208 mg O_2/L and 100 mg Cl^-/L, the standard deviation was ±10 mg O_2/L (coefficient of variation, 4.8%).

5220 D. Closed Reflux, Colorimetric Method

1. General Discussion

a. Principle: See Section 5220B.1*a*. Colorimetric reaction vessels are sealed glass ampules or capped culture tubes. Oxygen consumed is measured against standards at 600 nm with a spectrophotometer.

b. Interferences and limitations: See Section 5220C.1*b*.

ıtus

Section 5220C.2.

:rophotometer, for use at 600 nm
ss opening adapter for ampule or
or 25-mm tubes.

:nts

stion solution: Add to about 500
led water 10.216 g $K_2Cr_2O_7$, pri-
ndard grade, previously dried at
r 2 h, 167 mL conc H_2SO_4, and
SO_4. Dissolve, cool to room tem-
and dilute to 1000 mL.

uric acid reagent: See 5220B.3*b*.

amic acid: See Section 5220B.3*f*.

ssium hydrogen phthalate stand-
Section 5220B.3*g*.

dure

atment of samples: Measure suit-
ıme of sample and reagents into
ampule as indicated in Table
Prepare, digest, and cool samples,
nd one or more standards as di-
Section 5220C.4.

surement of dichromate reduction:
ooled samples, blank, and stand-
ral times and allow solids to settle
easuring absorbance. Dislodge sol-
adhere to container wall by gentle
and settling. Insert unopened tube
ıle through access door into light
spectrophotometer set at 600 nm.
sorbance and compare to calibra-
ve. Use optically matched culture tubes or ampules for greater sensitivity; discard scratched or blemished glassware.

c. Preparation of calibration curve: Prepare at least five standards from potassium hydrogen phthalate solution with COD equivalents from 20 to 900 μg O_2/L. Make up to volume with distilled water; use same reagent volumes, tube, or ampule size, and digestion procedure as for samples. Prepare calibration curve for each new lot of tubes or ampules or when standards prepared in ¶ 4*a* differ by ≥5% from calibration curve.

5. Calculation

$$\text{COD as mg } O_2/\text{L} = \frac{\text{mg } O_2 \text{ in final volume} \times 1000}{\text{mL sample}}$$

6. Precision and Bias

Forty-eight synthetic samples containing potassium hydrogen phthalate and NaCl were tested by five laboratories. At an average COD of 193 mg O_2/L in the absence of chloride, the standard deviation was ±17 mg O_2/L (coefficient of variation 8.7%). At an average COD of 212 mg O_2/L and 100 mg Cl^-/L, the standard deviation was ±20 mg O_2/L (coefficient of variation, 9.6%).

7. Bibliography

JIRKA, A.M. & M.J. CARTER. 1975. Micro semi-automated analysis of surface and wastewaters for chemical oxygen demand. *Anal. Chem.* 47:1397.

HIMEBAUGH, R.R. & M.J. SMITH. 1979. Semimicro tube method for chemical oxygen demand. *Anal. Chem.* 51:1085.

629

5310 TOTAL ORGANIC CARBON (TOC)*

5310 A. Introduction

1. General Discussion

The organic carbon in water and wastewater is composed of a variety of organic compounds in various oxidation states. Some of these carbon compounds can be oxidized further by biological or chemical processes, and the biochemical oxygen demand (BOD) and chemical oxygen demand (COD) may be used to characterize these fractions. The presence of organic carbon that does not respond to either the BOD or COD test makes them unsuitable for the measurement of total organic carbon. Total organic carbon (TOC) is a more convenient and direct expression of total organic content than either BOD or COD, but does not provide the same kind of information. If a repeatable empirical relationship is established between TOC and BOD or COD, then TOC can be used to estimate the accompanying BOD or COD. This relationship must be established independently for each set of matrix conditions, such as various points in a treatment process. Unlike BOD or COD, TOC is independent of the oxidation state of the organic matter and does not measure other organically bound elements, such as nitrogen and hydrogen, and inorganics that can contribute to the oxygen demand measured by BOD and COD. TOC measurement does not replace BOD and COD testing.

To determine the quantity of organically bound carbon, the organic molecules must be broken down to single carbon units and converted to a single molecular form that can be measured quantitatively. TOC methods utilize heat and oxygen, ultraviolet irradiation, chemical oxidants, or combinations of these oxidants to convert organic carbon to carbon dioxide (CO_2). The CO_2 may be measured directly by a nondispersive infrared analyzer, it may be reduced to methane and measured with a flame ionization detector, or CO_2 may be titrated chemically.

2. Fractions of Total Carbon

The methods and instruments used in measuring TOC analyze fractions of total carbon (TC) and measure TOC by two or more determinations. These fractions of total carbon are defined as: inorganic carbon (IC)—the carbonate, bicarbonate, and dissolved CO_2; total organic carbon (TOC)—all carbon atoms covalently bonded in organic molecules; dissolved organic carbon (DOC)—the fraction of TOC that passes through a 0.45-μm-pore-diam filter; nondissolved organic carbon (NDOC)—also referred to as particulate organic carbon, the fraction of TOC retained by a 0.45-μm filter; purgeable organic carbon (POC)—also referred to as volatile organic carbon, the fraction of TOC removed from an aqueous solution by gas stripping under specified conditions; and nonpurgeable organic carbon (NPOC)—the fraction of TOC not removed by gas stripping.

In most water samples, the IC fraction is many times greater than the TOC fraction. Eliminating or compensating for IC interferences requires multiple determinations to measure true TOC. IC interference can be eliminated by acidifying samples to

*Approved by Standard Methods Committee, 1985

Bioscience, Inc.
Environmental Products and Services

THE MICRO-COD TEST METHOD
AMPULES OR TWIST-TUBE VIALS

1. Preheat a laboratory oven or reactor to 150 degrees Celsius.

2. Unseal a COD ampule by snapping the top colorbreak or remove the cap from a COD twist-tube.

3. Carefully add 2.5 mls of sample down the side of the vessel such that it forms a layer on top of the reagents.

4. Carefully flame-seal the ampule or replace the twist-tube cap.

5. Thoroughly mix the contents of the sealed vessel by shaking.

6. Process standards and blanks exactly as the samples.

7. Place the vessel in an oven or reactor capable of maintaining 150 degrees celcius ± 2 degrees for 2 hours.

8. Remove vessels from the oven or reactor and allow to cool.

9. Allow any suspended precipitate to settle and wipe the outside of the vessel clean.

10. Use method 10A. for standard range reagent (20-900 mg/l COD) or use method 10B. for low range reagent (5-50mg/l COD).

    A. Set the wavelength of the spectrophotometer to 600 nm, and using a procedural blank, zero the absorbance reading.

    B. Use the highest standard (50mg/l COD) to set the spectrophotometer to zero absorbance at 440 nm.

11. Read the absorbance of each standard and sample on the spectrophotometer.

12. Prepare a graphic calibration curve by plotting the absorbance of the standards versus their known concentrations. Compare sample absorbances to the graphic calibration curve to determine COD concentrations.

PART II. GENERAL OPERATIONS

Section A Sample Collection, Preservation and Storage

Collection, preservation and storage of water samples are critical to the results of water quality analyses. The data are only as valid as the water sample.

A sampling program must be planned to satisfy the objectives of the study yet remain within the limitations of available manpower, time and money. The survey should use the minimum number of samples that adequately represent the effluent or body of water from which they are taken. The number of samples and location of sampling sites should be determined prior to the survey and must satisfy the requirements needed to establish water quality standard or effluent permit violations.

The microbiologist should participate in the planning which specifies the microbiological tests needed, the number of analyses to be performed, and the equipment required. Consideration should be given to the weather and other local conditions prior to the formulation of a final plan. For example, seasonal variations in water temperature and flows would be important factors in deciding when to study the effects of thermal pollution on bacteria. Sample collectors must know the exact location of the sampling sites and be fully trained in the aseptic technique of sample collection as well as the use of any specialized sampling equipment. The sample collector is responsible for the recording of all pertinent information about the sample that might be significant in the evaluation and interpretation of the laboratory data or that might be necessary in potential enforcement action.

This Section is organized as follows:

1. Sample Containers
2. Sampling Techniques

   Composite Sampling
   Surface Sampling by Hand
   Surface Sampling by Weighted Bottle Frame
   Depth Sampling
   Soil Sampling
   Sediment Sampling
   Water Tap Sampling
3. Sample Identification and Handling
4. Chain of Custody Procedures
5. Selection of Sampling Sites and Frequency

   Potable Water Supplies
   Lakes and Impoundments
   Stream Sampling
   Marine and Estuarine Sampling
   Domestic and Industrial Waste Discharges
   Recreational Waters
   Shellfish-Harvesting Waters
   Frequency of Sampling
6. Preservation and Transit of Samples

631

1. Sample Containers

1.1 Sample Bottles: Bottles must be resistant to sterilizing conditions and the solvent action of water. Wide-mouth borosilicate glass bottles with screw-cap or ground-glass stopper or heat-resistant plastic bottles may be used if they can be sterilized without producing toxic materials (see suggested sample containers in Figure II-A-1). Screw-caps must not produce bacteriostatic or nutritive compounds upon sterilization.

1.2 Selection and Cleansing of Bottles: Sample bottles should be at least 125 ml volume for adequate sampling and for good mixing. Bottles of 250 ml, 500 ml and 1000 ml volume are often used for multiple analyses. Discard bottles which have chips, cracks, and etched surfaces. Bottle closures must be water-tight. Before use, thoroughly cleanse bottles and closures with detergent and hot water, followed by a hot water rinse to remove all trace of detergent. Then rinse them three times with laboratory-pure water (II-B,6). A test for the biological examination of glassware where bacteriostatic or inhibitory residues may be present, is described in Part IV-A, 5.1.

1.3 Dechlorinating Agent: The agent must be placed in the bottle when water and wastewater samples containing residual chlorine are anticipated. Add sodium thiosulfate to the bottle before sterilization at a concentration of 0.1 ml of a 10 percent solution for each 125 ml (4 oz.) sample volume (1). This concentration will neutralize approximately 15 mg/l of residue chlorine.

1.4 Chelating Agent: A chelating agent should be added to sample bottles used to collect samples suspected of containing >0.01 mg/liter concentrations of heavy metals such as copper, nickel or zinc, etc. Add 0.3 ml of a 15 percent solution of ethylenediaminetetraacetic acid (EDTA) tetrasodium salt, for each 125 ml (4 oz.) sample volume prior to sterilization (2, 3).

1.5 Wrapping Bottles: Protect the tops and necks of glass stoppered bottles from contamination by covering them before sterilization with aluminum foil or kraft paper.

1.6 Sterilization of Bottles: Autoclave glass or heat-resistant plastic bottles at 121 C for 15 minutes. Alternatively, dry glassware may be sterilized in a hot air oven at 170 C for not less than two hours. Ethylene oxide gas sterilization is acceptable for plastic containers that are not heat-resistant. Sample bottles sterilized by gas should be stored overnight before being used to allow the last traces of gas to dissipate. See Part II-B, 3 for sterilization procedures.

1.7 Plastic Bags: The commercially-available bags (Whirl-pak) are a practical substitute for plastic or glass sample bottles in sampling soil or sediment. See Figure II-A-1. The bags are sealed in manufacture and opened only at time of sampling. The manufacturer states that such bags are sterilized.

2. Sampling Techniques

Samples are collected by hand or with a sampling device if (1) depth samples are required or (2) the sampling site has difficult access such as a manhole, dock, bridge or bank adjacent to a surface water.

2.1 Chlorinated Samples: When samples, such as treated waters, chlorinated wastewaters or recreational waters, are collected, the sample bottle must contain a dechlorinating agent (see this Section, 1.3).

2.2 Composite Sampling: In no case should a composite sample be collected for bacteriological examination. Data from individual samples show a range of values. A composite sample will not display this range. Individual results will give information about industrial process variations in flow and compo-

632

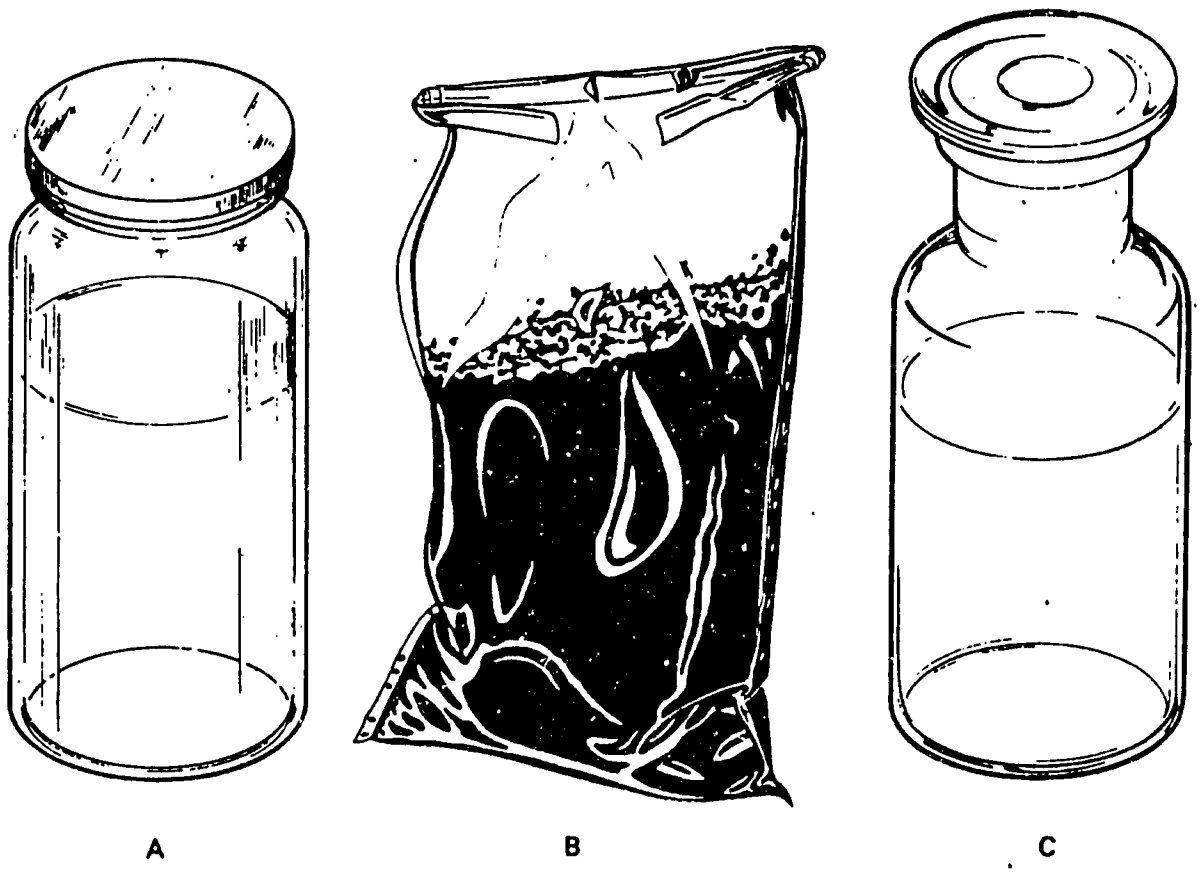


FIGURE II-A-1. Suggested Sample Containers

A Screw-cap Glass or Plastic Bottle.

B Plastic Bag (Whirl-pak).

C Glass Stoppered Bottle.

sition. Also, one or more portions that make up a composite sample may contain toxic or nutritive materials and cause erroneous results.

2.3 Surface Sampling by Hand: A grab sample is obtained using a sample bottle prepared as described in 1. above. Identify the sampling site on a chain of custody tag if required, or on the bottle label and on a field log sheet (see 3). Remove the bottle covering and closure and protect from contamination. Grasp the bottle at the base with one hand and plunge the bottle mouth down into the water to avoid introducing surface scum. Position the mouth of the bottle into the current away from the hand of the collector and away from the side of the sampling platform or boat (see Figure II-A-2). The sampling depth should be 15 to 30 cm (6 to 12 inches) below the water surface. If the water body is static, an artificial current can be created, by moving the bottle horizontally in the direction it is pointed and away from the sampler. Tip the bottle slightly upwards to allow air to exit and the bottle to fill. After removal of the bottle from the stream, pour out a small portion of the sample to allow an air space of 2.5 to 5 cm (1 to 2 inches) above each sample for proper mixing of the sample before analyses. Tightly stopper and label the bottle.

2.4 Surface Sampling by Weighted Bottle Frame: When sampling from a bridge or other structure above a stream or body of water, the sample collector places the bottle in a weighted frame (see Figure II-A-3) that holds the bottle securely. Remove cover and lower the device to the water. It is preferable to use nylon rope which does not absorb water and will not rot. Face the bottle mouth upstream by swinging the sampling device first downstream, and then allow it to drop into the water, without slack in the rope. Pull the sample device rapidly upstream and out of the water, thus simulating the scooping motion of grab sampling described in 2.3. Take care not to dislodge dirt or other material that might fall into the open bottle from the sampling platform.

2.5 Depth Sampling: Several additional devices are needed for collection of depth samples from lakes, reservoirs, estuaries and the oceans. These depth samplers require lowering the sampling device and/or container to the desired depth, then opening, filling, and closing the container and returning the device to the surface. Although depth measurements are best made with a pre-marked steel cable, the sample depths can be determined by premeasuring and marking the nylon rope at intervals with a non-smearing ink, paint, or fingernail polish. The following list of depth samplers is not inclusive but can serve as a guide:

2.5.1 ZoBell J-Z Sampler: This sampler described by ZoBell in 1941 (4) was designed for deep sea sampling but is also used in fresh waters. Figure II-A-4 shows its general appearance. It has a metal frame (A), a heavy metal messenger (B), a sealed glass tube (C) attached to a rubber tube (D), and a sterile 350 ml glass bottle (E) or a collapsible neoprene rubber bulb for shallow waters. The messenger (B) is released at the surface when the sampler reaches the desired depth, and breaks the glass tubing (C) at a file mark. The bent rubber tubing (D) then straightens out and the water is drawn in several inches from the sampler. A partial vacuum created by autoclaving of the sealed unit draws the water into the bottle.

2.5.2 Niskin Sampler: This is sometimes called a sterile-bag or "Book" sampler (see Figure II-A-5) (5). A messenger triggers the opening of two plates (A) in V-fashion by spring power, and causes the sterile plastic bag (B) to inflate. At the same time a plastic filler tube (C) leading to the plastic container is cut by a guillotine knife (D) and the bag fills with water. The bag is then automatically sealed with a clamp (E) and the apparatus is brought to the surface. Samplers are available that will hold 1, 2, 3, or 5 liters of water.

2.5.3 New York Dept. of Health Depth Sampler: This device (see Figure II-A-6) depends upon a vane (A) and lever (B) mechanism to lift the glass stopper (C) as water inertia is applied by a sharp upward tug on the line (D) attached to the apparatus. As the stopper is lifted, the bottle fills before the detachment of the stopper from the vane occurs and closes the sample bottle (6).

634

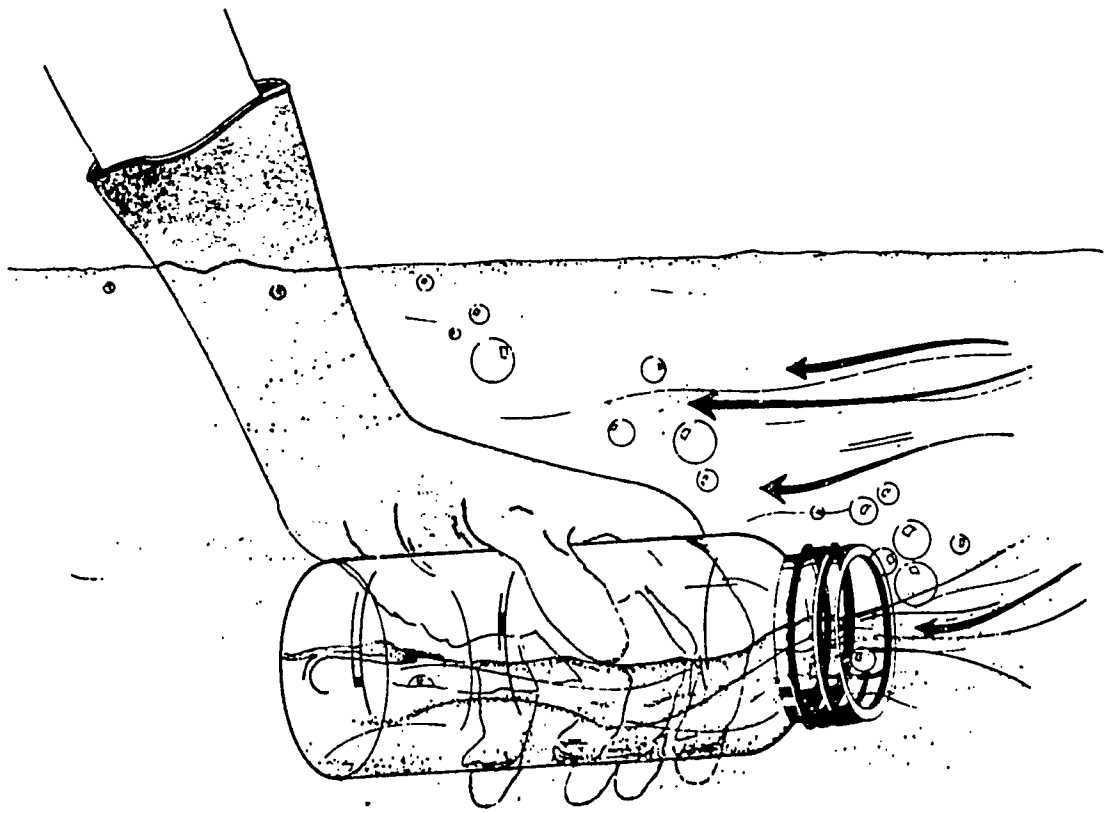


FIGURE II-A-2. Demonstration of Technique Used in Grab Sampling of Surface Waters.

635

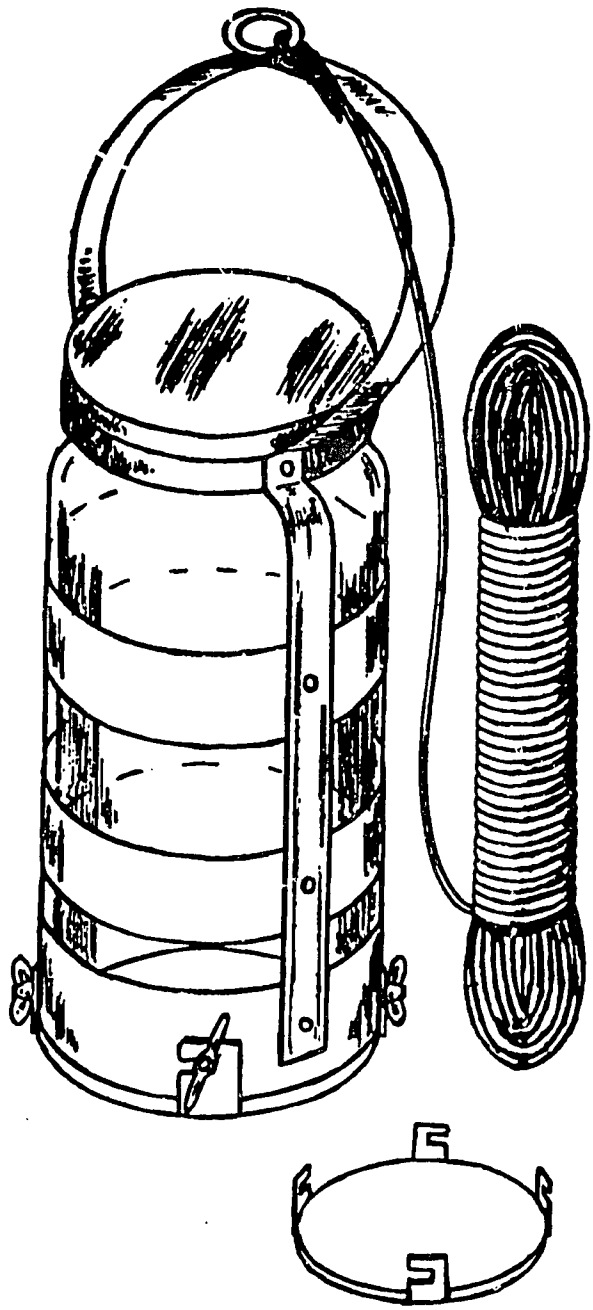


FIGURE II-A-3. Weighted Bottle Frame and Sample Bottle for Grab Sampling.

636

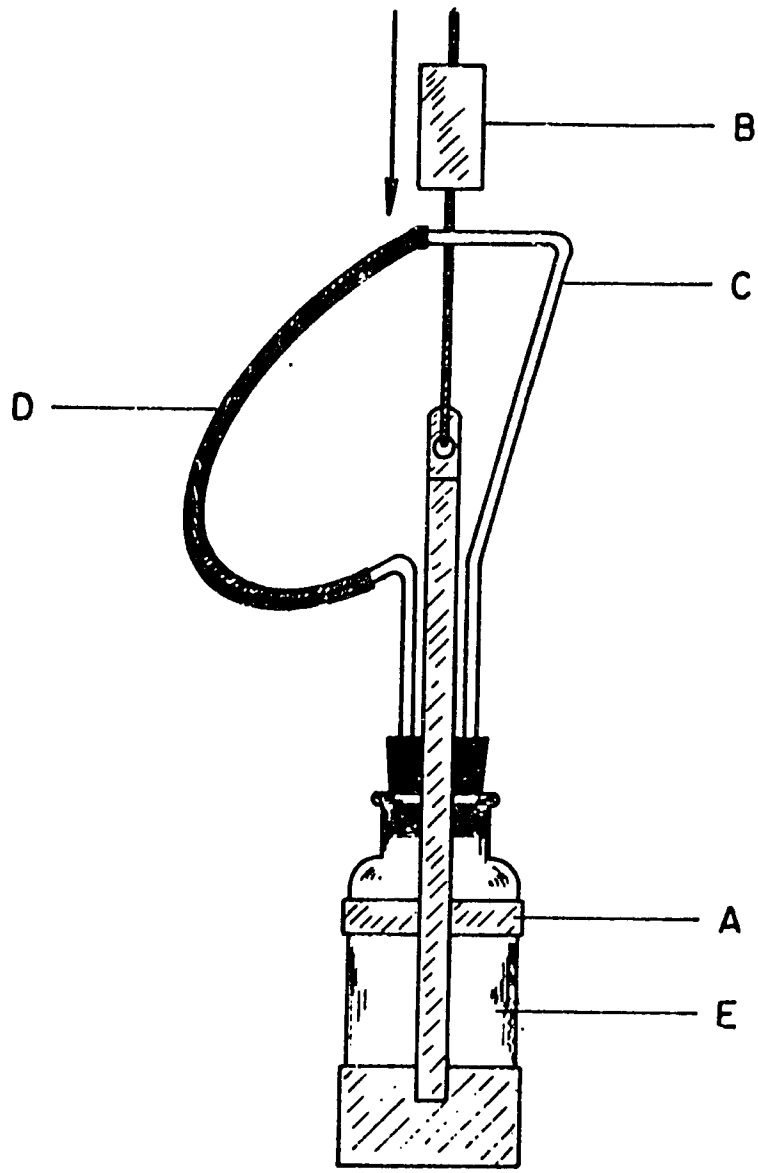


FIGURE II-A-4. Zobell J-Z Sampler. (A) metal frame, (B) messenger, (C) glass tube, (D) rubber tube and (E) sterile sample bottle.

631

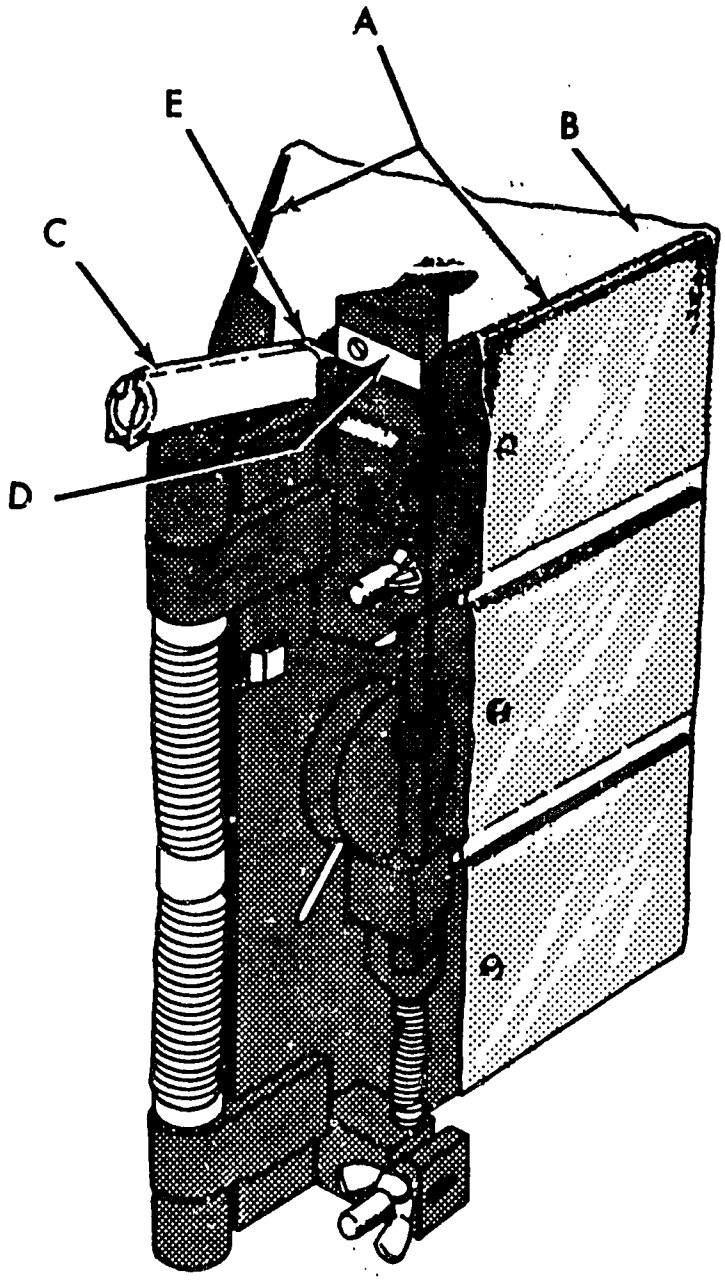


FIGURE II-A-5. Niskin Depth Sampler. (A) hinged plates, (B) plastic bag, (C) plastic filler tube in sheath, (D) guillotine knife and (E) closure clamp.

638

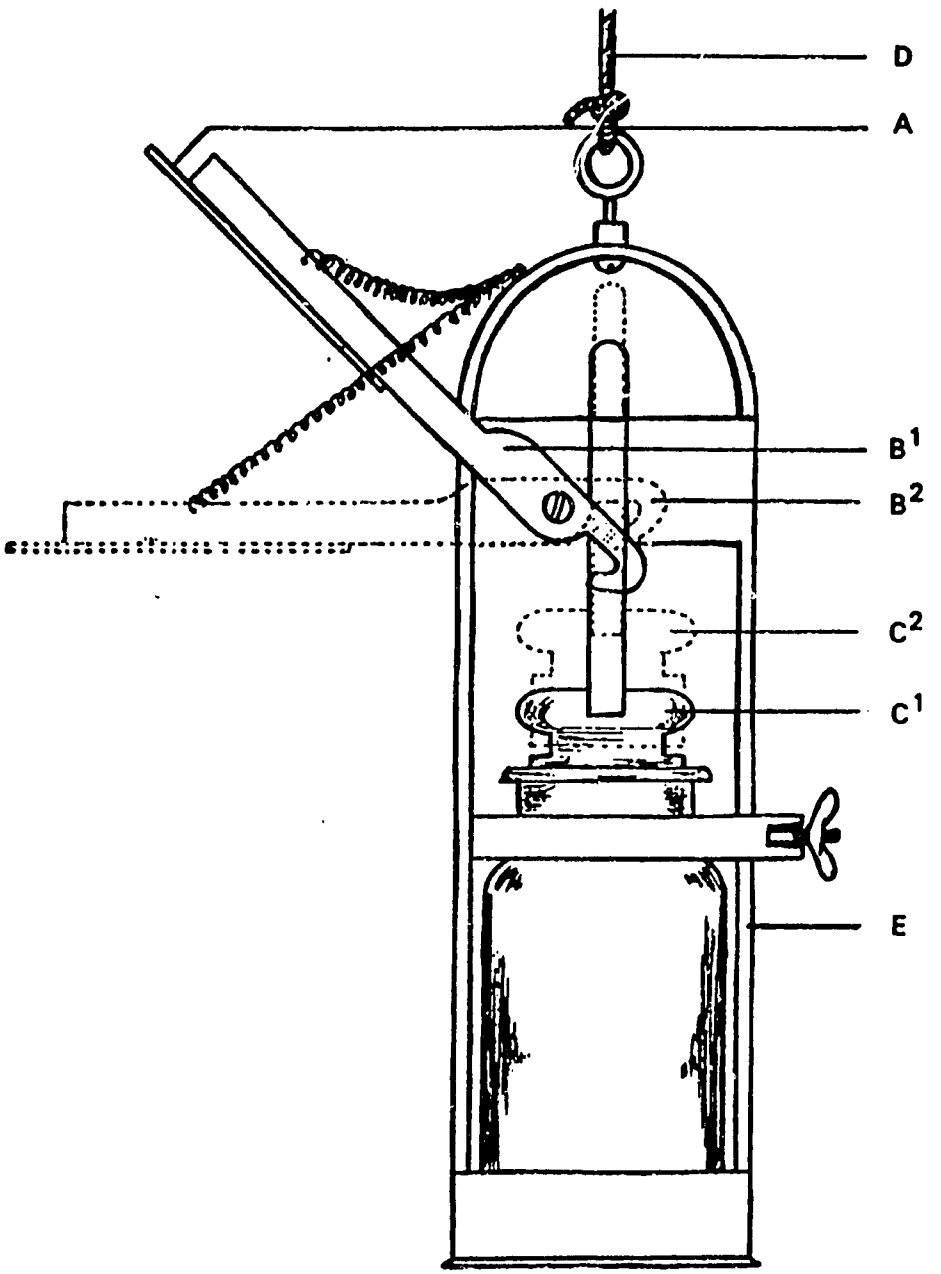


FIGURE II-A-6. New York State Dept. of Health Depth Sampler: (A) vane, (B^1) lever in closed position, (B^2) lever in open position, (C^1) glass stopper in closed position, (C^2) glass stopper in open position, (D) suspension line, and (E) metal frame.

634

2.5.4 Kemmerer Sampler (7): This depth sampler (see Figure II-A-7) has been used without sterilization to collect bacteriological water samples in high pollution areas. The sampler consists of a cylindrical brass or plastic tube (F) that contains a rubber stopper or valve at either end (D and G). The valves are connected to a rod (E) that passes through the center of the cylinder. The device is lowered into the water in the open position, and a water sample is trapped in the cylinder when the valves are closed by a dropped messenger (B). The Kemmerer sampler should not be used for collecting bacteriological samples without obtaining data that support its use without sterilization.

2.6 Sediment Sampling with Van Donsel-Geldreich Sampler (8): This device (see Figure II-A-8) collects sediment or mud in sterile "Whirl-Pak" plastic bags (A) down to 60 foot depth. The bag mouth is wrapped over a nosepiece (B), and the bag is kept closed during descent to the bottom by a bag clamp bar (H). As the mud plate (D) contacts the bottom, the nosepiece (B) is driven into the sediment by the weight (C) of the sampler. As the nosepiece (B) moves downward, the bag (A) slides through the bag clamp bar (H), opens, and fills with sediment. The bag is sealed when the double noose (F) tied to the bottom of the bag is pulled, before the apparatus is returned to the surface.

2.7 Water Tap Sampling: Make certain that samples are not collected from spigots that leak around their stems, or from spigots that contain aeration devices or screens within the faucet. For samples taken from direct water main connections, the spigot should be flushed for 2-3 minutes to clear the service line. For wells equipped with hand or mechanical pumps, pump the water to waste for five minutes before the sample is collected. Remove the cap aseptically from the sample bottle. Hold the sample bottle upright near the base while it is being filled. Avoid splashing. Do not rinse the bottle with the sample; fill it directly to within 2.5 cm (1 inch) from the top. Replace bottle closure and hood covering. Caution must be used to prevent contaminating the sample with finger, gloves or other materials. If the well does not have pumping machinery, collect the sample using a weighted sterilized sample bottle, such as described in 2.4 above, and shown in Figure II-A-3. Care must be taken to avoid contaminating the sample with the surface scum from the water surface.

2.8 Soil Sampling

2.8.1 Selection of the sampling site is based on knowledge of the area and the purposes of the analyses, i.e., surface sampling for natural background, surface contamination, or below surface sampling to monitor treatment effect such as irrigation, or stormwater runoff.

The actual sites for sampling and the number of points to be sampled must be predetermined by the survey objectives. Soil sampling has the advantage of permitting the survey planners to lay out a stable grid network for sampling and resampling over a given time period.

2.8.2 If a surface sample is desired, scrape the top one inch of soil from a square foot area using a sterile scoop or spoon.

If a subsurface sample is desired, use a sterile scoop or spatula to remove the top surface of one inch or more from a one foot square area. Use a second sterile scoop or spoon to take the sample.

Place samplings in a sterile one quart screw-cap bottle until it is full. Depending on the amount of moisture, a one quart bottle holds 300–800 grams of soil. Label and tag the bottle carefully and store at 4 C until analyzed..

3. Sample Identification and Handling

3.1 Specific details on sample identification are entered on a permanent label. Take care in transcribing sampling information to the label, because the enforcement action may depend upon evidence of primary labeling. See 4. in This Section. Labels must be clean,

1640

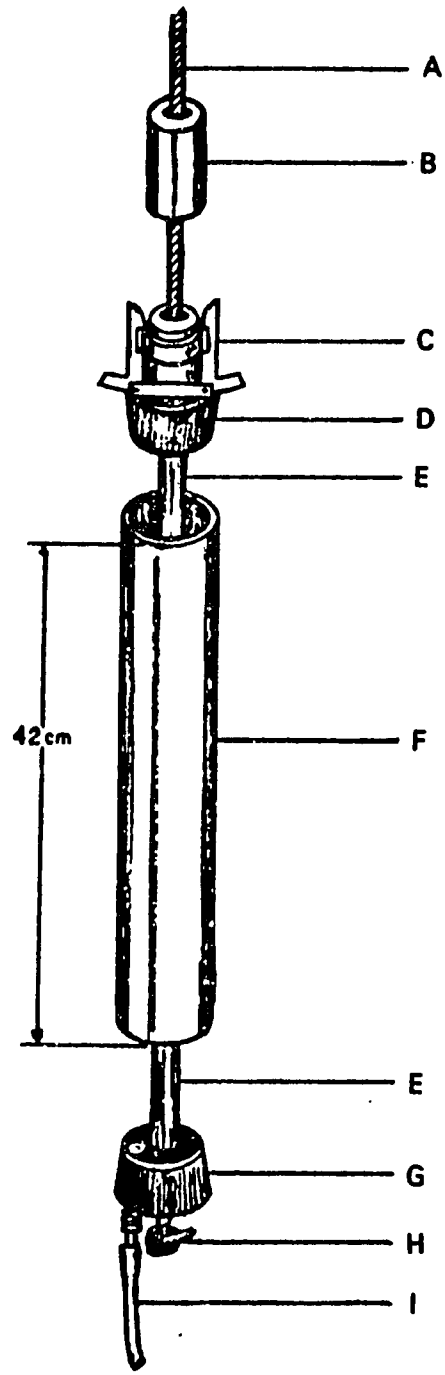


FIGURE II-A-7. Kemmerer Depth Sampler. (A) nylon line, (B) messenger, (C) catch set so that the sampler is open, (D) top rubber valve, (E) connecting rod between the valves, (F) tube body, (G) bottom rubber valve, (H) knot at the bottom of the suspension line and (I) rubber tubing attached to the spring loaded check valve.

641

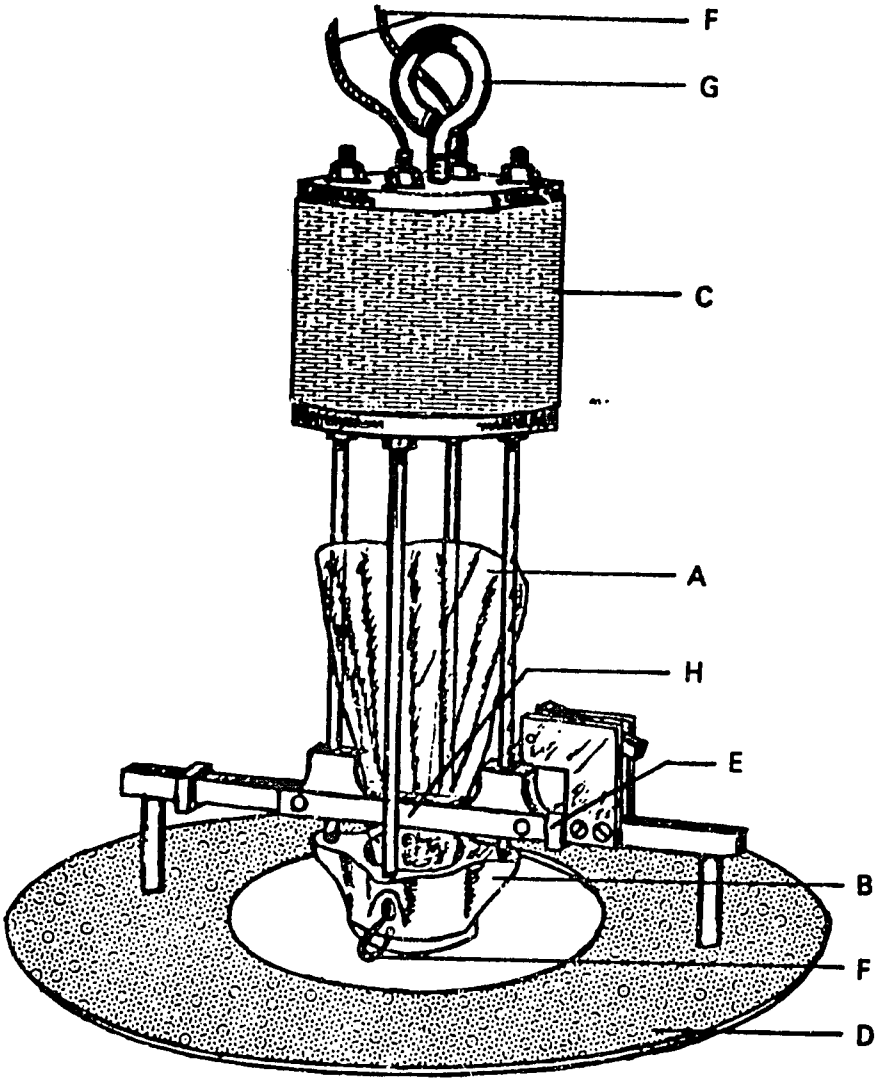


FIGURE II-A-8. Van Donsel-Geldreich Sediment Sampler. (A) sterile "Whirl-Pak" plastic bag, (B) nose piece, (C) weight, (D) mud plate, (E) slide bar, (F) part of the double noose, (G) attachment for the suspension line and (H) bag clamp bar.

-1042

waterproof, non-smearing and of sufficient size for the necessary information. Label must be securely attached to the sample bottle, but removable when necessary. Do not accept insufficiently or improperly labeled samples for examination. A sample label showing the minimum information required is pictured in Figure II-A-9.

3.2 Field Data Record: A field record should be completed on each sample to record the full details on sampling and other pertinent remarks such as flooding, rain or extreme temperature which are relevant to interpretation of results. This record also provides a back-up record of sample identification. One example is shown in Figure II-A-10.

3.3 Marking Device: A marking pen or other device must be non-smearing if wetted, and maintain a permanent legible mark.

3.4 Transport Container: Insulated ice containers in which the sample can be held, are recommended.

3.5 Storage of Samples: A refrigerator is necessary for storage of samples at the laboratory. The temperature range of the refrigerator is 1-4 C.

4. Chain of Custody Procedures

4.1 General: An agency must demonstrate the reliability of its evidence in pollution cases by proving the chain of possession and custody of samples which are offered for evidence or which form the basis of analytical results introduced into evidence. It is imperative that the office and laboratory prepare written procedures to be followed whenever evidence samples are collected, transferred, stored, analyzed, or destroyed.

4.1.1 The primary objective of these procedures is to create an accurate written record which can be used to trace the possession of the sample from the moment of its collection through its introduction into evidence. A sample is in custody if it is:

(a) in actual physical possession, or

(b) in view after being in physical possession, or

(c) in physical possession and locked up so that no one could tamper with it.

4.1.2 Personnel should receive copies of study plans and know the contents prior to the study. A pre-study briefing shall be held to appraise participants of the objectives, sample locations and chain of custody procedures. After chain of custody samples are collected, a de-briefing is held in the field to determine adherence to chain of custody procedures and whether additional samples are required.

4.2 Rules for Sample Collection

4.2.1 Handle the samples as little as possible.

4.2.2 Obtain stream and effluent samples using standard microbiological sampling techniques.

4.2.3 Attach sample tag or label (Figure II-A-9) to the sample container. The tag or label should contain as a minimum: serial number of label, location, date and time taken, type of sample, sequence number (first sample of the day – sequence No. 1, second sample, sequence No. 2, etc), analyses required and sample collector. The tags must be filled out legibly in waterproof ink.

4.2.4 Use a bound notebook to record field measurements and other pertinent information necessary to refresh the sampler's memory if the person later becomes a witness in an enforcment proceeding. A separate set of field notebooks should be maintained for each study and stored in a safe place where it can be protected and accounted for. A sample log sheet with a standard format should be established to minimize field entries and include the date, time, survey, type of samples, volume of each sample, type of analyses, label and sample numbers, sample location, field measurements such as temperature, conductivity, DO, pH, and any other pertinent

EPA, NATIONAL ENFORCEMENT INVESTIGATIONS CENTER

Station No.	Date	Time	Sequence No.

Station Location

_____Grab
_____Comp.

_____BOD _____Metals
_____Solids _____Oil and Grease
_____COD _____D.O.
_____Nutrients _____Bact.
_____Other

Samplers:

Remarks/Preservative:

SERIAL TAG #

FIGURE II-A-9. Example of a Sample Label.

SERIAL SHEET NO. _____

Samplers: _____

FIELD DATA RECORD

STATION	SAMPLE NUMBER	DATE OF COLLECTION	TIME (HRS) SAMPLE TAKEN	TIME (HRS) SAMPLE RECEIVED BY LAB	pH	TEMP °C	OTHER	REMARKS

US EPA, NEIC-Denver

FIGURE II-A-10. Field Data Record.

644

information or observation (Figure II-A-11). The entries should be signed by the sample collector. The responsibility for preparing and storing sample notebooks during and after a study should be assigned to a study coordinator, or his designated representative.

4.2.5 A field collector is responsible for the samples collected until properly dispatched to the receiving laboratory or turned over to an assigned custodian. He must assure that each container is in his physical possession or in his view at all times, or stored in a locked place where no one can tamper with it.

4.2.6 Color slides or photographs should be taken of the sample location and any visible water pollution. The signature of the photographer, time, date, and site location must be written on the back of the photo. Such photographs should be handled according to the established chain of custody procedures to prevent alteration.

4.3 Transfer of Custody and Shipment

In transfer of custody procedures, each custodian of samples must sign, record and date the transfer. Most regulatory agencies develop chain of custody procedures tailored to their needs. These procedures may vary in format and language but contain the same essential elements. Historically, sample transfer under chain of custody has been on a sample by sample basis which is awkward and time-consuming. However, EPA's National Enforcement Investigation Center (NEIC), Denver has set a precedent with its bulk transfer of samples. Bulk transfer is speedier, reduces paperwork and the number of sample custodians. The following description of chain of custody is essentially that of NEIC-Denver (9).

4.3.1 Samples must be accompanied by a Chain of Custody Record which includes the name of the study, collector's signature, station number, station location, date, time, type of sample, sequence number, number of containers and analyses required (Figure II-A-12). When turning over the possession of samples, the transferor and transferee sign, date and note time on the sheet. This record sheet allows transfer of custody of a group of samples in the field, to the mobile laboratory or to the NEIC-Denver laboratory. When a custodian transfers a portion of the samples identified on the sheet to the field mobile laboratory, the individual samples must be noted in the column with the signature of the person relinquishing the samples. The field laboratory person receiving the samples acknowledges receipt by signing in the appropriate column.

4.3.2 If a custodian has not been assigned, the field custodian or field sampler has the responsibility for packaging and dispatching samples to the laboratory for analysis. The "Dispatch" portion of the Chain of Custody Record must be filled out, dated, and signed.

4.3.3 Samples must be carefully packed in shipment containers such as ice chests, to avoid breakage. The shipping containers are padlocked for shipment to the receiving laboratory.

4.3.4 Packages must be accompanied by the Chain of Custody Record showing identification of the contents. The original must accompany the shipment. A copy is retained by the survey coordinator.

4.3.5 If samples are delivered to the laboratory when appropriate personnel are not there to receive them, the samples must be locked in a designated area within the laboratory so that no one can tamper with them. This same person must return to the laboratory, unlock the samples and deliver custody to the appropriate custodian.

4.4 Laboratory Custody Procedures

4.4.1 The laboratory shall designate a "sample custodian" and an alternate to act in his absence. In addition, the laboratory shall set aside as a "sample storage security area", an isolated room with sufficient refrigerator space, which can be locked or just a locked refrigerator in smaller laboratories.

4.4.2 Samples should be handled by the minimum possible number of persons.

645

SERIAL SHEET NO. _____

FOR _____ SURVEY, PHASE _____, DATE _____

TYPE OF SAMPLE _____ SAMPLERS: (Signature) _____

ANALYSES REQUIRED

STATION NUMBER	STATION DESCRIPTION	TOTAL VOLUME	TYPE CONTAINER	PRESERVATIVE	NUTRIENTS	BOD	COD	TOC	TOTAL SOLIDS	SUSPENDED SOLIDS	ALKALINITY	DO	pH*	CONDUCTIVITY*	TEMPERATURE*	TOTAL COLIFORM	FECAL COLIFORM	TURBIDITY	OIL AND GREASE	METALS	BACTI	PESTICIDES	HERB	TRACE ORGANICS	PHENOL	CYANIDE

REMARKS

*Field Measurements

US EPA, NEIC-Denver

FIGURE II-A-11. Sample Log Sheet.

SERIAL SHEET NO. _____

ENVIRONMENTAL PROTECTION AGENCY
Office Of Enforcement
NATIONAL ENFORCEMENT INVESTIGATIONS CENTER
Building 53, Box 25227, Denver Federal Center
Denver, Colorado 80225

CHAIN OF CUSTODY RECORD

SURVEY

SAMPLERS: (Signature)

STATION NUMBER	STATION LOCATION	DATE	TIME	SAMPLE TYPE Water Comp.	SAMPLE TYPE Water Grab.	SAMPLE TYPE Air	SEQ NO	NO. OF CONTAINERS	ANALYSIS REQUIRED

Relinquished by: (Signature)	Received by: (Signature)	Date/Time
Relinquished by: (Signature)	Received by: (Signature)	Date/Time
Relinquished by: (Signature)	Received by: (Signature)	Date/Time
Relinquished by: (Signature)	Received by Mobile Laboratory for field analysis: (Signature)	Date/Time
Dispatched by: (Signature) Date/Time	Received for Laboratory by:	Date/Time
Method of Shipment:		

Distribution: Orig. – Accompany Shipment
1 Copy – Survey Coordinator Field Files

GPO 854-609

FIGURE II-A-12. Chain of Custody Record.

647

4.4.3 Incoming samples shall be received only by the custodian, who will indicate receipt by signing the Chain of Custody Record Sheet accompanying the samples and retaining the sheet as a permanent record. Couriers picking up samples at the airport, post office, etc. shall sign jointly with the laboratory custodian.

4.4.4 Immediately upon receipt, the custodian places samples in the sample room, which will be locked at all times except when samples are removed or replaced by the custodian. To the maximum extent possible, only the custodian should be permitted in the sample room.

4.4.5 The custodian shall ensure that microbiological samples are properly stored and maintained at 1-4 C.

4.4.6 Only the custodian will distribute samples to personnel who are to perform tests.

4.4.7 The analyst records information in his laboratory notebook or analytical worksheet, describing the sample, the procedures performed and the results of the testing. The notes shall be dated and indicate who performed the tests. The notes shall be retained as a permanent record in the laboratory and should include any abnormalities which occurred during the testing procedure. In the event that the person who performed the tests is not available as a witness at time of trial, the government may be able to introduce the notes in evidence under the Federal Business Records Act.

4.4.8 Standard methods of laboratory analyses shall be used as described in the "Guidelines Establishing Test Procedures for Analysis of Pollutants," (10) and amendments. If laboratory personnel deviate from standard procedures, they should be prepared to justify their decision during cross-examination.

4.4.9 Laboratory personnel are responsible for the care and custody of a sample once it is handed over to them and should be prepared to testify that the sample was in their possession and view or secured in the laboratory at all times from the moment it was received from the custodian until the tests were run.

4.4.10 Once the sample testing is completed, microbiological samples can be discarded but identifying tags and laboratory record should be returned to the custodian. Other documentation of work will also be given to the custodian.

4.4.11 Tags and laboratory records of tests may be destroyed only upon the order of the Laboratory Director, who will first confer with the Chief, Enforcement Specialist Office, to make certain that the information is no longer required.

5. Selection of Sampling Sites and Frequency

These will be described for potable and recreational waters, streams, lakes, reservoirs, estuarine, and marine waters as well as domestic and industrial wastewaters.

5.1 Potable Water Supplies

An expanded program to maintain the sanitary quality of potable water supplies has been recently established by the National Interim Primary Drinking Water Regulations (11). The sampling program includes examination of water as it enters and flows throughout the distribution system. For application of the EPA Drinking Water Standards, the frequency of sampling and the location of sampling points are established jointly by the utility, the Reporting Agency, and the Certifying Authority. Additionally, the laboratory, the methods of analyses, and the technical competence of personnel must be inspected and approved by the Reporting Agency and the Certifying Authority.

5.1.1 Sampling Water Supplies: Figure II-A-13 shows how reservoirs and lakes used as water supplies are sampled: (A) at inlets, (B) at other possible sources of pollution, (C) at the draw-off point, (D) at quarter point intervals around the draw-off point at about the same depth and (E) at the reservoir outlet.

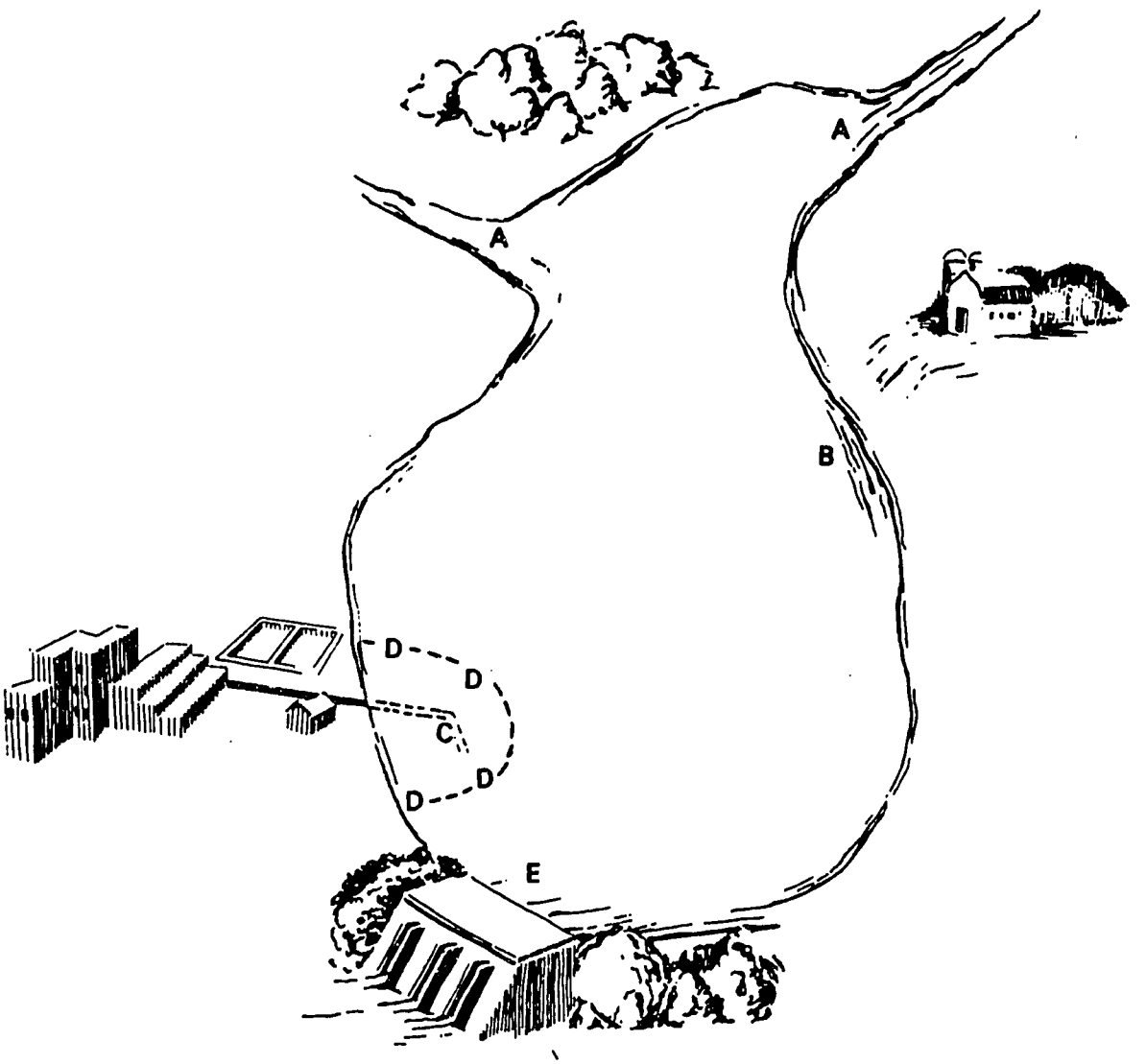


FIGURE II-A-13. Sampling a Water Supply Reservoir. (A) influent stream, (B) possible agricultural contamination, (C) water plant intake. (D) multi-point sampling around intake and (E) reservoir outlet.

649

5.1.2 Sampling Treatment Systems: Sampling should be representative of the distribution system and include sites such as municipal buildings, public schools, airports and parks, hydrants, restaurants, theaters, gas stations, industrial plants and private residences. A systematic coverage of such points in the distribution system should insure the detection of contamination from breaks in waterlines, loss of pressure or crossconnections. The sampling program should also include special sampling locations such as dead-end distribution lines that are sources of bacterial contamination (12).

5.1.3 Sample Frequency: The minimum number of samples which must be collected and examined each month is based upon the population density served by the distribution system (Table II-A-1). Samples should be collected at evenly spaced time intervals throughout the month. In the event of an unsatisfactory sample, repetitive samples must be collected until two consecutive samples yield satisfactory quality water. Repetitive samples from any single point or special purpose samples must not be counted in the overall total of monthly samples.

5.1.4 Standard Sample: The standards for microbiological quality are based upon the number of organisms allowable in a standard sample. A standard sample for the membrane filter technique is at least 100 ml. For the MPN test, a standard sample consists of five standard portions of either 10 ml or 100 ml.

5.2 Lakes and Impoundments

Figure II-A-14 shows the range of sampling points in a recreational impoundment or lake: (A) inlets, (B) source of pollution, (C) grid or transect across the long axis of the water body, (D) bathing beach and (E) outlet.

5.3 Stream Sampling

The objectives of the initial survey dictate the location, frequency and number of samples to be collected.

5.3.1 Selection of Sampling Sites: A typical stream sampling program includes sampling locations upstream of the area of concern, upstream and downstream of waste discharges, upstream and downstream from tributary entrances to the river and upstream of the mouth of the tributary. For more complex situations, where several waste discharges are involved, sampling includes sites upstream and downstream from the combined discharge area and samples taken directly from each industrial or municipal waste discharge. Using available bacteriological, chemical and discharge rate data, the contribution of each pollution source can be determined. See Figure II-A-14 and II-A-15.

5.3.2 Small Streams: Small streams should be sampled at background stations upstream of the pollution sources and at stations downstream from pollution sources. Additional sampling sites should be located downstream to delineate the zones of pollution. Avoid sampling areas where stagnation may occur (backwater of a tributary) and areas located near the inside bank of a curve in the stream which may not be representative of the main channel.

5.3.3 Large Streams and Rivers: Large streams are usually not well mixed laterally for long distances downstream from the pollution sources. Sampling sites below point source pollution should be established to provide desired downstream travel time and dispersal as determined by flow rate measurements. Particular care must be taken to establish the proper sampling points as shown in Figure II-A-15: Sampling point (A) is the upper reach control station, (B) monitors a non-point source of pollution, (C) samples the waste discharge as it enters the stream, (D) shows quarter-point sampling below the pollution to detect channeling, (D) also serves as an upstream monitor on the tributary measured as (E), and (F) monitors the downstream effect of the tributary after mixing. Occasionally, depth samples are necessary to determine vertical mixing patterns.

60

TABLE II-A-1

Sampling Frequency for Drinking Waters Based on Population

Population served:	Minimum number of samples per month	Population served:	Minimum number of samples per month
25 to 1,000	1	90,001 to 96,000	95
1,001 to 2,500	2	96,001 to 111,000	100
2,501 to 3,300	3	111,001 to 130,000	110
3,301 to 4,100	4	130,001 to 160,000	120
4,101 to 4,900	5	160,001 to 190,000	130
4,901 to 5,800	6	190,001 to 220,000	140
5,801 to 6,700	7	220,001 to 250,000	150
6,701 to 7,600	8	250,001 to 290,000	160
7,601 to 8,500	9	290,001 to 320,000	170
8,501 to 9,400	10	320,001 to 360,000	180
9,401 to 10,300	11	360,001 to 410,000	190
10,301 to 11,100	12	410,001 to 450,000	200
11,101 to 12,000	13	450,001 to 500,000	210
12,001 to 12,900	14	500,001 to 550,000	220
12,901 to 13,700	15	550,001 to 600,000	230
13,701 to 14,600	16	600,001 to 660,000	240
14,601 to 15,500	17	660,001 to 720,000	250
15,501 to 16,300	18	720,001 to 780,000	260
16,301 to 17,200	19	780,001 to 840,000	270
17,201 to 18,100	20	840,001 to 910,000	280
18,101 to 18,900	21	910,001 to 970,000	290
18,901 to 19,800	22	970,001 to 1,050,000	300
19,801 to 20,700	23	1,050,001 to 1,140,000	310
20,701 to 21,500	24	1,140,001 to 1,230,000	320
21,501 to 22,300	25	1,230,001 to 1,320,000	330
22,301 to 23,200	26	1,320,001 to 1,420,000	340
23,201 to 24,000	27	1,420,001 to 1,520,000	350
24,001 to 24,900	28	1,520,001 to 1,630,000	360
24,901 to 25,000	29	1,630,001 to 1,730,000	370
25,001 to 28,000	30	1,730,001 to 1,850,000	380
28,001 to 33,000	35	1,850,001 to 1,970,000	390
33,001 to 37,000	40	1,970,001 to 2,060,000	400
37,000 to 41,000	45	2,060,001 to 2,270,000	410
41,001 to 46,000	50	2,270,001 to 2,510,000	420
46,001 to 50,000	55	2,510,001 to 2,750,000	430
50,001 to 54,000	60	2,750,001 to 3,020,000	440
54,001 to 59,000	65	3,020,001 to 3,320,000	450
59,001 to 64,000	70	3,320,001 to 3,620,000	460
64,001 to 70,000	75	3,620,001 to 3,960,000	470
70,001 to 76,000	80	3,960,001 to 4,310,000	480
76,001 to 83,000	85	4,310,001 to 4,690,000	490
83,001 to 90,000	90	4,690,001 or more	500

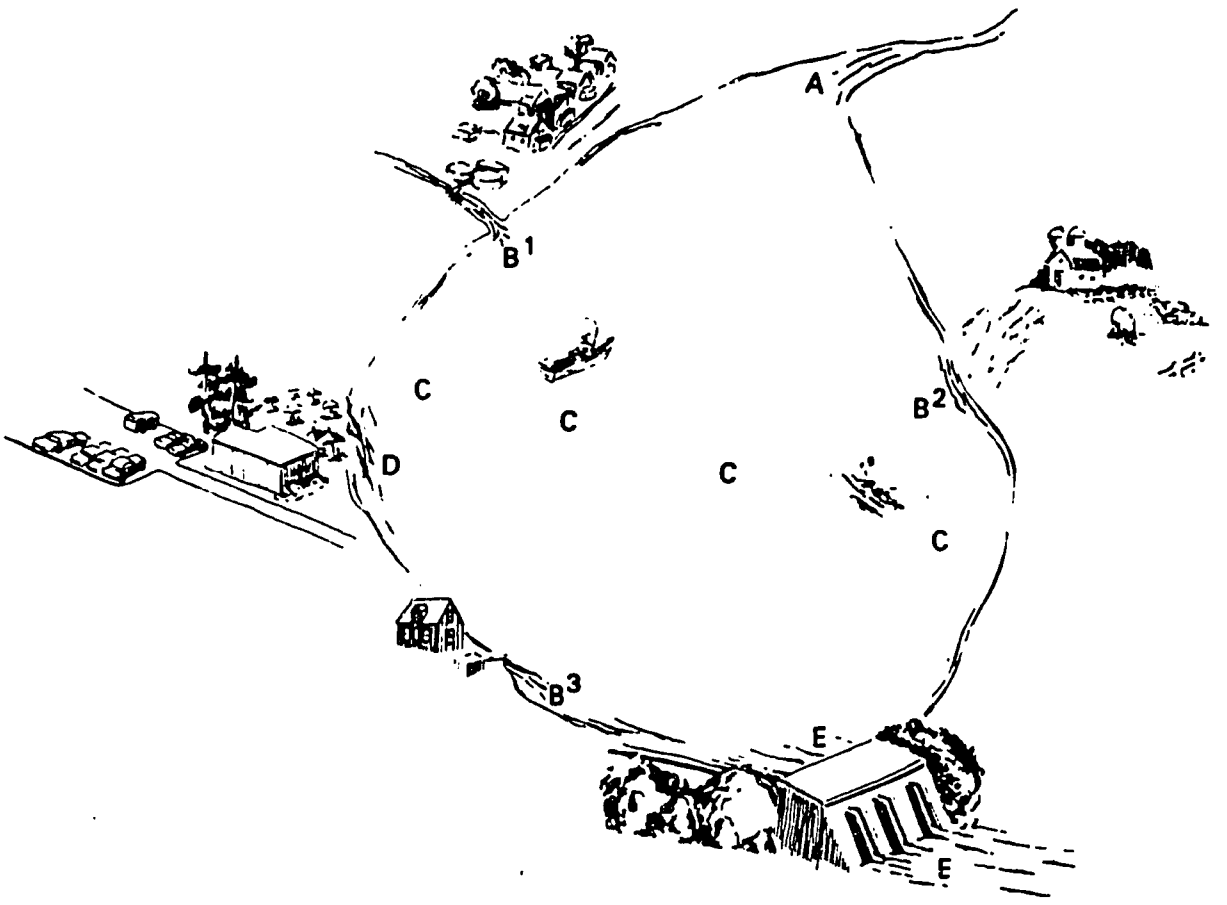


FIGURE II-A-14. Sampling a Lake or Impoundment. (A) inlets, (B) potential source of pollution, (B^1) village, (B^2) agricultural run-off, (B^3) home septic tank, (C) multi-point transect, (D) bathing beach and (E) outlet above and below dam.

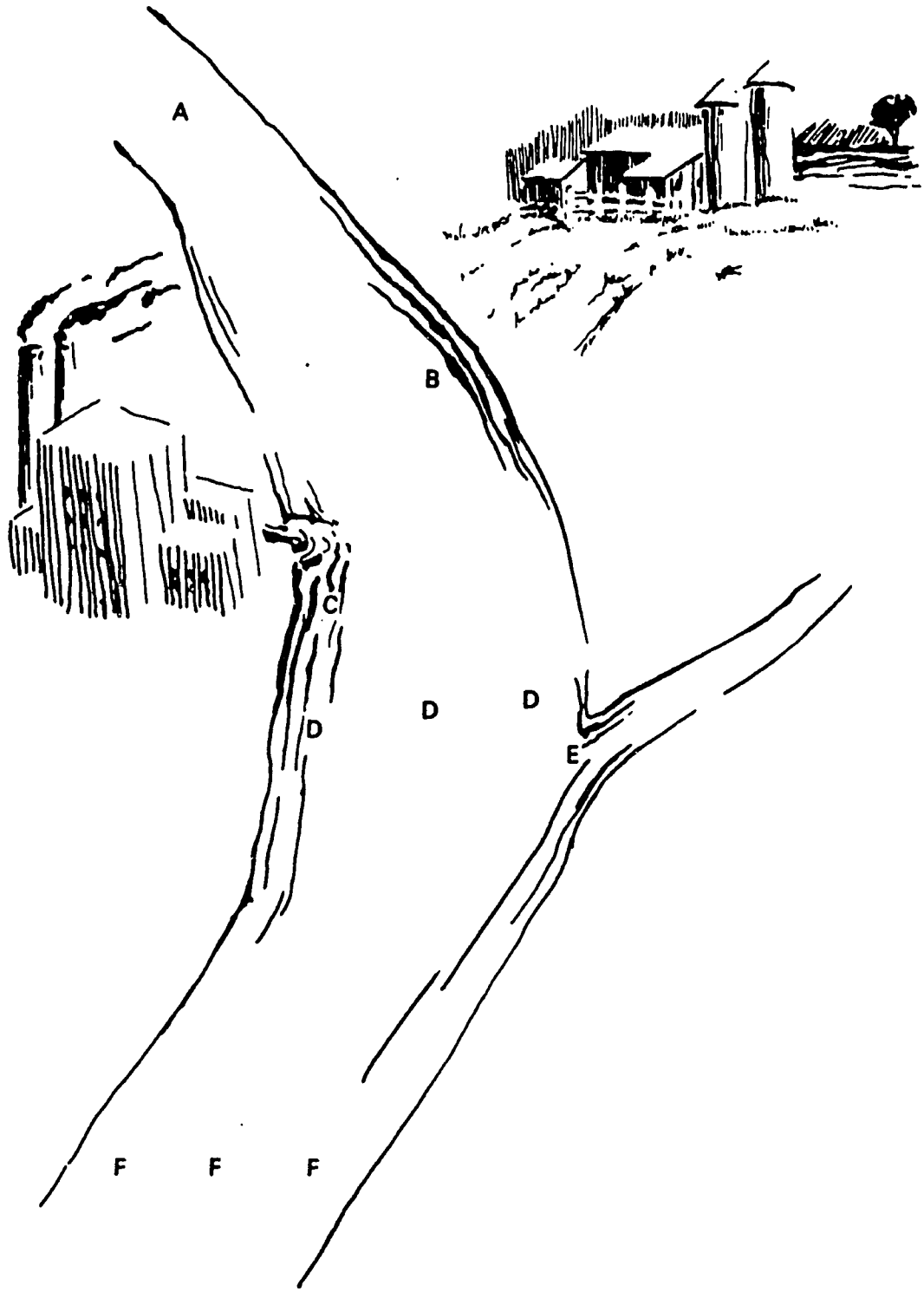


FIGURE II-A-15. Sampling a Large Stream. (A) control station, (B) agricultural pollution, (C) industrial discharge, (D) quarterpoint transect (E) tributary, and (F) downstream monitoring.

653

5.4 Marine and Estuarine Sampling

Sampling marine and estuarine waters requires the consideration of other factors in addition to those usually recognized in fresh water sampling. They include tidal cycles, current patterns, bottom currents and countercurrents, stratification, seasonal fluctuations, dispersion of discharges and multi-depth samplings.

The frequency of sampling varies with the objectives. When a sampling program is started, it may be necessary to sample every hour around the clock to establish pollutional loads and dispersion patterns. The sewage discharges may occur continuously or intermittently.

When the sampling strategy for a survey is planned, data may be available from previous hydrological studies done by Coast Guard, Corps of Engineers, National Oceanic and Atmospheric Administration (NOAA), U.S. Geological Survey, or university and private research investigations. In a survey, float studies and dye studies are often carried out to determine surface and undercurrents. Initially depth samples are taken on the bottom and at five feet increments between surface and bottom. A random grid pattern for selecting sampling sites is established statistically.

5.4.1 Marine Sampling: In ocean studies, the environmental conditions are most diverse along the coast where shore, atmosphere and the surf are strong influences. The shallow coastal waters are particularly susceptible to daily fluctuations in temperature and seasonal changes.

Sampling during the entire tidal cycle or during a half cycle may be required. Many ocean studies such as sampling over the continental shelf involve huge areas and no two areas of water are the same.

Selection of sampling sites and depths are most critical in marine waters. In winter, cooling of coastal waters can result in water layers which approach 0 C. In summer, the shallow waters warm much faster than the deeper waters. Despite the higher temperature, oxygen concentrations are higher in shallow than in deeper waters due to greater water movement, surf action and photosynthetic activity from macrophytes and the plankton.

Moving from the shallow waters to the intermediate depths, one observes a moderation of these shallow water characteristics. In the deeper waters, there is a marked stablization of conditions. Water temperatures are lower and more stable. There is limited turbulence, little penetration of light, sparse vegetation and the ocean floor is covered with a layer of silts and sediments.

5.4.2 Estuarine Sampling: When a survey is made on an estuary, samples are often taken from a boat, usually making an end to end traverse of the estuary. Another method involves taking samples throughout a tidal cycle, every hour or two hours from a bridge or from an anchored boat at a number of fixed points.

In a large bay or estuary where many square miles of area are involved, a grid or series of stations may be necessary. Two sets of samples are usually taken from an area on a given day, one at ebb or flood slack water, and the other three hours earlier, or later, at the half tidal interval. Sampling is scheduled so that the mid-sampling time of each run coincides with the calculated occurrence of the tidal condition.

In locating sampling sites, one must consider points at which tributary waters enter the main stream or estuary, location of shellfish beds and bathing beaches. The sampling stations can be adjusted as data accumulate. For example, if a series of stations half mile apart consistently show similar values, some of these stations may be dropped and other stations added in areas where data shows more variability.

Considerable stratification can occur between the salt water from the sea and the fresh water supplied by a river. It is essential when starting a survey of an unknown estuary to find out whether there is any marked stratification. This can be done by chloride determinations at different locations and depths. It is possible for

654

stratification to occur in one part of an estuary and not in another.

On a flood tide, the more dense salt water pushing up into the less dense fresh river water will cause an overlapping with the fresh water flowing on top. A phenomenon called a salt water wedge can form. As a result, stratification occurs. If the discharge of pollution is in the salt water layer, the contamination will be concentrated near the bottom at the flood tide. The flow or velocity of the fresh water will influence the degree of stratification which occurs. If one is sampling only at the surface, it is possible that the data will not show the polluted underflowing water which was contaminated at a point below the fresh water river. Therefore, where stratification is suspected, samples at different depths will be needed to measure vertical distribution.

5.5 Domestic and Industrial Waste Discharges

It is often necessary to sample secondary and tertiary wastes from municipal waste treatment plants and various industrial waste treatment operations. In situations where the plant treatment efficiency varies considerably, grab samples are collected around the clock at selected intervals for a three to five day period. If it is known that the process displays little variation, fewer samples are needed. In no case should a composite sample be collected for bacteriological examination. The NPDES has established wastewater treatment plant effluent limits for all dischargers. These are often based on maximum and mean values. A sufficient number of samples must be collected to satisfy the permit and/or to provide statistically sound data and give a fair representation of the bacteriological quality of the discharge.

5.6 Recreational Waters

5.6.1 Bathing Beaches: Sampling sites at bathing beaches or other recreational areas should include upstream or peripheral areas and locations adjacent to natural drains that would discharge stormwater, or run-off areas draining septic wastes from restaurants, boat marinas, or garbage collection areas (12). Samples of bathing beach water should be collected at locations and times of heaviest use. Daily sampling, preferably in the afternoon, is the optimum frequency during the season. Weekends and holidays which are periods of highest use must be included in the sampling program. Samples of estuarine bathing waters should be obtained at high tide, ebb tide and low tide in order to determine the cyclic water quality and deterioration that must be monitored during the swimming season.

5.6.2 Swimming Pools: Swimming pool water should be monitored at least daily during maximum use periods, preferably at the overflow. It is important to test swimming pool samples for neutralization of residual chlorine at pool side to assure that the dechlorinating agent was effective.

5.7 Shellfish-Harvesting Waters

Water overlying shellfish-harvesting areas should be sampled during periods of most unfavorable hydrographic conditions, usually at low tide after heavy precipitation. However, shellfish beds are sometimes exposed during low tide and must be sampled during other tidal conditions. Procedures for sampling of shellfish and water in shellfish growing areas are governed by the National Shellfish Sanitation Program's Manual of Operations (13).

5.8 Frequency of Sampling

The frequency of sampling depends upon the type of pollution that is to be measured. Cyclic pollution and its duration are measured as frequently as practical immediately downstream from the source. Uniform pollution loads are measured at greater distances downstream from the source and at less frequent time intervals than cyclic pollution. Climatic and tidal conditions must be considered in marine and estuarine sampling. A common approach for short-term studies is to collect samples from each site daily and advance the sampling intervals one hour during each 24-hour period to obtain data for a 7–10 day study.

655

Often the numbers of samples to be collected are specified by NPDES permits, drinking water regulations, or by state requirements. Some standards require a minimum number of samples to be collected each month. Other standards are less explicit and simply indicate that the geometric mean coliform density shall not exceed a certain level each month, with no more than 10%, 20%, etc. of samples exceeding a certain value. Where the number of samples required is undetermined, a sufficient number should be collected to measure the variations in stream conditions.

6. Preservation and Transit of Samples

The adherence to sample preservation and holding time limits is critical to the production of valid data. Samples exceeding these limits should not be analyzed. The following rules must be observed.

6.1 Storage Temperature and Handling Conditions

Bacteriological samples should be iced or refrigerated at a temperature of 1-4 C during transit to the laboratory. Insulated containers are preferable to assure proper maintenance of storage temperature. Care should be taken that sample bottles are not totally immersed in water during transit or storage.

6.2 Holding Time Limitations

Although samples should be examined as soon as possible after collection, they should not be held longer than six hours between collection and initiation of analyses (14). This limit is applied to fresh waters, seawaters and shellfish-bed waters. The exception is water supply samples mailed in from water treatment systems. Current regulations permit these samples to be held up to 30 hours.

6.2.1 Despite the establishment of a six hour limit, sewage samples, organically-rich wastes and marine waters are particularly susceptible to rapid increases or die-away and hence should be held for the shortest time possible to minimize change.

6.2.2 Temporary Field Laboratories: In situations where it is impossible to meet the six hour maximum holding time between collection and processing of samples, the use of temporary field laboratories located near the collection site should be considered.

6.2.3 Delayed Incubation Procedure: If sampling and transit conditions require more than six hours, and the use of field laboratories is impossible, the delayed incubation procedure for total and fecal coliforms and fecal streptococci should be considered.

6.2.4 Public Transportation: Occasionally, commercial forms of transit such as airlines, buslines or couriers can be used to transport samples contained in ice chests to the laboratory. These should be considered only when storage time and temperature requirements and the proper disposition of the samples can be assured.

REFERENCES

1. Public Health Laboratory Service Water Subcommittee, 1953. The effect of sodium thiosulfate on the coliform and *Bacterium coli* counts of non-chlorinated water samples. J. Hyg. 51:572.

2. Shipe, E. L. and A. Fields, 1954. Comparison of the molecular filter technique with agar plate counts for enumeration of *Escherichia coli* in various aqueous concentrations of zinc and copper sulfate. Appl. Microbiol. 2:382.

3. Shipe, E. L. and A. Fields, 1956. Chelation as a method for maintaining the coliform index in water supplies. Public Health Rep. 71:974.

4. Zobell, C. E., 1941. Apparatus for collecting water samples from different depths for bacteriological analysis. J. Marine Research 4:173.

5. Niskin, S., 1962. Water sampler for microbiological study. Deep Sea Research 9:501.

6. Fuhs, G. Wolfgang, 1977. Personal Communication. Director of the Environmental Health Center, Division of Lab and Research, New York State Health Department, Albany, New York.

7. Welch, P.S. 1948. Limnological Methods. Blakiston Company, Philadelphia, PA.

8. Van Donsel, D. J. and E. E. Geldreich, 1972. Relationships of Salmonella to fecal coliforms in bottom sediments. Water Research 5:1079.

9. Wills, Carroll, 1975 (June). Chain of Custody Procedures, National Enforcement Investigation Center-Denver, Colorado, U.S. EPA.

10. Guidelines Establishing Test Procedures for the Analysis of Pollutants. 40 CFR Part 136, 52780, as amended, December 1, 1976.

11. National Interim Primary Drinking Water Regulations, 40 Code of Federal Regulations, Amendments to Part 141, December 24, 1975.

12. Geldreich, E. E., 1975. Handbook for Evaluating Bacteriological Water Laboratories, (2nd ed.) U.S. Environmental Protection Agency, Municipal Environmental Research Laboratory, Cincinnati, Ohio. EPA-670/9-75-006.

13. Hauser, L. S. (ed.), 1965. National Shellfish Sanitation Program. Manual of Operations, Part I: Sanitation of shellfish growing areas. U.S. Public Health Service, Washington, D.C.

14. Public Health Laboratory Service Water Subcommittee, 1953. The effect of storage on the coliform and *Bacterium coli* counts of water samples. Storage for six hours at room and refrigerator temperatures. J. Hyg. 51:559.

PART II. GENERAL OPERATIONS

Section B Laboratory Equipment, Techniques and Media

Most equipment and supplies described in This Manual are available in well-equipped bacteriology laboratories. Other items specific for the membrane filter or multiple-tube dilution methods are used only in water laboratories. This Section describes the required equipment, media and preparation techniques used in the laboratory or in the field. The contents include:

1. Equipment and Supplies
2. Cleaning Glassware
3. Sterilization
4. Preparation and Use of Culture Media
5. Media Composition

   General Use Media
   MF Media for Coliforms
   MPN Media for Coliforms
   Media for Fecal Streptococci
   Media for *Salmonella* and Other Enterics
   Medium for Actinomycetes
6. Laboratory Pure Water
7. Dilution Water

1. Equipment and Supplies

1.1 Inoculating Needles and Loops: Needles and loops of nichrome, platinum or platinum-iridium wire are used to transfer microbes aseptically from one growth medium to another. A 24–26 gauge nichrome wire is recommended. The loop diameter should be at least 3 mm. They are sterilized by heating to redness in a gas flame or in an electric incinerator. Resterilization is not required for replicate transfers of the same bacteria or bacteria-containing materials to a sterile medium. Sterile, disposable hardwood applicator sticks or plastic loops can be used to inoculate fermentation tubes.

1.2 Plastic petri dishes (50 × 12mm) with tight-fitting lids are preferred for MF procedures because they retain humidity and are more practical for use in plastic bags submersed in a water bath incubator. Petri dishes (60 × 15 mm) with loose-fitting lids can be used in incubators with controlled humidity or in plastic boxes with tight covers, containing moist towels.

1.3 Incubators: Incubators are constant temperature air chambers or water baths which provide controlled temperature environment for microbiological tests. The incubator must control temperature within specified tolerance limits of the tests. The air type incubator can be water-jacketed, a dry air unit or aluminum block equipped with thermostat-controlled heating units that maintain 35 C ± 0.5 C. Overcrowding of incubators with plates and tubes must be avoided, because this will interfere with the constancy of the desired

temperature. Water bath or aluminum block type incubators used in fecal coliform tests must control the incubation temperature of 44.5 C ± 0.2 C. Covers are required. An accurate thermometer in the water bath monitors temperature. Recording thermometers are recommended for use in the water baths.

1.4 Colony Counter: A standard counting device equivalent to the Quebec Colony Counter that provides good visibility and magnification of at least 1.5 diameters on a non-glare ruled guide plate is recommended.

1.5 pH Equipment: Electrometric pH meter must be accurate to at least 0.1 pH unit.

1.6 Balances: For routine weighing of media and reagents, use a single pan top loader balance having a sensitivity of 0.1 gram at a load of 150 grams. For weighings of less than 2 grams, use a four place analytical balance having a sensitivity of 1 mg at a load of 10 grams.

1.7 Media Preparation Utensils: Use suitable non-corrosive utensils of plastic, glass, stainless steel, or non-chipped enamel. Utensils must be chemically clean before use to prevent contamination of media with chemicals such as chlorine, copper, zinc, chromium and detergents.

1.8 Pipets and Graduated Cylinders

1.8.1 Pipets: Because transfer pipets are an important element in any microbiological method, they must be properly used. If mouth-pipetting is practiced for non-polluted waters, pipets should be cotton-plugged for safety. Blow-out pipets are not to be used.

The major types of transfer pipets (non-volumetric) used in the laboratory are based on the method of draining:

(a) To Contain (TC) Pipets: Pipets calibrated to hold or contain the exact amount specified by the calibration. Pipet must be completely emptied to provide the stated volume. Examples: Transfer, Micro and Dual Purpose Pipets.

(b) To Deliver (TD) Pipets: Pipets designed to release the exact calibrated amount when the pipet tip is held vertically against the receiving vessel wall until draining stops. Examples: bacteriological, Mohr, serological, and volumetric pipets.

To Contain and To Deliver pipets are indicated by the letters TC or TD marked respectively on the neck with other calibration information.

(c) Blow-Out Pipets: These pipets are intended for rapid use in serology and are emptied by forceful blow-out. Because the blow-out action always produces an aerosol such pipets should never be used in the microbiology laboratory. The pipets do not deliver the calibrated amount until they are completely emptied.

Blow-Out pipets are marked with a double band etching or fired-in marking on the neck.

(d) Dual Purpose Pipets: A new pipet which combines three calibrations. The pipet has an upper graduation mark which is the To Deliver and Blow-Out line, a lower graduation which is the To Contain line, and carries the double band for Blow-Out.

For microbiological analyses, only bacteriological, serological or Mohr pipets are recommended for use in the To Deliver (TD) mode. Dual purpose or serological pipets which must be used as blow-out pipets, are not recommended for microbiology because of the danger of infection through aerosol and the possible mix-up of TD, TC and blow-out type pipets with subsequent misuse and improper delivery.

(e) Pipet Standards/Specifications: There are several tolerance specifications used to characterize measuring pipets. The analyst should be aware of these limits. When accuracy of measurements is critical, use only pipets within the following Class A limits:

NBS Specification for Mohr Measuring Pipets, Class A Volume Tolerances Circular 602 and Federal Specification NNN-P-350

Capacity/Grad (ml)	Tolerance (ml)
1/10 in 1/100	±0.0025
2/10 in 1/100	±0.004
1 in 1/100	±0.01
1 in 1/10	±0.01
2 in 1/10	±0.01
5 in 1/10	±0.02
10 in 1/10	±0.03
25 in 1/10	±0.05

For routine use, the following tolerances are acceptable:

APHA Specification, Bacteriological Pipets

Capacity/Grad (ml)	Tolerance (ml)
1 in 1	±0.025
1.1 in 1.1, 1.0	±0.025
2.2 in 2.2, 2.1 2.0, 1.0	±0.040
11 in 11	±0.10

USPHS Specification, Bacteriological Pipets for Water Analyses

Capacity/Grad (ml)	Tolerance (ml)
11 in 11, 10, 1	±0.06

1.8.2 Pipetting Devices: Mechanical pipetting devices are recommended for all purposes. Although several devices have recently become available, some are not practical for water analyses because they are too slow and cumbersome. A finger-mounted safety pipettor which can be fabricated in the laboratory (1, 2) is recommended as the most efficient. It can be fitted with a mouthpiece, hand-held bulb or attached to a vacuum pump. Figure II-B-1 shows the finger-held model and Figure II-B-2 shows an enlargement of the plastic ring suction device.

1.8.3 Graduates: For normal laboratory operations, graduates are used for measuring volumes greater than 10 ml. When extreme accuracy is required above 10 ml, volumetric pipets up to 200 ml and volumetric flasks from 10 ml to 6 liters are available. The tolerances given below are acceptable for graduates.

1.9 Pipet Container: Only aluminum, stainless steel, pyrex glass or other non-corrosive heat-resistant containers, either cylindrical or rectangular in shape, should be used. Pipets may be also wrapped in kraft paper. Copper or copper alloy containers must not be used.

1.10 Dilution (Milk Dilution) Bottles or Tubes: Borosilicate or other non-corrosive glass bottles with screw-caps and inert liners.

1.11 Fermentation Tubes and Vials: The fermentation tubes should be large enough to contain the media and inocula in no more than half of the tube depth. There should be sufficient media in the outer fermentation tube to fill the enclosed inverted vial after sterilization and partially submerge it.

NBS Specification, Tolerance of Graduates, in ml

Volume	Demarcations	NBS Class A	NNN-C-940 Type 1 Style 1
2000	20		±10.0
1000	10		±5.0
500	5	±1.3	±2.6
250	2	±0.8	±1.4
100	1	±0.4	±0.6
50	1	±0.26	

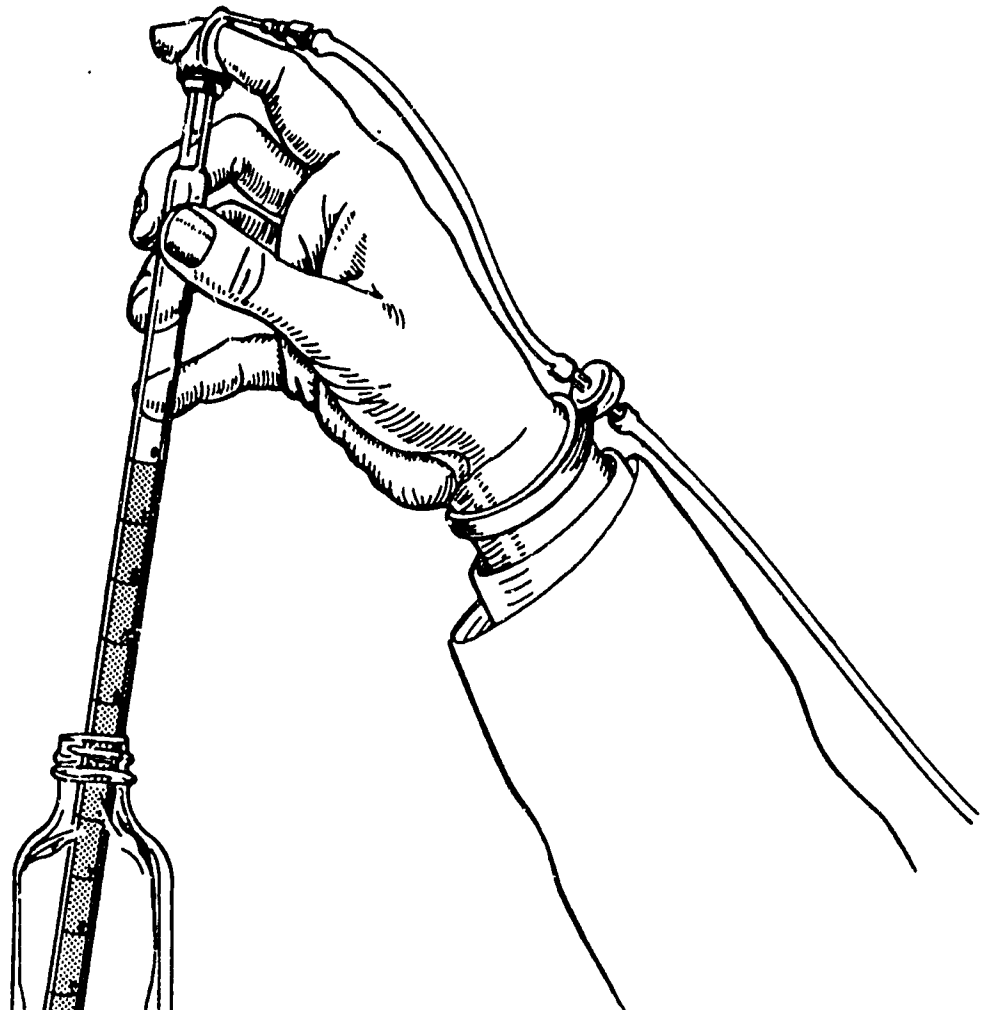


FIGURE II-B-1. Finger-Mounted Pipetting Device.

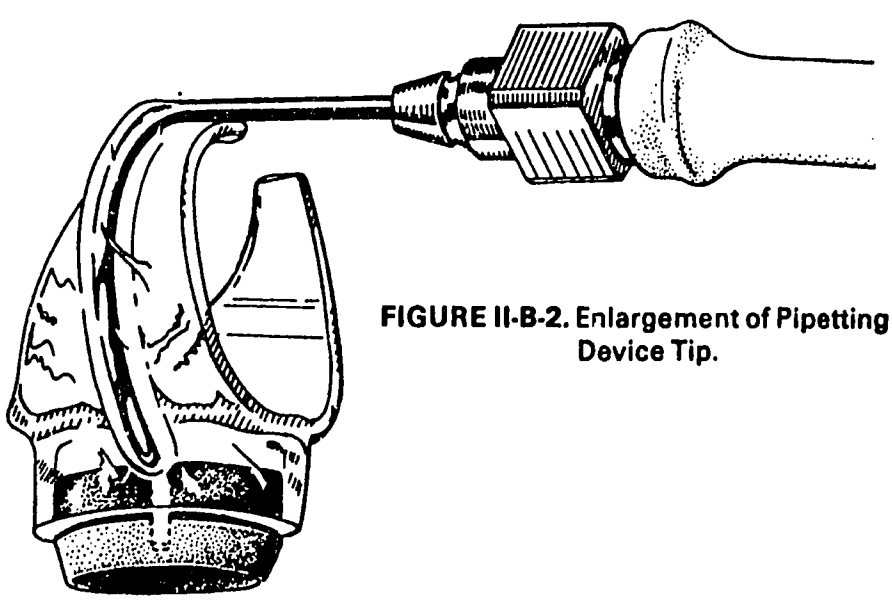


FIGURE II-B-2. Enlargement of Pipetting Device Tip.

66

1.12 Sample Bottles: Sample bottles should be borosilicate glass or plastic, resistant to sterilizing conditions and the solvent action of water and which do not produce toxic substances upon sterilization. Wide-mouth ground-glass stopper or screw-cap bottles are acceptable. Bottles equipped with screw-caps are acceptable provided that bacteriostatic or nutritive compounds are not produced from caps or liners. Bottles should be at least 125 ml volume.

2. Cleaning Glassware

In microbiology, clean glassware is crucial to insure valid results. Previously used or new glassware must be thoroughly cleaned with a phosphate-free laboratory detergent and hot water, then rinsed repeatedly with hot water, followed by at least three rinses with laboratory pure water. To determine whether the detergent used contains inhibitory residues, the test procedure for detergent suitability should be performed each time a new type detergent is purchased. See Part IV-A, 5.1.

3. Sterilization

Sterilization is the process that eliminates living organisms from treated substances or objects. Disinfection is the destruction or removal of the infectious agents by chemical or physical means. Usually chemical agents are used as disinfectants (germicide and bactericide are synonymous with the term, disinfectant). Pasteurization is a form of disinfection used for materials which may be altered or damaged by excessive heat. Low heat is applied once or repeatedly to sensitive liquids to destroy vegetative cells. Sterilization can be accomplished by moist heat, dry heat, incineration, filtration, radiation or by the use of the chemical agent, ethylene oxide. Bottles should have loosened caps for penetration of steam or gas.

3.1 Moist Heat: The autoclave is used in the laboratory for moist heat sterilization. It is normally operated at 15 lbs. per sq. in. steam pressure for 15 minutes, producing a temperature inside the autoclave of 121.6 C (250F) at sea level. Steam under pressure provides effective sterilization since it has good penetration power and coagulates microbial protoplasm. The temperature in the autoclave should be monitored. The relationship between the pressure of steam and the temperature is shown in Table II-B-1. The autoclave is used to sterilize solid and liquid media, contaminated materials, discarded cultures, glassware of all types, filtering units, etc. Pressure cookers and vertical autoclaves are not recommended.

3.2 Dry Heat: The hot-air oven is used to sterilize glassware such as petri dishes, pipets, sample bottles and flasks, hardwood applicator sticks, and other articles, but not liquids or materials which will evaporate or deteriorate. Since moisture is not present in the oven, a temperature of 165-170 C (329-338 F) is required for a 2 hour period.

3.3 Incineration: Contaminated materials that are combustible may be disposed of by burning. This method is also used for sterilizing inoculating needles and loops, and flaming the lips of test tubes and flasks.

3.4 Filtration. Filtration is used to sterilize liquids that are heat-sensitive. Filters include those composed of: asbestos-cellulose (Seitz filter), cellulose esters (0.22 μm MF or molecular filter), unglazed porcelain (Chamberland-Pasteur filter), or diatomaceous earth (Berkfeld filter). Although filters are normally used to remove bacteria, porosities small enough for the removal of viruses are available.

3.5 Ultraviolet Radiation: Ultraviolet light includes radiations between 150 and 4000 Angstrom units (A.U.), but radiations less than 1800 A.U. are absorbed by atmospheric oxygen. The greatest killing effect on microorganisms occurs at 2600 A.U. Commercial germicidal ultraviolet lamps emit primarily 2537 A.U. which has 85 percent of the germicidal ability of 2600 A.U. Ultraviolet radiation at the germicidal wavelength is used to sterilize laboratory equipment such as membrane filter units, inoculating rooms, bacteriological hoods and glove boxes. See Part IV-A, 4.2, for monitoring procedures.

3.6 Ethylene Oxide Chemical Sterilization: Low temperature ethylene oxide

TABLE II-B-1

Relationship of Steam Pressure to Temperature in the Autoclave

Pounds of pressure of steam per square inch	Corresponding Temperature In Degrees*	
	Celsius	Fahrenheit
0	100.0	212
5	108.3	227
10	115.5	240
15	121.6	250
20	126.6	260
25	130.5	267
30	134.4	274

*Correct at sea level and only if all air is evacuated from the sterilizing chamber since a mixture of steam and air at a given pressure gives a temperature that is less than that of pure steam only.

gas sterilization is used to sterilize plastics, rubber goods, delicate instruments and other materials that would be damaged by the high temperature of the steam pressure autoclave. Vent sterilized materials according to operating instructions.

4. Preparation and Use of Culture Media

The preparation of culture media and solutions is a critical aspect of water quality testing. In many laboratories media are prepared by nonprofessional personnel. If such personnel are properly trained and guided, they can perform the required tasks efficiently and reliably. However, the supervisor should monitor media preparation tc maintain quality control. Use commercial dehydrated media which require only weighing and dissolving of the powder in laboratory pure water for preparation and are much more likely to have uniform high quality than media compounded in the laboratory. See IV-A, 7.3-7.5 for quality control on preparation of media.

4.1 Supplies of Dehydrated Media: The laboratory worker should keep a record of the lot numbers of commercial media in use. The data of receipt of media and the date of opening should be recorded in the quality control log. It is suggested that supplies of dehydrated media be purchased for anticipated use over the next year. Whenever practical, one-quarter pound bottles should be purchased to insure minimal exposure of contents to atmosphere. When a new lot number of medium is used, the contents should be tested for expected performance characteristics (see Part IV-A for details). Stocks of dehydrated media should be stored in a cool, dry place away from sunlight.

4.2 Rehydration of Media: In this Manual, dry ingredients are added to 1 liter (1000 ml) of laboratory pure water. However, liquids such as ethanol are added to graduate and brought up to volume with laboratory pure water. Care must be taken to completely dissolve and mix the ingredients before dispensing the medium into bottles, tubes or flasks. If heat is necessary, it must be applied with caution and for the shortest possible time. Direct heat, boiling water bath and flowing steam are used selectively according to the type and volume of medium as described below:

4.2.1 MF Broths and Agars: Small volumes of broth are rehydrated in a boiling water bath for 5 minutes. Agars and larger volumes of broth require direct heating to the first bubble of boil. Such heating must be applied with stirring and constant attention until agar is dissolved.

4.2.2 Other Broths: Some heat may be required to dissolve the ingredients prior to autoclaving. Heat can be applied by flame or waterbath.

4.2.3 Other Agars: Heat is required for complete solution. For large volumes, direct heat will effect solution more rapidly but must be applied with stirring and constant attention to prevent scorching.

4.3 Sterilization: Media must be dissolved before autoclaving, to insure timing for complete sterilization. The specific recommendations for sterilization are described in subsection 5, Composition of Media, in This Section.

The following general recommendations can be made:

Group	Sterilization
MF and *Salmonella* Media	Heat to boiling only
Media Containing Blood	Autoclave, cool, then add blood
Litmus Milk and Other Milk-containing Media	115 C for 20 minutes (10 lbs. pressure)
Most Carbohydrate-Containing Media for Fermentation Reactions such as Phenol Red Broth, Triple Sugar Iron Agar	118 C for 15 minutes (12 lbs. pressure) or 121 C for 12 minutes

MPN, some *Salmonella* Media and General Use Media such as Trypticase Soy Broth and Agar, Nutrient Broth and Agar	121 C for 12-15 minutes (15 lbs. pressure)

As soon as the autoclave pressure has fallen to zero, the sterilized media should be removed from the autoclave for cooling before use or storage. Refrigerated media should be allowed to come to room temperature before use. Incubate MPN tubed media overnight and discard tubes showing bubbles. Media that have been poured into petri dishes should be used on the day of preparation or refrigerated. Water loss from evaporation can be prevented by storing plates in plastic bags. Plates should be stored inverted. See Part IV-A, 7.9 for storage limits.

Autoclaves should be inspected routinely to insure proper functioning of pressure gauges and thermometers. The use of commercially available temperature indicator devices within the sterilizer (heat-resistant spore preparations, chemically-impregnated tapes, vials containing chemicals, etc.) is recommended to insure sterility (see Part IV-A for details).

5. Composition of Media

The formulas for media used in this Manual and for other more commonly-used media are given in detail in this Section. Normally, these media should not be prepared from basic ingredients when suitable commercial media are available.

The catalogue numbers cited are those for ¼ lb. size if available, from the two U.S. manufacturers whose media were used in most of the method development work. This listing is not restrictive; other sources can be utilized if the user confirms that the formulas are the same as those cited here and that the media produce comparable results.

Culture media used for the examination of water and wastewaters are described separately for:

5.1 General Use Media
5.2 MF Media for Coliforms
5.3 MPN Media for Coliforms
5.4 Media for Fecal Streptococci
5.5 Media for *Salmonella* and Other Enterics
5.6 Medium for Actinomycetes

5.1 General Use Media

5.1.1 Nutrient Agar (Difco 0001–02, BBL 11471)

Use: This medium is used to cultivate pure culture isolates for subsequent gram stain and other examinations and for general cultivation of microorganisms that are not fastidious.

Composition:

Peptone	5.0 g
Beef Extract	3.0 g
Agar	15.0 g

Final pH: 6.8 ± .1

Preparation: Add 23 grams of nutrient agar per liter of laboratory pure water and mix well. Heat in boiling water bath to dissolve the agar completely. Dispense in screw-cap tubes, bottles or flasks and sterilize for 15 minutes at 121 C (15 lbs. pressure). Remove tubes and slant.

5.1.2 Nutrient Broth (Difco 0003–02, BBL 11478)

Use: General laboratory use for the cultivation of non-fastidious microorganisms.

Composition:

Peptone	5.0 g
Beef Extract	3.0 g

Final pH: 6.8 ± .1

5.5.5 Brilliant Green Agar (BBL 11072, Difco 0285-02)

Use: As a primary plating medium for the isolation of salmonellae.

Composition:

Yeast Extract	3.0	g
Polypeptone or Proteose Peptone	10.0	g
Sodium Chloride	5.0	g
Lactose	10.0	g
Saccharose (Sucrose)	10.0	g
Phenol Red	0.08	g
Brilliant Green	0.0125	g
Agar	20.0	g

Final pH: 6.9 ± .2

Preparation: Add 58 grams of brilliant green agar to 1 liter of cold laboratory pure water and heat to boiling. Dispense in screw-cap flasks and sterilize for 15 minutes at 121 C (15 lbs. pressure). Pour into sterile petri dishes.

Warning: A longer period of sterilization will reduce the selectivity of the medium.

5.5.6 Xylose Lysine Brilliant Green (XLBG) Agar

Use: *Salmonella* Differentiation.

Composition of XL Agar Base:

BBL 11836

Xylose	3.5	g
L-Lysine	5.0	g
Lactose	7.5	g
Saccharose (Sucrose)	7.5	g
Sodium Chloride	5.0	g
Yeast Extract	3.0	g
Phenol Red	0.08	g
Agar	13.5	g

Difco 0555-02

Xylose	3.75	g
L-Lysine	5.0	g
Lactose	7.5	g
Saccharose (Sucrose)	7.5	g
Sodium Chloride	5.0	g
Yeast Extract	3.0	g
Phenol Red	0.08	g
Agar	15.0	g

Final pH: 7.4 ± .2

Preparation: Add 45 or 47 grams of XL agar base to 1 liter of cold laboratory pure water. Heat in a boiling water bath to dissolve the agar. Prior to sterilization, add 1.25 ml of 1% aqueous brilliant green. Sterilize for 15 minutes at 121 C (15 lbs. pressure). Cool the sterilized medium to about 45-50 C and add 20 ml of a solution containing 34% sodium thiosulfate and 4% ferric ammonium citrate. Pour into sterile petri dishes.

5.5.7 Xylose Lysine Desoxycholate (XLD) Agar

Use: Differentiation of *Salmonella*.

Composition:

BBL 11837

Xylose	3.5	g
L-Lysine	5.0	g
Lactose	7.5	g
Saccharose (Sucrose)	7.5	g
Sodium Chloride	5.0	g
Yeast Extract	3.0	g
Phenol Red	0.08	g
Agar	13.5	g
Sodium Desoxycholate	2.5	g
Sodium Thiosulfate	6.8	g
Ferric Ammonium Citrate	0.8	g

Difco 0788-02

Xylose	3.75	g
L-Lysine	5.0	g
Lactose	7.5	g
Saccharose (Sucrose)	7.5	g
Sodium Chloride	5.0	g
Yeast Extract	3.0	g
Phenol Red	0.08	g
Agar	15.0	g
Sodium Desoxycholate	2.5	g
Sodium Thiosulfate	6.8	g
Ferric Ammonium Citrate	0.8	g

Final pH: 7.4 ± .2

loosely and sterilize for 15 minutes at 117 C (10 lbs. pressure).

5.5.17 H Broth (Difco 0451-02)

Use: Preparation of H agglutinating antigens of members of genus, *Salmonella*.

Composition:

BBL 11288

Thiotone (peptone)	10.0	g
Beef Extract	3.0	g
Dextrose	1.0	g
Sodium Chloride	5.0	g
Dipotassium Phosphate	2.5	g

Difco 0451–02

Thiotone (Peptone)	5.0	g
Tryptone	5.0	g
Beef Extract	3.0	g
Dextrose	1.0	g
Sodium Chloride	5.0	g
Dipotassium Phosphate	2.5	g

Final pH: 7.2 $\pm$ .2

Preparation: Add 21.5 grams of H broth to 1 liter of laboratory pure water, mix well and dissolve by warming. Dispense 5 ml amounts in screw-cap test tubes. Sterilize for 15 minutes at 121 C (15 lbs. pressure).

5.6 Medium for Actinomycetes

Starch-Casein Agar

(Medium may not be commercially available).

Use: Isolation of actinomycetes from water or soil.

Composition:

Soluble Starch (or Glycerol)	10.0	g
Casein (Vitamin-free)	0.3	g
Sodium Nitrate	2.0	g
Sodium Chloride	2.0	g
Dipotassium Phosphate	2.0	g
Magnesium Sulfate•7 H_2O	0.05	g
Calcium Carbonate	0.02	g
Iron Sulfate•7 H_2O	0.01	g
Agar	15.0	g

Final pH: 7.0–7.2

Preparation: Weigh out ingredients and add in turn to 1 liter of laboratory pure water in a two liter flask. Dissolve ingredients using gentle heat. Add the agar last and place in a boiling water bath. Heat and stir occasionally until dissolved. Dispense 250 ml volumes in 500 ml screw-cap flasks and 17 ml volumes in screw-cap tubes. Sterilize for 15 minutes at 121 C (15 lbs. pressure).

6. Laboratory Pure Water

Distilled or deionized water free of nutritive and toxic materials is required for preparation of media and dilution/rinse water.

6.1 Distilled Water Systems: Water distillation units can produce good grades of pure water. Stills are dependable and long-lived if maintained and cleaned properly. Use of softened water as the source water increases the interval between cleanings of the still. Stills characteristically produce a good grade of water which gradually deteriorates as corrosion, leaching and fouling set in. There is no sudden loss of water quality unless a structural failure occurs. Stills are efficient in removing dissolved chemicals but not dissolved gases. Fresh laboratory pure water may contain chlorine and ammonia. On storage, ammonia will increase and CO_2 will appear from air contamination. Distilled water systems should be monitored continuously for conductance and analyzed monthly for chlorine, ammonia and standard plate count and at least annually for trace metals. See Table IV-A-3.

6.2 Deionized Water Systems: Deionization systems produce a good grade of pure water and can produce an ultrapure water when combined with filtration and activated carbon in a recirculating system.

In contrast to distilled water systems, deionization systems do not gradually deteriorate. Rather, they continue to produce the same quality water for a long period of time until the resins and/or carbon are exhausted, whereupon the quality deteriorates quickly. Deionized water systems should be monitored continually with a conductance meter and analyzed monthly for ammonia/amines, total organic carbon, specific organic pollutants and Standard Plate Count and at least annually for metals. Amines may elute from the resin. Organic carbon results from organic chemicals in the water or from bacterial growth in the columns. Use of a 0.22 μm final filter is recommended to remove bacterial contamination. See Table IV-A-3.

Avoid the sudden loss of good quality water by continuously monitoring performance of the system, anticipating the remaining life of cartridges and replacing them before failure occurs.

6.3 Quality Control

Pure water systems should be monitored carefully as a part of the intralaboratory QC program. The water quality should meet the standards set in this Manual, Part IV-A, 5.2-5.4.

7. Dilution Water

Dilution water is used to reduce the number of microbial cells/unit volume of sample so that the density of cells is low enough to permit enumeration or manipulation by the technique of choice: pour-plate, spread plate, MF or MPN.

The ideal dilution water is neutral in effect. It maintains bacterial populations without stimulating cell growth and reproduction, damaging cells or reducing their ability to survive, grow or reproduce. Its basic purpose is to simulate the chemical conditions of the natural environment which are favorable to cell stability.

Microbiologists have tried different approaches to obtain an ideal diluent. Some workers have copied the natural environment by use of sterile fresh or marine waters as diluents, but these are non-standard. Other workers have used tap waters with the same lack of uniformity and an added potential for toxicity. Certainly for comparability of microbiological data the dilution water must be uniform between laboratories. The chemical elements and compounds required in natural conditions to insure a balance of cell solutes and maintain cell turgidity must be reproduced in the laboratory.

Inorganic constituents such as sodium, potassium, magnesium, phosphate, chloride and sulfate, and soluble organics such as peptone are used in synthetic dilution waters. pH is usually held to a near-neutral reaction. Two standard dilution waters are:

7.1 Phosphate Buffered Dilution Water

7.1.1 Stock Phosphate Buffer Solution

Potassium Dihydrogen Phosphate (KH_2PO_4)	34.0	g
Laboratory Pure Water	500	ml

Adjust the pH of the solution to 7.2 with 1 N NaOH and bring volume to 1000 ml with laboratory pure water. Sterilize by filtration or autoclave for 15 minutes at 121 C (15 lbs. pressure).

7.1.2 Storage of Stock Solution: After sterilization of the stock solution store in the refrigerator until used. Handle aseptically. If evidence of mold or other contamination appears, the stock solution should be discarded and a fresh solution prepared.

7.1.3 Working Solution

Stock Phosphate Buffer	1.25	ml
$MgCl_2$ Solution (38 g/liter)	5	ml
Laboratory Pure Water	1000	ml

Final pH: 7.2 ± 0.1

7.2 Peptone Dilution Water

Composition:

Peptone	1.0	g

Laboratory Pure Water 1000 ml

Final pH: 7.0 ± 0.1

7.3 Preparation of Dilution and Rinse Water

Dispense 102 ml volumes of phosphate buffer or peptone dilution water into borosilicate glass, screw-cap dilution bottles scribed at 99 ml. Loosen screw-caps on bottles and sterilize at 121 C for 15 minutes (15 lbs. pressure). Final volumes after sterilization should be 99 ± 2 ml. Cool and separate bottles outside of the 99 ml ± 2 ml limit. Tighten screw-caps after sterilization and store in a cool place.

Prepare dilution water for rinsing in 500 ml or larger volumes and autoclave for 30 minutes or more. Bottles or flasks must be separated sufficiently in the autoclave to permit easy access for steam.

REFERENCES

1. Songer, J. R., J. F. Sullivan and J. W. Monroe, 1971. Safe, convenient pipetting device. Appl. Microbiol. 21:1097.

2. Songer, J. R., D. T. Braymen and R. G. Mathis, 1975. Safe, convenient pipetting station. Appl. Microbiol. 30:887.

3. Taylor, R., R. Bordner and P. Scarpino, 1973. Delayed incubation membrane-filter test for fecal coliforms. Appl. Microbiol. 25:363.

4. Taylor, W. I., 1965. Isolation of Shigellae. I. Xylose lysine agars; new media for isolation of enteric pathogens. Am. J. Clin. Path. 14:471.

GENERAL REFERENCES

Difco Laboratories. General Conditions Pertaining to the Cultivation of Microorganisms; and, Preparation of Media from Dehydrated Culture Media. Difco Manual. 9th Edition. Difco Laboratories, Detroit, MI. p. 16 (1953).

Baltimore Biological Laboratories. General Suggestions for Use of Media: Dehydrated-Prepared. BBL Manual of Products and Laboratory Procedures. 5th Edition, BBL, Division of Becton-Dickinson and Co., Cockeysville, MD. p. 88 (1968).

Geldreich, E. E., 1975. Handbook for Evaluating Water Laboratories (2nd Edition). EPA-670/9-75-006, U.S. Environmental Protection Agency, Cincinnati, OH p. 83.

American Public Health Association, 1976. Standard Methods for the Examination of Water and Wastewater (14th Edition). American Public Health Association, Inc. p. 892.

169

PART II. GENERAL OPERATIONS

Section C Techniques for Isolation and Enumeration of Bacteria of Sanitary Significance

This Section describes the fundamental laboratory procedures needed for microbiological analyses of water and wastewater. *Although experienced microbiologists and technicians may not require the depth of information and the degree of detail given in this Section, it is provided to serve the technical personnel who are new to environmental microbiology.* The procedures included are:

1. **Preparation for Analyses**
2. **Streak Plate, Pour Plate and Spread Plate Methods**
3. **Membrane Filtration Method**
4. **Most Probable Number Method**
5. **Staining Procedures**
6. **Shipment of Cultures**

The specific details unique to tests are described in separate Sections of Part III: Standard Plate Count, Total Coliform, Fecal Coliform, Fecal Streptococci, *Salmonella* and Actinomycetes. Refer to Part IV, A & C for details of quality control on these analyses.

1. Preparation for Analyses

1.1 Preparation of Data Sheets

Select a standard format for bench sheet or card and use to record pertinent data: sample identification, sampling conditions, chlorine residual, temperature, pH, sampling site, station number, date and time of collection, sample collector, required chain of custody information, data and time received and analyzed, time elapsed from sample collection, analyses performed, sample volume(s), analyst, and laboratory identification numbers. (See Figures II-C-1, 2 and 3).

1.2 Disinfection of Work Area

Wipe the work area before and after use with laboratory-strength disinfectant and allow surface to dry before use. See Table V-C-3.

Keep a covered container of iodophor or quaternary ammonium disinfectant available for emergency use. Phenolics are acceptable if analyses for these compounds are not performed as part of laboratory work.

1.3 Pretreatment of Samples

Prior to dilution of samples for analyses, the analyst should examine the sample for free chlorine residual and possible uneven distribution of microorganisms.

610

MICROBIOLOGY LABORATORY RECORD
MEMBRANE FILTER ANALYSIS

PROJECT: _____ NO: _____

SAMPLE SOURCE: _____

SAMPLING DATE: _____ TIME: _____ FILTRATION DATE: _____ TIME: _____

COLLECTOR: _____ TEMP: _____ pH: _____ ANALYST: _____

VOL.	COUNT	COUNT/100 ml		VOL.	COUNT	COUNT/100 ml		VOL.	COUNT	COUNT/100 ml	

Remarks: _____ Remarks: _____ Remarks: _____

EPA-209 (Cin)
(Rev- 4-76)

FIGURE II-C-1. Microbiological Bench Card for MF Analyses.

MICROBIOLOGY LABORATORY RECORD
COLIFORM MPN ANALYSIS

Sample # _____ Date _____ Time _____ EST DST River _____

Location _____

Tide _____ Rain past 24 Hrs. _____ Depth _____ Water Temp. °C _____ Secchi Disc. _____

Dilution					MPN/100 ml
Coliform Pres. LST 24 Hrs.					
48 Hrs.					
Coliform Conf. BGLB 48 Hrs.					
Fecal Coliform EC 24 Hrs.					

AFO, Region III, USEPA

FIGURE II-C-2. Bench Card for MPN Analyses.

BACTERIAL INDICATOR ORGANISMS OF POLLUTION

STATION _____ BENCH NO _____

LOCATION _____

DATE HOURS SAD NO. _____

COLLECTED . . . . . ANALYST

EXAMINED . . . . . _____

M P N

DIL. ml.	PRESUMPTIVE LTB 35°C		CONFIRMED B.G.L.B. 35°C		EC 44.5°C	MEMBRANE FILTER	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	VOLUME FILTERED	COLONY COUNT
10							TOTAL COLIFORMS
10							FECAL COLIFORMS
10							
10						ORGANISMS PER 100 ML.	
10						TOTALS _____	
						FECALS _____	
						SALMONELLA _____	
						ISOLATION _____	
						SEROTYPE(S) _____	

CONFIRMED MPN . . . . . PER 100 ML

FECAL COLIFORM MPN . . . . . PER 100 ML.

REMARKS _____

SERL, Region IV, US EPA

FIGURE II-C-3. Combined Microbiological Bench Card

677

1.3.1 Water Samples with High Solids

(a) Blending of sediments, primary effluents, sludge and highly turbid waters is essential for representative subsampling.

(b) Blend the entire water sample containing particulates in a Waring-type blender. Use only autoclavable pyrex glass, stainless steel or plastic blender containers with safety screw covers to prevent release of aerosols.

(c) Limit blending to no more than 30 seconds at about 5000 RPM to avoid overheating or shearing damage.

(d) Dilute sediments or soils containing limited amounts of water at a 1:1, 1:2 ratio or more with dilution water to ensure good blending action and to reduce heat generation. Use of a large blender container rather than smaller units also reduces heat.

1.3.2 Dry Solid Samples

(a) Mix sample thoroughly and weigh 50 grams aliquot in a tared weighing pan. Dry at 105-110 C to constant weight. The final weight is used in calculating numbers of organisms/gram dry solids.

(b) Prepare the initial dilution by weighing out a second aliquot of 11 grams of original sample. Add to a 99 ml volume of buffered dilution water for a 1:10 dilution and blend sample aseptically in a Waring-type blender at 5000 rpm for 30 seconds. Use only a pyrex glass, stainless steel or plastic blender container with safety screw lid to prevent release of aerosols.

(c) Transfer an 11 ml sample of the 1:10 dilution to a second dilution bottle containing 99 ml buffered dilution water and shake vigorously about 25 times. Repeat this process until the desired dilution is reached.

1.3.3 Analytical Method

Although high solids samples with low microbial densities may require MPN or pour plate procedures, high density samples such as polluted soils, sludges and feces are diluted so that the MF method is applicable.

1.4 Dilution of Samples

1.4.1 Necessity for Dilutions: Dilutions of the original sample of water, wastewater or other material are often necessary to reduce the number of bacterial cells to measureable levels or to isolate single cells for purification and identification (see Part II-B, 7 for details on dilution water).

1.4.2 Serial Dilutions: A known quantity of the sample (usually 1.0 ml, but other volumes can be used) is transferred through a series of known volumes (e.g., 9 or 99 ml) of dilution water. This procedure is repeated until the desired bacterial density is reached. After dilution of the sample, the bacteria are enumerated using the membrane filtration, pour plate, streak plate, or the most probable number technique.

For ease of calculation and preparation, serial dilutions are usually prepared in succeeding ten-fold volumes called decimal dilutions. The decimal dilution procedure is shown in Figure II-C-4.

1.4.3 Special Dilutions for Membrane Filtration Procedures: The normally accepted limits for colonies per plate in membrane filtration methods (20–60, 20–80 or 20–100) require that decimal dilution series be modified to assume an MF plate count within the accepted limit.

The recommended method for obtaining counts within these limits is to filter dilution volumes of the decimal series which have a factor of 3, 4 or 5 among them (see Table II-C-1 for details).

1.4.4 Filtration Volumes for Membrane Filter Analyses: For sample volumes of 1-10 ml, add 20 ml of dilution water to the funnel before adding sample, to evenly disperse cells.

1.4.5 Preparation of Dilutions: Shake sample bottle vigorously (about 25 times in 7 seconds) to evenly distribute the bacteria. Take care to secure the screw-cap and prevent leakage during shaking.

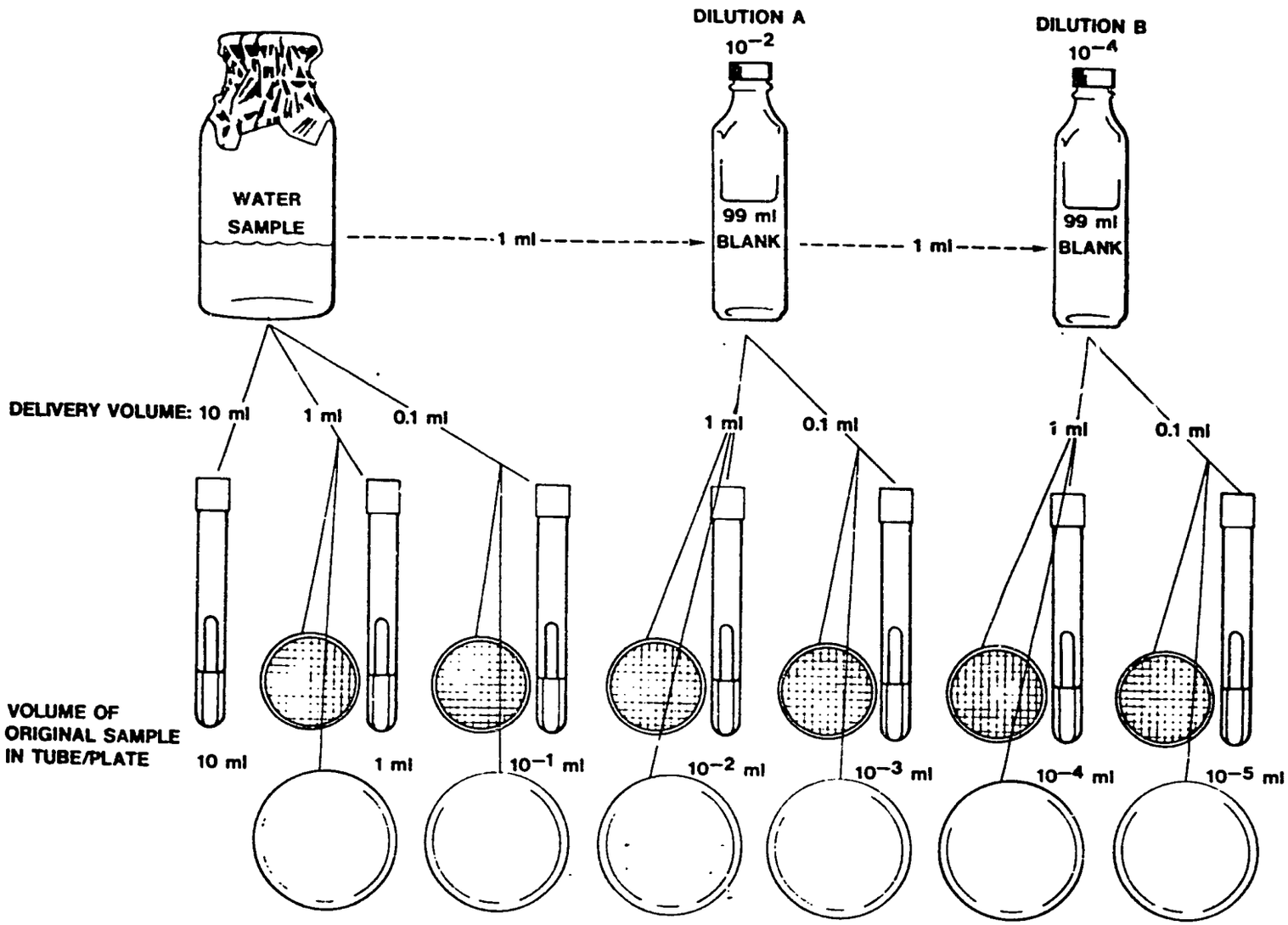


FIGURE II-C-4. Preparation of Decimal Dilution.

TABLE II-C-1

Recommended Filtration Volumes of Samples in MF Analyses

MF Count Range 20–60		MF Count Range 20–80		MF Count Range 20–100	
Sample vol., ml	added as:	Sample vol., ml	added as:	Sample vol., ml	added as:
.01	1 ml of 10^{-2}	.04	4 ml of 10^{-2}	.01	1 ml of 10^{-2}
.03	3 ml of 10^{-2}	.15	1.5 ml of 10^{-1}	.05	5 ml of 10^{-2}
.1	1 ml of 10^{-1}				
.3	3 ml of 10^{-1}	.5	5 ml of 10^{-1}	.25	2.5 of 10^{-1}
1.0	1 ml sample				
3	3 ml sample	2.0	2 ml sample	1.25	1.25 ml sample
10	10 ml sample	8.0	8 ml sample	6	6 ml sample
30	30 ml sample	30	30 ml sample	30	30 ml sample

(a) Withdraw 1.0 ml or 0.1 ml of original sample to test samples directly for a 1×10^0 ml and 1×10^{-1} ml volumes, respectively.

(b) Transfer a second 1.0 ml volume to a 99 ml dilution water bottle (Dilution A). Shake sample vigorously about 25 times and withdraw 1.0 ml or 0.1 ml of diluted sample for testing of 1×10^{-2} and 1×10^{-3} ml sample volumes. Theiresultant dilution is calculated as follows:

$$\frac{\text{Volume of Sample}}{\text{Volume of Dilution Blank} + \text{Volume of Sample}} = \text{Dilution Ratio}$$

or, [illegible]

$$\frac{1.0 \text{ ml}}{99.0 + 1.0 \text{ ml}} = \frac{1}{100}$$

or 10^{-2} (Dilution A)

(c) When 1.0 ml is transferred from dilution bottle A to a second dilution bottle (B), the dilution ratio for bottle B dilution shown in Figure II-C-4 is the product of the individual dilutions as follows:

$$A \times B = \text{Final or Total Dilution Ratio}$$

or

$$\frac{1}{100} \times \frac{1}{100} = \frac{1}{10{,}000}$$

Volumes of 0.1 ml can be tested directly from each serial dilution to provide intermediate dilutions.

(d) Alternatively, if an initial sample volume of 11 ml is transferred into the first 99 ml volume dilution blank, an intermediate dilution can be obtained with the added precision resulting from measurements of 1 ml volumes serially as opposed to the measurement of 0.1 ml volumes in (c).

(e) Different dilutions can be obtained by varying sample and dilution preparations. For example, if 2.0 ml of the sample is transferred to a 98.0 ml dilution blank, the final dilution ratio is calculated as follows:

$$\frac{2.0 \text{ ml}}{98.0 + 2.0 \text{ ml}} = \frac{2}{100} = \frac{1}{50}$$

(f) If 0.5 ml is added to a dilution blank containing 99.5 ml of buffer, the dilution ratio is calculated as follows:

$$\frac{0.5 \text{ ml}}{99.5 + 0.5 \text{ ml}} = \frac{0.5}{100} = \frac{1}{200}$$

(g) Varying the final volume tested will also permit modification of dilutions without increasing the number of dilution bottles as follows:

A 1:200 dilution can be obtained by testing 0.5 ml of a 1:100 dilution.

A 1:500 dilution can be obtained by testing 2 ml of a 1:1000 dilution.

1.4.6 Prompt Use of Dilutions: The potential toxicity of phosphate dilution water and the stimulatory effect of peptone dilution water increase rapidly with time. Therefore, dilutions of samples should be tested as soon as possible after make-up and should be held no longer than 30 minutes after preparation.

2. Streak, Pour and Spread Plate Methods

2.1 Summary: There are three plate dilution techniques commonly used for isolating and/or enumerating single colonies of bacteria: the streak plate, pour plate and spread plate procedures. These techniques described herein use solid or melted agar plating media to dilute out the microorganisms so that individual species or cells can be selected or counted from mixed cultures. Because colonies can originate from more than one cell, results may be reported as colony-forming-units (CFUs).

2.1.1 Streak Plate Method: To obtain a pure culture the analyst dilutes and isolates bacteria from a mixed culture by drawing a small amount of the bacterial growth lightly across the surface of an agar plate in a pattern with an inoculating needle or loop.

In one suggested pattern, the plate is streaked in parallel lines over half of the surface, rotated a quarter turn, streaked again, rotated another quarter turn and streaked once more. The inoculum is progressively diluted with each successive streak, and eventually single cells are deposited on the agar surface. After suitable incubation, single isolated colonies develop in the path of the streak. (see Figure II-C-5).

2.1.2 Pour Plate Method: The analyst dilutes and isolates cells in a bacterial suspension by consecutively transferring a portion of the original sample through a series of dilution water blanks. After an appropriate series of dilutions, the original bacterial population is diluted out to a countable level, as described in 1.4. Aliquots of the diluted sample are added to sterile petri dishes and mixed with melted agar. After the agar solidifies, the plates are inverted and incubated for a predetermined time. Surface or subsurface colonies will develop in some of the agar plates. These colonies can be counted to provide a quantitative value for the bacterial density of the original sample, or they can be picked for further qualitative study.

2.1.3 Spread Plate Method: The analyst isolates bacterial cells by delivering a small volume of a diluted sample onto a solid agar plate and spreading the inoculum by use of a sterile glass rod bent at an angle of about 120°. The inoculum is spread uniformly by holding the stick at a set angle on the agar and rotating the agar plate or rotating the stick until the inoculum is distributed evenly.

2.2 Scope and Application

2.2.1 Streak and pour plate methods provide the means to separate individual bacteria so that each cell will develop into an isolated colony in or on a solid medium. The methods can isolate specific bacteria by the use of selective or differential media.

The streak plate is only qualitative but the pour plate procedure can be used to quantitate bacteria present in a sample as in the Standard Plate Count Method (see Part III-A). The spread plate method provides a quantitative method for aerobic surface growth of cultures against which other surface growth methods such as the MF technique can be compared.

2.2.2 Because the volumes tested with the spread plate technique are limited to 0.1–0.5 ml, the sample must be diluted to contain at least 40–2000 cells/ml in order to have a counting range of 20–200.

2.2.3 In the pour plate technique, test volumes are limited to 0.1–2 ml per 100 ml petri dish so the sample must be diluted to contain 60–3000 cells/ml to have a 30–300 counting range.

2.3 Apparatus and Materials

2.3.1 Incubator set at 35 ± 0.5 C.

2.3.2 Water bath set at 44–46 C for tempering agar.

2.3.3 Colony Counter, Quebec darkfield model or equivalent.

2.3.4 Hand tally or electronic counting device.

2.3.5 Thermometer which has been checked against a National Bureau of Standards-Certified Thermometer or one of equivalent accuracy.

2.3.6 Inoculating needle and loop.

2.3.7 Pipet containers of stainless steel, aluminum or pyrex for glass pipets.

2.3.8 Petri dish containers of stainless steel, aluminum or pyrex glass for glass petri dishes.

2.3.9 Glass spreader rods.

2.3.10 Sterile T.D. bacteriological or Mohr pipets, glass or plastic of appropriate volume.

2.3.11 Sterile 100 mm × 15 mm petri dishes, glass or plastic.

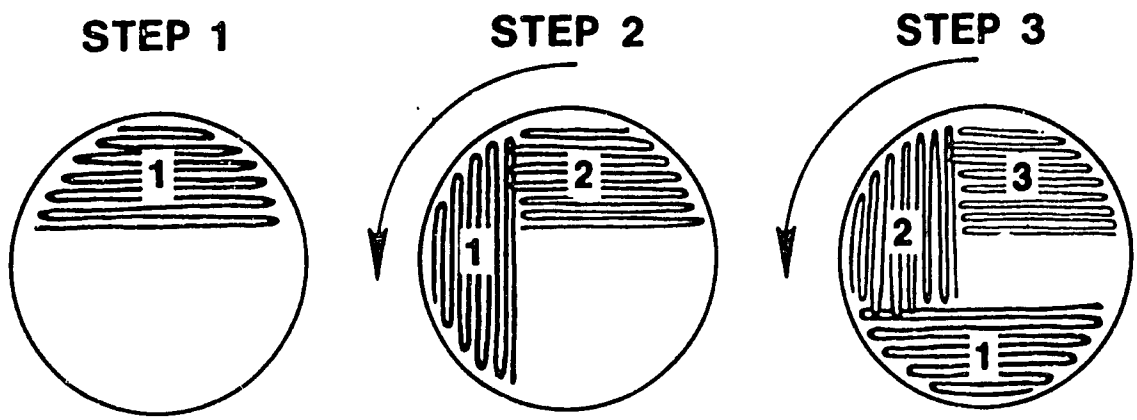


FIGURE II-C-5. Suggested Pattern for Preparing a Streak Plate.

2.3.12 Dilution (milk dilution) bottles, pyrex, marked at 99 ml volume, screw-cap with neoprene rubber liner.

2.3.13 Bunsen/Fisher type burner or electric incinerator.

2.4 Media

2.4.1 Sterile agar medium dispensed in bulk quantities in screwcapped bottles or flasks.

2.4.2 Sterile dilution water in bottles containing 99 $\pm$ 2 ml volumes.

2.5 Streak Plate Procedures

2.5.1 Melt the nonselective agar, such as nutrient agar or Trypticase soy agar, temper at 44-46 C, and add about 15 ml agar to each sterile petri dish. Allow to harden and dry for best results in streaking.

2.5.2 Bend an inoculation needle or loop at an angle about 1 cm from the needle tip to prevent cutting of the agar during streaking. Sterilize the needle by heating it to redness in a flame, and air-cool.

2.5.3 Remove screw cap and pick a small amount of growth from an isolated colony or from a mass of growth.

2.5.4 Draw the needle containing the bacteria back and forth across the surface of a previously-poured and hardened agar plate in a specific pattern, such as shown in Figure II-C-5. Streak 1/3–1/2 of the agar surface. Flame and cool needle after each step and inoculate plate further by drawing the needle across the area previously streaked.

2.5.5 Rotate the plate one quarter turn clockwise as each step is completed to make the streaking easier. Streaking patterns other than the model shown in Figure II-C-5 can be used; the objective is simply to deposit fewer and fewer cells along the streak until single cells are deposited on the agar surface. After incubation, these cells will develop into well-separated pure colonies of bacteria, each theoretically arising from a single bacterium.

2.5.6 After streaking, incubate the petri dishes at 35 C for 24 hours (or other appropriate conditions) in an inverted position to prevent condensation of water on the agar surface. Moisture interferes with development of isolated colonies by spreading bacterial growth over the agar surface.

2.5.7 For further purification, examine plates after incubation for single, well-isolated colonies. Pick typical colonies using a sterile inoculating needle, suspend cells in dilution water, and restreak on an agar plate, repeating steps 2.5.2–2.5.6. Isolated, single colonies from a plate containing like colonies may be considered to be pure.

2.5.8 Streaking may also be done on selective media, such as Endo or EMB agars or on selective/differential media e.g., in *Salmonella* testing.

2.6 Pour Plate Procedure

2.6.1 Shake the sample bottle vigorously about 25 times to disperse the bacteria. During shaking, close cap tightly to prevent leakage of sample and the danger of contamination.

2.6.2 Using a 1.1 ml bacteriological pipet, prepare the initial dilution by pipeting 1 ml of the sample into a 99 ml dilution water blank. This initial dilution represents a 10^{-2} dilution (see this Section, 1.4, Dilution of Samples).

2.6.3 Using the same pipet transfer 0.1 and 1.0 ml from the undiluted sample to two separate petri dishes.

2.6.4 Shake the 1:100 dilution bottle vigorously again and pipet 0.1 and 1.0 ml of the 1:100 dilution into two petri dishes using another sterile 1.1 ml pipet.

2.6.5 Pour aseptically 12–15 ml of the melted agar medium cooled to 44–46 C into each petri dish. Mix agar and inoculum by rotation, being careful to prevent spillover of

agar. One recommended technique uses a sequence of five rotations to the left, five to the right and five forward and backward. Allow the agar to solidify on a level surface.

2.6.6 Invert the dishes and incubate at the specific temperature and time. After incubation, well-isolated surface and subsurface colonies should develop in some of the plates.

2.6.7 When the pour plate technique is used quantitatively, count plates containing between 30 and 300 colonies. This is the technique used in the Standard Plate Count Method described in Part III-A. Pour plate counts are reported as the count per ml.

$$\text{Count/ml} = \frac{\text{Sum of Plate Counts}}{\text{Total Volume of Sample in ml}}$$

2.7 Spread Plate Procedure

2.7.1 Prepare the appropriate melted agar. Pour about 15 ml of the melted agar into each 100 mm petri dish. Keep covers opened slightly until agars have hardened and moisture or condensation have evaporated. Close dishes and store in refrigerator. Warm at room temperature before use.

2.7.2 Prepare a series of dilutions based upon the estimated concentration of bacteria so that 0.1-0.5 ml of inoculum will give a 20-200 count (equivalent to a 40-2000 count/ml in the diluted sample). The dilutions should bracket the estimated density of bacteria. The analyst must remember that if only 0.1 ml volume is tested, it must be plated on the agar plate marked with the next higher dilution; for example, 0.1 ml of the 10^{-1} dilution onto the surface of the plate marked 10^{-2}. Inoculate agar plates.

2.7.3 Remove the glass spreader from alcohol and flame. Cool for 15 seconds. Test glass rod on edge of agar to verify safe temperature before use. This step can be simplified by making and sterilizing a number of glass spreaders.

2.7.4 Place cool glass spreader on agar surface next to inoculum. Position spreader so that the tip forms a radius from the center to the plate edge. Holding spreader motionless, rotate plate several revolutions, or hold plate and move the spreader in a series of sweeping arcs. The purpose is to spread the inoculum uniformly over the entire surface of the agar.

2.7.5 Lift the glass spreader from the agar and place in alcohol solution. Cover plate partially, leaving open slightly to evaporate excess moisture for 15-30 minutes.

2.7.6 When agar surfaces are dry, close dishes, invert them and incubate as required for the specific test.

2.7.7 After incubation at the proper time and temperature, isolated surface colonies should develop in one or more dilutions within the acceptable counting range of 20-200 collonies. The maximum recommended number of colonies/spread plate is fewer than for other plate techniques because surface colonies are larger than subsurface colonies and crowding can result at lower count levels.

2.7.8 Count the colonies by normal techniques and report on a count/ml or count/100 ml basis dependent on the use of the data.

2.8 Reporting Results

2.8.1 Significant Figures: To prevent false precision in the reporting of counts, the plate counts must be limited to the digit(s) known definitely plus one digit which is in doubt. These combined digits are termed the Significant Figures (S.F.).

(a) For example, if an analyst reports a plate count of 124 to three significant figures he is indicating that he is certain of the first two digits, 1 and 2, but is uncertain whether the last digit is 3, 4 or 5. If the analyst were reporting that same number to two significant figures, he would report the first figures, 1, as certain, the second figure, 2, as uncertain, and the third figure, 4, as unknown. Hence he would report it as 120, inserting the zero only as a spacer. Large counts of 1200, 12,000 and

12,000,000 only contain two significant figures. Of course, zeros can be significant in counts of 10, 50, 105, etc.

(b) In plate count and MF methods, the number of significant digits which can be reported are dictated by the method itself as follows: within the acceptable counting range of the method itself, i.e., 20–60, 20–80, 20–100 or 30–300 the actual number of colonies observed is the best estimate of the true density. The number of significant figures are equal to the number of colonies.

TABLE II-C-2

Number of Significant Figures (S.F.) Reported

Actual Colony Count	Pour Plate/ Spread Plate Method	Membrane Filtration Method
1 - 9	1 S.F.	1 S.F.
10 - 99	2 S.F.	2 S.F.
100 - 300	3 S.F.	—

2.8.2 Rounding Off Counts: Since plate counts must be limited to the number of significant figures obtainable by the method, the non-zero number which is not significant should be treated by the standard scientific convention:

(a) If the insignificant digit is less than five, replace it with a zero, e.g., 3530 becomes 3500.

(b) If the insignificant digit is five, round the preceding significant digit to the nearest even number, e.g., with two S.F., 3450 becomes 3400, and 3550 becomes 3600.

(c) If the insignificant digit is greater than five, drop the digit and increase the preceding significant number by one, e.g., 3480 becomes 3500.

3. Membrane Filtration Method

3.1 Summary: The membrane filter method provides a direct count of bacterial colonies on the surface of the filter. The sample is filtered as soon as possible after collection. After the sample is filtered, the membrane filter is placed on a nutrient medium formulated to encourage growth of the bacteria for which the test is designed and to suppress the growth of other microorganisms. After incubation under the specified conditions, the bacteria retained on the surface of the membrane develop into visible colonies. The medium and the temperature of incubation influence the kinds and appearance of bacteria that develop. Two-step enrichment and delayed incubation MF procedures can also be used. The two-step procedure involves an acclimation period on another medium before the selective growth step.

3.2 Scope and Application

Membrane filter methods are preferred over MPN or other techniques, where applicable because of the following advantages.

3.2.1 Advantages

(a) One of the primary advantages of this method is its speed. Definitive results for total and fecal coliforms can be obtained in 22–26 hours, whereas 48–96 hours are required for the multiple-tube fermentation method.

(b) Considerably larger, more representative water samples can be examined than with the MPN. With waters of low bacterial densities such as finished waters, larger sample aliquots can be used to enhance the reliability of the results.

(c) The precision is greater with the MF than the multiple-tube technique because the former makes a direct count of colonies/unit volume.

(d) The method represents savings in time, labor, space, supplies and equipment.

(e) Because of its portability, this procedure is also very practical for field studies.

3.2.2 Limitations: Although the majority of water samples can be tested by membrane filtration, there are limitations with certain samples and some problems with membrane filters themselves.

(a) Some samples contain large quantities of colloidal materials or suspended solids such as iron, manganese or alum flocs or clay (1). Other samples may contain algae. These substances can clog the filter pores and prevent filtration or can cause the development of spreading bacterial colonies. When the bacterial counts of such samples are high, a smaller volume or a higher sample dilution can be used to minimize the effect of sample turbidity. The membrane filter method may be used with samples containing turbidity by filtration of several smaller replicate sample volumes and compositing the results. However, with waters of high turbidity and low bacterial count, the membrane filter method may not be applicable. In the latter situation the multiple-tube procedure should be used.

(b) Large non-specific populations may mask the appearance of indicators on selective media such as M-Ehdo MF medium (4).

(c) Industrial wastewaters may contain zinc, copper, or other heavy metallic compounds (2) which adsorb onto the membrane surface and interfere with subsequent bacterial development (1, 2, 3).

(d) MF analyses require preparation of MPN tubed media for verification.

(e) Inhibition may result in seawater or from toxic materials such as chlorine or phenols.

(f) Indicator organisms stressed in the environment may be poorly recovered (5).

3.3 Apparatus and Materials

3.3.1 Incubator set at 35 ± 0.5 C for total coliform test (Part III-B) and fecal streptococci test (Part III-D).

3.3.2 Water bath or other type of incubators such as the aluminum heat sink incubator, or equivalent, set at 44.5 ± 0.2 C for fecal coliform test (Part III-C).

3.3.3 Stereoscopic microscope, with magnification of 10–15 ×, wide-field type.

3.3.4 A microscope lamp producing diffuse light from cool, white fluorescent lamps adjusted to give maximum color or sheen appearance.

3.3.5 Hand tally.

3.3.6 Pipet container of stainless steel, aluminum or pyrex glass for glass pipets.

3.3.7 Graduated cylinders covered with aluminum foil or kraft paper and sterilized.

3.3.8 Membrane filtration units (filter base and funnel), glass, plastic or stainless steel, see Figure II-C-6. These are wrapped with aluminum foil or kraft paper and sterilized. See Figure II-C-7 for an exploded view of a stainless steel MF assembly and filter.

3.3.9 Ultraviolet sterilizer for the filter funnel is optional (6).

3.3.10 Line vacuum, electric vacuum pump or aspirator is used as a vacuum source. In an emergency or in the field, a hand pump or a syringe can be used. Such vacuum-producing devices should be equipped with a check valve to prevent the return flow of air.

3.3.11 Vacuum filter flask, usually 1 liter, with appropriate tubing. Filter manifolds to hold a number of filter bases are optional.

3.3.12 Safety trap flask placed between the filter flask and the vacuum source.

3.3.13 Forceps, straight or curved, with smooth tips to permit easy handling of filters without damage.

3.3.14 Alcohol, ethanol or methanol, in small wide mouthed vials, for sterilizing forceps.

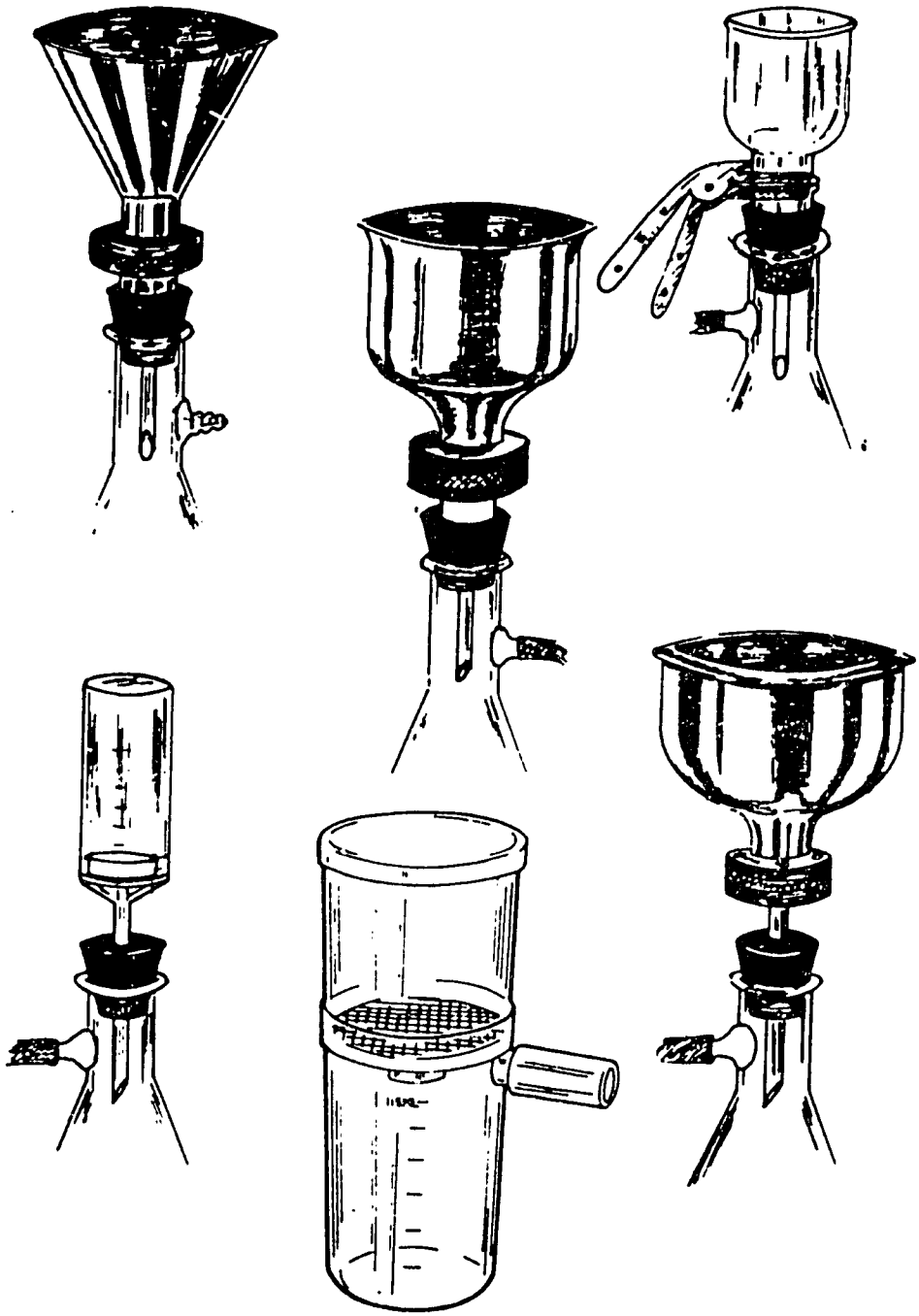


FIGURE II-C-6. Membrane Filtration Units Made by Various Manufacturers for Detection of Bacteria in Aqueous Suspensions.

633

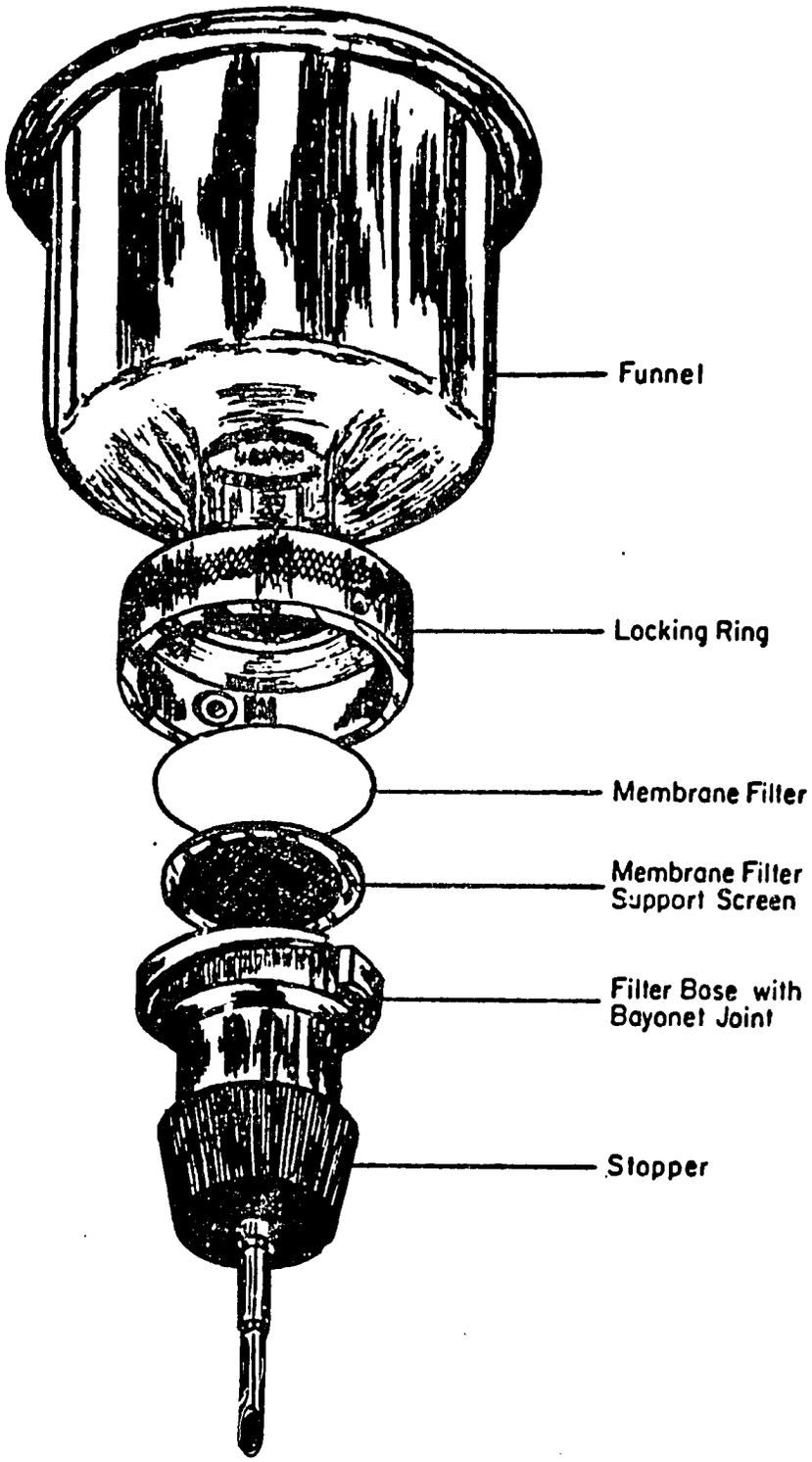


FIGURE II-C-7. Exploded View of a Stainless Steel Membrane Filtration Unit.

3.3.15 Bunsen or Fisher type burner or electric incinerator unit.

3.3.16 Sterile T.D. bacteriological or Mohr pipets, glass or plastic, of appropriate volume.

3.3.17 Sterile petri dishes, 50 × 12 mm, with tight-fitting lids and 60 × 15 mm, with loose-fitting lids glass or plastic.

3.3.18 Dilution bottles (milk dilution) pyrex, marked at 99 ml, screw-cap with neoprene liners.

3.3.19 Membrane filters, white, grid marked, 47 mm diameter, with 0.45 μm ± .02 μm pore size or other pore size recommended by the manufacturer for water analyses.

3.3.20 Absorbent pads of cellulosic paper, 47 mm diameter. The paper should be of high quality and free of sulfites or other substances that could inhibit bacterial growth.

3.3.21 Waterproof plastic bags.

3.3.22 Inoculation loops, at least 3 mm diameter, or needles, nichrome or platinum wire, 26 B&S gauge, in suitable holder. Disposable applicator sticks or plastic loops as alternatives to inoculation loops.

3.3.23 Media: Media required for a specific test should be prepared in pre-sterilized erlenmeyer flasks with metal caps, aluminum foil covers, or screw caps.

3.3.24 Dilution Water

(a) Sterile buffered or peptone dilution water dispensed in 99 ± 2 ml amounts in screw-capped dilution bottles.

(b) Sterile dilution water prepared in larger volumes for wetting membranes before addition of the sample and for rinsing the funnel after sample filtration.

3.4 Procedure

3.4.1 Prepare the required media as outlined in Part II-B. If the medium is an agar, cool to room temperature. Use sterile forceps for manipulation of absorbent pads and membrane filters, contacting the outer edges only, to avoid touching the filtering area or damaging the membrane filter surface. Sterilize forceps by immersing the tips in ethanol and flaming. Place absorbent pad in bottom of 50 or 60 mm petri dish. Add 1.8–2.0 ml broth to the sterile absorbent pad. Saturate but do not flood the pad. Tip the petri dish to drain off excess. If agar medium is used, add about 5–6 ml (to a depth of 2–3 mm) in the petri dish.

3.4.2 Arrange petri dishes in rows according to the dilution series. Mark each dish to identify the sample, volume or dilution to be filtered.

3.4.3 Using sterile forceps, place a membrane filter, grid-side up, on the porous plate of the filter base.

3.4.4 Attach the funnel to the base of the filter unit, taking care not to damage or dislodge the filter, The membrane filter is now fitted between the funnel and the base.

3.4.5 Shake the sample container vigorously about 25 times.

3.4.6 Prepare at least three sample increments according to 1.4.3, in this Section. Measure the desired volume of sample into the funnel with the vacuum turned off. To measure the sample accurately and to obtain good distribution of colonies on the filter surface, the following methods are recommended:

(a) Sample volumes of 20 ml or more: Measure the sample in a sterile graduated cylinder and pour it into the funnel. Rinse the graduate twice with sterile dilution water, and add the rinse water to the funnel. For potable waters, 100 ml volumes may be measured directly in a precalibrated funnel.

(b) Sample volumes of 10-20 ml: Measure the sample with a sterile 10 ml or 20 ml pipet into the funnel.

(c) Sample volumes of < 10 ml: Pour about 10 ml of sterile dilution water into the funnel and add the sample to the sterile water using appropriate sterile pipet.

(d) Sample volumes of less than 0.1 ml: Prepare appropriate dilutions in sterile dilution water and proceed as applicable in steps (b) or (c) above.

(e) The time elapsing between preparation of sample dilutions and filtration should be minimal and never more than 30 minutes.

3.4.7 Turn on the vacuum to filter the sample. Leave the vacuum on and rinse down the funnel walls at least twice with 20–30 ml of sterile dilution water. Turn off vacuum.

3.4.8 Remove the funnel from the base of the filter unit. An ultraviolet sterilizer unit can be used to hold and sterilize the funnel between filtrations. At least 2 minutes exposure time is required for funnel decontamination (6). Protect eyes from UV irradiation with glasses, goggles, or an enclosed UV chamber (7).

3.4.9 Holding the membrane filter at its edge with a sterilized forceps gently lift and place the filter grid-side up in the culture dish. Slide the filter onto the absorbent pad or agar, using a rolling action to avoid trapping air bubbles between the membrane filter and the underlying pad or agar. Reseat the membrane if non-wetted areas occur due to air bubbles.

3.4.10 Invert the petri dishes and incubate at the appropriate temperature in an atmosphere with close to 100% relative humidity for the required time.

3.5 Counting Colonies: The grid lines are used in counting the colonies.

3.5.1 Count the colonies for the parameter of interest following a preset plan such as shown in Figure II-C-8. Some colonies will be in contact with grid lines. A suggested procedure to reduce error in counting these colonies is shown in Figure II-C-9. Count the colonies in the squares indicated by the arrows.

3.5.2 The fluorescent lamp should be nearly perpendicular to the membrane filter. Count colonies individually, even if they are in contact with each other. The technician must learn to recognize the difference between two or more colonies which have grown into contact with each other and single, irregularly shaped colonies which sometimes develop on membrane filters. The latter colonies are usually associated with a fiber or particulate material and the colonies conform to the shape and size of the fiber or particulates. Colonies which have grown together almost invariably show a very fine line of contact.

3.6 Calculation of Results: Select the membrane filter with the number of colonies in the acceptable range and calculate count per 100 ml according to the general formula:

$$\text{count per 100 ml} = \frac{\text{No. colonies counted}}{\text{Volume of sample filtered, in ml}} \times 100$$

3.6.1 Counts Within the Acceptable Limits

(a) The acceptable range of colonies which is countable on a membrane is a function of the parameter as shown in Table II-C-3.

(b) Assume that filtration of volumes of 50, 15, 5, 1.5, and 0.5 ml produced colony counts of 200, 110, 40, 10, 5, respectively.

(c) An analyst would not actually count the colonies on all filters. By inspection he would select the membrane filter(s) with 20–80 coliform colonies and then limit his actual counting to such membranes.

(d) After selecting the best membrane filter for counting, in this case the MF with a 40 colony count, the analyst counts colonies according to the counting procedures in 3.6 and applies the general formula as follows:

$$\text{Colonies per 100 ml} = \frac{40}{5} \times 100 = 800$$

686

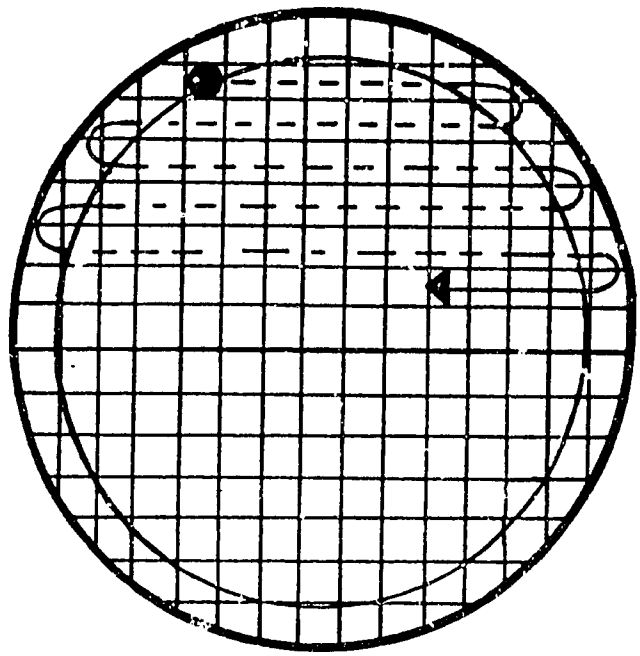


FIGURE II-C-8. Colony-Counting Pathway. (The inner circle indicates the effective filtering area, dashed line indicates the pathway.)

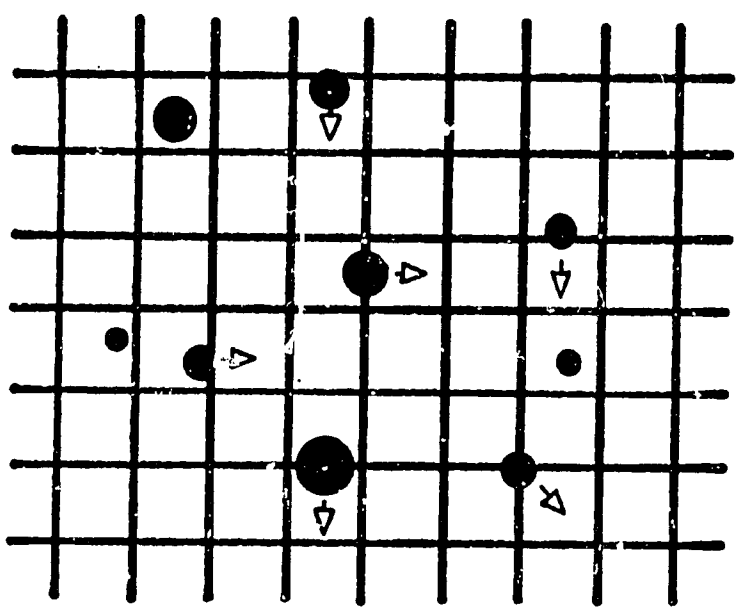


FIGURE II-C-9. Enlarged Portion of Grid-Marked Square of Filter. (Colonies are counted in squares indicated by the arrow.)

TABLE II-C-3

Acceptable Limits*

Parameters	Lower	Upper	Remarks
Total coliform bacteria	20 (0 for potable waters)	80	Limit, 200 colonies
Fecal coliform bacteria	20	60	of all types
Fecal streptococci	20	100	

* Colony counts < or > the limits cited above must be identified as outside of this range.

3.6.2 More Than One Acceptable Count

(a) If there are acceptable counts on replicate plates, carry counts independently to final reporting units, then calculate the arithmetic mean of these counts to obtain the final reported value.

For example, 1 ml volumes produce coliform counts of 26 and 36 or counts of 2600 and 3600/100 ml:

$$\frac{2600 + 3600}{2} = 3100$$

and value = 3100/100 ml

(b) If more than one dilution, independently carry counts to final reporting units, then average for final reported value.

For example, assume that volumes of 0.3, 0.1, 0.03 and 0.01 ml produced coliform colony counts of TNTC (Too Numerous To Count), 75, 30 and 8, respectively. In this example, two volumes, 0.1 and 0.03 produce colonies in the acceptable counting range.

Independently carry each MF count to a count per 100 ml:

$$\frac{75}{0.1} \times 100 = 75{,}000/100 \text{ ml}$$

$$\frac{30}{0.03} \times 100 = 100{,}000/100 \text{ ml}$$

Then calculate the arithmetic mean of these counts to obtain the final reported value:

$$\frac{75{,}000 + 100{,}000}{2} = 87{,}500$$

Report as: 88,000/100 ml.

3.6.3 If All MF Counts are Below the Lower Limit, Select the Most Nearly Acceptable Count (for non-potable waters)

For example, assume a count in which sample volumes of 1, 0.3 and .01 ml produced colony counts of 14, 3, and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 14, and report with a qualifying remark:

$$\frac{14}{1.0} \times 100 = 1400$$

Report as: Estimated Count, 1400 per 100 ml.

3.6.4 If Counts from All Membranes are Zero, Calculate Using Count from Largest Filtration Volume

For example, sample volumes of 25, 10, and 2 ml produced colony counts of 0, 0, and 0, respectively, and no actual calculation is possible, even as an estimated report. Calculate the number of colonies per 100 ml that would have been reported if there had been one colony on the filter representing the largest filtration volume, thus:

$$\frac{1}{25} \times 100 = 4$$

68

Report as: < (Less than) 4 colonies per 100 ml.

3.6.5 If All Membrane Counts are Above the Upper Limit, Calculate Count with Smallest Volume Filtered

For example, assume that the volumes 1, 0.3, and 0.01 ml produced colony counts of TNTC, 150, and 110 colonies. Since all colony counts are above the recommended limit, use the colony count from the smallest sample volume filtered and estimate the count as:

$$\frac{110}{0.01} \times 100 = 1{,}100{,}000$$

Report as: Estimated Count

1,100,000 per 100 ml.

3.6.6 If Colonies are Too Numerous To Count, Use Upper Limit Count with Smallest Filtration Volume

Assume in Example 3.6.5 that the volumes 1.0, 0.3, and 0.01 ml, all produced too many colonies to show separated colonies, and that the laboratory bench record showed TNTC (Too Numerous to Count).

Use 80 colonies (Upper Limit Count for Total Coliform) as the basis of calculation with the smallest filtration volume, thus:

$$\frac{80}{0.01} \times 100 = 800{,}000$$

Report as: > (Greater Than) 8000,000 per 100 ml.

3.6.7 If there is no result because of confluency, lab accident, etc. Report as: No Result and specify reason.

3.6.8 Reporting Results: Report bacterial densities per 100 ml of sample. See Figure II-C-1 and II-C-3 for examples of forms for reporting results.

3.7 Verification: A verified membrane filter test establishes the validity of colony differentiation on a selective medium and provides support evidence of colony interpretation.

3.7.1 A percent verification can be determined for any colony validation test:

$$\frac{\text{No. of colonies meeting verification test}}{\text{No. of colonies subjected to verification}} \times 100 = \text{Percent verification}$$

3.7.2 Verification is required for all positive samples from potable waters.

3.7.3 Verification is also recommended for establishing quality control in research for use with new test waters, new procedures or new technicians, for identifying unusual colony types and as support for data used in legal actions.

3.7.4 The worker is cautioned not to apply the percentage of verification determined for one sample to other samples.

3.7.5 The careful worker may also pick non-typical colonies and follow the verification procedure to determine that false negative colonies do not occur.

3.8 Significant Figures: See 2.8.1.

3.9 Repeatability and Reproducibility of Counts: Analysts should be able to duplicate their own colony counts on the same membrane within 5% and the counts of other analysts within 10%. Failure to agree within these limits should trigger a review of procedures.

4. Most Probable Number (MPN) Method

4.1 Summary: The Most Probable Number procedure estimates the number of specific organisms in water and wastewater by the use of probability tables.

Decimal dilutions of samples are inoculated in series into liquid tube media. Positive tests are indicated by growth and/or fermentative gas production. Bacterial densities are based on combinations of positive and nega-

681

tive tube results read from the MPN table. The MPN procedure may be carried to three stages of completion:

4.1.1 The Presumptive Test provides a preliminary estimate of bacterial density based on enrichment in minimally-restrictive tube media. The results of this test are never used without further analyses.

4.1.2 The Confirmed Test, the second stage of the MPN, is the usual extent of testing. Growth from each positive Presumptive Test tube is inoculated into a more selective inhibitory medium. The tubes are incubated at the prescribed temperature and time, the positive reactions noted and counts calculated from the MPN table.

4.1.3 The Completed Test is the third stage of the MPN used for total coliform analyses only. Positive tubes from the Confirmed Test are submitted to additional tests to verify the identification of the isolated microorganisms. Although the Completed Test provides the greatest reliability, the amount of time and the workload restrict its use to periodic substantiation of Confirmed Test results, to other QC checks on methodology and analysts, and to research.

4.2 Scope and Application

4.2.1 Advantages: The MPN procedure has the advantages inherent in liquid nutrient media.

(a) The Presumptive and Confirmed Tests require only observing and recording of gas/no gas for coliforms and growth/no growth for fecal streptococci. The tests require minimal experience, training or interpretation by the analyst.

(b) Water samples with high turbidity or large numbers of algae have no apparent deleterious effect on the tube reactions.

(c) If a toxic substance is present in the sample, the resultant 1:10 or 1:100 dilution of that sample in the liquid broth may reduce the toxicity to the point of no effect.

(d) The MPN may be the only method applicable to problem sample materials such as bottom sludges, muds, soils and sediments (with blending).

4.2.2 Limitations: The MPN procedure has disadvantages:

(a) This method is ordinarily limited to a maximum sample volume of 10 ml per tube, but 100 ml portions are used in shellfish waters.

(b) The time required for the test may be as long as 96 hours for a Confirmed Test result.

(c) The MPN tables are probability calculations and inherently have poor precision and contain a 23% bias at the 5 tube, three dilution levels normally used.

(d) The man-hour requirements to prepare glassware and media and to perform the tests are significant.

(e) Relatively large amounts of bench space, incubator space and tube/rack storage space are required.

(f) The procedure does not lend itself to field work. As compliance monitoring of water quality and effluent standards becomes a major legal requirement, the time, precision and equipment limitations cited in (b), (c), (d) and (e) above are more serious for the large number of field analyses which will be required.

(g) Background organisms or toxic constituents in 10 ml volumes of marine water can interfere and be undetected.

4.2.3 The minimum MPN test that is acceptable for water and wastewater analyses is the Confirmed Test because of the high probability of false positive reactions in the Presumptive Test.

690

4.3 Apparatus and Materials

4.3.1 Water bath or air incubator set at 35 ± 0.5 C for total coliform and fecal streptococci tests. Water bath at 44.5 ± 0.2 for fecal coliform test.

4.3.2 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

4.3.3 Inoculation loops, 3 mm diameter, and needle of nichrome or platinum wire, 26 B&S gauge, in suitable holder.

4.3.4 Disposable applicator sticks or plastic loops as alternatives to inoculation loops in 4.3.3 above.

4.3.5 Compound microscope, oil immersion, 1000×.

4.3.6 Culture tube racks, 10 × 5 openings; each opening to accept 25 mm diameter tubes.

4.3.7 Gas burner, Bunsen/Fisher types or electric incinerator unit.

4.3.8 Sterile T.D. bacteriological or Mohr pipets, glass or plastic, of appropriate sizes.

4.3.9 Dilution bottles (milk dilution), pyrex glass, marked at 99 ml volume, screw cap with neoprene liner.

4.3.10 Pyrex culture test tubes 150 × 25 or 150 × 20 mm containing inverted fermentation vials, 75 × 10 mm and proper closures.

4.3.11 Gram stain solutions (optional). See This Section 5.3.

(a) Hucker's Crystal Violet Solution (stain).

(b) Lugol's Iodine Solution (mordant).

(c) Acetone Alcohol (decolorizer).

(d) Safranin Solution (counterstain).

4.3.12 Glass microscope slides, 2.5 × 7.6 cm (1 × 3 inches).

4.4 Media: Appropriate media dispensed in test tubes or in fermentation test tubes. See Part IIB. for specific media.

4.5 Dilution Water: Sterile 99 ± 2 ml volumes in screw-cap pyrex glass bottles. See Part II-B, 7.

4.6 Presumptive Test

4.6.1 Shake the sample or dilution container vigorously about 25 times.

4.6.2 To perform the Presumptive Test, arrange a series of three or more rows of culture tubes containing the test medium in a rack, providing for five replicates in each row. Use five rows for samples of unknown density. Inoculate each successive row with decreasing decimal dilutions of the sample. For example, in testing polluted waters for total coliforms, the initial sample inoculations might be 0.1, 0.01, 0.001, 0.0001, 0.00001 ml of original sample into successive rows each containing five replicate volumes. This series of sample volumes would yield determinate results from test waters containing up to 16,000,000 organisms per 100 ml by use of the MPN tables.

When removing sample aliquots or dilutions for further inoculations, do not insert the pipet tip more than 2.5 cm (1 inch) below the surface of the sample.

4.6.3 Incubate tubes for 24 ± 2 hours at 35 C. A positive presumptive test is gas production for the coliforms or growth for fecal streptococci. After 24 hours incubation, examine the tubes for gas formation and/or growth. Inoculate positive tubes into Confirmed Test media. If there is no gas or growth reincubate these negative tubes for an additional 24 hours.

4.6.4 If the Presumptive tubes are negative after 48 ± 3 hours, discard tubes. If the Presumptive tubes are positive, the cultures are verified in the Confirmed Test. Record the negative and positive results.

4.7 Confirmed Test

4.7.1 The Confirmed Test is performed by verifying positive tubes from the Presumptive Test at 24 and 48 hours. If Presumptive tubes are positive at 24 hours, confirm them at that time.

4.7.2 A positive test is indicated by gas production for the coliform bacteria or growth for fecal streptococci. After 24 ± 2 hours incubation, examine the tubes for gas formation and/or growth. If there is no gas/growth, reincubate these negative tubes for a second 24 hours.

4.7.3 After 48 hours ± 3 hours examine tubes for gas and/or growth, record positive and negative results. Discard negative tubes. Retain positive tubes if the test is to be carried to completion for total coliform tests.

4.7.4 The fecal coliform MPN test is performed by inoculating EC Broth tubes with growth from all positive Presumptive tubes and incubating them at the elevated temperature of 44.5 C for 24 hours. Gas production is the positive reaction.

4.7.5 Passage of positive Presumptive cultures through the Confirmed Test completes the MPN series for fecal streptococci and fecal coliform bacteria.

4.7.6 In routine practice, most sample examinations for total coliform are terminated at the end of the Confirmed Test. However, for quality control, at least five percent of the Confirmed Test samples (and a minimum of one sample per test run) should be carried through the Completed Test.

4.8 Completed Test for Total Coliform MPN

Positive Confirmed Test cultures may be subjected to final Completed Test identification through application of further culture tests, as follows:

4.8.1 Streak Levine's EMB agar plates from each positive confirmatory tube and incubate at 35 C for 24 hours. Pick typical coliform colonies (or atypical colonies if no typical colonies are present), inoculate into lauryl tryptose fermentation tubes and incubate at 35 C for 24-48 hours. The formation of gas in any amount in the fermentation tubes constitutes a positive Completed Test for total coliforms.

Typical colonies show a golden green metallic sheen or reddish purple color with nucleation.

Atypical colonies are red, pink or colorless, unnucleated and mucoid.

4.8.2 Optional Gram Stain Procedure

The gram stain test has been used in the Completed MPN Test for demonstrating gram negative, nonsporeforming rods from isolated colonies. Although the gram stain procedure is proposed for revision of the 15th edition of *Standard Methods,* it provides a final check on results and remains useful for evaluating questionable colony types.

After incubation of the EMB agar plates for 24 hours at 35 C (in 4.8.1) pick at least two typical colonies (or atypicals if no typical colonies are present) and inoculate onto nutrient agar slants. Incubate for 24 hours at 35 C, and proceed as in 5, this Section.

4.9 Calculation of MPN Value

The calculated density of the Confirmed or Completed Test may be obtained from the MPN table based on the number of positive tubes and reactions in each dilution.

4.9.1 Table II-C-4 illustrates the MPN indices and 95% Confidence Limits for general use.

4.9.2 Table II-C-5 shows the MPN indices and limits for potable water testing.

4.9.3 Three dilutions are necessary to formulate the MPN code. For example in Table II-C-4 if five 10 ml, five 1.0 ml, and five 0.1 ml portions are used as inocula and positive results are observed in five of the 10 ml inocula, three of the 1.0 inocula, and none of the 0.1

TABLE II-C-4

Most Probable Number Index and 95% Confidence Limits for Five Tube, Three Dilution Series (8, 9)

No. of Tubes Giving Positive Reaction out of			MPN Index per 100 ml	95% Confidence Limits		No. of Tubes Giving Positive Reaction out of			MPN Index per 100 ml	95% Confidence Limits	
5 of 10 ml Each	5 of 1 ml Each	5 of 0.1 ml Each		Lower	Upper	5 of 10 ml Each	5 of 1 ml Each	5 of 0.1 ml Each		Lower	Upper
0	0	0	<2								
0	0	1	2	<0.5	7	4	2	1	26	9	78
0	1	0	2	<0.5	7	4	3	0	27	9	80
0	2	0	4	<0.5	11	4	3	1	33	11	93
						4	4	0	34	12	93
1	0	0	2	<0.5	7						
1	0	1	4	<0.5	11	5	0	0	23	7	70
1	1	0	4	<0.5	11	5	0	1	31	11	89
1	1	1	6	<0.5	15	5	0	2	43	15	110
1	2	0	6	<0.5	15	5	1	0	33	11	93
						5	1	1	46	16	120
2	0	0	5	<0.5	13	5	1	2	63	21	150
2	0	1	7	1	17						
2	1	0	7	1	17	5	2	0	49	17	130
2	1	1	9	2	21	5	2	1	70	23	170
2	2	0	9	2	21	5	2	2	94	28	220
2	3	0	12	3	28	5	3	0	79	25	190
						5	3	1	110	31	250
3	0	0	8	1	19	5	3	2	140	37	340
3	0	1	11	2	25	5	3	3	180	44	500
3	1	0	11	2	25	5	4	0	130	35	300
3	1	1	14	4	34	5	4	1	170	43	490
3	2	0	14	4	34	5	4	2	220	57	700
3	2	1	17	5	46	5	4	3	280	90	850
3	3	0	17	5	46	5	4	4	350	120	1,000
4	0	0	13	3	31	5	5	0	240	68	750
4	0	1	17	5	46	5	5	1	350	120	1,000
4	1	0	17	5	46	5	5	2	540	180	1,400
4	1	1	21	7	63	5	5	3	920	300	3,200
4	1	2	26	9	78	5	5	4	1600	640	5,800
4	2	0	22	7	67	5	5	5	≥2400		

TABLE II-C-5

Most Probable Number Index and 95% Confidence Limits for Testing Potable Waters

Number of Positive Tubes from five 10 ml Portions	MPN Index/100 ml	95% Confidence Limits	
		Lower	Upper
0	<2.2	0	6.0
1	2.2	0.1	12.6
2	5.1	0.5	19.2
3	9.2	1.6	29.4
4	16.	3.3	52.9
5	>16.	8.0	Infinite

ml inocula, the coded results of the test are 5-3-0. The code is located in the MPN Table, and the MPN index of 79 per 100 ml is recorded.

4.9.4 When the series of decimal dilutions is other than 10, 1.0 and 0.1 ml, select the MPN value from Table II-C-4 and calculate according to the following formula:

$$\text{MPN (From Table)} \times \frac{10}{\text{Largest Quantity Tested}}$$

$$= \text{MPN/100 ml}$$

As an example, five out of five 0.01 ml portions, two out of five 0.001 ml portions, and zero out of five 0.0001 ml portions from a sample of water, gave positive reactions. From the code 5–2–0 in MPN Table (Table II-C-4), the MPN index 49 is adjusted for dilutions:

$$49 \text{ (From Table)} \times \frac{10}{0.01} = 49{,}000$$

The final corrected MPN Value = 49,000/100 ml.

4.9.5 If more than the above three sample volumes are inoculated, the three significant dilutions must be determined. The significant dilutions are selected using the following rules:

(a) Only three dilutions are used in the code for calculating an MPN value.

(b) To obtain the proper three dilutions, select the smallest sample volume giving all positive results and the two succeeding lessar sample volumes. See Table II-C-6, Test 1 and 2.

(c) If less than three dilutions show positive tubes, select the three highest sample volumes which will include the dilutions with the positive tubes. See Table II-C-6, Test 3.

(d) If there are positive tubes in the dilutions higher than these dilutions selected, positive results are moved up from these dilutions sample volume to increase the positive tubes in the highest dilution selected. See Table II-C-6, Test 4.

(e) There should be no negative results in higher sample volumes than those chosen. However, if negative tubes are present, e.g., 4/5, 5/5, 3/5 and 0/5 the highest sample volume with all positive tubes must be used along with the next two lower sample volumes. See Table II-C-6, Test 5.

(f) If all tubes are positive, choose the three highest dilutions. See Table II-C-6, Test 6.

(g) If all tubes are negative, choose the three lowest dilutions. See Table II-C-6, Test 7.

(h) If positive tubes skip a dilution, select the highest dilution with positive tubes and the two lower dilutions. See Table II-C-6, Test 8.

(i) If only the middle dilution is positive, select this dilution and one higher and lower dilution. See Table II-C-6, Test 9.

4.9.6 A number of theoretically possible combinations of positive tube results are omitted in Table II-C-4 because the probability of their occurrence is less than 1%. If such unlikely tube combinations occur in more than 1% of samples, review the laboratory procedures for errors and note sample types. Collect fresh samples for analyses.

4.9.7 The MPN can also be computed for each sample based upon the number of positive and negative Presumptive, Confirmed or Completed Tests, and the total number of milliliters tested (10). MPN/100 ml =

$$\frac{\text{No. of Positive Tubes} \times 100}{\sqrt{(\text{No. of ml in Negative Tubes}) \times (\text{No. of ml in All Tubes})}}$$

Example: From a sample of water, five out of five 10 ml portions, two out of five 1.0 ml portions, and zero out of five 0.1 ml portions

TABLE II-C-6

Selection of Coded Results, Five Tube Series

Test	Positive Tubes/ml Sample Volume 10	1.0	0.1	0.01	0.001	0.0001	Code
1	5*	3	0	0	0	0	5-3-0
2	5	5	4	0	0	0	5-4-0
3		4	1	0	0	0	4-1-0
4	5	5	4	1	1	0	5-4-2
5	4	5	3	0	0		5-3-0
6		5	5	5	5	5	5-5-5
7		0	0	0	0	0	0-0-0
8		4	0	2	0	0	4-0-2
9		0	1	0	0	0	0-1-0

*Underlines indicate positive tube series selected for code.

gave positive results. Therefore, MPN/100 ml =

$$\sqrt{\frac{7 \times 100}{(3.5) \times (55.5)}} = 50.22$$

or

MPN/100 ml = 50

4.10 Reporting Results: Report the MPN value for water samples on the basis of 100 ml of sample. Report the MPN values of solid type samples on the basis of 1 gram of dry weight sample.

Examples of bench forms are shown in Figures II-C-1, 2 and 3.

4.11 Precision and Accuracy

4.11.1 The precision of the MPN value increases proportionately with the number of replicates tested.

4.11.2 Multiple-tube values are generally high because MPN tables include a 23% positive bias.

4.11.3 MPN numbers represent only a statistical estimate of the true bacterial density in the sample. The 95% Confidence Limits for each MPN value included in the Tables II-C-4 and 5 show the limited precision of these estimates.

5. Staining Procedures

5.1 Preparation of Bacterial Smears

5.1.1 Place a small drop of laboratory pure water on a clean slide.

5.1.2 Using a sterilized inoculating needle, pick a small amount of growth from the agar slant. Mix the bacteria with the drop of water on the slide and spread evenly over an area the size of a quarter. Use loop for broth cultures.

5.1.3 Air-dry the smear and fix by quickly passing the slide several times through a portion of the flame.

5.2 Gram Stain

Gram staining is a general test for characterization of bacteria and for examination of culture purity.

5.2.1 Prepare and fix a bacterial smear as in 5.1. For quality control, prepare a separate smear of known gram positive cocci and gram negative rods.

5.2.2 Flood the smear with ammonium oxalate-crystal violet stain for one minute.

5.2.3 Wash the slide in a gentle stream of tap or pure water and flood with Lugol's iodine solution (mordant). Allow it to remain for one minute.

5.2.4 Wash the slide in water and blot dry.

5.2.5 Decolorize with acetone alcohol, either by adding it dropwise on the tilted slide until the blue color stops flowing from the smear, or by gently agitating the slide up and down in a beaker containing the alcohol wash for about 30 seconds.

5.2.6 Flood the smear with the safranin counterstain for 10 seconds. Wash and air-dry.

5.2.7 Examine under the oil immersion objective. Gram positive cells retain the crystal violet stain and are blue in color. Gram negative cells are decolorized by the acetone alcohol so that they accept the safranin countarstain and appear pink to red.

5.3 Stain Solutions

Only those stains and dyes which are certified by the National Biological Stain Commission should be used.

5.3.1 Loeffler's Methylene Blue

(a) Methylene Blue-ethyl alcohol solution.

(1) Dissolve 0.3 grams of methylene blue (90% dye content) in 30 ml of 95% ethyl alcohol (Solution A).

(2) Dissolve 0.01 grams of potassium hydroxide in 100 ml of laboratory pure water (Solution B).

(3) Mix solution A and B.

5.3.2 Solutions for Gram Staining

(a) Ammonium oxalate-crystal violet solution:

(1) Dissolve 2 grams crystal violet (approximately 85% dye content) in 20 ml of 95% ethyl alcohol (Solution A).

(2) Dissolve 0.8 grams ammonium oxalate in 80 ml laboratory pure water (Solution B).

(3) Mix Solutions A and B.

(4) Filter through cheesecloth or coarse filter paper.

(5) Problems with the gram stain technique are frequently traceable to the ammonium oxalate-crystal violet solution. In the event that decolorization is insufficient, the amount of crystal violet in the solution can be reduced to as little as 10% of the recommended amount.

(b) Lugol's iodine: Dissolve 1 gram iodine crystals and 2 grams potassium iodide in about 5 ml of pure water. After crystals are in solution, add sufficient laboratory pure water to bring the final solution to a volume of 300 ml.

(c) Acetone-Alcohol: Combine fifty ml volumes of acetone and 95% ethyl alcohol.

(d) Safranin: Dissolve 2.5 grams of safranin in 100 ml of 95% ethyl alcohol. Store as a stock solution. For working solution, add 10 ml of stock to 100 ml of laboratory pure water, mix and store.

6. Shipment of Cultures

6.1 Confirmation or further identification to serotype may be required if the bacteriological data are to be used for specific needs such as enforcement cases, epidemiological studies, tracing sources of pollution or scientific publication. The selected cultures should be sent to an official typing center or state health laboratory with pertinent information for the confirmatory identification. This service is usually available if the cultures are of public health significance, but permission should be obtained from the reference laboratory before sending cultures.

Observe the following instructions:

6.1.1 Send only pure cultures.

6.1.2 Provide a culture with discernable growth on brain heart infusion agar, blood agar base or nutrient agar stab in a screw-cap tube or vial sealed with a cork soaked in hot paraffin. Triple sugar iron agar or other sugar-containing agars should not be used.

6.1.3 Complete the reference laboratory form which requires information on the source of the culture, tests completed, results obtained and identification of the originating laboratory. Include form with cultures, but on outside of secondary container.

6.2 Shipping Regulations for Cultures: Transportation (DOT) regulations apply to surface and air transportation, but shipment of cultures beyond 100–200 miles is only practical by air. Air freight service is limited in many areas, hence passenger-carrying aircraft must be used for safe and quick service. Strict shipping regulations are imposed on such passenger service shipments (11). Packaging and labeling of the cultures must conform with current federal shipping regulations for etiological agents described in: 49 CFR 173.387 (12) and 42 CFR 72.25 (c) (13). The requirements in 6.2.1–6.2.8 that follow are also shown in Figure II-C-10.

6.2.1 Place each culture in a securely closed, watertight PRIMARY CONTAINER (screw-cap test tube or vial) and seal the cap with tape.

6.2.2 Wrap the PRIMARY CONTAINER with sufficient absorbent material (paper towel, tissue, etc.) to absorb the entire contents should breakage or leakage occur.

6.2.3 Place the wrapped, sealed, PRIMARY CONTAINER in a durable, watertight SECONDARY CONTAINER (screw-cap metal mailing tube or sealed metal can). Screw-cap metal mailing tubes should be sealed with tape. Several PRIMARY CONTAINERS of cultures, each individually wrapped in absorbent material, may be placed in the SECONDARY CONTAINER provided that the total aggregate volume does not exceed 50 ml. (NOTE: Multiple secondary containers of cultures, which individually meet the packaging requirements for shipment of 50 ml or less, can be overpacked in a single outer shipping container, provided that the total aggregate volume does not exceed 4000 ml).

6.2.4 Data forms, letters and other information identifying or describing the cultures should be placed around the outside of the SECONDARY CONTAINER. DO NOT ENCLOSE WITHIN THE SECONDARY CONTAINER.

6.2.5 Place the SECONDARY CONTAINER and information form in an OUTER MAILING TUBE OR BOX.

6.2.6 Place an address label and ETIOLOGIC AGENT/MICROBIOLOGICAL CULTURES label on the outer mailing tube or box.

6.2.7 Individual primary containers of greater than 50 ml of culture material require special packaging and cannot be transported on passenger-carrying aircraft.

6.2.8 International shipments must also conform to the added regulations: US Post Office Publication 51, Air Cargo Restricted Articles Tariff 6-D and DOT Regulations 49 CFR, Section 173.

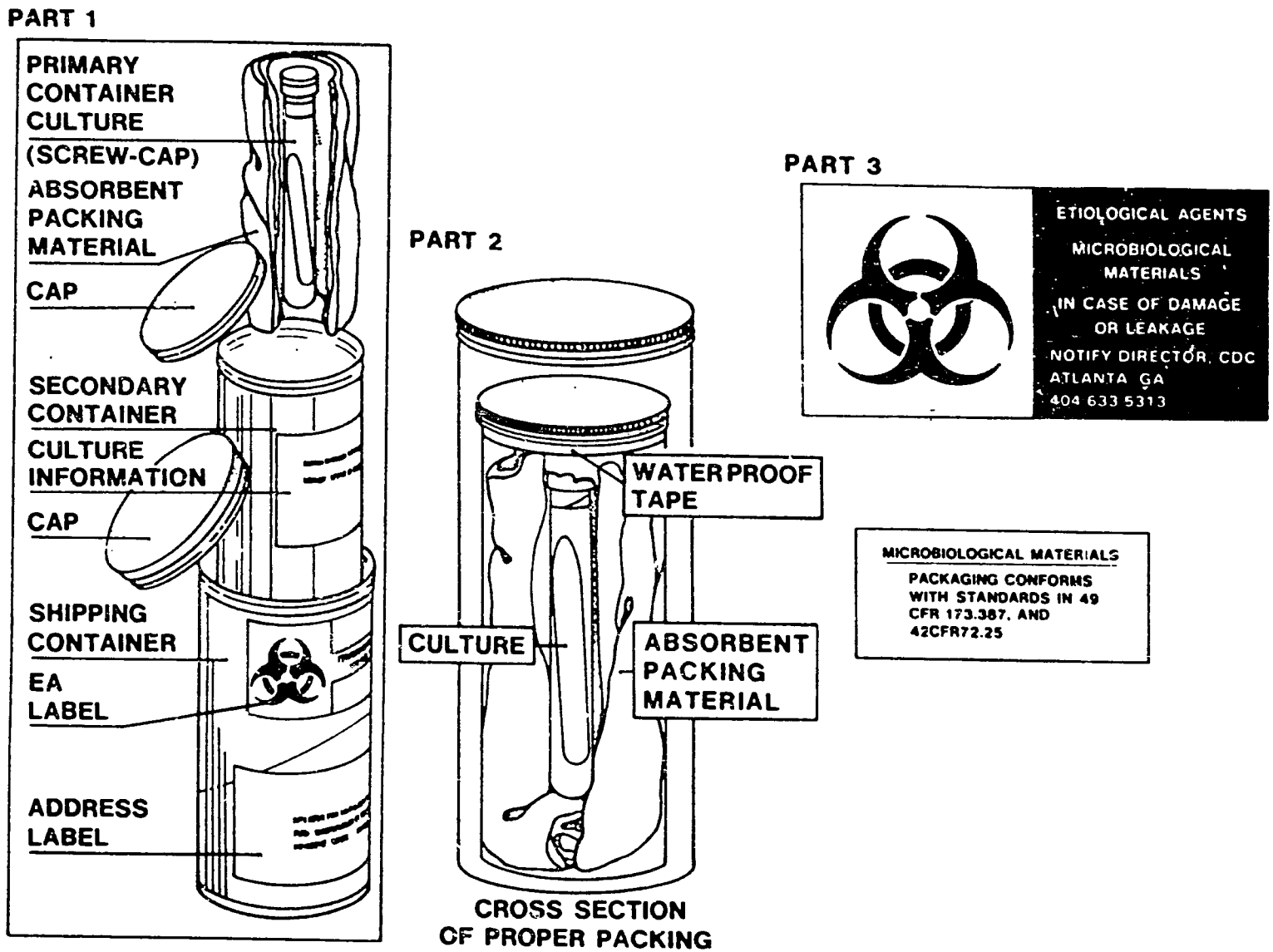


FIGURE II-C-10. Packaging and Labelling of Microbiological Cultures for Shipment.

REFERENCES

1. Clark, H. F., P. W. Kabler and E. E. Geldreich, 1957. Advantages and limitations of the membrane filter procedures. Water Sewage Works 104:385.

2. Shipe, E. L. and A. Fields, 1954. A comparison of the molecular filter technique with agar plate count for enumeration of *Escherichia coli*. Appl. Microbiol. 2:382.

3. Shipe, E. L. and G. M. Cameron, 1954. A comparison of the membrane filter with the most probable number method for coliform determinations from several waters. Appl. Microbiol. 2:85.

4. Geldreich, E. E., H. L. Jeter and J. A. Winter, 1967. Technical considerations in applying the membrane filter procedure. Health Laboratory Science 4:113.

5. Proceedings of the Symposium on the Recovery of Indicator Organisms Employing Membrane Filters. Jan 20–21, 1975. Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268. (1977).

6. Rhines, C. E. and W. P. Cheevers, 1965. Decontamination of membrane filter holders by ultraviolet light. J. Am. Water Works Association 57:500.

7. Manning, H., 1975. AQC Newsletter #26, July 1975. U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, OH 45268. p. 15.

8. Swaroop, S., 1938. Numerical estimation of B. coli by dilution method. Indian J. Med. Research 26:353.

9. Swaroop, S., 1951. The range of variation of the most probable number of organisms estimated by the dilution method. Indian J. Med. Research 39:107.

10. Thomas, H. A., Jr., 1942. Bacterial densities from fermentation tubes. J. Am. Water Works Association 34:572.

11. Morbidity and Mortality Report, 1975. Center for Disease Control, Public Health Service, USDHEW, Atlanta, GA 24:49.

12. Title 49, Code of Federal Regulations (CFR), Part 173.

13. Interstate Quarantine, regulations of the shipment of etiologic agents. Title 42, Code of Federal Regulations, (CFR), Part 72, Section 25.

PART II. GENERAL OPERATIONS

Section D Selection of Analytical Methods

This Section discusses the selection of methods for monitoring water and wastewater in response to the Laws, the microbiological standards that have been established, and the criteria that have been recommended to enforce the laws. The major problems that have developed in the application of the methods are identified and solutions are given where they are available.

1. Methodology

   1.1 National Interim Primary Drinking Water Regulations
   1.2 NPDES Guidelines
   1.3 Marine Sanitation Regulations
   1.4 Water Quality Standards
   1.5 Water Quality Criteria
   1.6 Alternate Test Procedures

2. Problems in Application

   2.1 Stressed Microorganisms
   2.2 Incomplete Recovery/ Suppression
   2.3 Interference by Turbidity
   2.4 Analysis of Ground Water
   2.5 Field Problems
   2.6 Method Modifications and Kits
   2.7 Changes in Membrane Filters and Methodology
   2.8 *Klebsiella* in Industrial Wastes

3. Recommendations for Methods in Waters and Wastewaters

1. Methodology

Test procedures have been specified and published in Federal Register for drinking water, wastewater discharges (NPDES) and vessel discharges.

1.1 National Interim Primary Drinking Water Regulations

Although the National Interim Primary Drinking Water Regulations (Title 40 CFR Part 141) state that the total coliform analyses can be performed by the membrane filter or MPN procedures, the MF procedure is preferred because large volumes of samples can be analyzed in a much shorter time, a critical factor for potable water. Samples containing excessive noncoliform populations or turbidity must be analyzed by the MPN technique. These regulations specify the testing of sample sizes of 100 ml for the MF technique and the testing of five replicate 10 or 100 ml volumes for the MPN procedure. The law directs that the samples be taken at points representative of the distribution system. The minimal schedules for the frequency of sampling are based on population and the required response is given for positive test results. A detailed description of the proposed criteria for interim certification of microbiology laboratories under

104

the Safe Drinking Water Act is given in Appendix B.

1.2 National Pollution Discharge Elimination System (NPDES) Guidelines

The NPDES established guidelines for analysis of pollutants under PL 92-500, Section 304 (g). The parameters and methods are described in 40 CFR Part 136, as amended (40 Code of Federal Regulations, Protection of the Environment, ch. 1 – Environmental Protection Agency, Part 136, Guidelines Establishing Test Procedures for the Analysis of Pollutants). The method must be specified and MPNs must be five tube, five dilution. See Table II-D-1.

1.3 Marine Sanitation Regulations

The regulations for marine sanitation devices (40 CFR Part 140) established performance standards and specified the analytical methods as those promulgated in 40 CFR Part 136, cited in 1.2 above.

1.4 Water Quality Standards

Water quality standards (limits) have been established by law for drinking water and certain sewage and industrial effluents. These standards and the reference sources are listed in Table II-D-2. A standard must be specified in the NPDES permit to be enforceable.

1.5 Water Quality Criteria

Water quality criteria have been recommended by the EPA for certain types of water classified according to use. These criteria are listed in Table II-D-3.

1.6 Alternate Test Procedures

The amendments to 304 (g) also provide procedures for approval of alternate methods. National approval for test methods is obtained by application to EPA through EMSL-Cincinnati while case by case approval is obtained by application through the EPA Regional Offices (40 CFR 136.4).

2. Problems in Application

Although the methods described in this Manual are judged the best available, there are difficulties in the application of methods in different geographical areas, in certain wastes and in some potable and surface waters. Additional problems can stem from the indiscriminate use of new and simplified equipment, supplies or media that have been proposed for use in these procedures.

2.1 Stressed Microorganisms

Some water and wastewater samples contain microorganisms which should reproduce but do not under the conditions of test. These organisms have been described as injured or stressed cells. The stress may be caused by temperature changes or chemical treatment such as chlorine or toxic wastes (1).

Stressed organisms are particularly important in environmental measurements because tests for bacterial indicators or pathogens can give negative responses, then recover later and multiply to produce dangerous conditions. Subsections 2.1.1 and 2.1.2 describe efforts to recover stressed microorganisms.

2.1.1 Ambient Temperature Effects

Extreme ambient temperatures stress microorganisms and reduce recovery of microbiological indicators. For example, in Alaska and other extremely cold areas, the severe change from cold stream temperature to 44.5 C temperature of incubation reduces recovery of fecal coliforms. The two-step MF test for fecal coliforms increases recoveries by use of a 2-hour acclimation on an enrichment medium at 35 C before normal incubation at 44.5 C.

In contrast, water samples from natural waters at high temperatures may include large numbers of non-coliform organisms which interfere with sheen production on MF's and with positive gas production in MPN analyses. An improved MF medium that provides greater selectivity is desirable but may not be possible without sacrificing recovery.

103

TABLE II-D-1

Approved Test Procedures for the Analysis of Pollutants (40 CFR 136)

Parameter per 100 ml	Method	Reference and Page No. SM[1]	USGS[2]	This Manual
Fecal Coliforms	MPN	922		Part III C, 5
	MF	937	45	Part III C, 2
Fecal Coliforms in presence of chlorine[3]	MPN	922		Part III C, 5
	MF	928, 937		Part III C, 2
Total Coliforms	MPN	916		Part III B, 4
	MF	928	35	Part III B, 2
Total Coliforms in presence of Chlorine	MPN	916		Part III B, 4
	MF	933		Part III B, 2
Fecal Streptococci	MPN	943		Part III D, 4
	MF	944	50	Part III D, 2
	Plate Count	947		Part III D, 5

[1] Standard Methods for the Examination of Water and Wastewater, 14th Edition, (1975).

[2] Slack, K. V., et.al. Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples. USGS Techniques of Water Resources Inv., Book 5, ch. A4 (1973).

[3] Since the MF technique usually yields low and variable recovery from chlorinated wastewaters, the MPN method will be required to resolve any controversies.

TABLE II-D-2

Water Quality Standards

Water or Wastewater	Microbiological Standards Coliforms/100 ml Total	Fecal	Reference Source
Potable Water	<5	—	PL 93-523
Chlorinated Effluents	—	200-400	PL 92-500
2° Treatment Wastes	—	200-400	40 CFR Part 133
Selected Industrial Wastes	—	200-400	PL 92-500
Leather and Tanning	—	400	40 CFR Part 425
Feed Lots	—	400	40 CFR Part 412
Meat Products	—	400	40 CFR Part 432
Beet Sugar	—	400	40 CFR Part 409
Canned Fruits and Vegetables	—	400	40 CFR Part 407
Textiles	—	400	40 CFR Part 410
Effluents from Marine Sanitation Devices with Discharges Type I	—	1000	40 CFR Part 140 and Amendments
Type II	—	200	40 CFR Part 140 and Amendments

105

TABLE II-D-3

Water Quality Criteria

Water or Wastewater	Statistical Measure	Microbiological Criteria Coliforms/100 ml Total	Fecal	Reference Source
Public Water Supply	log $\overline{X}$	20000	2000	A
Recreational Water: Primary Contact	log $\overline{X}$/30 days		200	B
	maximum/30 days, in 10% of Samples		400	B
General Contact	log $\overline{X}$/30 days		1000	B
	maximum/30 days, in 10% of Samples		2000	B
Agricultural Water	monthly $\overline{X}$	5000	1000	B
Shellfish-Raising Waters	Daily Median	70	14	C & D
	Highest 10% of Daily Values	230	43	

A Water Quality Criteria, EPA. March, 1973. Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

B Water Quality Criteria, FWPCA, April 1, 1968. Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

C National Shellfish Sanitation Program Manual of Operation. U.S. Dept. of HEW, 1965. Public Health Service Publ. No. 33. Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

D Quality Criteria for Water, July 1976, O.W.H.M., US EPA.

106

2.1.2 Chlorinated Effluents and Toxic Wastes

Although thiosulfate is added to all samples suspected of containing chlorine, to neutralize its toxic effects, the membrane filter procedure yields poor recovery of coliforms from chlorinated effluents as compared to MPN recovery (1-6). A recent amendment to 40 CFR 136 added Coliform bacteria (Fecal) in the presence of chlorine, as a specific parameter and recommended analysis by the MF or MPN techniques (7). A qualifying statement appended to the method in 40 CFR Part 136 requires the five tube, five dilution MPN and states: "Since the membrane filter technique usually yields low and variable recovery from chlorinated wastewaters, the MPN method will be required to resolve any controversies." Therefore, the MPN procedure should be used in analysis of chlorinated effluents where the data may be challenged by legal or enforcement actions. The MF may be used currently for self-monitoring situations. (See Table II-D-1).

Proposed changes in MF materials and procedures include new membrane filter formulations, an agar overlay technique, modified media and twostep methods (1). Present modifications of the MF method have not produced recoveries of fecal coliforms from chlorinated effluents equivalent to MPN recoveries. Thorough evaluation and approval of proposed procedures by EPA are required before changes will be acceptable.

Certain types of wastes show recovery problems for total and fecal coliforms:

1. Primary and Chlorinated-Primary Waste Effluents.

2. Chlorinated-Secondary and Chlorinated-Tertiary Waste Effluents.

3. Industrial wastes containing toxic metals or phenols.

When turbidity and low recovery prevent the application of the MF technique to coliform analyses of primary and secondary effluents or industrial wastes containing toxic materials, the MPN procedure is required. However, the two-step MF procedure for total coliforms described in this Manual and in *Standard Methods* is acceptable for toxic wastes.

If the MF procedure is applied to chlorinated or toxic samples, the laboratory should require data from at least 10 samples collected over 1 week of plant processing (but not less than 5 calendar days) to show comparability of the MF to the MPN technique. See Part IV-C, 3 for details.

2.2 Incomplete Recovery/Suppression

When coliforms are present in low numbers in drinking water, high levels of non-coliforms can suppress growth or mask detection. This problem may appear as a mass of confluent growth on a membrane filter or as spots of sheen in this confluent growth. In the MPN procedure, presumptive tubes may show heavy growth with no gas bubbles, dilution skips or unusual tube combinations. When these negative presumptive tubes are transferred to BGLB, they confirm in this more restrictive medium, indicating that the coliform gas production in the Presumptive Test was suppressed by non-coliforms.

2.3 Interference by Turbidity

The tendency of bacteria to clump and adhere to particles can produce inaccurate results in the analysis of water samples. The National Interim Primary Drinking Water Regulations (NIPDWR) specify one turbidity unit as the primary maximum allowable level but permit up to five turbidity units if this level does not interfere with disinfection or microbiological analyses. Turbidity can interfere with filtration by causing a clumping of indicators or clogging of pores. The turbidity as organic solids can also provide nutrients for bacterial growth and subsequently produce higher counts. The type of particles variably affects the filtration rate; for example, clay, silt or organic debris clog more easily than sand. Background organisms may also be imbedded

in the particles and interfere with the coliform detection.

2.4 Analysis of Ground Water

Although total coliforms are a valid measure of pollution, their use as indicators in analyzing ground waters and rural community supplies may not sufficiently describe the water quality. For example, ground waters frequently contain high total counts of bacteria with no coliforms. Such waters pass Interim Drinking Water Regulations but technical judgment must conclude these are not acceptable as potable waters.

2.5 Field Problems

Assurance of data validity demands sample analyses within the shortest time interval after collection. This need requires field analyses using either a mobile laboratory or field kit equipment. Since a mobile laboratory may not be available for a survey, it is likely that at least a part of the analyses will need to be completed in an onsite facility. If the analyses can be done using membrane filtration techniques, field kits such as Millipore's Water Laboratory and MF Portable Incubator (heat sink) are particularly helpful for rapid set-up and analyses of limited samples. However, if large numbers of samples are tested per day or the survey covers more than a few days, the heat-sink incubator is impractical because of limited capacity and high cost. In such surveys, a mobile laboratory utilizing water-jacketed incubators is more practical.

2.6 Method Modifications and Kits

Commercial manufacturers continue to offer proprietory kits and method modifications to speed or simplify the procedures used in coliform and fecal coliform analyses, primarily for field use. Most of these units have not been demonstrated to produce results comparable to the official procedures. If not tested to the satisfaction of EPA, such method modifications and kits cannot be used for establishing total or fecal coliform numbers for permits under NPDES or for total coliform numbers under the Safe Drinking Water Act. The procedure required for acceptance of an alternative procedure is described in 40 CFR Parts 136.4 and 136.5, as amended.

2.7 Changes in Membrane Filters and Methodology

There is an expected pattern of changes in materials and methodology used in the manufacture of membrane filters. The changes may or may not be announced by the manufacturer. Therefore, it is important for the laboratory to monitor membrane performance as described in Section A of Quality Control in this Manual.

These changes include modification of formulations and the replacement of the 0.45 μm pore MF by a 0.7 μm retention pore MF for improved recovery. Tests by independent investigators show that several MF's give comparable recovery (5, 6, 8, 9), however, enrichment or two-temperature incubations are needed before recoveries approach the MPN values (See 2.1.2 in this Section).

This discussion of problems with new methodology and membrane materials should not be interpreted as indicating that EPA discourages new developments. Rather EPA encourages the MF supply industry to test and examine procedures, to innovate and to research. The membrane filter manufacturers should be commended and encouraged to continue their efforts toward solving problems and improving materials and techniques in water microbiology.

2.8 *Klebsiella* in Industrial Wastes

Klebsiella bacteria (part of the coliform group) multiply in certain industrial wastes, are not differentiated from fecal coliforms by MF and MPN procedures and consequently are included in the results. These recoveries have been reported in textile, paper and pulp mills and other wastes. Objections have been raised to the application of fecal coliform standards

TABLE II-D-4

Selection of Methods for Problem Samples

Problem Area	Parameter Chosen	Method of Choice
Shellfish-harvesting waters	Total coliform	MPN*
	Fecal Coliform	MPN*
Marine & Estuarine Waters	Total Coliform	MF/MPN
	Fecal Coliform	MF/MPN
Treated Industrial Wastes (non-chlorinated, non-toxic) Low Solids Wastes	Fecal Coliform	MF
Toxic Industrial Wastes (metals, phenolics) and High Solids Wastes	Fecal Coliform	MPN or alternate procedure, tested and approved**
Primary and Chlorinated-Primary Municipal/Industrial Effluent	Total Coliform	MPN or alternate procedure, tested and approved**
	Fecal Coliform	MPN or alternate procedure, tested and approved**
Chlorinated-Secondary Effluent	Total Coliform	Two-Step MF
	Fecal Coliform	MPN or alternate procedure, tested and approved**

*MPN recommended to conform with the MPN method specified for examination of shellfish.

**Requires proof of comparability under EPA's specified test regime that the alternate procedure (MF, streak plate, etc.) is valid. See This Manual, IV-C, 3.

to these wastes because *Klebsiella* originate from other than sanitary sources. However, EPA does consider large numbers of *Klebsiella, Aeromonas* and other noncoliforms as indicators of organic pollution. Further, these organisms do occur in low densities in human and animal wastes.

3. Recommendations for Methods in Waters and Wastewaters

The amended Federal Water Pollution Control Act, the Marine Protection, Research and Sanctuaries Act and the Safe Drinking Water Act require recommendations on analytical methodology. Generally, the membrane filter methods are preferred over MPN and other techniques, where proven applicable.

In Table II-D-4, problem samples are identified and the analytical method recommended for parameters of choice.

REFERENCES

1. Bordner, R. H., C. F. Frith and J. A. Winter, eds., 1977. Proceedings of the Symposium on Recovery of Indicator Organisms Employing Membrane Filters, U.S. Environmental Protection Agency, EPA-600-19-77-024, EMSL-Cincinnati, Cincinnati, OH 45268.

2. Lin, S. D., 1973. Evaluation of coliform tests for chlorinated secondary effluents. JWPCF, 45:3:498.

3. Greene, R. A., R. H. Bordner and P. V. Scarpino, 1974. Applicability of the membrane filter and most probable number coliform procedures to chlorinated wastewaters. Paper G87 given at 74th Annual Meeting of the American Society for Microbiology, May 12-17, 1974, Chicago, IL.

4. Rose, R. E., E. E. Geldreich and W. Litsky, 1975. Improved membrane filter method for fecal coliform analysis. Appl. Microbiol. 29:4:532.

5. Lin, S. D., 1976. Evaluation of Millipore HA and HC membrane filters for the enumeration of indicator bacteria. Appl. Environ. Microbiol. 32:300.

6. Green, B. L., E. Clausen and W. Litsky, 1975. Comparison of the new Millipore HC with conventional membrane filters for the enumeration of fecal coliform bacteria. Appl. Microbiol. 30:697.

7. Guidelines for Establishing Test Procedures, 40 Code of Federal Regulations (CFR) Part 136, Published in Federal Register, 40, 52780, Dec. 1, 1976.

8. Tobin, R. S. and B. J. Dutka, 1977. Comparison of the surface structure, metal binding, and fecal coliform recoveries of nine membrane filters. Appl. Environ. Microbiol. 34:69.

9. Lin, S. D., 1977. Comparison of membranes for fecal coliform recovery in chlorinated effluents. JWPCF, 49:2255.

STANDARD PROCEDURES

FOR

SPECIFIC ANALYTICAL METHODS

PART III. ANALYTICAL METHODOLOGY

Section B Total Coliform Methods

This section describes the enumerative techniques for total coliform bacteria in water and wastewater. The method chosen depends upon the characteristics of the sample. The Section is divided as follows:

1. Definition of the Coliform Group
2. Single-Step, Two-Step and Delayed-Incubation Membrane Filter (MF) Methods
3. Verification
4. Most Probable Number (MPN) Method
5. Differentiation of the Coliform Group by Further Biochemical Tests

1. Definition of the Coliform Group

The coliform or total coliform group includes all of the aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose in 24-48 hours at 35 C. The definition includes the genera: *Escherichia, Citrobacter, Enterobacter, and Klebsiella.*

2. Single-Step, Two-Step and Delayed-Incubation Membrane Filter Methods

2.1 Summary: An appropriate volume of a water sample or its dilution is passed through a membrane filter that retains the bacteria present in the sample.

In the single-step procedure the filter retaining the microorganisms is placed on M-Endo agar, LES M-Endo agar or on an absorbent pad saturated with M-Endo broth in a petri dish. The test is incubated at 35 C for 24 hours.

In the two-step enrichment procedure the filter retaining the microorganisms is placed on an absorbent pad saturated with lauryl tryptose (lauryl sulfate) broth. After incubation for 2 hours at 35 C, the filter is transferred to an absorbent pad saturated with M-Endo broth, M-Endo agar, or LES M-Endo agar, and incubated for an additional 20–22 hours at 35 C. The sheen colonies are counted under low magnification and the numbers of total coliforms are reported per 100 ml of original sample.

In the delayed-incubation procedure, the filter retaining the microorganisms is placed on an absorbent pad saturated with M-Endo preservative medium in a tight-lidded petri dish and transported from field site to the laboratory. In the laboratory, the filter is transferred to M-Endo growth medium and incubated at 35 C for 24 hours. Sheen colonies are counted as total coliforms per 100 ml.

117

2.2 Scope and Application: The total coliform test can be used for any type of water or wastewater, but since the development of the fecal coliform procedure there has been increasing use of this more specific test as an indicator of fecal pollution. However, the total coliform test remains the primary indicator of bacteriological quality for potable water, distribution system waters, and public water supplies because a broader measure of pollution is desired for these waters. It is also a useful measure in shellfish-raising waters.

Although the majority of water and wastewater samples can be examined for total coliforms by the single-step MF procedure, coliforms may be suppressed by high background organisms, and potable water samples may require the two-step method.

If the membrane filtration method is used to measure total coliforms in chlorinated secondary or tertiary sewage effluents the two-step enrichment procedure is required. However, it may be necessary to use the MPN method because of high solids in the wastes or toxicity from an industrial waste (see Part II-D, this Manual).

The delayed-incubation MF method is useful in survey monitoring or emergency situations when the single step coliform test cannot be performed at the sample site, or when time and temperature limits for sample storage cannot be met. The method eliminates field processing and equipment needs. Also, examination at a central laboratory permits confirmation and biochemical identification of the organisms as necessary. Consistent results have been obtained with this method using water samples from a variety of sources (1, 2). The applicability of this method for a specific water source must be determined in preliminary studies by comparison with the standard MF method.

2.3 Apparatus and Materials

2.3.1 Water jacket, air, or heat sink incubator that maintains 35 ± 0.5 C. Temperature is checked against an NBS certified thermometer or equivalent. Incubator must have humidity control if loose-lidded pertri dishes are used. See Part II-B, 1.2.

2.3.2 A binocular (dissection) microscope, with magnification of 10 or 15×, and a daylight type fluorescent lamp angled to give maximum sheen appearance.

2.3.3 Hand tally.

2.3.4 Pipet container of stainless steel, aluminum or pyrex glass for glass pipets.

2.3.5 Sterile 50–100 ml graduated cylinders covered with aluminum foil or kraft paper,

2.3.6 Sterile, unassembled membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper. Portable field filtration units are available.

2.3.7 Vacuum source.

2.3.8 Vacuum filter flask with appropriate tubing. Filter manifolds which hold a number of filter bases can also be used.

2.3.9 Ultraviolet sterilizer for MF filtration units (optional).

2.3.10 Safety trap flask between the filter flask and the vacuum source.

2.3.11 Forceps with smooth tips.

2.3.12 Methanol or ethanol, 95%, in small vial, for flaming forceps.

2.3.13 Bunsen/Fisher burner or electric incinerator.

2.3.14 Sterile TD bacteriological or Mohr pipets, glass or plastic, of appropriate size.

2.3.15 Sterile petri dishes with tight-fitting lids, 50 × 12 mm or loose-fitting lids 60 × 15 mm, glass or plastic.

2.3.16 Dilution bottles (milk dilution), pyrex, marked at 99 ml volume, screw cap with neoprene rubber liner.

2.3.17 Membrane filters, white, grid-marked, 47 mm diameter, with 0.45 µm ± 0.02 µm pore size, or other pore size, as recommended by manufacturer for water analyses.

2.3.18 Absorbent pads.

2.3.19 Inoculation loops, at least 3 mm diameter, or needles, nichrome or platinum wire, 26 B&S gauge, in suitable holder.

2.3.20 Disposable applicator sticks or plastic loops as alternatives to inoculation loops.

2.3.21 Shipping tubes, labels, and packing materials for mailing delayed incubation plates.

2.4 Media: Media are prepared in presterilized erlenmeyer flasks with metal caps, aluminum foil covers, or screw caps.

2.4.1 M-Endo broth or agar (See Part II-B, 5.2.2).

2.4.2 LES M-Endo agar (See Part II-B, 5.2.4).

2.4.3 Lauryl tryptose broth (See Part II-B, 5.3.1).

2.4.4 Brilliant green lactose bile broth (See Part II-B, 5.3.2).

2.4.5 M-Endo holding medium (See Part II-B, 5.2.3).

2.4.6 Sodium benzoate, U.S.P., for use in the delayed incubation procedure (See Part II-B, 5.2.3).

2.4.7 Cycloheximide (Actidione – Upjohn, Kalamazoo, MI) for use as antifungal agent in delayed incubation procedure (See Part II-B, 5.2.3).

2.5 Dilution Water (See Part II-B, 7 for preparation).

2.5.1 Sterile dilution water dispensed in 99 ± 2 ml amounts in screw-capped dilution bottles.

2.5.2 Sterile dilution water prepared in 1 liter or larger volumes for wetting membranes before addition of small sample volumes and for rinsing the funnel after sample filtration.

2.6 Procedure: Refer to the general procedure in Part II-C for more complete details.

2.6.1 Single-Step Procedure

(a) Prepare the M-Endo broth, M-Endo agar or LES M-Endo agar as directed in Part II-B.

(b) Place one sterile absorbent pad in the bottom half of each petri dish. Pipet 1.8–2.0 ml M-Endo broth onto the pad to saturate it. Pour off excess broth. Alternatively, pipet 5–6 ml of melted agar into each dish (2–3 mm) and allow to harden before use. Mark dishes and bench forms with sample identities and volumes.

(c) Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and the base.

(d) Shake the sample bottle vigorously about 25 times and measure the desired volume of sample into the funnel. Select sample volumes based on previous knowledge to produce membrane filters with 20–80 coliform colonies. See Table II-C-1. If sample volume is < 10 ml, add 10 ml of sterile dilution water to the filter before adding sample.

It is desirable to filter the largest possible sample volumes for greatest accuracy. However, if past analyses of specific samples have resulted in confluent growth, "too numerous to count" membranes, or lack of sheen from excessive turbidity, additional samples should be collected and filtration volumes adjusted to provide isolated colonies from smaller volumes. See 2.7.2 in this Section for details on adjusting sample volumes for potable waters.

The suggested method for measuring sample volumes is described in Part II-C, 3.4.6.

(e) Filter sample and rinse the sides of the funnel at least twice with 20–30 ml of sterile dilution water. Turn off the vacuum and remove the funnel from the filter base. Aseptically remove the membrane filter from the filter base and place grid-side up on the agar or pad.

(f) Filter samples in order of increasing sample volume, filter potable waters first.

(g) If M-Endo broth is used, place the filter on an absorbent pad saturated with the broth. Reseat the membrane, if air bubbles occur, as evidenced by non-wetted areas on the membrane. Invert dish and incubate for 24 ± 2 hours at 35 ± 0.5 C in an atmosphere with near saturated humidity.

(h) If M-Endo agar or LES M-Endo agar is used, place the inoculated filter directly on the agar surface. Reseat the membrane if bubbles occur. Invert the dish and incubate for 24 ± 2 hours at 35 ± 0.5 C in an atmosphere with near saturated humidity.

(i) If tight-lidded dishes are used, there is no requirement for near-saturated humidity.

(j) After incubation remove the dishes from the incubator and examine for sheen colonies.

(k) Proceed to 2.7 for Counting and Recording Colonies.

2.6.2 Two-Step Enrichment Procedure

(a) Place a sterile absorbent pad in the top of each petri dish.

(b) Prepare lauryl tryptose broth as directed in Part II-B. Pipet 1.8–2.0 ml lauryl tryptose broth onto the pad to saturate it. Pour off excess broth.

(c) Place a sterile membrane filter on the filter holder, grid-side up and attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and the base.

(d) Shake the sample bottle vigorously about 25 times to obtain uniform distribution of bacteria. Select sample volumes based on previous knowledge to produce membrane filters with 20–80 coliform colonies. See Table II-C-1. If sample volume is < 10 ml, add 10 ml of sterile dilution water to filter before adding sample.

(e) Filter samples in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations. The methods of measurement and dispensation of the sample into the funnel are given in Part II-C, 3.4.6.

(f) Turn on the vacuum to filter the sample through the membrane, rinse the sides of the funnel at least twice with 20–30 ml of sterile dilution water. Turn off vacuum and remove funnel from base.

(g) Remove the membrane filter aseptically from the filter base and place grid-side up on the pad in the top of the petri dish. Reseat MF if air bubbles are observed.

(h) Incubate the filter in the petri dish without inverting for 1 1/2 – 2 hours at 35 ± 0.5 C in an atmosphere of near saturated humidity. This completes the first step in the Two-Step Enrichment Procedure.

(i) Prepare M-Endo broth, M-Endo agar, or LES M-Endo agar as directed in Part II-B.

If M-Endo broth is used, place a new sterile absorbent pad in the bottom half of the dish and saturate with 1.8-2.0 ml of the M-Endo broth. Transfer the filter to the new pad. Reseat MF if air bubbles are observed. Remove the used pad and discard.

If M-Endo or LES M-Endo agar is used, pour 5-6 ml of agar into the bottom of each petri dish and allow to solidfy. The agar medium can be refrigerated for up to two weeks.

115

(j) Transfer the filter from the lauryl tryptose broth onto the Endo medium. Reseat if air bubbles are observed.

(k) Incubate dishes in an inverted position for an additional 20–22 hours at 35 ± 0.5 C. This completes the second step in the Two-Step Enrichment Procedure.

(l) Proceed to 2.7 Counting and Recording.

2.6.3 Delayed Incubation Procedure

(a) Prepare the M-Endo Holding Medium or LES Holding Medium as outlined in Part II-B, 5.2.3 or 5.2.5. Saturate the sterile absorbent pads with about 2.0 ml of holding broth. Pour off excess broth. Mark dishes and bench forms with sample identity and volumes.

(b) Using sterile forceps place a membrane filter on the filter base grid side up.

(c) Attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and base.

(d) Shake the sample vigorously about 25 times and measure into the funnel with the vacuum off. If the sample is < 10 ml, add 10 ml of sterile dilution water to the membrane filter before adding the sample.

(1) Select sample volumes based on previous knowledge to produce counts of 20–80 coliform colonies. See Table II-C-1.

(2) Follow the methods for sample measurement and dispensation given in Part II-C, 3.4.6

(e) Filter the sample through the membrane and rinse the sides of the funnel walls at least twice with 20–30 ml of sterile dilution water.

(f) Turn off the vacuum and remove the funnel from the base of the filter unit.

(g) Aseptically remove the membrane filter from the filter base and place grid side up on an absorbent pad saturated with M-Endo Holding Medium or LES Holding Medium.

(h) Place the culture dish in shipping container and send to the examining laboratory. Coliform bacteria can be held on the holding medium for up to 72 hours with little effect on the final counts. The holding period should be kept to a minimum.

(i) At the examining laboratory remove the membrane from the holding medium, place it in another dish containing M-Endo broth or agar medium, and complete testing for coliforms as described above under 2.6.1.

2.7 Counting and Recording Colonies: After incubation, count colonies on those membrane filters containing 20–80 golden-green metallic surface sheen colonies and less than 200 total bacterial colonies. A binocular (dissection) microscope with a magnification of 10 or 15× is recommended. Count the colonies according to the general directions given in Part II-C, 3.5.

2.7.1 The following general rules are used in calculating the total coliform count per 100 ml of sample. Specific rules for analysis and counting of water supply samples are given in 2.7.2.

(a) Countable Membranes with 20–80 Sheen Colonies, and Less Than 200 Total Bacterial Colonies: Select the plate counts to be used according to the rules given in Part II-C, 3.6, and calculate the final value using the formula.

Total Coliforms/100 ml:

$$\frac{\text{No. of Total Coliform Colonies Counted}}{\text{Volume in ml of Sample Filtered}} \times 100$$

(b) Counts Greater Than the Upper Limit of 80 Colonies: All colony counts are above the recommended limits. For example, sample volumes of 1, 0.3, and 0.01 ml are filtered to

116

produce total coliform colony counts of TNC, 150, and 110 colonies.

Use the count from the smallest filtration volume and report as a greater than count/100 ml. In the example above:

$$\frac{110}{0.01} \times 100 = 1{,}100{,}000$$

or > 1,100,000 coliforms/100 ml.

(c) Membranes with More Than 200 Total Colonies (Coliforms plus Non-coliforms).

(1) Estimate sheen colonies if possible, calculate total coliform density as in (a) above.

Report as: Estimated Count/100 ml.

(2) If estimate of sheen colonies is not possible, report count as Too Numerous to Count (TNTC).

(d) Membranes with Confluent Growth

Report as: Confluent Growth and specify the presence or absence of sheen.

2.7.2 Special Rules for Potable Waters

(a) Countable Membranes with 0-80 Sheen Colonies, and Less than 200 Total Colonies

Count the sheen colonies per volume filtered. Calculate and report the number of Total Coliforms/100 ml.

(b) Uncountable Membranes for Potable Water Samples

If 100 ml portions of potable water samples cannot be tested because of high background counts or confluency, multiple volumes of less than 100 ml can be filtered. For example, if 60 colonies appear on the surface of one membrane through which a 50 ml portion of the sample was passed, and 50 colonies on a second membrane through which a second 50 ml portion of the sample was passed, the colonies are totaled and reported as 110 total coliforms per 100 ml.

If filtration of multiple volumes of less than 100 ml still results in confluency or high background count, the coliforms may be present but suppressed. These samples should be analyzed by the MPN Test. This MPN check should be made on at least one sample for each problem water once every three months.

(c) Membranes with Confluent Growth

For potable water samples, confluence requires resampling and retesting.

(d) Verification. Because unsatisfactory samples from public water supplies containing 5 or more coliform colonies must be verified, at least 5 colonies need to be verified for each positive sample. Reported counts are adjusted based on verification.

(e) Quality control procedures are specified by EPA under the law, and described in Appendix C in this Manual.

2.7.3 Reporting Results: Report total coliform densities per 100 ml of sample. See Figure II-C-3 for an example of a bench form for reporting results. A discussion on significant figures is given in Part II-C, 2.8.

2.8 Precision and Accuracy: There are no established precision and accuracy data available at this time.

3. Verification

Verification of total coliform colonies from M-Endo type media validates sheen as evidence of coliforms. Verification of representative numbers of colonies may be required in evidence gathering or for quality control procedures. The verification procedure follows:

3.1 Using a sterile inoculating needle, pick growth from the centers of at least 10 well-isolated sheen colonies (5 sheen colonies per plate for potable waters). Inoculate each into a tube of lauryl tryptose broth and incubate 24-48 hours at 35 C ± 0.5 C. Do not transfer exclusively into brilliant green bile lactose broth. However, colonies may be transferred to LTB and BGLB simultaneously.

3.2 At the 24 and 48 hour readings, confirm gas-positive lauryl tryptose broth tubes by inoculating a loopful of growth into brilliant green lactose bile broth and incubate for 24–48 hours at 35 ± 0.5 C. Cultures that are positive in BGLB are interpreted as verified coliform colonies (see Figure III-B-1).

3.3 If questionable sheen occurs, the worker should also verify these colonies.

4. Most Probable Number (MPN) Method

4.1 Summary: This method detects and estimates the total coliforms in water samples by the multiple fermentation tube technique. The method has three stages: the Presumptive, the Confirmed, and the Completed Tests. In the Presumptive Test, a series of lauryl tryptose broth fermentation tubes are inoculated with decimal dilutions of the sample. The formation of gas at 35 C within 48 hours constitutes a positive Presumptive Test for members of the total coliform group. However, the MPN must be carried through the Confirmed Test for valid results. In this test, inocula from positive Presumptive tubes are transferred to tubes of brilliant green lactose bile (BGLB) broth. The BGLB medium contains selective and inhibitive agents to suppress the growth of all non-coliform organisms. Gas production after incubation for 24 or 48 hours at 35 C constitutes a positive Confirmed Test and is the point at which most MPN tests are terminated. The Completed Test begins with streaking inoculum from the positive BGLB tubes onto EMB plates and incubating the plates for 24 hours at 35 C. Typical and atypical colonies are transferred into lauryl tryptose broth fermentation tubes and onto nutrient agar slants. Gas formation in the fermentation tubes and presence of gram-negative rods constitute a positive Completed Test for total coliforms. See Figure III-B-2. The MPN per 100 ml is calculated from the MPN table based upon the Confirmed or Completed test results.

4.2 Scope and Application

4.2.1 Advantages: The MPN procedure is a tube-dilution method using a nutrient-rich medium, which is less sensitive to toxicity and supports the growth of environmentally-stressed organisms. The method is applicable to the examination of total coliforms in chlorinated primary effluents and under other stressed conditions. The multiple-tube procedure is also better suited for the examination of turbid samples, muds, sediments, or sludges because particulates do not interfere visibly with the test.

4.2.2 Limitations: Certain non-coliform bacteria may suppress coliforms or act synergistically to ferment lauryl tryptose broth and yield false positive results. A significant number of false positive results can also occur in the brilliant green bile broth when chlorinated primary effluents are tested, especially when stormwater is mixed with the sewage (3). False negatives may occur with waters containing nitrates (4). False positives are more common in sediments.

4.3 Apparatus and Materials

4.3.1 Water bath or air incubator set at 35 ± 0.5 C.

4.3.2 Pipet containers of stainless steel, aluminum, or pyrex glass for glass pipets.

4.3.3 Inoculation loops, at least 3 mm diameter and needles of nichrome or platinum wire, 26 B & S gauge, in suitable holders.

4.3.4 Disposable sterile applicator sticks or plastic loops as alternatives to inoculating loops.

4.3.5 Compound microscope, oil immersion.

4.3.6 Bunsen/Fisher burner or electric incinerator unit.

4.3.7 Sterile TD Mohr or bacteriological pipets, glass or plastic, of appropriate size.

118

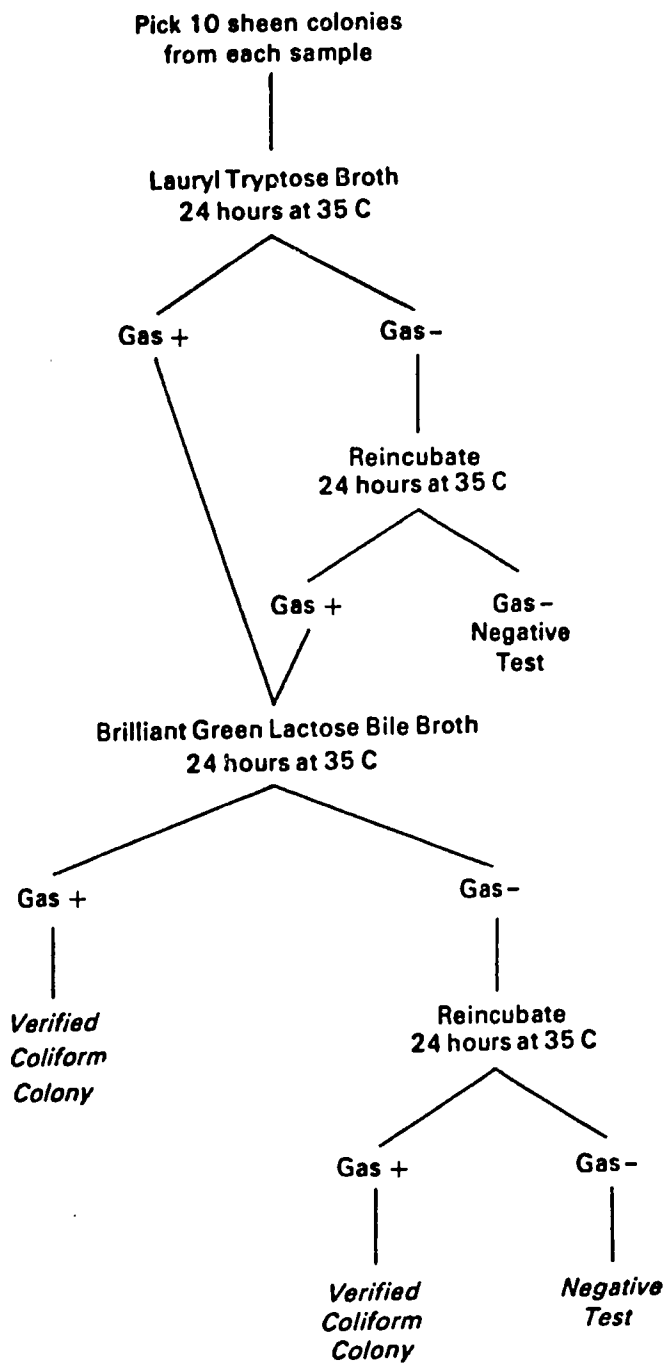


FIGURE III-B-1. Verification of Total Coliform Colonies on the Membrane Filter

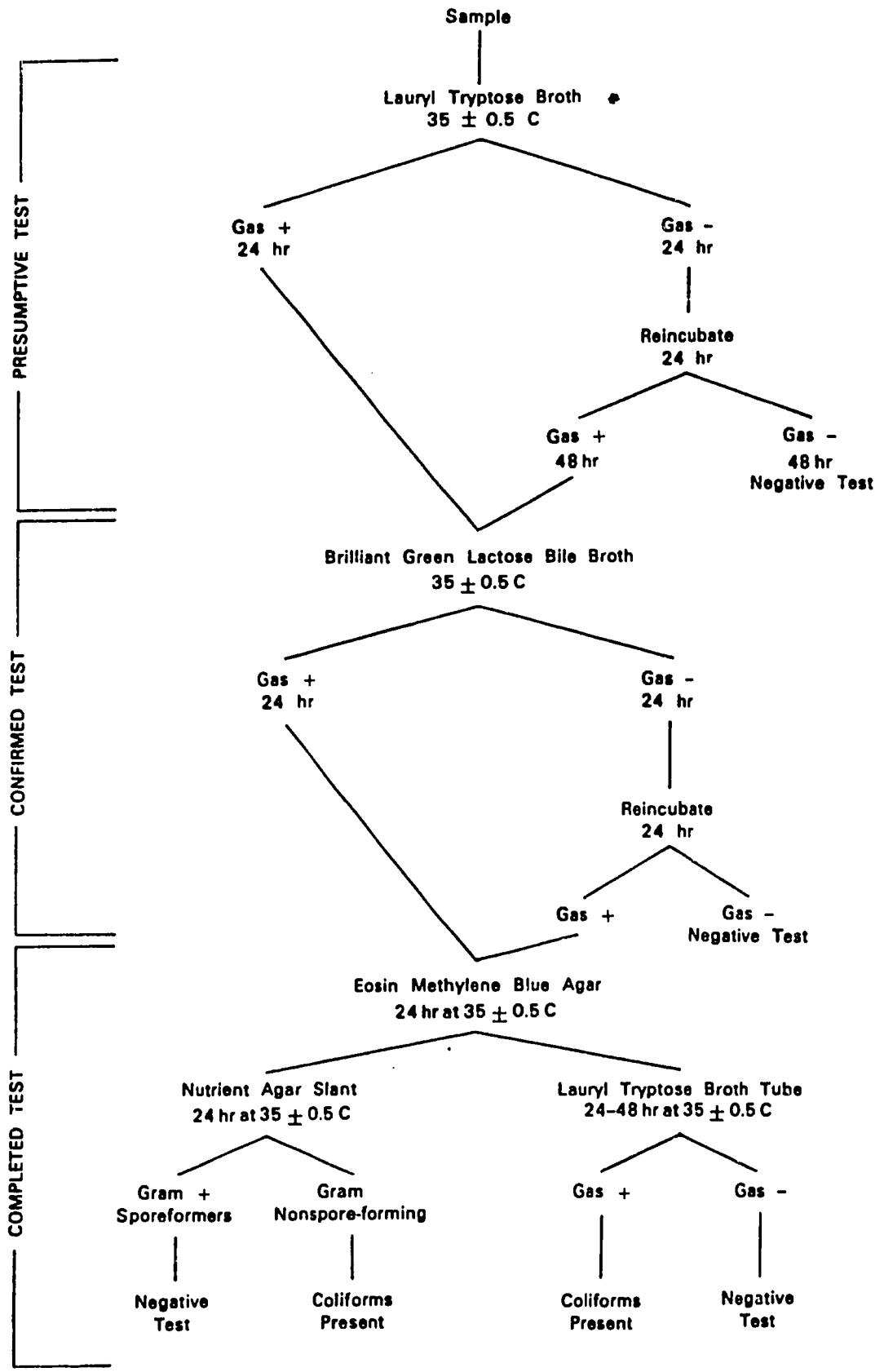


FIGURE III-B-2. Flow Chart for the Total Coliform MPN Test

110

4.3.8 Pyrex culture tubes, 150 × 25 mm or 150 × 20 mm, containing inverted fermentation vials, 75 × 10 mm with caps.

4.3.9 Culture tube racks to hold fifty, 25 mm diameter tubes.

4.3.10 Dilution bottles (milk dilution) pyrex glass, 99 ml volume, screw cap with neoprene rubber liners.

4.4 Media

4.4.1 Presumptive Test: Lauryl tryptose broth. See Part II-B, 5.3.1 . Lactose broth is not used because of false positive reactions.

4.4.2 Confirmed Test: Brilliant green bile broth. (See Part II-B, 5.3.2).

4.4.3 Completed Test:

(a) Eosin methylene blue agar (see Part II-B, 5.3.3).

(b) Nutrient agar or plate count agar slants (see Part II-B, 5.1.1 and 5.1.5).

4.5 Dilution Water: Sterile dilution water dispensed in 99 ± 2 ml amounts preferably in screw-capped bottles. (See Part II-B, 7).

4.6 Procedure: Part II-C describes the general MPN procedure in detail.

4.6.1 Prepare the media for Presumptive, Confirmed or Completed Tests selected. (See Part II-B, 5.3).

4.6.2 Presumptive Test (See Figure III-B-2): To begin the Presumptive Test, arrange fermentation tubes of lauryl tryptose broth in rows of 5 tubes each in the tube rack. Select sample volumes and clearly label each bank of tubes to identify the sample and volume inoculated.

(a) For potable waters, five portions of 10 ml each or five portions of 100 ml each are used.

(b) For relatively-unpolluted waters the sample volumes for the five rows might be 100, 10, 1, 0.1 and 0.01 ml, respectively; the latter two volumes delivered as dilutions of original sample.

(c) For known polluted waters the initial sample inoculations might be 0.1, 0.01, 0.001, 0.0001, and 0.00001 ml of original sample delivered as dilutions into successive rows each containing five replicate volumes. This series of sample volumes will yield determinate results from a low of 200 to a high of 16,000,000 organisms per 100 ml.

(d) Shake the sample and dilutions vigorously about 25 times. Inoculate each 5-tube row with replicate sample volumes in increasing decimal dilutions and incubate at 35 C ± 0.5 C.

(e) After 24 ± 2 hours incubation at 35 C, gently agitate the tubes in the rack and examine the tubes for gas. Any amount of gas constitutes a positive test. If there is no gas production in the tubes, reincubate for an additional 24 hours and reexamine for gas. Positive Presumption tubes are submitted directly to the Confirmed Test. Results are recorded on laboratory bench forms.

(f) If a laboratory using the MPN test on water supplies finds frequent numbers of Presumptive test tubes with heavy growth but no gas, these negative tubes should be submitted to the Confirmed Test to check for suppression of coliforms.

(g) If The Presumptive Test tubes are gas-negative after 48 ± 3 hours, they are discarded and the results recorded as negative Presumptive Tests. Positive Presumptive tubes are verified by the Confirmed Test.

(h) If the fecal colifrom test is to be run, (Part III-C), the analyst can inoculate growth from positive Presumptive Test tubes into EC medium at the same time as he inoculates the Confirmed Test Medium.

4.6.3 Confirmed Test (See Figure III-B-2)

(a) Carefully shake each positive Presumptive tube. With a sterile 3 mm loop or a sterile

applicator stick, transfer growth from each tube to BGLB. Gently agitate the tubes to mix the inoculum and incubate at 35 ± 0.5 C.

(b) After 24 ± 2 hours incubation at 35 C examine the tubes for gas. Any amount of gas in BGLB constitutes a positive Confirmed Test. If there is no gas production in the tubes (negative test) reincubate tubes for an additional 24 hours. Record the gas-positive and gas-negative tubes. Hold the positive tubes for the Completed Test if required for quality control or for checks on questionable reactions.

(c) After 48 ± 3 hours reexamine the Confirmed Test Tubes. Record the positive and negative tube results. Discard the negative tubes and hold the positive tubes for the Completed Test if required as in (b) above.

(d) In routine practice most sample analyses are terminated at the end of the Confirmed Test. However, the Confirmed Test data should be verified by carrying 5% of Confirmed Tests with a minimum of one sample per test run through the Completed Test.

(e) For certification of water supply laboratories, the MPN test is carried to completion (except for gram stain) on 10 percent of positive confirmed samples and at least one sample quarterly.

4.6.4 Completed Test (See Figure III-B-2)

Positive Confirmed Test cultures may be subjected to final Completed Test identification through application of further biochemical and culture tests, as follows:

(a) Streak one or more EMB agar plates from each positive BGLB tube. Incubate the plates at 35 ± 0.5 C for 24 ± 2 hours.

(b) Transfer one or more well-isolated typical colonies (nucleated with or without a metallic sheen) to lauryl tryptose broth fermentation tubes and to nutrient or plate count agar slants. Incubate the slants for 24 ± 2 or 48 ± 3 hours at 35 ± 0.5 C. If no typical colonies are present, pick and inoculate at least two atypical (pink, mucoid and unnucleated) colonies into lauryl tryptose fermentation tubes and incubate tubes for up to 48 ± 3 hours.

(c) The formation of gas in any amount in the fermentation tubes and presence of gram negative rods constitute a positive Completed Test for total coliforms.

4.6.5 Special Considerations for Potable Waters

Sample Size – For potable waters the standard sample shall be five times the standard portion which is either 10 milliliters or 100 milliliters as described in 40 CFR 141 (5).

Confirmation – If a laboratory using the MPN test on water supplies finds frequent numbers of Presumptive test tubes with heavy growth but no gas, these negative tubes should be submitted to the Confirmed Test to check for suppression of coliforms.

Completion — In water supply laboratories, 10% of all samples and at least one sample quarterly must be carried to completion but no gram stain of cultures is required.

4.7 Calculations: The results of the Confirmed or Completed Test may be obtained from the MPN table based on the number of positive tubes in each dilution. See Part II-C, 4.9 for details on calculation of MPN results.

4.7.1 Table II-C-4 illustrates the MPN index and 95% Confidence Limits for combinations of positive and negative results when five 10 ml, five 1.0 ml, and five 0.1 ml volumes of sample are tested.

4.7.2 Table II-C-5 provides the MPN indices and limits for the five tube, single volumes used for potable water supplies.

4.7.3 When the series of decimal dilutions is other than those in the tables select the MPN value from Table II-C-4 and calculate according to the following formula:

$$\text{MPN (From Table)} \times \frac{10}{\text{Largest Volume Tested}}$$

$$= \text{MPN/100 ml}$$

122

4.8 Reporting Results: Report the MPN values per 100 ml of sample. See an example of a report form in Figures III-D-2 and III-D-3.

4.9 Precision and Accuracy: The precision of the MPN value increases with increased numbers of replicates tested. A five tube, five dilution MPN is recommended for natural and waste waters. Only a five tube, single volume series is required for potable waters.

5. Differentiation of the Coliform Group by by Further Biochemical Tests

5.1 Summary: The differentiation of the members of the coliform group into genera and species is based on additional biochemical and cultural tests (see Table III-B-1). These tests require specific training for valid results.

5.2 Apparatus and Materials

5.2.1 Incubator set at 35 ± 0.5 C.

5.2.2 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

5.2.3 Inoculation loop, 3 mm diameter and needle.

5.2.4 Bunsen/Fisher type burner or electric incinerator.

5.2.5 Sterile TD Mohr and bacteriological pipets, glass or plastic, of appropriate volumes.

5.2.6 Graduates, 25 - 500 ml.

5.2.7 Test tubes, 100 × 13 mm or 150 × 20 mm with caps, in racks.

5.2.8 Reagents

(a) Indole Test Reagent: Dissolve 5 grams para-dimethylamino benzaldehyde in 75 ml isoamyl (or normal amyl) alcohol, ACS grade, and slowly add 25 ml conc HCl. The reagent should be yellow and have a pH below 6.0. If the final reagent is dark in color it should be discarded.

Some brands are not satisfactory and others become unsatisfactory after aging. Both amyl alcohol and benzaldehyde compound should be purchased in as small amounts as will be consistent with the volume of work anticipated. Store the reagent in the dark in a brown bottle with a glass stopper.

(b) Methyl Red Test Reagent: Dissolve 0.1 gram methyl red in 300 ml of 95% ethyl alcohol and dilute to 500 ml with distilled water.

(c) Voges-Proskauer Test Reagents

(1) Naphthol solution: Dissolve 5 grams purified alphanaphthol (melting point 92.5 C or higher) in 100 ml absolute ethyl alcohol. This solution must be freshly prepared each day.

(2) Potassium hydroxide solution: Dissolve 40 grams KOH in 100 ml distilled water.

(d) Oxidase Test Reagents

(1) Reagent A: Weigh out 1 gram alphanapthol and dissolve in 100 ml of 95% ethanol.

(2) Reagent B: Weigh out 1 gram para-aminodimethylaniline HCl (or oxylate) and dissolve in 100 ml of distilled water. Prepare frequently and store in refrigerator.

5.3 Media

5.3.1 Tryptophane broth for demonstrating indole production in the Indole Test. (See Part II-B, 5.1.9 (a) for preparation).

5.3.2 MR-VP broth (buffered glucose) to demonstrate acid production by methyl red color change in the Methyl Red Test and to demonstrate acetyl methyl carbinol production in the Voges-Proskauer test. (See Part II-B, 5.1.9 (b) for preparation).

5.3.3 Simmon's Citrate Agar to demonstrate utilization of citrate as a sole source of carbon. (See Part II-B, 5.1.9 (c) for preparation).

TABLE III-B-1

Differentiation of the Coliform and Related Organisms Based Upon Biochemical Reactions

Bacterium	Tests								
	Indole	Methyl Red	Voges-Proskauer	Citrate	Cytochrome Oxidase	Ornithine Decarboxylase	Lysine Decarboxylase	Arginine Dehydrolase	Motility
Escherichia coli	+	+	–	–	–	V	V	V	±
Citrobacter freundii	–	+	–	+	–	V	V	V	+
Enterobacter aerogenes	–	–	+	+	–	+	+	–	+
Klebsiella	±	–	+	±	–	–	+	–	–
Pseudomonas	–	–	–	+	+	–	–	–(+)[1]	+
Aeromonas	+	–	+	+	+	+	–	+	+

V = variable

( )[1] = reaction of *P. aeruginosa*

124

5.3.4 Nutrient agar slant for oxidase test. (See Part II-B, 5.1.1 for preparation).

5.3.5 Decarboxylase medium base containing lysine HCl, arginine HCl or ornithine HCl to demonstrate utilization of the specific amino acids. (See Part II-B, 5.5.14 for preparation).

5.3.6 Motility test medium (Edwards and Ewing). (See Section II-B, 5.1.10 for preparation).

5.3.7 Multitest Systems (optional to Single Test Series)

(a) API Enteric 20 (Analytab Products, Inc.).

(b) Enterotube (Roche Diagnostics).

(c) Inolex (Inolex Biomedical Division of Wilson Pharmaceutical and Chemical Corp.).

(d) Minitek (Baltimore Biological Laboratories, Bioquest).

(e) Pathotec Test Strips (General Diagnostics Division of Warner-Lambert Company).

(f) r/b Enteric Differential System (Diagnostic Research, Inc.).

5.4 Procedure

5.4.1 Biochemical tests should always be performed along with positive and negative controls. See Table IV-A-5.

5.4.2 Indole Test

(a) Inoculate a pure culture into 5 ml of tryptophane broth.

(b) Incubate the tryptophane broth at 35 ± 0.5 C for 24 ± 2 hours and mix well.

(c) Add 0.2–0.3 ml test reagent to the 24 hour culture, shake and allow the mixture to stand for 10 minutes. Observe and record the results.

(d) A dark red color in the amyl alcohol layer on top of the culture is a positive indole test; the original color of the reagent, a negative test. An orange color may indicate the presence of skatole and is reported as a ± reaction.

5.4.3 Methyl Red Test

(a) Inoculate a pure culture into 10 ml of buffered glucose broth.

(b) Incubate for 5 days at 35 C.

(c) To 5 ml of the five day culture, add 5 drops of methyl red indicator.

(d) A distinct red color is positive and distinct yellow, negative. Orange color is dubious, may indicate a mixed culture and should be repeated.

5.4.4 Voges Proskauer Test: This procedure detects the production of acetyl methyl carbinol which in the presence of alphanapthol and potassium hydroxide develops a reddish color.

(a) Use a pure culture to inoculate 10 ml of buffered glucose broth or 5 ml of salt peptone glucose broth or use the previously inoculated buffered glucose broth from the Methyl Red Test.

(b) Incubate the inoculated salt peptone glucose broth or the buffered glucose broth at 35 ± 0.5 C for 48 hours.

(c) Add 0.6 ml naphthol solution and 0.2 ml KOH solution to 1 ml of the 48 hour salt peptone or buffered glucose broth culture in a separate clean test tube. Shake vigorously for 10 seconds and allow the mixture to stand for 2–4 hours.

(d) Observe the results and record. A pink to crimson color is a positive test. Do not read after 4 hours. A negative test may develop a copper or faint brown color.

5.4.5 Citrate Test

(a) Lightly inoculate a pure culture into a tube of Simmon's Citrate Agar, using a needle to stab, then streak the medium. Be careful not to carry over any nutrient material.

(b) Incubate at 35 C for 48 hours.

(c) Examine agar tube for growth and color change. A distinct Prussian blue color in the presence of growth indicates a positive test; no color change is a negative test.

5.4.6 Cytochrome Oxidase Test (Indophenol): The cytochrome oxidase test can be done with commercially-prepared paper strips or on a nutrient agar slant as follows:

(a) Inoculate nutrient agar slant and incubate at 35 C for 18–24 hours. Older cultures should not be used.

(b) Add 2–3 drops of reagent A and reagent B to the slant, tilt to mix and read reaction within 2 minutes.

(c) Strong positive reaction (blue color slant or paper strip) occurs in 30 seconds. Ignore weak reactions that occur after 2 minutes.

5.4.7 Decarboxylase Tests (lysine, arginine and ornithine)

(a) The complete decarboxylase test series requires tubes of each of the amino acids and a control tube containing no amino acids.

(b) Inoculate each tube lightly.

(c) Add sufficient sterile mineral oil to the broths to make 3–4 mm layers on the surface and tighten the screw caps.

(d) Incubate for 18–24 hours at 35 C and read. Negative reactions should be reincubated up to 4 days.

(e) Positive reactions are purple and negative reactions are yellow. Read the control tube without amino acid first; it must be yellow for the reactions of the other tubes to be valid. Positive purple tubes must have growth as evidenced by turbidity because uninoculated tubes are also purple; nonfermenters may remain alkaline throughout incubation.

5.4.8 Motility Test

(a) Stab-inoculate the center of the tube of Motility Test Medium to at least half depth.

(b) Incubate tubes 24–48 hours at 35 C.

(c) Examine tubes for growth. If negative, reincubate at room temperature for 5 more days.

(d) Non-motile organisms grow only along the line of inoculation. Motile organisms grow outward from the line of inoculation and spread throughout the medium producing a cloudy appearance.

(e) Addition of 2, 3, 5 triphenyl tetrazolium chloride (TTC) will aid recognition of motility. Growth of microorganisms reduces TTC and produces red color along the line of growth.

5.4.9 Additional Biochemical Tests: If other biochemical tests are necessary to further identify enteric bacteria, for example specific carbohydrate fermentation, see the Table III-E-5, Biochemical Characteristics of Enterobacteriaceae.

5.4.10 Multitest Systems: Multitest systems are available which use tubes containing agar media that provide numerous biochemical tests, plastic units containing a series of dehydrated media, media-impregnated discs and reagent-impregnated paper strips. Some of the systems use numerical codes to aid identification. Others provide computerized identification of bacteria. A number of independent investigators have compared one or more multitest systems with conventional or traditional biochemical tests. Some of the earlier systems have been improved. Most of the recent studies report the correct identification of high percentages of isolates. The systems are described in Part III-E, 5.6.

126

REFERENCES

1. Geldreich, E. E., P. W. Kabler, H. L. Jeter and H. F. Clark, 1955. A delayed incubation membrane filter test for coliform bacteria in water. Amer. Jour. Public Health 45:1462.

2. Brezenski, F. T. and J. A. Winter, 1969. Use of the delayed incubation membrane filter test for determining coliform bacteria in sea water. Water Res. 3:583.

3. Geldreich, E. E., 1975. Handbook for Evaluating Water Bacteriological Laboratories (2nd ed.), EPA-670/9-75-006. U.S. Environmental Protection Agency, Cincinnati, Ohio.

4. Tubiash, H., 1951. The Anaerogenic Effect of Nitrates and Nitrites on Gram-negative Enteric Bacteria. Amer. Jour. Public Health 41:833.

5. National Interim Primary Drinking Water Regulations, 40 Code of Federal Regulations (CFR) Part 141.14 (b) and (c), Published in Federal Register, 40, 59566, December 24, 1975.

PART III. ANALYTICAL METHODOLOGY

Section C Fecal Coliform Methods

The direct membrane filter (MF), the delayed-incubation MF and the multiple-tube, most probable number (MPN) methods can be used to enumerate fecal coliforms in water and wastewater. For a general description of the fundamental laboratory techniques refer to Part II-C. The method chosen depends upon the characteristics of the sample. The Section is divided as follows:

1. **Definition of the Fecal Coliform Group**
2. **Direct Membrane Filter (MF) Method**
3. **Delayed-Incubation Membrane Filter Method**
4. **Verification**
5. **Most Probable Number (MPN) Method**

1. Definition of the Fecal Coliform Group

1.1 The fecal coliforms are part of the total coliform group. They are defined as gram-negative nonspore-forming rods that ferment lactose in 24 $\pm$ 2 hours at 44.5 $\pm$ 0.2 C with the production of gas in a multiple-tube procedure or produce acidity with blue colonies in a membrane filter procedure.

1.2 The major species in the fecal coliform group is *Escherichia coli*, a species indicative of fecal pollution and the possible presence of enteric pathogens.

2. Direct Membrane Filter (MF) Method

2.1 Summary: An appropriate volume of a water sample or its dilution is passed through a membrane filter that retains the bacteria present in the sample. The filter containing the microorganisms is placed on an absorbent pad saturated with M-FC broth or on M-FC agar in a petri dish. The dish is incubated at 44.5 C for 24 hours. After incubation, the typical blue colonies are counted under low magnification and the number of fecal coliforms is reported per 100 ml of original sample.

2.2 Scope and Application

2.2.1 Advantages: The results of the MF test are obtained in 24 hours. Up to 72 hours are required for the multiple-tube fermentation method. The M-FC method provides direct enumeration of the fecal coliform group without enrichment or subsequent testing. Over 93% of the blue colonies that develop in this test using M-FC medium at the elevated temperature of 44.5 C $\pm$ 0.2 C are reported to be fecal coliforms (1). The test is applicable to the examination of lakes and reservoirs, wells and springs, public water supplies, natural bathing waters, secondary non-chlorinated effluents from sewage treatment plants, farm ponds, stormwater runoff, raw municipal sewage, and feedlot runoff. The MF test has been used with varied success in marine waters.

2.2.2 Limitations: Recent data (2, 3) indicate that the single-step MF fecal coliform procedure may produce lower results than those obtained with the fecal coliform

128

multiple-tube procedure, particularly for chlorinated effluents. Since chlorination stresses fecal coliforms and significantly reduces recovery, this method should not be used with chlorinated wastewater. Disinfection and toxic materials such as metals, phenols, acids or caustics also affect recovery of fecal coliforms on the membrane filter. Any decision to use this test for stressed microorganisms requires parallel MF/MPN evaluation based on the procedure described in Part IV-C, 3.

Recently-proposed solutions to problems of lower recovery (2, 4, 5, 6) include the use of two-step incubation, two-step incubation overlay and/or enrichment techniques and modification of membrane filter structures.

2.3 Apparatus and Materials

2.3.1 Water bath, aluminum heat sink, or other incubator that maintains a stable 44.5 ± 0.2 C. Temperature is checked against an NBS certified thermometer or one of equivalent accuracy.

2.3.2 Binocular (dissecting type) microscope, with magnification of 10–15× and daylight-type fluorescent lamp.

2.3.3 Hand tally.

2.3.4 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

2.3.5 Graduated cylinders, covered with aluminum foil or kraft paper before sterilization.

2.3.6 Sterile, unassembled membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil and kraft paper.

2.3.7 Vacuum source.

2.3.8 Vacuum filter flask, with appropriate tubing. Filter manifolds which hold a number of filter bases can also be used.

2.3.9 Safety trap flask between the filter flask and the vacuum source.

2.3.10 Forceps with smooth tips.

2.3.11 Ethanol, 95% or methanol, in small vial, for sterilizing forceps.

2.3.12 Bunsen/Fisher burner or electric incinerator.

2.3.13 Sterile TD bacteriological or Mohr pipets, glass or plastic, of appropriate size.

2.3.14 Sterile petri dishes, 50 × 12 mm plastic with tight-fitting lids.

2.3.15 Dilution bottles (milk dilution), pyrex glass, marked at 99 ml volume, screw-cap with neoprene rubber liner.

2.3.16 Membrane filters, white, grid marked, 47 mm diameter, 0.45 ± 0.02 μm pore size or other pore size recommended by manufacturer for water analyses. The Millipore HC MF, not the HA, is recommended.

2.3.17 Absorbent pads.

2.3.18 Water-proof plastic bags.

2.3.19 Inoculation loops, 3 mm diameter, or needle of nichrome or platinum wire, 26 B&S gauge, in suitable holder.

2.3.20 Disposable applicator sticks or plastic loops as alternatives to inoculation loops.

2.3.21 Ultraviolet sterilizer for MF filtration units (optional).

2.4 Media

2.4.1 M-FC broth or agar prepared in presterilized erlenmeyer flasks (See Part II-B, 5.2.1).

2.4.2 Lauryl tryptose broth prepared in 10 ml volumes in fermentation tubes (see Part II-B, 5.3.1) for verification.

129

2.4.3 EC medium prepared in 10 ml volumes in fermentation tubes (see Part II-B, 5.3.4) for verification.

2.5 Dilution Water (See Part II,B, 7 for preparation).

2.5.1 Sterile buffered dilution water or peptone water dispensed in 99±2 ml amounts in screw-capped dilution bottles.

2.5.2 Sterile buffered water or peptone water prepared in 500 ml or larger volumes for wetting membranes before addition of the sample, and for rinsing the funnel after sample filtration.

2.6 Procedure: The general membrane filter procedure is described in detail in Part II-C.

2.6.1 Prepare the M-FC broth or agar medium as outlined in Part II-B, 5.2.1. Saturate the sterile absorbent pads with about 2.0 ml of broth or add 5–6 ml of M-FC agar to the bottom of each 50 × 12 mm petri dish (to a depth of 2–3 mm). Pour off excess liquid from broth-saturated pads. Mark dishes and bench forms with sample identity and sample volumes.

2.6.2 Using a sterile forceps place a sterile membrane filter on the filter base, grid side up.

Attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and base.

2.6.3 Shake the sample vigorously about 25 times and measure the sample into the funnel with the vacuum off. If sample volume is < 10 ml, add 10 ml of sterile dilution water to the filter before adding the sample.

2.6.4 Sample volumes for fecal coliform enumeration in different waters and wastewaters are suggested in Table III-C-1. These volumes should provide the recommended count of 20–60 colonies on a membrane filter. Fecal coliform levels are generally lower than total coliform densities in the same sample; therefore larger volumes are sampled.

2.6.5 Do not filter less than 1.0 ml of undiluted sample.

2.6.6 Filter the sample and rinse the sides of the funnel walls at least twice with 20–30 ml of sterile dilution water.

2.6.7 Turn off the vacuum and remove the funnel from the filter base.

2.6.8 Aseptically remove the membrane filter from the filter base. Place the filter, grid side up, on the absorbent pad saturated with M-FC Broth or on M-FC agar, using a rolling motion to prevent air bubbles.

2.6.9 Incubate the petri dishes for 24 ± 2 hours at 44.5 ± 0.2 C in sealed waterproof plastic bags submerged (with the petri dishes inverted) in a waterbath, or without plastic bag in a heat-sink incubator. MF cultures should be placed in incubator within 30 minutes of filtration.

2.6.10 After 24 hours remove dishes from the incubator and examine for blue colonies.

2.7 Counting and Recording Colonies: Select those plates with 20-60 blue (sometimes greenish-blue) colonies. Non-fecal colonies are gray, buff or colorless and are not counted. Pinpoint blue colonies should be counted and confirmed. The colonies are counted using a microscope of 10-15× and a fluorescent lamp. Use of hand lens or other simple optical devices of lower magnification make difficult the identification and differentiation of typical and atypical blue colonies.

2.7.1 The general counting rules are given in Part II-C, 3.5. The following rules are used in calculating the fecal coliform count per 100 ml of sample:

(a) Countable Membranes with 20–60 Blue Colonies. Count all blue colonies using the formula:

$$\frac{\text{No. of Fecal Coliform Colonies Counted}}{\text{Volume in ml of Sample Filtered}} \times 100 = \text{fecal coliform count/100 ml}$$

730

TABLE III-C-1

Suggested Range of Sample Volumes for Fecal Coliform Tests
Using the Membrane Filter Method

Sample Source	100	30	10	3	1	0.3	0.1	0.03	0.01	0.003	0.001	0.0003	0.0001
Swimming Pools	X												
Wells, Springs	X	X											
Lakes, Reservoirs	X	X	X										
Water Supply Intakes			X	X	X	X	X						
Bathing Beaches			X	X	X	X	X						
River Water					X	X	X	X	X				
Chlorinated Sewage Effluent					X	X	X	X	X				
Raw Sewage									X	X	X	X	X

127

For example, if 40 colonies are counted after the filtration of 50 ml of sample, the calculation is:

$$\frac{40}{50} \times 100 = \underline{80 \text{ fecal coliforms/100 ml}}.$$

(b) Countable Membranes With Less Than 20 Blue Colonies. Report as: Estimated Count/100 ml and specify the reason.

(c) Membranes With No Colonies. Report the count as: Less than (calculated value)/100 ml, based upon the largest single volume filtered.

For example, if 10, 3 and 1 ml are filtered and all plates show zero counts, select the largest volume, apply the general formula and report the count as a < (less than) value:

$$\frac{1}{10} \times 100 = 10$$

or < 10 fecal coliforms/100 ml.

(d) Countable Membranes With More Than 60 Blue Colonies. Calculate count from highest dilution and report as a > value.

(e) Uncountable Membranes With More Than 60 Colonies. Use 60 colonies as the basis of calculation with the smallest filtration volume, e.g., 0.01 ml:

$$\frac{60}{0.01} \times 100 = 600{,}000$$

Report as: > 600,000 fecal coliforms/100 ml.

2.7.2 Reporting Results. Report fecal coliform densities per 100 ml. See discussion on significant figures in Part II-C, 2.8.1.

2.8 Precision and Accuracy

2.8.1 Ninety-three percent of the blue colonies that develop on M-FC medium at the elevated temperature of 44.5 ± 0.2 C were verified as fecal coliform (1).

2.8.2 Laboratory personnel should be able to duplicate their own colony counts on the same plate within 5%, and the counts of other analysts on the same plate within 10%.

3. Delayed-Incubation Membrane Filter (MF) Method

3.1 Summary: Bacteria are retained on 0.45 μm filters after passage of selected sample volumes through the filters. The filters are placed on M-VFC broth (a minimum growth medium) and transported from field sites to the laboratory. In the laboratory, the filters are transferred to the M-FC medium and incubated at 44.5 C for 24 hours. Blue colonies are counted as fecal coliforms.

3.2 Scope and Application

3.2.1 Advantages: The delayed incubation MF method is useful in survey monitoring or emergency situations when the standard fecal coliform test cannot be performed at the sample site, or when time and temperature limits for sample storage cannot be met. The method eliminates field processing and equipment needs. Also, examination at a central laboratory permits confirmation and biochemical identification of the organisms as necessary. Consistent results have been obtained with this method using water samples from a variety of sources (7).

3.2.2 Limitations: The applicability of this method for a specific water source must be determined in preliminary studies by comparison with the standard MF method. For example, limited testing has indicated that the delayed-incubation method is not as effective in saline waters (7).

3.3 Apparatus and Materials

3.3.1 Water bath, aluminum heat sink, or equivalent incubator that maintains a 44.5 ± 0.2 C temperature.

3.3.2 Binocular (dissection) microscope, with magnification 10 or 15×, binocular, wide-field type. A microscope lamp producing

diffuse daylight from cool white fluorescent lamps.

3.3.3 Hand tally.

3.3.4 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

3.3.5 Graduated cylinders, covered with aluminum foil or kraft paper before sterilization.

3.3.6 Sterile unassembled membrane filtration units (filter base and funnel), glass, plastic or stainless steel wrapped with aluminum foil or kraft paper.

3.3.7 Vacuum source.

3.3.8 Filter flask to hold filter base, with appropriate tubing. Filter manifold to hold a number of filter bases can also be used. In the field, portable field kits are also used.

3.3.9 Safety trap flask between the filtering flask and the vacuum source.

3.3.10 Forceps with smooth tip.

3.3.11 Ethanol, 95% or methanol, in small vial, for sterilizing forceps.

3.3.12 Bunsen/Fisher type burner.

3.3.13 Sterile TD bacteriological or Mohr pipets, glass or plastic, in appropriate volumes.

3.3.14 Sterile petri dishes, 50 × 12 mm plastic with tight-fitting lids.

3.3.15 Dilution bottles (milk dilution), pyrex glass, 99 ml volume, screw-caps with neoprene rubber liners.

3.3.16 Membrane filters, white, grid marked, 47 mm in diameter, 0.45±0.02 μm pore size, or other pore size recommended by the manufacturer for water analyses. The Millipore HC MF, not the HA is recommended.

3.3.17 Shipping tubes, labels, and packing materials for mailing delayed incubation plates.

3.3.18 Ultraviolet sterilizer for MF filtration units (optional).

3.4 Media: The following media are prepared in pre-sterilized erlenmeyer flasks with metal caps, aluminum foil covers, or screw-caps:

3.4.1 M-VFC holding media (see Part II-B, 5.2.6).

3.4.2 M-FC broth or agar (see Part II-B, 5.2.1).

3.5 Dilution Water

3.5.1 Sterile dilution water dispensed in 99 ± 2 ml volumes in screw-capped bottles.

3.5.2 Sterile dilution water prepared in large volumes for wetting membranes before the addition of the sample, and for rinsing the funnel after sample filtration.

3.6 Procedure: The general membrane filter procedure is described in detail in Part II-C.

3.6.1 Prepare the M-VFC holding medium as outlined in Part II-B, 5.2.6. Saturate the sterile absorbent pads with about 2.0 ml of M-VFC broth. Pour off excess broth. Mark dishes and bench forms with sample identity and volumes.

3.6.2 Using sterile forceps place a membrane filter on the filter base grid side up.

3.6.3 Attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and base.

3.6.4 Shake the sample vigorously about 25 times and measure into the funnel with the vacuum off. If the sample is < 10 ml, add 10 ml of sterile dilution water to the membrane filter before adding the sample.

(a) Sample volumes for fecal coliform enumeration in different waters and wastewaters are suggested in Table III-C-1. These volumes should produce membrane filters with a recommended count of 20–60 colonies.

(b) Follow the methods for sample measurement and dispensation given in Part II-C, 3.4.6.

3.6.5 Filter the sample through the membrane and rinse the sides of the funnel walls at least twice with 20–30 ml of sterile dilution water.

3.6.6 Turn off the vacuum and remove the funnel from the base of the filter unit.

3.6.7 Aseptically remove the membrane filter from the filter base and place grid side up on an absorbent pad saturated with VFC medium.

3.6.8 Place the culture dish in shipping container and send to the examining laboratory. Fecal coliform bacteria can be held on the VFC holding medium for up to 72 hours with little effect on the final counts. The holding period should be kept to a minimum.

3.6.9 At the examining laboratory remove the membrane from the holding medium, place it in another dish containing M-FC broth or agar medium, and complete testing for fecal coliforms as described above under 2.6.

3.7 Counting and Recording Colonies: After the required incubation select those plates with 20-60 blue (sometimes greenish-blue) colonies. Gray to cream colored colonies are not counted. Pin-point blue colonies are not counted unless confirmed. The colonies are enumerated using a binocular microscope with a magnification of 10 or 15×.

Refer to 2.7.1, for rules used in reporting the fecal coliform MF counts.

3.8 Reporting Results: Record densities as fecal coliforms per 100 ml. Refer to Part II-C, 2.8, for discussions on the use of significant figures and rounding off values.

3.9 Precision and Accuracy: As reported in 2.8, this Section.

4. Verification

Verification of the membrane filter test for fecal coliforms establishes the validity of colony differentiation by blue color and provides supporting evidence of colony interpretation. The verification procedure corresponds to the fecal coliform MPN (EC Medium) test.

4.1 Pick from the centers of at least 10 well-isolated blue colonies. Inoculate into lauryl tryptose broth and incubate 24-48 hours at 35 ± 0.5 C.

4.2 Confirm gas-positive lauryl tryptose broth tubes at 24 and 48 hours by inoculating a loopful of growth into EC tubes and incubating for 24 hours at 44.5 ± 0.2 C. Cultures that produce gas in EC tubes are interpreted as verified fecal coliform colonies (see Figure III-C-1).

4.3 A percent verification can be determined for any colony-validation test:

$$\frac{\text{No. of colonies meeting verification test}}{\text{No. of colonies subjected to verification}} \times 100 = \text{Percent verification}$$

Example: Twenty blue colonies on M-FC medium were subjected to verification studies shown in Figure III-C-1. Eighteen of these colonies proved to be fecal coliforms according to provisions of the test:

$$\text{Percent verification} = \frac{18}{20} \times 100 = 90\%$$

4.4 A percent verification figure can be applied to the direct test results to determine the verified fecal coliform count per 100 ml:

$$\frac{\text{Percent verification}}{100} \times \text{count per 100 ml} = \text{Verified fecal coliform count}$$

Example: For a given sample, by the M-FC test, the fecal coliform count was found to be

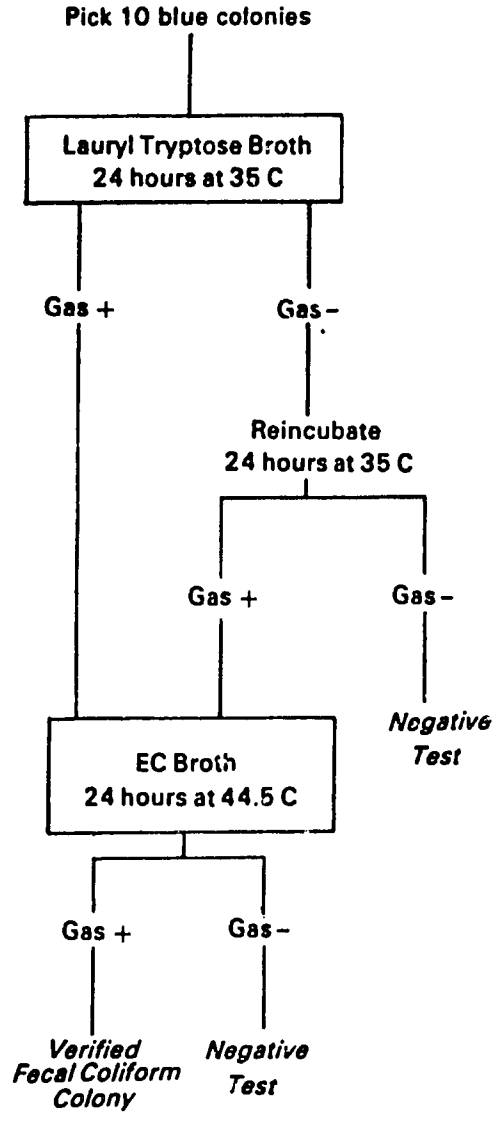


FIGURE III-C-1. Verification of Fecal Coliform Colonies on the Membrane Filter

735

42,000 organisms per 100 ml. Supplemental studies on selected colonies showed 92% verification.

$$\text{Verified fecal coliform count} = \frac{92}{100} \times 42{,}000 = 38{,}640$$

Rounding off = 39,000 fecal coliforms per 100 ml

The worker is cautioned not to apply percentage of verification determined on one sample to other samples.

5. Most Probable Number (MPN) Method

5.1 Summary: Culture from positive tubes of the lauryl tryptose broth (same as presumptive MPN Method, Part III-B) is inoculated into EC Broth and incubated at 44.5 C for 24 hours (see Figure III-C-2). Formation of gas in any quantity in the inverted vial is a positive reaction confirming fecal coliforms. Fecal coliform densities are calculated from the MPN table on the basis of the positive EC tubes (8).

5.2 Apparatus and Materials

5.2.1 Incubator that maintains 35 ± 0.5 C.

5.2.2 Water bath or equivalent incubator that maintains a 44.5 ± 0.2 C temperature.

5.2.3 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

5.2.4 Inoculation loop, 3 mm diameter and needle of nichrome or platinum wire, 26 B & S gauge, in suitable holder. Sterile applicator sticks are a suitable alternative.

5.2.5 Sterile pipets T.D., Mohr or bacteriological, glass or plastic, of appropriate size.

5.2.6 Dilution bottles (milk dilution), pyrex, 99 ml volume, screw-cap with neoprene liners.

5.2.7 Bunsen or Fisher-type burner or electric incinerator unit.

5.2.8 Pyrex test tubes, 150 × 20 mm, containing inverted fermentation vials, 75 × 10 mm, with caps.

5.2.9 Culture tube racks to hold fifty, 25 mm diameter tubes.

5.3 Media

5.3.1 Lauryl tryptose broth (same as total coliform Presumptive Test medium) prepared in 10 ml volumes in appropriate concentration for sample volumes used. (Part II-B, 5.3.1).

5.3.2 EC medium prepared in 10 ml volumes in fermentation tubes (Part II-B, 5.3.4).

5.4 Dilution Water: Sterile buffered or peptone dilution water dispensed in 99 ± 2 ml volumes in screw-capped bottles.

5.5 Procedure: Part II-C describes in detail the general MPN procedure. See Figure III-C-2.

5.5.1 Prepare the total coliform Presumptive Test medium, (lauryl tryptose broth) and EC medium. Clearly mark each bank of tubes, identifying the sample and the volume inoculated.

5.5.2 Inoculate the Presumptive Test medium with appropriate quantities of sample following the Presumptive Test total coliform procedure, (Part III-B).

5.5.3 Gently shake the Presumptive tube. Using a sterile inoculating loop or a sterile wooden applicator, transfer inocula from positive Presumptive Test tubes at 24 and 48 hours to EC confirmatory tubes. Gently shake the rack of inoculated EC tubes to insure mixing of inoculum with medium.

5.5.4 Incubate inoculated EC tubes at 44.5 ± 0.2 C for 24 ± 2 hours. Tubes must be placed in the incubator within 30 minutes after inoculation. The water depth in the water bath incubator must come to the top level of the culture medium in the tubes.

5.5.5 The presence of gas in any quantity in the EC confirmatory fermentation tubes af-

136

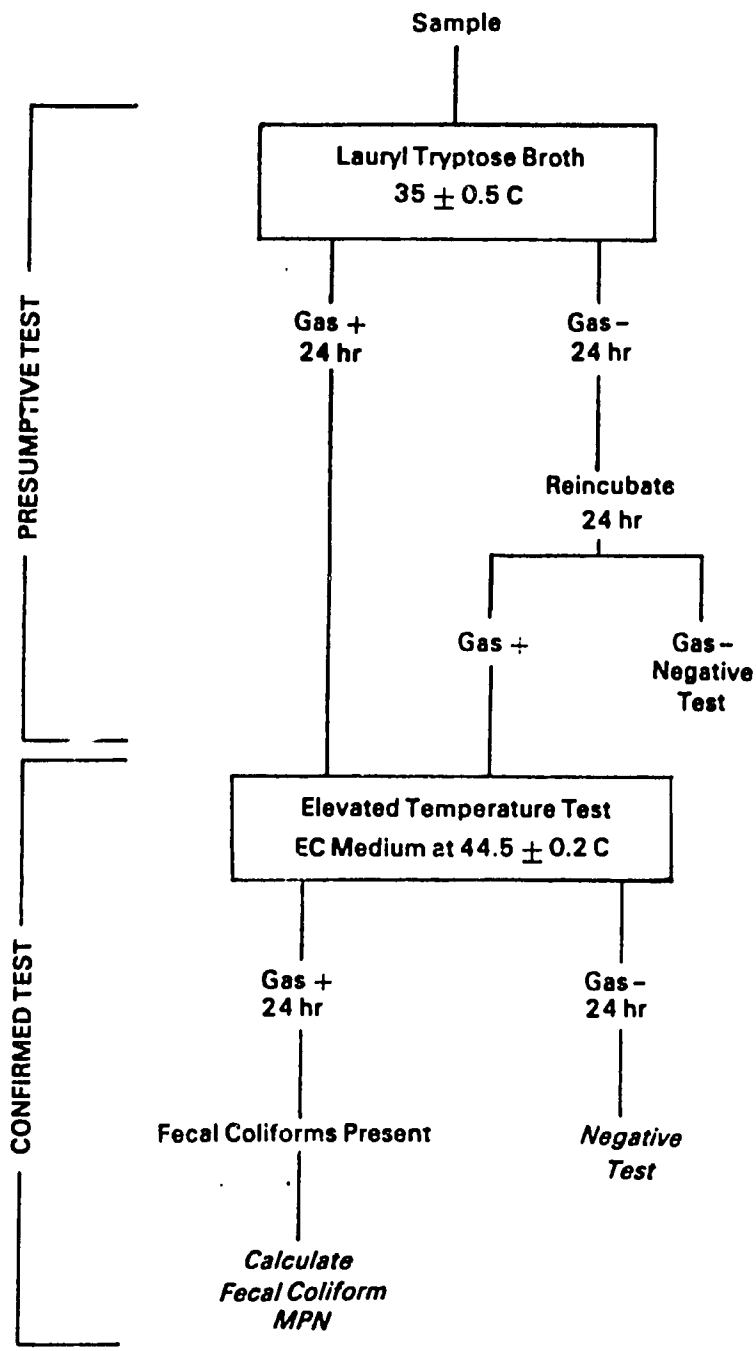


FIGURE III-C-2. Flow Chart for the Fecal Coliform MPN Tests.
(EPA, 1978)

ter 24 ± 2 hours constitutes a positive test for fecal coliforms.

5.6 Calculations

5.6.1 Calculate fecal coliform densities on the basis of the number of positive EC fermentation tubes, using the table of most probable numbers (MPN).

5.6.2 The MPN results are computed from three dilutions that include the highest dilution with all positive tubes and the next two higher dilutions. For example, if five 10 ml, five 1.0 ml, and five 0.1 ml sample portions are inoculated initially into Presumptive Test medium, and positive EC confirmatory results are obtained from five of the 10 ml portions, three of the 1.0 ml portions, and none of the 0.1 ml portions, the coded result of the test is 5-3-0. The code is located in the MPN Table II-C-4, and the MPN per 100 ml is recorded. See Part II-C, 4.9 for rules on selection of significant dilutions.

5.7 Reporting Results: Report the fecal coliform MPN values per 100 ml of sample.

5.8 Precision and Accuracy: The precisions of the MPN counts are given as confidence limits in the MPN tables. Note that the precision of the MPN value increases with increased numbers of replicates per sample tested.

REFERENCES

1. Geldreich, E. E., H. F. Clark, C. B. Huff, and L. C. Best, 1965. Fecal-coliform-organism medium for the membrane filter technique. J. Amer. Water Works Assoc. 57:208.

2. Bordner, R. H., C. F. Frith and J. A. Winter, (Editors), 1977. Proceedings of the Symposium on the Recovery of Indicator Organisms Employing Membrane Filters. U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, OH. (EPA-600/9-77-024).

3. Lin, S. D., 1973. Evaluation of coliform tests for chlorinated secondary effluents. J. Water Pollution Control Federation 45:498.

4. Lin, S. D., 1976. Evaluation of Millipore HA and HC membrane filters for the enumeration of indicator bacteria. Appl. Environ. Microbiol. 32:300.

5. Lin, S. D.,1976. Membrane filter method for recovery of fecal coliforms in chlorinated sewage effluents. Appl. Environ. Microbiol. 32:547.

6. Green, B. L., E. M. Clausen, and W. Litsky, 1975. Two-temperature membrane filter method for enumeration of fecal coliform bacteria from chlorinated effluents. Appl. Environ. Microbiol. 33: 1259.

7. Taylor, R. H., R. H. Bordner, and P. V. Scarpino, 1973. Delayed-incubation membrane-filter test for for fecal coliforms. Appl. Microbiol. 25:363.

8. Geldreich, E. E., 1966. Sanitary Significance of Fecal Coliforms in the Environment, U.S. Dept. of the Interior, FWPCA, WP-20-3, 122 pp.

PART IV. QUALITY ASSURANCE

Section A Laboratory Operations

Section A describes the checks and monitoring procedures that should be performed on materials, supplies, instrumentation and the physical facility. These checks should be documented completely and recorded as performed. See Part V-A, 1.2 for details on this documentation.

1. Sample Collection and Handling
2. Laboratory Facilities
3. Laboratory Personnel
4. Laboratory Equipment and Instrumentation
5. General Laboratory Supplies
6. Membrane Filters
7. Culture Media

1. Sample Collection and Handling

The acquisition of valid data begins with collection of a representative water sample or other environmental material being tested. Samples must be maintained as closely as possible to original condition by careful handling and storage.

Sample sites and a sampling frequency are selected to provide data representative of the characteristics and the variability of the water quality at that station. The most important quality control factor in sampling is the immediate analysis of the sample. If the sample cannot be analyzed at once, it should be refrigerated and analyzed within six hours. Recommended procedures for collecting, transporting and handling water and wastewater samples are described separately in Part II-A of this Manual.

2. Laboratory Facilities

2.1 Ventilation: Laboratories should be well-ventilated and free of dust, drafts and extreme temperature changes. Central air conditioning has advantages: 1) The incoming air is filtered, reducing contamination of the laboratory and culture work. 2) The uniform temperature control of air conditioning permits stable operation of incubators. 3) With closed windows, drafts and air currents which can cross-contaminate surroundings and work areas during warm weather are minimized. 4) Low humidity reduces moisture problems with media, chemicals, analytical balances and other instrumentation.

2.2 Space Utilization: Ideally the areas provided for the preparation and sterilization of media, glassware and equipment should be separated from the laboratory working area but located close enough for convenience. In public health laboratories that analyze many

different types of samples a separate work area is desirable for water analysis. Special work areas such as an absolute barrier or vented laminar flow hood (see Part V-C) are often used for dispensing and preparing sterile media and tissue cultures, for transferring microbial cultures or for working with pathogenic materials. In smaller laboratories it may be necessary to carry out these separate activities in different sections of the same room. However, limited facilities and restricted work space may seriously hamper the quality of the work and influence the validity of results. Visitors and through traffic should be discouraged in work areas. Through traffic can be prevented by laboratory design.

2.3 Laboratory Bench Areas: Sufficient clean bench space should be available for the analyses to be performed efficiently. For routine work, 6 linear feet is the recommended minimum work area for each analyst. Research work or other analyses using specialized equipment may require significantly more space per worker. These estimates of bench space are exclusive of work areas used for preparatory and supporting activities. Laboratory lighting should be even, screened to reduce glare, and provide about 100 footcandle light intensity at working surfaces.

Bench tops should be set at heights of 36–38 inches with a depth of 28–30 inches. This height is comfortable for work in a standing or sitting position. Desk tops or sit-down benches are set at 30–31 inches height to accommodate microscopy, plate counting, calculations and writing activities. Bench tops should be stainless steel, epoxy plastic or other smooth impervious material which is inert, corrosion-resistant and has minimum seams.

2.4 Walls and Floors: Walls should be covered with waterproof paint, enamel or other surface material that provides a smooth finish which is easily cleaned and disinfected. Floors should be covered with good quality tiles or other heavy duty material which can be maintained with skid-proof wax. Bacteriostatic agents contained in some wall or floor finishes increase the effectiveness of disinfection.

2.5 Monitoring for Cleanliness in Work Areas: High standards of cleanliness should be maintained in work areas. The laboratory can be monitored for cleanliness by one or more of the procedures described below. Since these monitoring procedures cannot recover all of the microorganism populations present, absolute limits are difficult to develop. Rather, the tests should be used regularly on a weekly or other basis to monitor counts in the same work areas over time or to make comparisons between different work areas.

2.5.1 RODAC Agar Plates (1)

Work areas and other surfaces can be checked by RODAC plates which contain general growth media for total counts or selective media for coliforms, enteric pathogens, streptococci, staphylococci, or other microorganisms cultured in the laboratory. RODAC is an acronym for Reproducible Organism Detection and Counting. The RODAC dish has a test area of about 25 cm with Quebec style grids embossed in the plate. It is specifically designed to enumerate the microbial population of flat solid surfaces by contact techniques.

(a) Purchase RODAC plates prefilled with desired test medium or prepare plates by filling the center well with about 16 ml of appropriate agar. When preparing agar in the laboratory, add 0.07% soy lecithin or 0.5% polysorbate (Tween 80) to the agar to neutralize the effect of the disinfectant on test surfaces. Cooling leaves a raised bed of agar about 1 mm higher than the rim of the dish, allowing contact of the sterile agar to the test surface for direct counts.

(b) To sample an area, remove the plastic cover and carefully press the agar to the solid surface being sampled. It is important that the entire agar layer contacts the test surface. Use a rolling motion with uniform pressure on the back of the plate to insure complete contact. Replace the cover and incubate in an inverted position for the appropriate time and temperature.

(c) Count the colonies with a Quebec colony counter and report as the number of colo-

nies per RODAC plate or number of colonies per 25 sq. cm.

2.5.2 Swab Method (1)

(a) The swab contact method can be used to monitor the contamination of work areas and especially those with cracks, corners, crevices and rough surfaces. Dacron swabs rinse out more easily than cotton ones. These may be purchased from Econ Microbiological Laboratory, 2716 Humboldt Avenue, South, Minneapolis, MN. Rayon swabs are available from Fuller Pharmaceutical Company, Minneapolis, MN, and Consolidated Laboratories Inc., Chicago Heights, IL. Swabs made of calcium alginate which is soluble in water, can also be used. These are available from Consolidated Laboratories, Inc., Chicago Heights, IL.

Rinse Solution Vials – Add 1.25 ml stock phosphate buffer solution, 5 ml of 10% aqueous sodium thiosulfate, 4 grams Asolectin (Associated Concentrates, 32–30 61st Street, Woodside, Long Island, NY 11377) and 10 grams Tween 20 or Tween 80 (Hilltop Research, Inc., Miamiville, OH 45147) to 500 ml distilled water; heat to solution in boiling water bath. Cool and make up to 1 liter. Dispense in screw-capped vials in 10 ml or other volumes and sterilize for 15 minutes at 15 lb. pressure (121 C). Because Asolectin is hygroscopic, store it in a desiccator and weigh quickly.

(b) To sample an area, open a sterile swab container, grasp the end of the stick and remove aseptically. Open a 10 ml vial of neutralizing buffer, moisten swab head, and press out excess solution against the inside of the vial. Hold the swab handle at a 30° angle against the sampling area. Rub the swab head slowly and throughly over approximately 8 sq. in. of surface area. Repeat procedure 3 times over the same area, turning the swab and reversing directions. Place the swab head in the neutralizing buffer vial, rinse briefly in solution and press out the excess liquid.

Test four more 8 sq. in. areas of surface, rinsing the swab in solution after each swabbing. After the fifth area has been swabbed, remove the excess liquid, place swab head in vial and break or cut with a sterile scissors leaving the swab head in the vial. Replace the cap and store vial at 4 C or on ice until analysis.

When using alginate swabs, rinse solution vials should contain 4.5 ml after sterilization. Prepare a 10% solution of sodium hexametaphosphate in appropriate vials and steam sterilize. Follow the above swabbing procedures but after swabbing the fifth 8 sq. in. area, deposit the swab head in the rinse vial and add 0.5 ml of sterile hexametaphosphate solution.

To begin plating, shake the vial vigorously to dislodge the bacteria from the swab. Pipet 1 ml and 0.1 ml aliquots of the rinse solution. The 1 ml portions represent a 1:10 dilution and the 0.1 ml portions represent a 1:100 dilution. Pour the plates with Standard Methods Agar. Mix and cool to solidify and incubate at 35 C for 48 hours.

(c) Count colonies and convert the value to the number/ml which equals the count per 8 sq. in. of area.

2.5.3 Air Density Plates (1)

The number of microorganisms in the laboratory air is directly proportional to the amount and kind of activity. These organisms affect results when the suspended cells contact materials and equipment or when they settle on exposed test materials and surfaces.

(a) The numbers and types of airborne microorganisms can be determined by exposing petri plates for a specified time at points where inoculating, filtering, plating and transfer work is done. This exposure method can be used to monitor total bacteria or the specific organisms being tested such as coliforms, enteric pathogens, streptococci, staphylococci, yeasts or molds.

(b) Pour the petri dishes with the appropriate agar and allow to harden. Store poured plates in the refrigerator if they are not used on the same day.

(c) Remove petri dish covers and place dishes top side up on sterile towels. Expose the

Regular large petri dish = 0.2576 ft²
∴ expose for 3.5 mins to report colonies per 1 sq. ft/minute

plates in selected work sites for 15 minutes and mark them with sample site identification. Replace covers. Incubate Standard Methods Agar at 35 C for 48 hours and other media for the specified time periods.

(d) Count the colonies and report the number per square foot. The number of organisms which settle in 15 minutes of exposure on a petri dish is equivalent to that for 1 sq. ft./minute because the area of a standard-size petri dish is approximately 1/15 sq. ft. The microbial density should not normally exceed 15 colonies per sq. ft. Air density plates should be taken weekly during peak work periods for routine monitoring.

2.6 Laboratory Maintenance: Laboratory benches, shelves, floors and windows should be cleaned on a scheduled basis. Proper maintenance is evidenced by lack of dust and soil build-up on shelves, in corners, etc. Floors should be wet-mopped and treated with a disinfectant solution to reduce contamination of air in the laboratory. Sweeping or dry-mopping should not be permitted in a microbiology laboratory. Work benches should be wiped down with disinfectant before and after each use and at the end of the day.

Every attempt should be made to maintain the laboratory areas free of clutter. Laboratory benches, space under benches, shelves and drawers accumulate equipment, materials and supplies at a steady rate. The disorder can interfere with laboratory operations. It can be controlled by making a directed effort to clean up work areas immediately after each use and by conducting a weekly clean-up of the laboratory. Discard unneeded materials and store equipment and supplies not in current use. The most important step in maintaining an orderly laboratory is to have ample storage space near-by.

3. Laboratory Personnel

Microbiologists, technicians and support personnel in the microbiology laboratory must have training and experience appropriate to the laboratory's analytical program. The variety and complexity of the tasks and tests performed determine the professional and on-the-job-training required.

3.1 Professional Microbiologist: The microbiologist directs and participates in the collection and storage of samples, the preparation of glassware, equipment and media, the analysis of a variety of waters and wastewaters and the evaluation of procedures as required. He takes part in the quality control program. He counts, tabulates and summarizes data and prepares or helps to prepare reports from the results. He should have at least a BS degree in microbiology or a BS/BA degree in biology with a minor in microbiology.

The professional microbiologist initially carries out routine tasks and progresses to more difficult work as he gains experience. When he reaches the senior level he performs the most complex duties.

3.2 Senior Grade and Supervisory Microbiologists: A senior grade microbiologist performs the duties described in 3.1 but carries out a wider range of assignments and more difficult tests. He participates in program planning and laboratory management and solves significant microbiological problems that require a higher than bench-level skill and knowledge. The experienced microbiologist consults and advises on procedural problems and trains personnel. He provides expert testimony, interprets results, prepares reports and recommends microbiological standards or actions for regulatory programs.

The supervisory microbiologist performs administrative functions of: planning and directing water quality monitoring programs, designing field surveys, advising administrative officers on policy matters related to microbiology and inspecting laboratory facilities. He is recognized for his authoritative scientific competence.

3.3 Technicians: The technician performs semi-professional and professional duties of limited scope and complexity. Typically, he assists professionals by doing the routine tests.

Under supervision, he performs tasks involving a series of steps. Technicians learn through on-the-job instruction and by performing standard tasks repetitively.

Technicians' tasks begin at the simplest level but can progress to the more detailed procedures performed by the professional microbiologist. However, these higher levels of skill and knowledge are generally limited to the specific areas in which they have received on-the-job-training.

3.4 Support Personnel: Laboratory aides and clerks provide the necessary support services to the laboratory. Aides prepare glassware, make media and sterilize materials. One or more aides are recommended for every bench microbiologist. Clerical duties include recording data, filing records, typing reports, ordering supplies, maintaining inventories, distributing mail and answering the telephone.

3.5 On-the-Job-Training and Experience: In addition to the formal academic training, the professional microbiologist should have experience in aquatic or environmental microbiology. Technical or on-the-job-training for at least two weeks is required for each parameter tested in water microbiology. The formal training of the technician ranges from high school to technical or academic training short of a degree in microbiology. Technical and scientific experience is often substituted for advanced formal training.

The microbiologist and technician should be encouraged to attend courses at centers of expertise such as universities, commercial manufacturers, US EPA facilities, the Center for Disease Control (CDC) of USPHS, FDA and other federal and state governmental agencies. Courses might include fluorescent antibody techniques, isolation and identification of the Enterobacteriaceae and laboratory safety.

3.6 Laboratory Supervison: Ideally, the laboratory should be directed by a professional microbiologist. However, in a small laboratory where the staff consists of a single non-professional technician, an approved consultant microbiologist should be available for guidance and assistance.

Work assignments in the laboratory should be clearly defined. The analyst should be trained in basic laboratory procedures and should perform well at a given level of responsibility before new assignments are made. The supervisor should periodically review procedures such as sample collecting and handling, media and glassware preparation, sterilization, routine testing procedures, counting, data handling, and quality control techniques. Problem areas should be identified and solved by the staff. Improved laboratory results will be the measure of effective personnel practice and training.

4. Laboratory Equipment and Instrumentation

Quality control of laboratory apparatus includes servicing and monitoring the operation of incubators, waterbaths, hot-air sterilizing ovens, autoclaves, water stills, refrigerators, freezers, etc. Each item of equipment should be tested to verify that it meets the manufacturer's claims and the user's needs for accuracy and precision. Maintenance should be performed on a regular basis by a technician who is familiar with the equipment.

4.1 A summary of recommended monitoring procedures for laboratory equipment is given in Table IV-A-1.

4.2 Monitoring Procedures for UV Lamps

4.2.1 Spread Plate Irradiation

This test should be run when a new UV lamp is installed and rerun quarterly to measure continuing effectiveness of the UV irradiation.

(a) Prepare 100 ml or more of plate count agar. Pour 10–15 ml of the melted agar into each 100 mm petri dish as needed. Keep covers opened slightly until agar has hardened

143

and moisture and condensation have evaporated. Close dishes and store in refrigerator until use.

(b) Prepare a series of dilutions of a coliform culture so that 0.5 ml of inoculum will give a 200–250 colony count (see Geldreich and Clark).

(c) Pipet 0.5 ml of the selected dilution to each agar plate.

(d) Remove a glass spreader-rod from the alcohol container and ignite by passing through flame. Let burn completely and cool for 15 seconds. Test glass rod on edge of agar to verify safe temperature before use. Glass spreaders may also be autoclaved.

(e) Place sterile, cool glass spreader on agar surface next to inoculum. Position spreader so that the tip forms a radius from the center to the plate edge. Holding stick motionless, rotate plate several revolutions, or hold the plate and move the stick in a series of sweeping arcs to spread the inoculum uniformly over the entire surface of the agar.

(f) Lift the glass spreader from the agar and place in disinfectant solution.

(g) Repeat the spread plate procedure with other petri plates.

(h) With cover removed, place agar spread plates under UV lamp at points where sterility is desired.

(i) Place one inoculated plate under ordinary laboratory lighting as a control.

(j) Expose plates for two minutes.

(k) Close plates and incubate at 35 C for 24 hours. Remove and examine for growth.

(1) Count all plates and record results. Control plate should contain 200–250 colonies. UV-irradiated plates should show 99% reduction in the count of the control plate. If count reduction is less than 80%, replace lamp.

4.2.2 Measurements of UV Light Intensity

To monitor performance of UV lamps, it is necessary to measure UV light intensity using the UV light meter manufactured for this purpose. The shortwave UV meter Model J-225 is available from UV Products, Inc., San Gabriel, CA 91778 for about $125. This instrument or equivalent is recommended.

(a) Measure and average light intensities of a new UV lamp at proper distances of use. Record results in QC log, noting readings and dates of installation.

(b) Monitor lamp intensity quarterly thereafter. Replace bulb when light is down to 80% of original intensity.

(c) Measure and average light intensities of replacement lamp at proper distances. Record readings and date of installation.

5. General Laboratory Supplies

5.1 Laboratory Glassware

5.1.1 A summary of procedures for maintenance of glassware is given in Table IV-A-2).

5.1.2 Glassware pH Check: Traces of some cleaning solutions are difficult to remove completely. Before using a batch of clean glassware, test several pieces for an alkaline or acid residue by adding and dispersing a few drops of bromthymol blue or other pH indicator and observing the color reactions. Bromthymol blue is particularly advantageous for this check because it shows color changes of yellow to blue green to blue, in the pH range 6.5 to 7.3.

To prepare bromthymol blue indicator, add 16 ml of 0.01 N NaOH to 0.1 gram of bromthymol blue. Dilute to 250 ml with distilled water (0.04% solution).

5.1.3 Test Procedure for Suitability of Detergents Used in Washing (2)

(a) Wash and rinse six, 100 mm diameter petri dishes in the usual manner. These are Group A.

(b) After normal washing, rinse a second group of 6 petri dishes 12 times with successive portions of non-toxic distilled water. These are Group B.

(c) Wash 6 petri dishes with the detergent wash water using detergent concentrations normally employed, and dry without rinsing. These are Group C.

(d) Sterilize dishes in the usual manner.

(e) Add the proper dilution (usually two different dilutions are used) of a water sample yielding 30–300 colonies to triplicate petri dishes from each Group (A, B and C). Proceed according to the Standard Plate Count Procedure in Part III-A.

(f) The results are interpreted as follows:

(1) Differences in bacterial counts of less than 15% among all Groups indicate the detergent has no toxicity or inhibitory effect.

(2) Differences in bacterial counts of 15% or more between Group A and B demonstrate that inhibitory residues are left on glassware after the normal washing procedure used.

(3) Disagreement in averages of less than 15% between Groups A and B, and greater than 15% between Groups A and C indicates that detergent used has inhibitory properties which are eliminated during routine washing.

5.1.4 Sterility Checks on Glassware

After sterilization of a load of bottles, flasks or tubes, test items of each type for sterility by adding to one of each aerobic or anaerobic broth medium (e.g., lauryl tryptose or fluid thioglycollate broth). Incubate and check for growth.

5.2 Pure Water Quality: Pure water systems are meant to produce the best possible water; however, the potential quality of the water will vary with the type of system used.

The quality of water obtainable from a pure water system differs with the system used and its maintenance. The acceptable limits are given in Table IV-A-3.

5.3 Water Suitability Test

5.3.1 Summary: The water suitability procedure of Geldreich and Clark (3) is a sensitive test for determination of toxic or stimulatory effects of distilled or deionized water on bacteria. It is based on the growth of *Enterobacter aerogenes* in a chemically defined medium. Reduction of 20% or more in the bacterial population compared to a control, is judged toxic. Increased growth greater than 300% is called stimulatory.

5.3.2 Scope and Application: The test is recommended for periodic use and as a special measure of water suitability. This test called the Test for Bacteriological Properties of Distilled water, in the 14th edition of *Standard Methods* is required annually of laboratories in the Interim Certification Program for Drinking Water Supplies.

However, the test is not easily done on an infrequent basis because it requires: work over a four day period to complete, an ultrapure control water and very pure reagents, and absolute cleanliness of culture flasks, petri dishes, test tubes and pipets, etc. It is a complex method that requires skill and experience, is very sensitive to toxicants and cannot be related directly to routine analytical results.

5.3.3 Apparatus and Materials

(a) Incubator set at 35 ± 0.5 C.

(b) Water bath for tempering agar set at 44-46 C.

(c) Colony counter, Quebec dark field model or equivalent.

(d) Hand tally or electronic counting device (optional).

(e) Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

(f) Petri dish containers of stainless steel or aluminum for glass petri dishes.

(g) Thermometer certified by National Bureau of Standards or one of equivalent accuracy, with calibration chart.

(h) Sterile TD (To Deliver) bacteriological or Mohr pipets, glass or plastic of appropriate volumes, see Part II-B, 1.8.1.

(i) Sterile 100 mm × 15 mm petri dishes, glass or plastic.

(j) Sterile pyrex glass flasks, 100 ml, 500 ml, and 1000 ml volume.

(k) Dilution bottles (milk dilution), pyrex glass, marked at 99 ml volume, screw cap with neoprene rubber liner.

(l) Bunsen/Fisher gas burner or electric incinerator.

(m) Inoculation loops, at least 3 mm diameter, or needles, nichrome or platinum wire, 26 B&S gauge, in suitable holder.

5.3.4 Media

(a) Sterile Plate Count Agar (Tryptone Glucose Yeast Agar) dispensed in tubes (15 to 20 ml per tube) or in bulk quantities in screw cap flasks or dilution bottles. See Part II-B, 5.1.5.

(b) Sterile buffered dilution water, 99 ± 2 ml volumes, in screw capped dilution bottles. See Part II-B, 7.

(c) Nutrient agar slants with slopes approximately 6.3 cm (2½ in) length in 125 × 16 mm screw cap tubes.

5.3.5 Reagents: Sensitivity of the test depends on the purity of the reagents used. Use reagents of the highest purity and prepare them in water freshly redistilled from a glass still.

(a) Sodium citrate solution: Dissolve 0.29 g sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$, in 500 ml redistilled water.

(b) Ammonium sulfate solution: Dissolve 0.60 g ammonium sulfate, $(NH_4)_2SO_4$, in 500 ml redistilled water.

(c) Salt mixture solution: Dissolve 0.26 g magnesium sulfate, $MgSO_4 \cdot 7H_2O$; 0.17 g calcium chloride, $CaCl_2 \cdot 2H_2O$; 0.23 g ferrous sulfate, $FeSO_4 \cdot 7H_2O$; and 2.50 g sodium chloride, NaCl, in 500 ml redistilled water.

(d) Phosphate buffer solution: Stock phosphate buffer solution, diluted 1:25 in redistilled water, see Part II-B, 7.1 in this Manual.

Boil reagent solution 1-2 min.to kill vegetative cells. Store in sterilized glass-stoppered bottles in the dark at 5 C for several months but test for sterility before each period of use. Prepare the salt-mixture solution without the ferrous sulfate for long-term storage. To use the mixture, add an appropriate amount of the freshly prepared and freshly boiled iron salt. Solutions with a heavy turbidity should be discarded and a new solution prepared. Bacterial contamination may cause turbidity in the phosphate buffer solution. Discard if this occurs.

5.3.6 Culture: Isolate a pure culture of *Enterobacter aerogenes* (IMViC type --++) from a polluted stream or sewage sample.

5.3.7 Procedure

(a) Collect 200 ml each, of the unknown Test Water and the Control Water (laboratory pure reference water) in sterile 500 ml screw cap flasks. Boil for 2 minutes and cool to room temperature.

(b) Label five, sterile 200 ml screw cap flasks as A, B, C, D, and E. Add the Test Water, Control Water, and reagents to each flask as described in the following table:

Reagents	Control Test (ml)		Optional Tests (ml)		
	Control Water A	Unknown Test Water B	Nutrient Available C	Nitrogen Source D	Carbon Source E
Sodium citrate solution	2.5	2.5	-	2.5	-
Ammonium sulfate solution	2.5	2.5	-	-	2.5
Salt-mixture solution	2.5	2.5	2.5	2.5	2.5
Phosphate buffer (7.3 ± 0.1)	1.5	1.5	1.5	1.5	1.5
Test Water	-	21.0	21.0	21.0	21.0
Control Water	21.0	-	5.0	2.5	2.5
Total volume	30.0	30.0	30.0	30.0	30.0

(c) Perform a Standard Plate Count on prepared reagents, Control Water, and Test Water as a check on contamination.

(d) On the day before performing the distilled-water suitability test, inoculate a strain of *E. aerogenes* onto a nutrient agar slant. Streak the entire agar surface to develop a continuous-growth film and incubate 18-24 hours at 35 C. To harvest viable cells, pipet 1-2 ml sterile dilution water from a 99 ml water blank onto the 18-24 hour culture. Emulsify the growth on the slant by gently rubbing the bacterial film with the pipet, being careful not to tear the agar; then pipet the suspension back into the original 99 ml water blank.

(e) Make a 1:100 dilution of the original bottle into a second water blank, a further 1:100 dilution of the second bottle into a third water blank, then 10 ml of the third bottle into a fourth water blank, shaking vigorously after each transfer. Pipet 1.0 ml of the fourth dilution ($1:10^{-6}$) into each of Flasks A, B, C, D, and E. This procedure should result in a final dilution of the organisms to a range of 30-80 viable cells from each ml of test solution.

(f) Variations among strains of the same organism, different organisms, media, and surface area of agar slopes will possibly necessitate adjustment of the dilution procedure in order to arrive at a specific density range between 30 and 80 viable cells. To establish the growth range numerically for a specific organism and medium, make a series of plate counts from the third dilution to determine the bacterial density. Then choose the proper volume from this third dilution, which, when diluted by the 30 ml in Flasks A, B, C, D, and E, will contain 30-80 viable cells/ml. If the procedures are standardized as to surface area of the slant and laboratory technic, it is possible to reproduce results on repeated experiments with the same strain of microorganism.

(g) Add a suspension of *Enterobacter aerogenes* (IMViC type --++) of such density that each flask will contain 30-80 cells/ml, prepared as directed above. Cell densities below this range result in ratios that are not consistent, while densities above 100 cells/ml decrease sensitivity to nutrients in the Test Water. Make an initial bacterial count by plating triplicate 1 ml portions from each culture flask in plate count agar. Mix well and incubate Test A through E at 35 C for 24 $\pm$ 2 hours. Prepare final plate counts from each flask, using dilutions of 1, 0.1, 0.01, 0.001, and 0.0001 ml.

5.3.8 Calculations

(a) For growth-inhibiting substances:

$$\text{Ratio} = \frac{\text{colony count/ml Flask B}}{\text{colony count/ml Flask A}}$$

A ratio of 0.8 to 1.2 (inclusive) shows no toxic substances; a ratio of less than 0.8 shows growth-inhibiting substances in the water sample.

(b) For nitrogen and carbon sources that promote growth:

$$\text{Ratio} = \frac{\text{colony count/ml Flask C}}{\text{colony count/ml Flask A}}$$

(c) For nitrogen sources that promote growth:

$$\text{Ratio} = \frac{\text{colony count/ml Flask D}}{\text{colony count/ml Flask A}}$$

(d) For carbon sources that promote bacterial growth:

$$\text{Ratio} = \frac{\text{colony count/ml Flask E}}{\text{colony count/ml Flask A}}$$

(e) Do not calculate ratios b, c, or d when ratio a indicates a toxic reaction. Ratios b, c, or d, in excess of 1.2 indicate an available source for bacterial growth.

5.3.9 Interpretation of Results

(a) The colony count from Flask A after 20-24 hours at 35 C, will depend on the number of organisms initially planted in Flask A and on the strain of *E. aerogenes* used in the test procedures. This is the reason the control, Flask A, must be run for each individual series of tests. However, for a given strain of *E. aerogenes* under identical environmental conditions, the terminal count

should be reasonably constant when the initial plant is the same. The difference between 30 and 80 cells in the initial plant in Flask A will produce a three-fold difference in the final counts, providing the growth rate remains constant. Thus, it is essential that the initial colony counts on Flask A and Flask B be approximately equal to secure accurate data.

(b) When the ratio exceeds 1.2, it may be assumed that growth-stimulating substances are present. However, this procedure is extremely sensitive and ratios up to 3.0 would have little significance in actual practice. Therefore, when the ratio is between 1.2 and 3.0, Tests C, D, and E do not appear to be necessary except in special circumstances.

(c) Usually Flask C will be very low and Flasks D and E will have a ratio of less than 1.2 when the ratio of Flask B to Flask A is between 0.8 and 1.2. The limiting factors of growth in Flask A are the nitrogen and organic carbon present. An extremely large amount of ammonia nitrogen with no organic carbon could increase the ratio in Flask D above 1.2, or the absence of nitrogen with high carbon concentration could give ratios above 1.2 in Flask E, with a B:A ratio between 0.8 and 1.2.

(d) A ratio below 0.8 indicates that the water contains toxic substances, and this ratio includes all allowable tolerances. As indicated in the proceding paragraph, the ratio could go as high as 3.0 from 1.2 without any undesirable consequences.

5.4 Use Tests for Media, Membranes and Laboratory Pure Water

The Use Test, a pragmatic approach to evaluation of materials and supplies, uses the routine methods of analysis to compare current and new batches or lots. Such tests operate on the theory that if a stimulatory or toxic effect cannot be demonstrated in actual test use, there is no effect.

When a new shipment or lot of culture medium, membrane filters or a new source of laboratory pure water is to be used, or at annual testing period for water, conduct comparison tests of the current lot in use (reference lot) against the new lot (test lot) as follows:

5.4.1 Use a single batch of pure water, glassware, membrane filters, or other needed materials as specified to control all other variables except the one under study.

5.4.2 Use the reference lot and the test lot, to conduct parallel pour plate or MF plate count tests on five natural or treated water samples according to standard procedures in this Manual.

When comparing sources of pure water, conduct the tests in parallel using the reference water and test water separately for all water purposes in the tests (dilution, rinse, media preparation).

5.4.3 After incubation, compare the bacterial colonies from the two lots for size and appearance. If the colonies on the test lot are atypical or noticeably smaller than colonies on the reference lot plates, record the evidence of inhibition or other problem, regardless of count differences.

5.4.4 Count plates and calculate the individual counts per ml or per 100 ml, as required for final reporting values.

5.4.5 Transform the final reporting values to logarithms.

5.4.6 Compile the log-transformed results for the two lots in parallel columns and calculate the + or − difference, d, between the two transformed results for each sample.

5.4.7 Calculate the mean, $\bar{d}$, and the standard deviation s_d of these differences.

5.4.8 Calculate the Student's t statistic, using the number of samples as n:

$$t = \frac{\bar{d}}{s_d/\sqrt{n}}$$

5.4.9 Use the critical value, 2.78, selected from a Student's t table at the .05 significance level for five samples (4 degrees of freedom), for comparison against the calculated value.

5.4.10 If the calculated t value does not exceed 2.78, the lots do not produce signifi-

145

cantly different results and the test lot is acceptable.

5.4.11 If the calculated t value exceeds 2.78, the lots produce significantly different results. If the test lot results exceed reference lot results, the test lot is more stimulatory. If the test lot results are less than the reference lot, the test lot is less stimulatory.

5.4.12 If condition 5.4.3 or 5.4.11 occurs, review test conditions, rerun the test and/or obtain different lots for testing and use.

5.5 Reagents in General: The quality of test reagents must be assured. They must be correctly prepared and properly stored. The following general rules should be followed:

5.5.1 Use ACS or AR grade chemicals that meet ACS specifications for preparing reagents. Impurities in uncertified or lesser grades of chemicals may inhibit bacterial growth, provide nutrients or fail to produce the desired reaction.

5.5.2 Date chemicals and reagents when received and when opened for use.

5.5.3 make reagents up to volume in volumetric flasks. For storage, transfer to good quality plastic (polyethylene, polypropylene or tetrafluoroethylene) or borosilicate glass bottles with polyethylene or other inert plastic stoppers or caps.

5.5.4 Identify prepared reagents with the generic name, the concentration, the date prepared and the initials of preparer.

5.5.5 Store reagents under the conditions recommended by the manufacturer.

5.5.6 Run positive and negative controls with each series of cultural or biochemical tests.

5.6 Serological Reagents

5.6.1 Evaluate serological antisera against known antigens and compare with antisera that have demonstrated acceptable reactivity. The quality of commercial serological reagents is subject to methodology changes and new knowledge in manufacturing processes. Continuity of reagent quality depends on compliance of each new reagent with minimum specifications.

5.6.2 Repeat quality control procedures each time that reagent batches are prepared, regardless of the expiration date given by the manufacturer.

5.6.3 Discard sera or antigens if contamination is discovered.

5.6.4 Select another working dilution if the level of activity has dropped.

5.7 Fluorescent Antibody Reagents

Highly specific reagents, antigens of high purity and very specific potent antibodies are required for fluorescent antibody techniques. The antisera must exhibit high staining intensities. Some sera may have high titers in one type of serological test, but demonstrate poor staining titer and vice versa.

5.7.1 Store desiccated fluorescent antibody (FA) reagents at 4 C. Prepare aliquots of the rehydrated conjugate in screw-cap glass vials, freeze and store at -20 C until working dilutions are prepared.

5.7.2 Test FA reagents for correct reactions with positive and negative controls before each use. Results of positive controls should be within one dilution of the average titer.

5.8 Dyes/Stains: Organic chemicals are used as selective agents (e.g., brilliant green in brilliant green lactose bile broth), as indicators in bacteriological media (phenol red lactose), and as bacteriological stains (gram stain). Dyes from any commercial supplier vary from lot to lot in percent dye, dye complex, insolubles and inert materials present. Because dyes for microbiological uses must be of proper strength and stability to produce the correct reactions, purchase only dyes which have been certified by the Biological Stain Commission for biological use.

5.8.1 Fluorescent Dyes: An important factor in the preparation of antisera-dye conju-

gates is the purity of the fluorescein dye. Infrared studies reveal that the purity of fluorescein isothiocyanate (FITC) commonly used in FA tests, ranges from 30 to 100% in commercial products. Ideally 100% pure FITC preparations are used. Dyes of less purity may be satisfactory if the weight used to label the protein component of the serum allows for the impurities, and if the impurities do not increase non-specific staining.

5.8.2 Check bacteriological stains before use with at least one positive and one negative control culture.

6. Membrane Filters

6.1 Government Specifications

6.1.1 The quality and performance of membrane filters vary with the manufacturer, type, brand, lot number and storage conditions. Membranes considered for purchase by federal agencies must conform to the government specifications published in 1974 Military Specifications (4). The detailed specifications required of the manufacturer by the federal government are an important advantage to other users of membrane filters because by meeting federal requirements, manufacturers realize it is most efficient to make all membranes to these specifications. Hence, membrane filters purchased from companies selling to federal agencies meet the federal specifications.

6.1.2 The specifications list requirements for pore size, porosity, flow rate, diffusibility, autoclavability, sterility, diameter, thickness, bacterial retention and bacterial cultivation. The gridlines must be easily visible, permanent and not imprinted into the surface so as to cause channelling of growth. The grid ink must not stimulate or inhibit growth.

6.1.3 The pads must have a specified diameter and thickness, limited acidity, no waxes, sulfites, and other stimulating or inhibitive materials, and must absorb uniformly a specified volume of medium.

6.2 Manufacturer's Quality Assurance

6.2.1 Some manufacturers certify that their membranes meet stated specifications on sterility, retention, recovery, pore size, flow rate, pH, total acidity, phosphate and other extractables.

6.2.2 Most manufacturers sell sterile membranes and those packaged for sterilization by the user. Membranes may be sterilized by autoclaving, ethylene oxide or irradiation.

6.3 User's Quality Assurance

6.3.1 In addition to the quality assurance provided by the manufacturer, the user should determine that the membranes perform satisfactorily by inspecting and testing each lot he orders. The membranes should not be misshapen or the gridlines distorted after autoclaving.

6.3.2 A membrane should allow good diffusibility of medium and be completely wetted within 15 seconds after it is placed on the medium substrate with no dry areas indicated by colorless or lighter-colored areas.

6.3.3 After incubation, the colonies should be of the expected size, with defined shape and clearly delineated edges. The color and morphology of the colonies must be typical of those defined by the test procedure.

6.3.4 The analyst should observe that the gridline ink does not "bleed" across the surface, or restrict colony development on or adjacent to the cross-hatching. Colonies should be evenly distributed across the membrane surface. Membranes containing sizable areas with no colony development are questionable.

6.4 ASTM Test Procedures

Because of variable recoveries of microorganisms on different membrane filters, there is a critical need for standard procedures for evaluating membrane filters. Procedures are being developed by Committee D-19.08.04 of ASTM for physical, chemical and microbiological characteristics of membrane filters. The procedures are intended for the larger laboratory and the manufacturer to test new batches

or lots of MF's. Brief descriptions of these tests for bacterial retention, inhibitory effects, recovery, extractables and flow rate follow.

6.4.1 Bacterial Retention Test

The test is based on filtration of a standard culture through a 0.45 μm MF into a broth. Sterile equipment and aseptic techniques must be used. Five randomly-selected membranes from five randomly-selected packages should be tested. Control membranes should be taken from the same package as the test membranes.

(a) Add 140 ml of double strength Trypticase soy or Tryptic soy broth to a liter vacuum flask and attach vacuum tubing. Wrap flask in kraft paper and sterilize.

(b) Using an 18 hour broth culture of *Serratia marcescens*, prepare a final dilution in 0.1% peptone, containing 1000 cells per 100 ml.

(c) Assemble a membrane filtration apparatus using a sterile vacuum flask containing the broth. Insert the test filter, turn on vacuum and pour in 20 ml of sterile rinse water to set the membrane. Filter 100 ml of the culture suspension. Rinse funnel twice with 20 ml of peptone water.

(d) After the filtration remove funnel and transfer the membrane filter to a Trypticase soy or Tryptic soy agar plate. Then aseptically remove membrane filteration base from the flask and insert a sterile stopper. Repeat filtrations four more times with sterile MF assemblies, culture flasks and membranes.

(e) Incubate agar plates and flasks for 48 hours at 35 C. Examine membranes for growth outside of filtering areas which indicates a leaky filtration assembly that could cause a false positive test. Turbidity in the culture flask indicates bacterial growth and failure of the membrane to retain the 0.2–0.3 μm sized bacteria, but does not indicate the numbers of organisms passing the filter.

(f) Turbidity indicates filter failure. Repeat the test and control procedures.

6.4.2 Inhibitory Effects: The test for inhibitory effects compares membrane filter counts on one or more test lots of membrane filters, with spread plate counts on Plate Count Agar using a specific pure culture of *Escherichia coli* (IMViC + + – –).

(a) Prepare a dilution of the stabilized 24-hour culture in 0.1% peptone dilution water so that 0.2 ml contains 30–150 viable cells. Add about 20 ml of sterile dilution water to the funnel before filtration.

(b) Filter five replicate sample volumes of 0.2 ml through five replicate membrane filters of each MF test lot and place each on a plate of Tryptic soy agar.

(c) Prepare the corresponding spread plates in random fashion to avoid a time effect, following a randomization table.

(d) With the same pipet, deliver five additional 0.2 ml volumes to the surface of five plates of Tryptic soy agar. The samples are distributed evenly over the surfaces of the plates with sterile glass rods.

(e) Incubate the membrane filters at 44.5 C and agar plates at 35 C for 24 hours. Count the colonies. Compare recoveries on spread plates and membrane filters. Acceptable filters should recover some set percentage of the spread plate count. This percentage should be established by each laboratory based on previous performance by a known acceptable lot.

6.4.3 Recovery (5): The procedure for recovery compares the fecal coliform counts on test membranes to the counts on spread plates using M-FC agar substrate. Four polluted waters and one raw sewage sample are analyzed.

(a) To determine sample test volumes, prepare serial dilutions of each sample in 0.1% peptone water to produce a suspension containing approximately 20–60 fecal coliforms per 0.1 ml. Hold original samples at 4 C. Determine the fecal coliform density of each of the samples or dilutions by membrane filter or spread plate test. Read the results after 22–24 hours incubation at 44.5 ± 0.2 C.

(b) If the fecal coliform density in the raw water samples is less than 10 per 0.1 ml, seed the sample with raw sewage, allow to stabilize at 4 C for 24 hours and test as in (a). Then proceed with the standard test procedure.

(c) Use the optimum dilutions from (a) or (b) for the test membranes and the spread plate controls.

(d) Perform the membrane filter tests according to the procedure described in Part II-C, 3 of This Manual.

(e) Prior to beginning the spread plate tests, air dry the surface of the M-FC agar contained in 100 ml petri dishes. Aseptically deliver 0.1 ml of the selected sample dilution to the agar surface and spread with a sterile bent glass rod. Allow the sample aliquot to be completely absorbed before inverting the dish.

(f) Alternate MF tests with spread plate controls to randomize systematic errors.

(g) Insert petri dishes into waterproof bags or seal with waterproof tape, and submerge in the waterbath incubator. Incubate for 22–24 hours at 44.5 $\pm$ 0.2 C. Record the temperature continuously during the incubation period. After incubation, remove plates and examine.

(h) Count the blue colonies. If more than one dilution was prepared, select the plates with between 10 and 100 colonies, but preferably with 20–60 colonies. Calculate the arithmetic mean of the five replicate fecal coliform counts and the five replicate spread plates. Determine percent recovery:

$$\frac{\text{MF } \bar{X} \text{ Count}}{\text{Spread Plate } \bar{X} \text{ Count}} \times 100 = \%\ \text{Recovery}$$

(i) Verification: Pick 20 blue colonies from each of 2 randomly-selected filters and 2 spread plates. If plates contain less than 20 colonies, pick all blue colonies. Verify the colonies in EC media as described in Part III-C, 4 of This Manual. To be acceptable, 80% of the colonies must verify. If the samples are known or suspected to have a high concentration of pseudomonads or aeromonads, perform the cytochrome oxidase test. As in the verification procedure, a minimum negative oxidase test confirmation of 80% should be achieved for the membranes to be considered acceptable.

6.4.4 Extractables

(a) Total Extractables

(1) Dry filters for 15 minutes at 70 C then bring to room temperature in a desiccator. Weigh to constant weight on a four-place analytical balance.

(2) Boil filters in 100 ml of distilled water for 20 minutes. Remove the filters and dry at 70 C for 60 minutes. Bring filters to room temperature in a desiccator and reweigh to constant weight. Weighings shall be to the nearest 0.1 milligram.

$$\frac{\text{Original Weight} - \text{Weight After Extraction}}{\text{Original Weight}} \times 100 = \text{Percent Extractables}$$

(b) Specific Extractables

(1) Immerse filter in ASTM Type 1 reagent grade water for 24 hours (6).

(2) Remove filter and assay the extract for metals, total organics, phosphorus and ammonia.

6.4.5 Flow Rate

Water flow rate shall be determined by timing the passage of 500 ml of particle-free distilled water through a filter at 25 C and at a differential pressure of 70 centimeters of mercury. Particle-free water for this test is produced by passage of a high purity water through a 0.2 μm membrane filter, three times in succession, at 25C and at a differential pressure of 177 cm of mercury.

$$\text{The flow rate} = \frac{\frac{60}{t} \times 500 \text{ ml}}{\text{EFA cm}^2}$$

$= \text{ml/min/cm}^2$

Where: t = experimental time in minutes for filtering 500 ml of particle-free distilled water

and

EFA = effective filtering area of a 47 mm diameter filter

An average flow rate reported by manufacturers for 0.45 μm pore size, 47 mm diameter filters is 65 ml/min/cm^2.

7. Culture Media

Since even the best cultural procedure is ineffectual if the medium is not prepared correctly, it is important to train personnel to use the best materials and techniques in media preparation, storage and application. Some factors that must be considered follow:

7.1 Ordering Media

7.1.1 Order media in quantities to last up to one year. Always use oldest stock first.

7.1.2 Whenever possible and practical, order media in 1/4 pound multiples rather than one pound bottles to insure sealed protection of the supply as long as possible. Most deterioration of media occurs after bottles are opened.

7.1.3 Maintain an inventory record of media: the dates received, sizes, number of units, etc. Review the inventory quarterly for necessary reordering. Date each bottle when received and when opened. Bottles should be inspected for color changes, caking or other indication of deterioration. Discard such bottles and reorder.

7.2 Holding Time Limits for Media

Because of the myriad of environmental conditions affecting media, and the unique composition and sensitivities of different media, it is impossible to establish universal time limits for holding unopened bottles of media. Therefore, a conservative and protective recommendation is to limit the storage of unopened bottles of cultural media to two years. This limit should insure good performance of media with proper storage conditions.

7.3 Preparation of Media

7.3.1 In high humidity areas, store opened bottles of media in a large hinged-door desiccator. Open bottles as briefly as possible during the weighing process and return to the desiccator immediately after use.

7.3.2 Discard opened bottles of media 6 months after initial use.

7.3.3 Weigh media to the nearest 0.1 gram on a single pan, top loader balance as quickly as possible.

7.3.4 Keep balance out of drafts and away from high humidity. Use a plastic shield around the balance to protect from drafts.

7.3.5 Clean the balance and surrounding area immediately after weighing media.

7.4 Solution of Media

7.4.1 Check cleanliness of glassware. Use bromthymol blue indicator to spot-check pH of glassware (5.1.2 in this Section).

7.4.2 Prepare media in a deionized or distilled water of proven quality. Ultra-pure water from a recirculating deionization system is recommended. Measure volumes with the greatest accuracy possible using the proper pipet or graduate (Part II-B, 1.8).

Check the pH of media after solution and after sterilization using a laboratory model pH meter. Enter results in the QC record book. The reading should be within 0.2 units of the stated value. If not, discard batch and remake. If the pH is still incorrect, use another bottle or batch. pH differences indicate a problem of distilled water quality, deterioration of

medium or improperly-prepared medium. The problem must be identified and corrected. The problem should be carefully documented in the quality control record book and reported to the manufacturer if the data indicate the medium as the source of error.

7.4.3 Note in the QC record any unusual color development, darkening, or precipitation of media. Check the sterilization time and temperature. If there is a drastic change in appearance, discard medium and remake it. If the problem still exists, remake the batch using a different lot of medium.

7.4.4 Containers for preparation of batches of broth or agar should be twice the volume of the medium being prepared.

7.4.5 Media should be stirred continuously while being heated to avoid burning. Agar media are particularly susceptible to scorching and boilover. The only insurance against scorching or boilover is use of a boiling water bath for small batches of media or constant attention while heating larger batches on a hot plate or burner. A combination hot plate and magnetic stirrer is recommended for solution of media.

7.4.6 Bottles, tubes or plates of prepared media are identified and dated.

7.5 Sterilization by Autoclave

7.5.1 Media should be sterilized for the minimal time specified by the manufacturer. The amount of time required to sterilize a medium in an autoclave will vary with the type and volume of medium and the size and shape of containers. See the table in Part II-B, 4.3 on sterilization.

7.5.2 Since the potential for damage to media increases with increased exposure to heat, the amount of lag time before the autoclave is at full pressure and temperature can be a critical factor in whether media are damaged. The danger from an extended heating period is reduced with use of a double-walled autoclave which allows the operator to maintain full pressure and temperature in the jacket between loads. As soon as the autoclave is loaded and closed, steam can be admitted to the chamber and in a relatively short time, full pressure and temperature are developed in the chamber. The total exposure time for media sterilized 15 minutes at 121 C should not exceed 45 minutes.

7.5.3 Avoid overcrowding in an autoclave which reduces its efficiency. Large volumes of media should be preheated to reduce the lag period before placing them in the autoclave.

7.5.4 Remove sterile media from the autoclave as soon as pressure is at zero.

7.5.5 Media must be discarded if contamination is suspected. Reautoclaving is not permitted.

7.5.6 A preventive maintenance contract is recommended for autoclaves. It should include checks on the accuracy of pressure and temperature gauges and recorders and operability of safety valve.

7.5.7 Check the effectiveness of sterilization weekly, using strips or ampuls of *Bacillus stearothermophilus* spores. Commercial packages of these spores are available in ampuls of growth-indicator media. Sterilization at 121 C for 12–15 minutes will kill the spores. Incubate the autoclaved cultures at 55–60 C and if growth occurs, sterilization is inadequate.

7.6 Sterilization by Filtration

7.6.1 Non-autoclavable solutions can be sterilized by membrane filtration. With careful preparation of the sterile filtration and receiving apparatus, passage of a solution through a 0.2 µm membrane filter will produce a sterile solution.

7.6.2 The filtration and subsequent sterile dispensations should be performed in a safety cabinet or bio-hazard hood.

7.7 Gaseous Sterilization

Equipment, supplies or other solid or dry materials which are sensitive to heat can be

154

sterilized by long exposure to ethylene oxide gas (ETO) using available commercial equipment. Check sterility with culture of *B. subtilus* var. *niger* spores.

7.8 Use of Agars, Broths, and Enrichment Media

7.8.1 Agars

(a) Agar plates to be used for streaking or spread plates are kept open slightly for 15 minutes after pouring or after taking out of refrigerated storage to evaporate free moisture which would cause confluent growth on streak plates.

(b) Agar plates used for MF and spread plate work must be free of lumps, uneven surfaces, pock marks, bubbles or foam which prevent good contact between the agar and the membrane or uniform growth on spread plates.

(c) Melted agars should be held in a tempering water bath at 44-46 C but no longer than three hours. As a safety precaution against the use of agars which are too hot and might kill cells, place a bottle of agar in the same boiling water or under the same autoclave conditions as the agars to be used. After the agar is melted, transfer agar to a tempering bath. Insert thermometer in the agar bottle and use it to determine when the temperature of the agars is at approximately 44-46 C and safe for use in pour plates.

7.8.2 Broths

(a) Handle sterile MPN fermentation tubes of lauryl tryptose broth or brilliant green bile broth carefully prior to use. Shaking can entrap air in the inner tubes and produce a false positive. Examine fresh tubes before use and discard any with a bubble.

(b) Reduced media such as thioglycollate broth oxidize in storage. Before use, these broths must be heated in boiling water for 20-30 minutes to reduce the medium.

7.8.3 Enrichments

(a) Bring the base medium to 44-46 C before addition of a labile constituent.

(b) Warm enrichments such as blood or serum to room temperature before adding to a base medium.

(c) Once labile material is added to a medium, prepare plates or tubes as soon as possible. Do not hold the batch medium in a water bath for more than 10 minutes.

7.9 Storage of Media

7.9.1 The recommended time limits for holding prepared media in the laboratory are:

MF broths in screw-cap flasks at 4 C	96 hours (Work Week)
MF agars in plates with tight-fitting covers at 4 C	Two Weeks
Agar or broths in loose-cap tubes, at 4 C	One Week
Agar or broths in screw-cap tubes, tightly closed, at 4 C	Three Months
Agar plates (non-MF) with loose-fitting covers, in sealed plastic bags, at 4 C	Two Weeks
Large volumes of agar in screw-cap flasks or bottles tightly-closed, at 4 C	Three Months

7.9.2 Store fermentation tube media in the dark at room temperature or 4 C. If refrigerated, incubate overnight at room temperature to detect false positive gas bubbles.

7.9.3 Since loss of moisture is a major problem of storage, screw-capped tubes and flasks are recommended. Prepoured agar

155

sterilized by long exposure to ethylene oxide gas (ETO) using available commercial equipment. Check sterility with culture of *B. subtilus* var. *niger* spores.

7.8 Use of Agars, Broths, and Enrichment Media

7.8.1 Agars

(a) Agar plates to be used for streaking or spread plates are kept open slightly for 15 minutes after pouring or after taking out of refrigerated storage to evaporate free moisture which would cause confluent growth on streak plates.

(b) Agar plates used for MF and spread plate work must be free of lumps, uneven surfaces, pock marks, bubbles or foam which prevent good contact between the agar and the membrane or uniform growth on spread plates.

(c) Melted agars should be held in a tempering water bath at 44-46 C but no longer than three hours. As a safety precaution against the use of agars which are too hot and might kill cells, place a bottle of agar in the same boiling water or under the same autoclave conditions as the agars to be used. After the agar is melted, transfer agar to a tempering bath. Insert thermometer in the agar bottle and use it to determine when the temperature of the agars is at approximately 44-46 C and safe for use in pour plates.

7.8.2 Broths

(a) Handle sterile MPN fermentation tubes of lauryl tryptose broth or brilliant green bile broth carefully prior to use. Shaking can entrap air in the inner tubes and produce a false positive. Examine fresh tubes before use and discard any with a bubble.

(b) Reduced media such as thioglycollate broth oxidize in storage. Before use, these broths must be heated in boiling water for 20–30 minutes to reduce the medium.

7.8.3 Enrichments

(a) Bring the base medium to 44–46 C before addition of a labile constituent.

(b) Warm enrichments such as blood or serum to room temperature before adding to a base medium.

(c) Once labile material is added to a medium, prepare plates or tubes as soon as possible. Do not hold the batch medium in a water bath for more than 10 minutes.

7.9 Storage of Media

7.9.1 The recommended time limits for holding prepared media in the laboratory are:

MF broths in screw-cap flasks at 4 C	96 hours (Work Week)
MF agars in plates with tight-fitting covers at 4 C	Two Weeks
Agar or broths in loose-cap tubes, at 4 C	One Week
Agar or broths in screw-cap tubes, tightly closed, at 4 C	Three Months
Agar plates (non-MF) with loose-fitting covers, in sealed plastic bags, at 4 C	Two Weeks
Large volumes of agar in screw-cap flasks or bottles tightly-closed, at 4 C	Three Months

7.9.2 Store fermentation tube media in the dark at room temperature or 4 C. If refrigerated, incubate overnight at room temperature to detect false positive gas bubbles.

7.9.3 Since loss of moisture is a major problem of storage, screw-capped tubes and flasks are recommended. Prepoured agar

756

plates can be sealed in plastic bags to retain moisture and refrigerated.

7.9.4 A simple check for loss of moisture in broth tubes can be made by marking the original level in several tubes of each batch and then observing the loss of moisture over time. If the estimated moisture loss exceeds ten percent, discard the tubes of broth.

7.9.5 Protect media containing dyes from light. If color changes are observed, discard the medium.

7.9.6 Prepared sterile broths and agars are available from commercial sources. Their use may be advantageous when analyses are done intermittently, when staff is not available for such preparation work, or when cost of their use can be balanced against other factors of laboratory operation. However, purchase of prepared media does not reduce the responsibility of the laboratory for checking the performance of the media, regardless of the stated quality control practices of the manufacturer.

7.10 Quality Control of Prepared Media

7.10.1 Maintain a book with a complete record of each batch of medium prepared. Include the date, the name of the medium and lot number, amount of medium weighed out and volume of medium prepared, the record of sterilization, pH measurements, pH adjustments made, special handling or preparation techniques, e.g., use of heat-sensitive compounds or components, and name of preparer.

7.10.2 Incubate five percent of each batch of medium for 2 days at 35 C and inspect for growth.

7.10.3 Check each batch of medium when used by inoculating 2 tubes or plates with pure cultures of species producing positive and negative reactions for that medium.

7.10.4 Test new batches of differential media by inoculating with organisms of known fermentative or other biochemical ability. Similarly, enrichment and selective media are tested for productivity of the desired microorganisms and inhibition of other microorganisms. Tables IV-A-4 and IV-A-5 list a group of organisms with the broths, agars, biochemical tests and reactions to which they can be applied.

7.10.5 Record sterility and positive/negative performance checks in the media preparation portion of the quality control log.

TABLE IV-A-1

Monitoring Laboratory Equipment

Item	Monitoring Procedure
1. Balance	a. An analytical balance with a sensitivity of 1 mg or less at a 10 g load should be used for weighing 2 g or less. For larger quantities, a balance with accuracy of 50 mg at a 150 g load should be used. b. Check balance monthly with a set of certified class S weights. c. Wipe balance and weights clean after each use. d. Protect weights from laboratory atmosphere and corrosion. e. Contract with a qualified expert for balance maintenance on an annual basis.
2. pH Meter	a. Compensate for temperature with each use. b. Date standard buffer solutions when first opened and check monthly with another pH meter. Discard the buffer solution if the pH is more than ± 0.1 pH unit from the manufacturer's stated value or if it is contaminated with microorganisms. c. Standardize with at least one standard buffer (pH 4.0, pH 7.0, or pH 10.0) before each use. d. Do not re-use buffer solution. e. Have meters inspected at least yearly as part of a maintenance contract.
3. Water Deionizer	a. Monitor water for conductance daily. Monitor trace metals and other toxic or nutritive compounds monthly. See Table IV-A-3. b. Replace cartridges as indicated by manufacturer or as indicated by analytical results. c. Monitor bacterial counts at exit point of unit. Replace the cartridges when standard plate count exceeds 10,000/ml.
4. Water Still	a. Drain and clean monthly according to instructions from the manufacturer. b. Drain and clean distilled water reservoir quarterly. c. Check distilled water continuously or daily using a conductance meter. See Table IV-A-3. d. Conduct chemical tests on water to detect toxicity or stimulation effect. See Table IV-A-3. e. Conduct standard plate counts monthly on stored water and clean out reservoir if count > 10,000/ml.

158

TABLE IV-A-1
(continued)

Monitoring Laboratory Equipment

Item	Monitoring Procedure
5. Dispensing Apparatus	a. Check accuracy of dispensation with an NBS class A, graduated cylinder at the start of each volume change and periodically throughout extended runs. b. Lubricate moving parts according to manufacturer's instructions or at least once per month. c. Correct immediately any leaks, loose connections or malfunctions. d. After dispensing each type of medium, pass a large volume of hot distilled water through dispenser to remove traces of agar or medium. e. At the end of the work day, break down unit into parts, wash well, rinse with distilled water and dry.
6. Ultraviolet Sterilizer	a. Remove plug from outlet and clean ultraviolet lamps monthly by wiping with a soft cloth moistened with ethanol. b. Test ultraviolet lamps with light meter quarterly; if they emit less than 80% of their rated initial output, replace them. c. Perform spread plate irradiation test quarterly, see This Section, 4.2.1.
7. Membrane Filter Equipment	a. Check funnel support for leaks. b. Check funnel and funnel support to make certain they are smooth. Discard funnel if inside surfaces are scratched. c. Clean thoroughly after each work day.
8. Spectrophotometer	a. Maintain quality control and calibration check as recommended by the manufacturer. b. Have inspected yearly by a factory maintenance man.
9. Centrifuge	a. Check brushes and bearings for wear every six months. b. Check rheostat control against a tachometer at various loadings every six months to ensure proper gravitational fields.

TABLE IV-A-1
(continued)

Monitoring Laboratory Equipment

Item	Monitoring Procedure
10. Microscope	a. Allow only trained technicians to use. b. Appoint one laboratory worker to be responsible for the care of the microscope. c. Clean optics and stage after every use. Use only lens paper for cleaning. d. Keep covered when not in use. e. Establish annual maintenance on contract. f. Maintain in one location if possible.
11. Microscope, Fluorescence	a. Allow only trained technicians to use microscope and light source. b. Keep a log of lamp operation time. c. Monitor lamp with meter. See Section 4.2.2. Replace the lamp when < 80% of original fluorescence is observed. d. Check lamp alignment, particularly if bulb has been changed. Realign the fluorescent light source if necessary. e. Use known 4+ fluorescence slides as controls.
12. Safety Cabinet (Hood)	a. Check filters monthly for plugging or obvious dirt accumulation. Clean or replace filter as needed. b. Check cabinet for leaks and for rate of air flow every three months. c. Expose blood agar plates to air flow for one hour once per month to measure contamination. d. Remove plug from the outlet and clean ultraviolet lamps every two weeks by wiping with a soft cloth moistened with ethanol. e. Test ultraviolet lamps quarterly with a light meter. If lamp emits less than 80% of the rated output, replace lamp. f. Perform maintenance as directed by the manufacturer. g. Purchase and use a pressure monitor control device to measure efficiency of air flow.

160

TABLE IV-A-1
(continued)

Monitoring Laboratory Equipment

Item	Monitoring Procedure
13. Thermometers and Recording Devices	a. Check the accuracy of thermometers and temperature recording instruments, in the monitoring range, at least annually against a certified thermometer or one of equivalent accuracy. Thermometer graduations should not exceed the 0.2 or 0.5 C deviation permitted in the analytical method. Check mercury columns for breaks. b. Record calibration checks in quality control (QC) record. Mark NBS calibration correction on each thermometer or on the outside of the incubator, refrigerator or freezer containing the thermometer. c. Record daily temperature checks on charts and keep for at least three years. A simple, one year chart is shown in Figure IV-A-1.
14. Water Bath	a. Check and record temperature daily. Bath must maintain the uniform temperature needed for the test in use. b. Maintain accurate thermometer completely immersed in water bath. c. A recording thermometer and alarm system are recommended. d. Clean monthly. e. Use only stainless steel, rubber, plastic-coated, or other corrosion-proof racks.
15. Refrigerator at 4 C	a. Check and record temperature daily. b. Clean monthly. c. Require identification and dating of all material. d. Defrost unit and discard outdated materials in refrigerator and freezer compartments every three months.
16. Hot Air Oven	a. Test performance with spore strips or suspensions quarterly. b. Equip and monitor sterilization with a thermometer accurate in 160-180 C range.
17. Freezers	a. Check temperature and record daily. b. Use of recording thermometer and alarm system recommended. c. Require identification and dating of all materials. d. Clean and defrost freezer every six months. Discard outdated materials.

161

TABLE IV-A-1
(continued)

Monitoring Laboratory Equipment

Item	Monitoring Procedure
18. Autoclave	a. Record temperature and pressure for each run. Recording thermometer recommended. b. Verify that autoclave maintains uniform operating temperature. c. Check operating temperature with a min/max thermometer on a weekly basis. d. Test performance with spore strips or suspensions weekly. If evidence of contamination occurs, check until the cause is identified and eliminated. e. Procure semi-annual preventive maintenance inspections.
19. Incubator (Air/Water-Jacket)	a. Check and record temperature daily. b. If partially-submersible glass thermometer is used, bulb and stem must be immersed in water to the mark on stem. c. Measure temperatures daily on top and bottom shelves. Periodically measure temperature on all shelves in use. d. Expand test points proportionately for walk-in incubators. e. Recording thermometer and alarm system are recommended. f. Locate incubator where room temperature does not go outside of the 16–27 C range.

161

Instrument _____ Temperature _____ Room _____

Read daily.
Record temperature in space provided.

Date	Jan.	Feb.	Mar.	Apr.	May	June	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Date
1													1
2													2
3													3
4													4
5													5
6													6
7													7
8													8
9													9
10													10
11													11
12													12
13													13
14													14
15													15
16													16
17													17
18													18
19													19
20													20
21													21
22													22
23													23
24													24
25													25
26													26
27													27
28													28
29													29
30													30
31													31

FIGURE IV-A-1. Equipment Operation Temperature Record.

TABLE IV-A-2

Glassware Maintenance

Item	Monitoring Procedure
1. Utensils and Containers for Media Preparation	Use utensils and containers of non-corrosive and non-contaminating materials such as pyrex glass, stainless, steel or aluminum.
2. Glassware (Reusable)	a. With each use, examine glassware especially screw-capped dilution bottles and flasks, for chipped or broken edges and etched surfaces. Discard chipped or badly-etched glassware. b. Inspect glassware after washing. If water beads excessively on the cleaned surfaces, run the glassware through again. c. Test for acid or alkaline residues by adding bromthymol blue indicator to representative glassware items (see 5.1.2 in This Section). d. Test for residual detergent by the test in 5.1.3, This Section.

TABLE IV-A-3

Laboratory Pure Water for Bacteriological Testing

Parameter	Ideal Monitoring Frequency	Limit
Chemical Tests		
Conductivity	Continuously or with each use	1-2 μmhos/cm at 25 C
pH	With each use	5.5-7.5
Total Organic Carbon	Monthly	< 1.0 mg/liter
Trace Metal, Single	Monthly	<0.05 mg/liter
Trace Metals, Total (Cd, Cr, Cu Ni, Pb, Zn)	Monthly	< 1.0 mg/liter
Ammonia/Amines	Monthly	<0.1 mg/liter
Free chlorine	With each use	<0.1 mg/liter
Biological Tests		
Standard Plate Count		
Fresh Water	Monthly	< 1000 bacteria/ml
Stored Water	Monthly	<10,000 bacteria/ml
Water Suitability Test	Yearly and when conditions change	Ratio: 0.8-3.0
Water Use-Test	Yearly and when conditions change	Calculated t value < 2.78

A10

TABLE IV-A-4

Quality Control of Media

Medium	Control Cultures	Expected Results
M-Endo MF Broth or Agar	*Escherichia coli*	Golden green metallic sheen
	Enterobacter aerogenes	Golden green metallic sheen
	Achromobacter sp	Red colonies
	Pseudomonas sp	Red colonies
	Salmonella sp	Red colonies if medium overheated
M-FC Broth or Agar	*E. coli*	Blue colonies
	K. pneumoniae	Blue colonies
	E. aerogenes	No growth
Brilliant Green Bile Lactose Broth	*E. coli*	Growth with gas
	E. aerogenes	Growth with gas
	C. freundii	Growth with gas
	Staph. aureus	No growth
Lauryl Tryptose Broth	*E. coli*	Growth with gas
	E. aerogenes	Growth with gas
	S. typhimurium	Marked to complete inhibition
	S. aureus	Marked to complete inhibition
Levine's Eosin Methylene Blue Agar	*E. coli*	Nucleated black colonies with golden green metallic sheen
	E. aerogenes	Pink colonies with dark centers
	C. freundii	Colorless colonies
	Salmonella sp	Colorless colonies
	Klebsiella sp	Large brown mucoid colonies
Xylose Lysine Desoxycholate Agar (XLD)	*Salmonella* sp	Red colonies, to red with black centers
	Klebsiella sp	Yellow colonies
	E. coli	Yellow colonies
	E. aerogenes	Yellow colonies
Bismuth Sulfite Agar	*Salm. typhosa*	Black colony with black or brownish-black zone, with or without sheen
	Other *Salmonella* sp	Raised green colonies
	Coliforms	Green colonies
Brilliant Green Agar	*Salmonella* sp	Pink-white opaque colonies surrounded by brilliant red zone
	E. coli	Inhibition or yellow green colonies
	P. vulgaris	Marked to complete inhibition or red colonies

TABLE IV-A-4
(continued)

Quality Control of Media

Medium	Control Cultures	Expected Results
KF Streptococcus Agar	*Strep. faecalis*	Pink to red colonies
	Strep. pyogenes	No growth
	S. aureus	No growth
	E. coli	No growth
PSE Agar	*S. faecalis*	Black colonies
	E. coli	No growth
	S. aureus	No growth

167

TABLE IV-A-5

Quality Control of Biochemical Tests

Test	Control Culture	Expected Results	
BHI Broth at pH 9.6	*Strep. faecalis* *Strep. mitis-salivarius*	Positive: Negative:	growth no growth
BHI Broth with 6.5% NaC1	*S. faecalis* *S. mitis-salivarius*	Positive: Negative:	growth no growth
Arginine Dehydrolase (Moeller's medium)	*Salm. typhimurium* *Salm. flexneri*	Positive: Negative:	alkaline reaction reddish violet color yellow color
Lysine Decarboxylase (Moeller's medium)	*S. typhimurium* *S. flexneri*	Positive: Negative:	alkaline reaction reddish violet color acid, yellow reaction
Ornithine Decarboxylase (Moeller's medium)	*S. typhimurium* *S. flexneri*	Positive: Negative:	alkaline reaction reddish violet color yellow color
Indole Production (Tryptophane Broth)	*Escherichia coli* *Salmonella* sp *Enterobacter aerogenes*	Positive: Negative: Negative:	red color orange/yellow color orange/yellow color
Methyl Red (Buffered Peptone Glucose Broth)	*E. coli* *E. aerogenes*	Positive: Negative:	red color no change
Voges-Proskauer (Buffered Peptone Glucose Broth)	*E. aerogenes* *E. coli*	Positive: Negative:	pink color no color change
Citrate Utilization (Simmons Citrate Broth)	*E. aerogenes* *E. coli*	Positive: Negative:	growth, change to blue color no color change, no growth
Urease Production (Christensen's Urea Agar)	*Proteus mirabilis* *Salmonella* sp	Positive: Negative:	color change, pink to red no color change
Catalase (BHI agar slant)	*S. aureus* *S. faecalis*	Positive: Negative:	bubbles no bubbles

TABLE IV-A-5
(continued)

Quality Control of Biochemical Tests

Test	Control Culture	Expected Results
Cytochrome Oxidase (Alpha-napthol and para-amino-dimethylaniline oxalate)	*Pseudomonas aeruginosa* *E. coli* *S. aureus*	Positive: blue color Negative: no change Negative: no change
Phenylalanine Deaminase (Phenylalanine Agar)	*P. mirabilis* *Salmonella* sp *E. coli*	Positive: green color Negative: no color change Negative: no color change
Malonate Utilization (Malonate Broth)	*K. pneumoniae* *E. coli*	Positive: blue color change Negative: no growth or color change
Milk, Methylene Blue, 0.1%	*S. faecalis* Group Q Streptococci *Strep. salivarius* *P. aeruginosa* *C. perfringens*	Positive: reduction of methylene blue Negative: no growth Negative: no growth Peptonization and digestion Acid, coagulation and gas
Nitrate Reduction (Potassium Nitrate Broth)	*E. coli* *P. aeruginosa*	Positive: red color change Negative: no color change
2, 3, 5-Triphenyl Tetrazolium Chloride in TG Agar	*S. faecalis* *Strep. faecium*	Positive: reduction of TTC (red color) Negative: no color change
Tellurite Agar	*S. faecalis* *S. faecium*	Growth No growth
Beta-Hemolysis in Blood Agar	*S. faecalis* var. *zymogenes* *S. faecalis*	Positive: lysis of red blood cells Negative: no lysis of red blood cells

		Slant	Butt	H_2S Production
Hydrogen Sulfide (Triple Sugar Iron Agar)	*E. coli*	A	A G	-
	P. vulgaris	A	A G	+
	S. typhimurium	K	A G	+

TABLE IV-A-5
(continued)

Quality Control of Biochemical Tests

Test	Control Culture	Expected Results		
		Slant	Butt	H_2S Production
Lysine (Lysine Iron Agar)	*S. typhimurium*	K	K	+
	S. flexneri	K	A	-
Gelatin Liquefaction at 20°C (Nutrient Gelatin)	*S. marcescens*	Positive:	liquefaction	
	S. faecalis var. *liquefaciens*	Positive:	liquefaction	
	S. faecalis	Negative:	no liquefaction	
	C. perfringens	Positive:	liquefaction	
	E. coli	Negative:	no liquefaction	

REFERENCES

1. American Public Health Association, 1972. Standard Methods for the Examination of Dairy Products, (13 ed.) American Public Health Association, Inc., Washington, DC. RODAC: p. 192; Swab: pp. 43-44; and Air: p. 44.

2. American Public Health Association, 1975. Standard Methods for the Examination of Water and Wastewater (14 ed.) American Public Health Association, Inc., Washington, DC. p. 885.

3. Geldreich, E. E. and H. F. Clark, 1965. Distilled water suitability for microbiological applications. J. Milk and Food Tech. 28:351.

4. Military specifications for Disk, Filtering, Microporous, Hydrosol Type, 47 mm diameter 100s. MIL-D-37005 (DSA-DM) 5 September, 1973. Directorate of Medical Materiel, Defense Personnel Supply Center, Dept. of Defense, Philadelphia, PA.

5. Tentative method for evaluating water testing membrane filters for fecal coliform recovery. D3508-76T. 1978 Annual Book of ASTM Standards, Part 31, p. 1127.

6. Standard specification for reagent water. D1193-77. 1978 Annual Book of ASTM Standards, Part 31, p. 20.