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Seafood Safety and Fish Decontamination
Technical and Health Implications

Final Report
Volume II

DEPURATION OF BACTERIAL & VIRAL CONTAMINANTS DISINFECTION USING HYDROGEN PEROXIDE & SILVER SURVIVAL OF HEPATITIS A VIRUS & SEAFOOD SAFETY SURVEY OF HEAVY METALS AND MICROBIAL PATHOGENS IN FRESH FISH IN ISRAEL

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Table of Contents

	Page
Abstract	3
Part I	4-36
Decontamination of Fish by Water Change	
Introduction	
Materials and Methods	
Results and Discussion	
Conclusions of Part I	
Part II	37-98
A Preliminary Results of the Toxicity of Hydrogen Peroxide (H_2O_2) and Silver Ions (Ag+) on Fish	
B Die-off Microorganisms (E coli, MS2 Phages and Polio 1) by Hydrogen Peroxide and Silver Ions	
Introduction	
Materials and Methods	
Results	
I. The Active Residual of H_2O_2 and Ag+ Ions in Different Kind of Water	
a At the Presence of Fish and Airing the Water	
b Without the Presence of Fish and Airing the Water	
c Comparison between the Residual and Active H_2O_2 with Aerated Water	
II. The Resistance of Fish to Different Levels of H_2O_2 and Aerated Water	
III. The Die-Off Kinetics of the Different Microorganisms in Clean and Maintenance Water.	
a. Die-Off Kinetics of E coli.	
b Die-Off Kinetics of MS2 and Poliovirus 1	
Conclusions of Part II	
References of Part I and Part II	

Part III 99-106

Survival of Hepatis A Virus (HAV), Poliovirus 1, F+ bacteriophages and E coli in PBS, Groundwater and Wastewater as it Pertains to Seafood Safety
- Introduction
- Materials and Methods
- Results
- References of Part III

Part IV 107-120

Microbes and Heavy Metals Detected in Freshwater Aquacultured Fish Marketed in Israel
- Abstract
- Introduction
- Meterials and Methods
- Results
- Discussion
- References

ABSTRACT

Fish grown in contaminated ponds can be exposed to a variety of human pathogenic microorganisms which may accumulate in its tissues This study was conducted in order to 1) examine the efficiency of changing the water, in the holding tanks, to reduce the level of microorganisms in various fish, tissues, 2) test for the presence of microbes and heavy metal in fish marketed in Israel

Fish contamination was accomplished by seeding 10^9, 10^{11}, 10^{10} cfu or pfu ml^{-1} of E coli, MS2 coliphage and poliovirus 1, respectively, into 100 l of water These microorganisms were tested in the skin, muscle, liver, spleen and the digestive tract (DT) A single inoculation of the test microorganisms was followed by 3-24 water change (tank volumes) and compared with self purification (control) experiments without changing the water The highest levels of microorganisms were found in the DT, 6h after inoculation for E coli and 12h after inoculation for MS2 and poliovirus 1 Lower levels of the microorganisms were found in the spleen, liver and on the skin and none were detected in the muscle In the decontamination experiments, no microorganisms were detected in the DT 5-6 days after inoculation, as compared to 11-21 days in the control experiments The results of this study indicate that faster decontamination of fish is achieved by repeated changing of the water in the holding tanks and thus may lower the risk of disease transmission by fish handing or consumption

Two species of fish, *Tilapia sp* and *Mugil sp* , grown in fresh water aquaculture and marketed in Israel were tested for the presence of microbes and heavy metals These species are known to tolerate heavy organic loads Samples of digestive tract (DT), liver, spleen, skin and muscle were assayed for *Aeromonas*, *Fecal coliforms*, and F+ coliphages DT and muscle were assayed also for enteroviruses The most frequent microorganism found was *Aeromonas*, followed by *Fecal coliforms*, and F+ coliphage In both fish species, the highest concentration of *Aeromonas* was present in the DT, followed by skin, liver, spleen and muscle *Fecal coliforms* were not found in any of the muscles sampled Enteroviruses were not detected in either DT or muscle Even though our results show that both microbial and heavy metal concentrations were within the acceptable Israeli ranges, we recommend that measures be taken in processing to prevent cross-contamination of edible tissue by DT and skin

PART I

Decontamination of Fish By Water Change

INTRODUCTION

I Preface

Fish have always been an important source of protein for humans The demand for fish protein is based partly on the fact that this protein is relatively cheap to produce In fact, it is expected that as the world's population increases so will the demand for fish protein

Fertilizing fish ponds with human and animal excreta is an ancient practice in many parts of the world including Asia, Europe, and the Middle East The use of domestic fish ponds, for example, can be seen in an Egyptian tomb that is dated to over four thousand years ago (McGarry, 1977) However, aquaculturalists from the Far East were the first to recognize that the addition of wastewater to fish ponds increases the fish yields (Prowse, 1962) Wastewater was also used for a similar purpose in the Middle Ages (Feachem et al , 1983) Nowadays in many areas of the world it is customary to enrich fishponds with wastewater of different levels of treatment (Edwards, 1985) In some areas, fish are grown in wastewater reservoirs and are used as biological filters to decrease the amount of organic material floating in the water (Henderson, 1979, Laventar and Teltech, 1986, Dotan et al , 1989) Hepher and Schroeder (1977) have shown that growing fish in wastewater reservoirs can cover the cost of collecting this water Moreover, Nkuchia (1994) recently analyzed the net benefits of using wastewater for irrigation only, for aqauculture only, and both for wastewater and aquaculture He found that using wastewater for aqauculture only to grow fish as human food (rather than animal food) has more benefits than using wastewater for irrigation only

II Advantages and disadvantages of using wastewater for growing fish

There are many advantages and a number of disadvantages associated with the use of wastewater for growing fish Some of the advantages are associated with

increases in the fish yields (Hepher and Schroeder, 1977, Dotan et al 1989, Fattal et al 1989 and Mancy et al , 1989)

Feldleit (1986) showed that a constant flow of wastewater into fish ponds increases the fish yields by a factor of four as compared with the national average in Israel This increase results from enrichment of the entire food chain with nutrients The presence of nutrients in wastewater is likely to result in the eutrophication of the body of water This may be a disadvantage in pools of natural waters, but is a significant advantage in fishponds (Mann, 1972) Data from fishfarms show that the addition of wastewater to the fish ponds decreases the need for food additives by 40% (Hepher and Schroeder, 1977) An experiment conducted in Israel by Moav, et al, (1977) showed that the organic material present in high concentrations in wastewater could be the only source of food for fish In addition, the fish that were fed by wastewater only had a lower fat content as compared with fish that were fed food additives Another advantage is that the fish grown in waters rich in organic material improve the quality of the water The fish are used as organic filters and they reduce the amount of plankton and solids floating in the water (Henderson, 1979) In Israel, fish were put into surface water reservoirs of the national water carrier in order to reduce the amount of organic material floating in the water Two years after these reservoirs were populated with fish the underwater flora disappeared Populating the reservoirs with fish also decreased the number of snails in the reservoirs to a minimum and caused the aftertaste and smell from blue algae to disappear (Laventar and Teltech, 1986) At a sewage treatment plant it was found that the removal of microorganisms from ponds where fish are raised is similar or higher than in oxidation ponds (Carpenter et al , 1974, Hejkal et al , 1983, Colleman et al , 1974) The utilization of phytoplankton by the fish prevented algae bloom and reduced the mortality rate of fish (Hepher and Schroeder, 1977, Schroeder, 1975) However, it is worth mentioning that the population of phytoplankton in the waters remained in equilibrium so that no

oxygen deficiency was expected and there was no need for airing the pond (Moav, et al 1977)

The disadvantages of using wastewater in fishponds are associated with the following

a Increased mortality of the fish or a decrease in their growth rate as a result of a hostile environment These conditions may result from adding uncontrolled wastewater to the pond (Feldleit, 1986)

b A health risk associated with 1) working in the fish industry, cultivating, and/or processing fish (Ahmed, 1991), 2) consumers who eat raw or cooked fish These consumers may be infected by pathogens originating from contaminated fish from wastewater (Higashi, 1985)

These limitations can be reduced by improving the quality of wastewater for fish Coleman et al (1974), Carpenter (1974) and Wolny (1966), for example, have described how fish grow well in treated wastewater In addition, the wastewater can be diluted with clean water in order to keep the oxygen at a level high enough to grow fish (Vaas, 1948), or wastewater can be added at a constant volume to the fishpond in order to compensate for water loss from seepage and evaporation

III Pathogens present in human excreta and raw wastewater

Feachem et al (1983) and Shuval et al (1986) provide a thorough review of the pathogenic microorganisms including viruses, bacteria, protozoa, and Helminths which may be found in untreated domestic wastewater The number and type of these microorganisms depends upon enteric disease morbidity in the community responsible for the discharge (Cooper, 1991)

In areas where industrial effluent is mixed with domestic wastewater, toxic chemicals may also create the potential for adverse toxicological effects The presence of pathogenic microorganisms and toxic chemicals in untreated wastewater creates the

potential for disease transmission and toxicological health effects where there is contact, ingestion, or inhalation of pathogens or chemicals (USEPA/USAID, 1992) In low income countries, infectious disease agents rather than chemicals are the main source of health concerns associated with untreated wastewater However, this should only be the case if wastewater strictly originates from domestic wastewater sources without the addition of chemical contaminants from industrial sources Besides the health concerns due to pathogens or chemicals, untreated wastewater may provide favorable conditions for spreading vector-borne diseases In addition raw wastewater can also create conditions for nuisances like unpleasant odors and encourage fly breeding (Edwards, 1992)

The principal infectious agents that may be present in raw wastewater can be broadly classified into four groups

1 Viruses Over 100 different enteric viruses capable of producing infections or disease are excreted by humans The most common viruses are the enteroviruses (polio, echo, and coxsackie), rotaviruses, reoviruses and hepatitis A virus (Hurst et al , 1989)
2 Bacteria The most common pathogen is the genus salmonella Less common genera are shigella, vibrio, mycobacterium, campylobacter, and others
3 Protozoa The most important one is Entamoeba histolytica, Giardia, and Cryptospordium
4 Helminths The most important helminths are intestinal worms including Ascaris lumbricoides, Taenia saginata and solium, trichuris trichina, and ancylostoma

IV Effect of environmental conditions on stress in fish

Fish are poikilothermic animals (cold blooded) who live in water Their existence depends on the quality of the water in which they live Fish that are raised in relatively small bodies of water, such as ponds, are sensitive to changes in their environment

Chemical, biological, physical or procedural changes may cause stress in fish It is generally accepted that stress is an important cause of disease outbreaks in fish following a decline in their immune systems This may enhance the penetration of bacteria and viruses to the fish organs and thus cause a variety of diseases in fish (Sniezko, 1974)

The following are some of the causes of stress in fish

1 Changes in the concentrations of the dissolved gases - Changes in the concentrations of the dissolved gases in the water, especially of oxygen and nitrogen, may cause damage to the fish's gills and skin This in turn "invites" a microbial invasion (Rucker, 1972) An insufficient supply of oxygen causes mortality in the fish, changes in the concentrations of microorganisms, and to high biooxygen demands (B O D) (Halley et al , 1967) When the concentration of dissolved oxygen is low, the sensitivity of fish to pathogens increases (Boyd, 1990)

2 Temperature - The temperature effects the following factors metabolic rate, immune system, fish reproduction, and the concentration of dissolved oxygen in the water These factors in turn effect the following B O D , toxicity of the contaminants which effect the growth of the fish, and the levels of pathogens and parasites (Eisa et al , 1985)

3 Eutrophication - Eutrophication is characterized by changes in dissolved oxygen levels and changes in the pH A decrease in the concentration of oxygen caused by eutrophication has a profound effect on the fish (Fry, 1969) Moreover, eutrophication causes algae bloom and increases in the concentrations of bacteria which in turn increases morbidity among the fish (Collins, 1970)

4 Wastewater - As mentioned before, domestic wastewater contains a large number of pathogens Wastewater, fish food, and fish excretions contribute to the multiplication of bacteria and to an increase in the B O D (Shotts et al 1972) Organic material decomposes at a rate that increases as the temperature increases The process requires oxygen and as such decreases the concentration of dissolved oxygen in the water (Hepher and Schroeder, 1977)

5 Metabolic byproducts of fish - Ammonia appears naturally in fishponds as a result of fish excretions and the decomposition of organic material In fishponds that have reached equilibrium the ammonia is either absorbed by phytoplankton or undergoes nitrification by anaerobic processes The toxic material to fish is predominantly non-ionic gaseous ammonia The relationship between gaseous ammonia and the total ammonias (the ions) depends on the pH and the temperature of the water (Chen, 1991) As the pH and temperature increase the toxicity of the ammonia on the fish increases The toxicity of ammonia is also increased by a decrease in the concentration of dissolved oxygen and by an increase in the density of the water (Downing and Merkens, 1955)

6 Industrial Pollution - Industrial pollution is caused by chemicals that reach the water Couch (1974) reports that exposure to chemicals frequently causes pathological changes in fish tissue This pehonomenon allows for the invasion of fish by pathogens which in turn leads to high fish mortality (Skimode, 1970, Starr and Jones, 1957, Burton et al , 1972, Chen, 1991)

7 Density - Density can be determined by the number of fish grown in a certain fish pond per the area of the pond or the volume of the water in the pond High density of the fish in the pond causes stress to the fish (Dotan et al, 1989, Fattal et al, 1992)

It is very difficult to define which of the above factors has a significant meaning on stress in fish For the most part, the stresses occur as complexes and their effects are either additive or synergistic (Sniezko, 1974)

V The penetration of pathogens into fish tissues

Pathogens are likely to penetrate the fish's tissues despite the fish's defense system The penetration occurs through the fish's skin, mouth, gills, and thin epithelium (Sniezko, 1974) With exposure to high patnogen concentrations, penetration through the gall bladder, the stomach cavity, and even to the eyes is possible (Munro, 1982)

The penetration of the different microorganisms in the fish organs largely depends on the concentration of these microorganisms in the water where the fish is grown When the concentration of these microorganisms reaches beyond certain threshold, the penetration of the microorganisms could reaches even the muscle This particularly happend when the fish showed signs of high stress (Buras et al, 1987)

1 Manner in which the pathogens enters through the fish's tissue

a Penetration through the skin - The fish's outer layer of skin is covered by a thick layer of mucous which prevents the adsorption and growth of microorganisms within the skin Skin wounds, however, allow for penetration of the fish's skin by bacteria, viruses, and fungi In an experiment, goldfish were exposed to a suspension of *Aeromonas hydrophila* after their skin was scraped with a blunt instrument No bacteria were found on the surface of the skin that had not been damaged On the other hand, within the wounds, a large number of bacteria had been adsorbed in the exposed injured areas at the compactum layer (Crouse-Eisnor et al , 1985)

b Penetration through the mouth and the digestive tract - Water and particles reach the stomach through the mouth Bacteria, viruses, and protozoa enter the fish in the same manner Some enter through the intestines and then pass from

the digestive tract to the inner organs such as the spleen, the liver and the kidneys (Sniezko, 1970, Buras et al , 1985, Fattal et al , 1992) In the digestive tract there is a high concentration of phagocytes which present the first barrier to foreign bodies that invade the fish After entry into the esophogus, the microorganisms can reach the blood stream or the lymphatic system and from there they enter the various lymphatic organs In the lymphatic organs further phagocytic activity takes place From there, the microorganisms that have not yet been phagocytized and are still in the blood stream can reach other tissues (Digirolamo et al, 1975) Canzonier (1971) assumes that the penetration is based on two different but related processes The first is the entry of contaminated food into the digestive tract In this case, the bacteria or viruses reach the digestive tract, pass through it, and are excreted in the feces The second process is long term exposure to small numbers of microorganisms In this case, the microorganisms remain in the digestive tract and in the lymphatic organs The remaining microorganisms will not be released quickly and, therefore will stay in the digestive tract for long periods of time Pain (1986) suggests the existence of cleanliness cells called haemocytes which capture microorganisms from the digestive tract and transfer them to other tissues The physiological system which is responsible for this phenomenon may be different in different species of fish and this has important public health implications

c Penetration through the gills - The penetration of fish by microorganisms may also occur through the gills Studies have shown that two hours after exposure of carp to a viruses, they could be detected in the gills and 5 days later could be found in the blood system (Ahne, 1978) It appears that the type of cells which existed in the gills serve as proper pathways to the blood system (Chilmoczyk, 1980)

2 Defense system of the fish

The defense system of the fish can be divided into the physiological system and the immune system (Ellis et al , 1976)

The physiological system is composed of the skin and a thick layer of mucous which prevents the adsorption and growth of microorganisms on the skin This layer protects the skin cells from mechanical, biological and chemical wounds and provides a selective transmission of metabolytes and pathogens between the fish and the environment (Krovacek et al , 1987) The immune system includes lymphocytes, granulocytes, and phagocytes all of which are found in the skin, gills, digestive tract, and the lymphatic organs These cells attack the microbiological invadors The lymphatic organs like the spleen and kidneys destroy the different antigens in an efficient manner (Lamers, 1985) In fact, the lymphatic organs that are most active in distancing foreign microorganisms are the spleen and kidneys These two organs have the highest concentrations of macrophages and lymphocytes (Buras et al , 1980) In a study of the phagocyte system, Ellis et al (1976) showed that carbon (particles) were phagocytized within 24 hours and most of the phagocytes contained carbon particles An hour after inoculation, carbon particles were found in the spleen and in the kidneys The phagocytic cells in the lymphatic organs represent the fish's potential initial defense mechanism (Buras et al , 1987) According to Ellis et al (1976), cells that are analogous to the copper cells are found in the livers of carp This fact hints at the role of these cells in the fish's defense system.

VI Health risks associated with contaminated fish

Fish can serve as a vehicle for the transmission of various diseases Fish contaminated by naturally-occuring toxins, bacteria, viruses, or parasites have caused numerous disease outbreaks Contamination of seafood with infectious agents may be due to several factors, such as organisms of fecal origin in polluted water (mainly

wastewater), naturally occuring aquatic microorganisms, and improper handling or preparation practices (Ahmed, 1991, Food and Drug Administration (FDA), 1994)

Assessing the health risks from seafood consumption is difficult due to the regional disparities between levels of environmental contamination, cultural practices, varying degrees of sanitation, and completely different methods of handling and preparing seafood In the United States, for example, 10 5% of all food-borne disease outbreaks between 1978 and 1987 were attributed to fish and shellfish consumption By comparison, however, chicken is 200 times more likely to cause disease than contaminated fish and 100 times more likely to result in the death of the consumer Significantly the consumption of raw or partially cooked shellfish is 100 times more likely to result in illness and there is a 250 fold increase in the risk of death (Nightingale, 1990)

1 Fish as pathogen carriers

Fish that come into contact with domestic wastewater are exposed to various human pathogens These pathogens (e g salmonella, shigella, or poliovirus) might penetrate the fish's tissues but not necessarily effect the fish themselves The pathogens can enter the digestive tract and settle in many of the fish's organs without causing any symptoms of illness in the fish (Buras et al , 1985) In this way the fish may serve as carriers of these pathogens and transmit them to the consumer and to individuals who work in the fisheries (Schewan, 1962, Guelin, 1962) Table A illustrates reported cases in which fish served as carriers of pathogens and/or microbiological indicators

Table A Reported cases in which fish served as carriers of microbiological indicators and/or pathogens

Type of fish	Isolated Micro-organisms	Organ from which they were isolated	Sampling location	Reference
Salmon	*Eentrobacter* *Aeromonas* *Acinobacter* *Enterococcus* *E coli*	Digestive tract	Streams in British Columbia	Trust and Sparrow, 1974
Carp	*E coli* *F streptococci*	Digestive tract Skin Muscle	Wastewater treatment plant	Hejkal et al , 1983
Carp and Tilapia	*E coli* *F coliforms* *Salmonella* *F streptococci* *Vibrio para-haemoliticus* Bacterio-phages Poliovirus	Several fish organs (especially the digestive tract)	Stabilization ponds (Israel)	Buras et al , 1987
Fresh-water fish	*Clostridium-botulinum* (type E) *Clostridium-tetani*	Not specified	Phillippines	Velazquez, 1980
Carp	*Salmonella*	Various organs	Laboratory experiments	Buras et al , 1985
Catfish	*Salmonella*	Digestive tract	Wastewater treatment plant	Janssen, 1970
Tilapia	*Salmonella*	Digestive tract Skin	Fishponds enriched with pig waste	Baker et al , 1983
Tilapia	*T coliforms* *F coliforms* Bacterio-phages *Salmonella* Parasites	Digestive tract Gills Liver Muscle	Fishponds enriched with sludge (Thailand)	Edwards et al, 1987 and Edwards 1992

Type of fish	Isolated Micro-organisms	Organ from which they were isolated	Sampling location	Reference
Tilapia	*T coliforms* *F coliforms*	Digestive tract	Fishponds enriched with domestic wastewater Thailand	Edwards et al, 1987 and Edwards 1992
Carp and Tilapia	*Aeromona* *E coli* *C perefringens* *Enterococcus*	Digestive tract Skin Liver Spleen	Wastewater ponds after treatment (Israel)	Dotan et al , 1989 Fattal et al , 1992 & 1993
Ayu	*Vibrio colera* (non 01)	not specified	River in Japan	Muroga et al , 1979
Carp, tilapia, and trout	*Aeromonas* *E coli* Enteroviruses polio 1 (immune type) *Enterococcus* F^+ coliphage	Digestive tract Gills Skin Liver Spleen	Wholesale market (Israel)	Nasser et al , 1992

In all the cases presented in the table the fish served as carriers and the authors did not report any visible signs of illness in the fish Results of a number of studies have shown that the level of microorganism contamination in the fish's tissue reflects the quality of the water in which the fish were grown (Nasser et al , 1992)

2 Diseases associated with eating fish

There are few reported cases of the passive transfer of disease causing pathogens from fish to humans (Edwards, et al , 1987) Fujino, et al (1951) reported an outbreak of food poisoning caused by eating sushi The Center for Disease Control in the United States publishes the "Morbidity and Mortality Weekly Report and Annual Summary of Food and Waterborne Diseases" (See reports for the years 1975-1993) Levin (1978) reviewed the literature on the incedents of disease outbreaks in fish and shellfish that were reported between 1963-1976

The diseases associated with eating fish and shellfish are generally divided into two categories A) diseases caused by chemical agents (toxins), and B) diseases caused by microbiological agents

A Diseases caused by chemical agents (toxins)

Naturally occuring seafood-borne toxins have affected consumers globally While these toxins are found in various parts of the world, most cases of poisoning seem to be limited to North America, Asia, and Europe Consequently, seafood-borne toxins have not been a major factor in seafood-related illnesses in Egypt, Israel, and other Middle-Eastern countries (Carr and Stem, 1993)

Seven types of toxins have been recognized as the most important ciguatera, scombroid, paralytic shellfish poisoning, neurotoxic shellfish poisoning, diarrhetic shellfish poisoning, puffer fish poisoning, and amnesic shellfish poisoning (Ahmed ed , 1991) These toxins are particularly insidious because they cannot be detected by

sensory inspection and are not inactivated by heat Natural intoxifications, with the exception of scombroid poisoning, are region-specific and often species-specific

All of the toxins mentioned above, with the exception of that responsible for scombroid poisoning, bioaccumulate in fish and shellfish as a result of food chain magnification and are, consequently, present in the organisms at harvest Scombroid poisoning, which has a wide geographic distribution and is present in various fish species, results from improper handling procedures These procedures allow bacteria to proliferate on the fish surface and subsequently convert free histidine to histamine in the fish tissues (Carr and Stem, 1993)

B Diseases caused by microbiological agents

This category of disease is generally divided into diseases caused by viruses, bacteria, and protozoa For details and review of the literature see Carr and Stem, 1993 The major points of the review are summarized below

1 Viruses

Seafood-borne viral outbreaks are almost exclusively associated with the consumption of shellfish (DeLeon and Gerba, 1990, Gerba, 1988) Very few viral outbreaks have been attributed to the contamination of finfish Shellfish are bottom dwellers and obtain their food by filtering the water, ingesting viruses either directly or through particles of food to which the viruses are attached These viruses may remain viable for long periods of time in the gut or mucus membranes of the shellfish Shellfish thus act as passive carriers for both viruses and bacterial pathogens (Gerba, 1988) More than 100 enteric viruses have been isolated from human feces Of these enteric viruses, mainly hepatitis A, non-A, non-B Hepatitis, Norwalk have been epidemiologically linked to seafood-related disease outbreaks

2 Bacteria

Different species of bacteria can serve as indicators of each source of contamination For example, pathogenic bacteria of the vibrio genera are normally resident in most aquatic ecosystems and, thus, do not necessarily indicate fecal contamination The presence of other species of bacteria from the genera salmonella shigella, and campylobacter indicate either cross contamination due to improper processing or the presence of fecal material in the waterbody In many cases it is difficult to asses whether adulteration of the food occurred as a result of fecal contamination in the environment or poor sanitation during handling and/or preparation

Species typically associated with seafood-borne disease outbreaks are vibrio cholerae, vibrio parahaemolyticus, and other vibrios and aeromonas However, certain bacteria which adulterate other foods such as chicken can also contaminate seafood For example, salmonella, campylobacter, escherichia, yersinia, shigella, clostridium, and staphylococcus have all been identified as causal organisms in seafood-borne disease outbreaks

3 Parasites

Parasites which might be associated with seafood-borne diseases are Giardia lamblia, the causative agent of giardias which has been traced to contaminated salmon Helminths contracted from seafood of concern to man can be broadly classified into three groups Cestodes, Trematodes, and Nematodes

VII Fishponds as breeding sites for mosquitos

The use of wastewater for enrichment of fishponds might provide another environmental problem i e breeding mosquitos, particularly culex pipien molastis, that are capable of living in wastewater or polluted water The important factor in breeding

of mosquitos and other vectors in the pond is growth of vegetation around the fish ponds (Curtis and Feachem 1981) This problem may be avoided if fish farmers use tilapia fish which have been shown to be suitable for ponds fertilized with wastewater These fish consume mosquito larvae and are effective in controlling mosquitos (Edwards, 1992)

VIII Wastewater quality criteria for public health protection

To protect public health against the harmful effects associated with the use of untreated wastewater guidlines and standards have been set for the safe use of wastewater in irrigation and aquaculture Guidelines are prepared by the international agencies like the WHO FAO, and the United Nation's Environment Program (UNEP) based on scientific criteria Individual countries are expected to use proposed guidlines to develop national standards based on the economic, social, technical, and political situation within the country (Hespahol and Prost, 1993) Guidlines and standards on wastewater quality are principally directed towards the pathogenic organisms The recommended microbiological quality for protecting public health in wastewater reuse for aquaculture are as follows

1 The use of indicator organisms

As mentioned previously, untreated wastewater contains a large number of diverse infectious agents Therefore, monitoring all of them would be impractical To overcome this limitation, coliforms are used as indicator organisms in monitoring the quality of wastewater (Cooper, 1991)

The concentration of indicator organisms is thought to have a semi-quantitative relationship with pathogens In practice, fecal coliforms are considered to be reasonable indicators of pathogens because their environmental survival and rates of removal or die-off in the treatment process are broadly similar (Mara and Caincross, 1989)

Coliforms are less effective as indicators of excreted viruses and are of very limited use as an indicator for protozoa and helminths for which no reliable indicators exist (WHO, 1989) Where laboratory facilities exist, it is important to monitor bacteriophages as indicators for enteroviruses in wastewater reuse It has been claimed that their removal occurs in parallel with suspended solids Monitoring of suspended solids is therefore useful in wastewater reuse projects (FAO, 1992) Since there are no adequate indicators for parasites wastewater guidelines and standards specify the minimum treatment requirement that is known to effectively remove these agents (Cooper, 1991)

2 World Health Organization guidelines for aquaculture

WHO published recommended *Health guidelines for the use of wastewater in irrigation and aquaculture* (WHO, 1989) The guidelines recommended that the microbiological quality of hellminths in wastewater for unrestricted irrigation use should not be more than one egg per liter and for fecal coliforms should not exceeded 1000 cfu/100 ml The recommended guideline for aquaculture for fecal coliforms is less than 1000 cfu/100 ml and for helminths, the absence of viable trematodes in a liter This standard is higher based on the assumption that pathogens may accumulate in the digestive tract and intraperitoneal fluid of fish and high standards of hygiene during handling, gutting, and cooking of fish were needed Bacteriological quality standards for fish muscles was suggested by Buras et al (1987) and later modified by Buras (1990) and are presented below

Total aerobic bacterial count in fish muscle tissue (bacteria/gram)	Fish quality
0-10	Very good
10-30	Medium
>50	Unacceptable

IX Measures taken to prevent transmission from fish

There are many measures that can be taken to reduce infectious diseases caused by the consumption of seafood (see Food and Drug Administration, 1994) The main measures are

1 Ensure thorough cooking of all fish
2 Educating consumers about the health risks associated with eating raw seafood even if it is considered a delicacy in some regions of the world
3 Proper handling procedures for fish
4 Adequate processing of domestic wastewater to reduce pathogens
5 Routine sampling of seafood in markets may help to prevent some disease outbreaks
6 Proper refrigeration or freezing of the fish after harvesting can prevent bacterial pathogens from multiplying and can kill some cestode larvae
7 Good sanitation during farming of the fish
8 Prevent high density and stress of farmed fish since this is very important factor in bacterial and viral penetration of fish tissues
9 Irradiation of live and fresh or frozen seafood can be effective in reducing some pathogens in fish tissues to safer levels
10 Depuration or purification of shellfish and fish is also a good and common technique used to reduce seafood-borne outbreaks (for more details see the following section)

X Purification of microbiologically contaminated fish

Many feasible options exist to minimize the risks associated with the consumption of seafood Reducing pathogen levels in the environment by treating domestic wastewater adequately is an important step, but may be impossible to implement on a worldwide basis, especially in those countries without adequate financial resources Moreover seafood is often harvested in polluted waters and exported to other areas, thus speeding contamination of these regions

In Israel, transfering the fish, prior to the marketing, from the growing fishpond to a holding pond for several days is common procedure The holding pond contains only fresh and clean water without any additive food It was found that transportation of starved fish can significantly reduce the mortality rate of fish, as compared with fed fish Does this procedure also reduce the microbiological counts in the fish tissues? Fattal et al, 1993, examined this procedure and found that there was no appreciable decrease of indicator microorganisms in fish tissue when the fish were kept for several days in these holding ponds

Depuration offers a potential alternative for cleaning fish and shellfish of bacterial and viral pathogens prior to consumption The methods used to depurate shellfish prior to marketing are well known and very successful However, there is a lack of awareness of the need to decontaminate fish prior to marketing Due to the success of the methods used to depurate shellfish and the physiological closeness between shellfish and fish, it is worthwhile to examine the possibility of using the same techniques used to depurate shellfish to depurate fish as well The optimal decontamination conditions change according to the fish species It is important to match the methods of depuration with the fish species being purified based on the characteristic conditions under which that species is grown In addition, one must consider the factors that effect the rate of purifying the microbiologically contaminated fish

1 Factors that effect the rate of depurating microbiologically contaminated fish

1 The way in which the fish was contaminated - Natural microbiological contamination is depurated more slowly than artificial contamination (i e in the laboratory) (Fattal et al , 1993)

2 Time period of exposure to contamination - The ability to depurate the fish that were exposed to contaminants for long periods of time is lower than that for fish that were exposed for shorter periods of time (Strauss, 1985, Fattal et al , 1993)

3 The physical state of the microorganisms - There is evidence that viruses that are adsorbed to particles or excretions are depurated more slowly than unadsorbed viruses (Durgin et al , 1981, Meinhold et al , 1982)

4 Level of contamination - When fish are heavily polluted, the depuration period is higher than when they are lightly polluted (Liu, 1967, Metcalf et al , 1980, Buras et al , 1987)

5 The type of microbiological contamination - Researchers have shown that the time required to depurate shellfish from viruses is longer than that for bacterias (Metcalf et al , 1980, Grohmann et al , 1981)

2 Common methods of depurating microbiologically contaminated shellfish

Richards (1988) surveyed the common methods used to depurate shellfish In the survey, he divides the methods into three groups

1 "Natural depuration" or "relaying" - In this method, the shellfish are removed from the contaminated water and transferred to clean water This method is intended for natural environmental conditions

2 Controlled purification - This process takes place in waters other than those in which the shellfish were raised The shellfish are depurated in tanks through which there is a constant flow of clean water This method is the most widely used today

3 "Flow through" - This process takes place on the seashore and depends on a constant supply of seawater or maintenance water

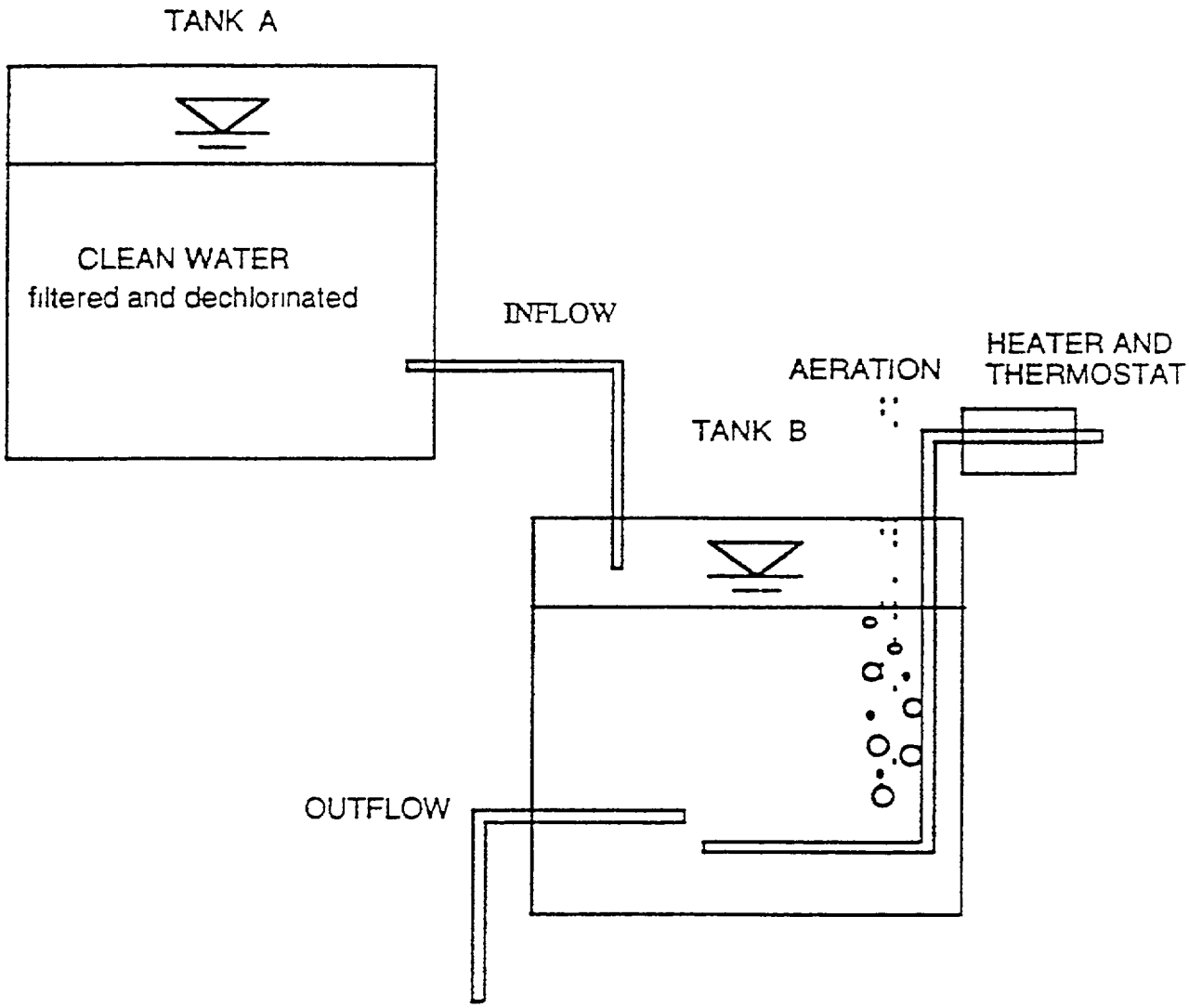
In this report, we have attempted to apply the methods commonly used for depurating shellfish to telapia fish We compared the self purification method with the active depuration by water changes method

The objectives of this study are 1) to study the bacterial and viral penetration uptake and accumulation in tissues of fish grown in polluted water, as a model of fish contamination by wastewater reuse in aquaculture, 2) to study the self purification in fish grown in polluted water, 3) to evaluate the effect of active depuration, by continuous water change, on fish decontamination

Materials and Methods

Tilapia-sarotherodom aureus x nilaticus hybrids were maintained in containers of 1 m^3, containing filtered aerated tap water at 25°C Fish were held for at least 7 days in the experimental container with no feeding prior to each experiment Decontamination experiments were performed in two tanks (A and B) of 100 liter each (see scheme 1 for layout of the depuration system) Tank B was the holding tank in which fish were kept and the contamination performed Tank A contained filtered tap water at 25°C as a supply of water for continuous water changes in tank B The concentration of the inoculated microorganisms in the water of tank B was 10^5 cfu/ml for E coli, 10^6 pfu/ml for MS2 phages and 10^5 pfu/ml for poliovirus 1 The retention time of water in tank B averaged 3 hrs Water changes were started 24 hours after the inoculation of the test microorganisms The control experiments were performed in tank B with no water changes At time intervals, 2 fish were dissected and the skin, muscle, liver, spleen and digestive tract were aseptically removed and homogenized in PBS (phosphate buffered saline) Samples were assayed on the same day for E coli and then the remaining samples were kept at -20°C for virus assay The decontamination experiments were repeated 3 times and the control experiments 2 times E coli (F18) enumeration was performed by the membrane filtration technique (Dufour et al 1981) MS2 coliphages were enumerated by the double-agar technique using S typhimurium (WG49) as the host (Adams, 1959, Havelaar and Hogeboom, 1984) Poliovirus 1, Lsc strain was cultivated and enumerated on BGM monolayers (Buffalo Green Monkey Kidney) Fish tissue homogenates were introduced onto monolayers with RPMI-1640

medium Infected monolayers were overlayed with RPMI-1640 medium containing 1% nobel agar Infected cultures were incubated at 37°C for 48 hrs and the developed plaques were counted (Guttmann-Bass, and Nasser, 1984)



Scheme 1 Layout Of The Active Depuration System (1)

(1) Water change experiments were performed in tanks (A & B) of 100 liter volume each Tank B was the holding tank in which fish were kept and contamination was performed Tank A contained cleaned, filtered and dechlorinated tap water at 25 ^{0}c as a supply of water for continuous water change in tank B Every three hours 100 liters of water from tank A passed tank B This is considered as 1 water change Water change in tank B started 24 hours after inoculation of the tested microorganisms in tank B

Results and Discussion

In the first annual report, covering the period of September 29, 1992 to September 30, 1993, we reported on the result concerning the multiple contamination experiment of fish and its kinetics (See Part IV in that report pages 18 to 27) The results presented in that report showed that the contamination level of the test microorganisms in the digestive tract was higher than that found in the water in which the fish were grown, and reaches their maximum levels 12 hours after each inoculation The maximum concentration of the microorganisms in the water reached 6 hours after each inoculation The concentration of microorganisms in the skin spleen and liver were much lower than in the water No microorganisms were found in fish muscle even after 11 inoculations with high liters of the microorganisms in the water (see materials and methods in this report)

In this report we are presenting data on decontamination experiments by water changes against control self purification experiments

Five different experiments were conducted two for self purification (for details see Appendix No 1 A and B and three for decontamination experiments see Appendix No 2, A, B and C) The total number of fish samples was 60 and the total number of water samples was 42 The results presented in this report is the log mean of the test microorganisms of the two self purification experiment vs the three decontamination experiments After conducting special statistical analysis we found that there were no significant differences between the results from turbid water and clean water in the

decontamination experiments (Appendix 2) Therefore the results from the turbid water and the clean water were combined and are presented in this section together

Figures 1 to 5 present the concentration of the test microorganisms in water (Figure 1), in the digestive tract (Figure 2), in the skin (Figure 3), in the liver (Figure 4) and in the spleen (Figure 5) The A figures present the self purification experiments and the B figures the water change experiments The concentration of the microorganisms is expressed as a log mean of 2 to 6 fish samples as cfu or pfu/ml, gr or cm^2

Figure 1a shows that the test microorganisms could be detected in the water (up to 2 6 log in polio 1), 18 days after inoculation in the control self purification experiments Figure 1b shows that the test microorganisms could be detected in the water 3 to 6 days after inoculation in the decontamination experiments (E coli was eliminated after 3 days, MS2 after 5 days and Polio 1 after 6 days) Figure 2a presents the microorganisms in the digestive tract (D T) in the control experiments MS2 was still detectable in the D T 21 days after inoculation, whereas E coli was undetectable at day 11 and polio 1 at day 21 Figure 2b shows that these microorganisms were undetectable 5 to 6 days after inoculations (E coli 5 days, MS2 and Polio 1 six days post inoculation) It is worth notice that the highest contamination was detected in the D T with levels of microorganisms similar to those in the seeded water Ellis et al, (1976) found that this phenomena is due to the fact that the wall of the digestive tract are concentrated with phagocyte cells which serve as an important defense mechanism for the fish Figure 3a shows that in the control experiments in the skin, MS2 was

undetectable at day 21, E coli and Polio 1 were undetectable at day 11 In decontamination experiments (Figure 3b) the test microorganisms were undetectable in the skin 4 to 6 days post inoculation Figure 4a shows that Polio 1 could not be detected in the liver at day 11 and MS2 and E coli at day 6 in the control experiments Figure 4b shows these microorganisms could not be detected in the liver at day 3 to 6 in the decontamination experiments Figure 5a shows that MS2 and Polio 1 could not be detected in the spleen at day 9 whereas E coli at day 3 in the control experiments Figure 5b shows that E coli could not be detected at the second day after inoculation in the spleen whereas MS2 at day 5 and polio at day 6 in the decontamination experiments

None of the test microorganisms were found in the muscle

The results of this study indicate the feasibility purifying fish by a simple technique of changing water Buras et al , (1987) found that, after 8 days of exposing the fish to salmonella, (8 days with 8 water changes) were needed to depurate the fish organs from this microorganism On the other hand, the results from this study – with control self purification experiments – were in agreement with the results obtained earlier in our laboratory (Fattal et al , 1993), where no appreciable decrease of indicator microorganisms was observed for 5-8 days after inoculation in fish maintained in a tank without changing the water Fast decontamination of fish is achieved, in this study by repeated changing of the water in the holding tank and this may lower the risk of disease transmission by fish handling or consumption In order to conserve water, the contaminated ambient water may be recycled through a process of filtration and disinfection

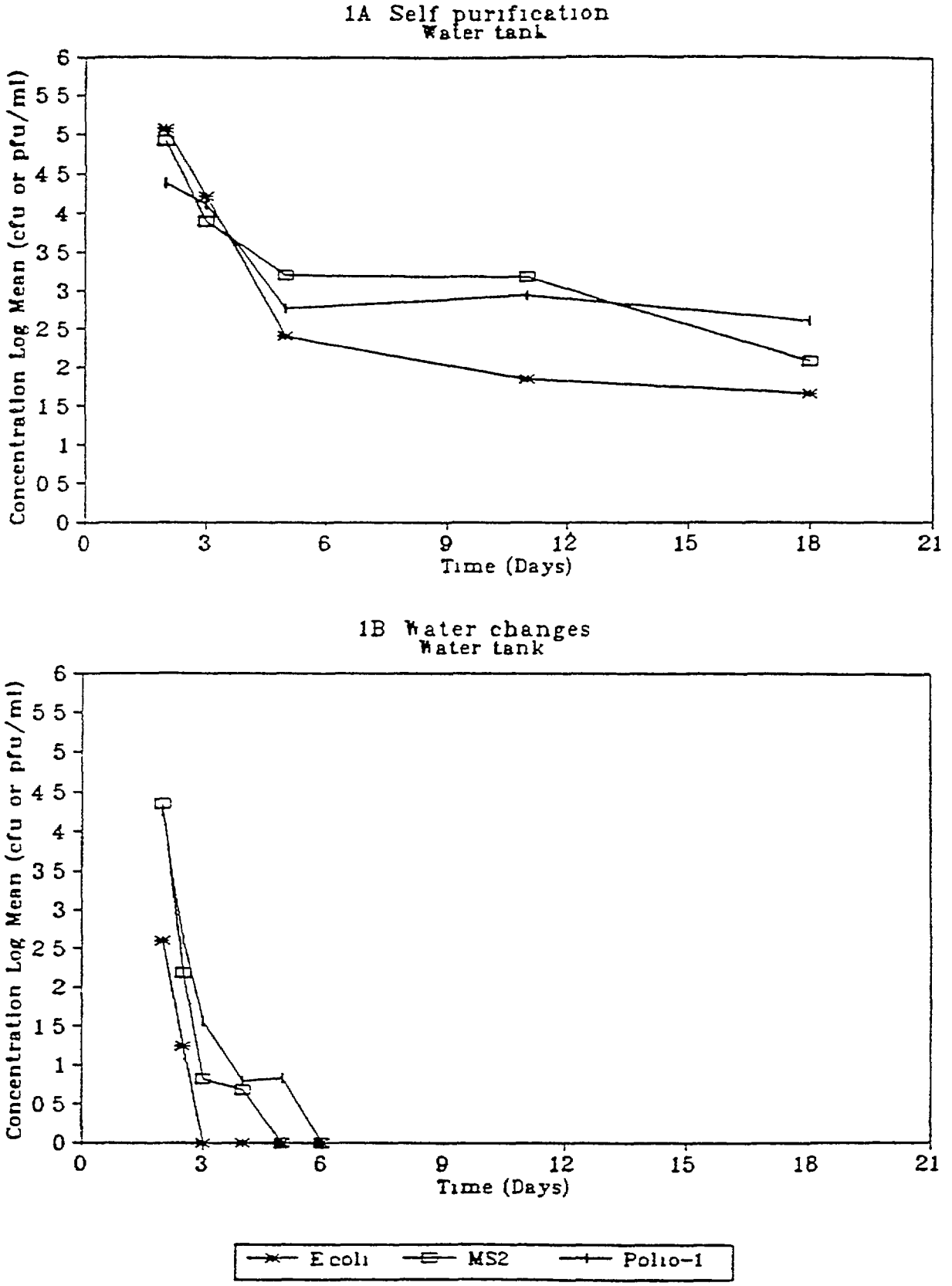


Figure 1 Residual concentration (log mean) of E coli, MS2 and Polio 1 in water in self purification (A) and water changes experiments (B).

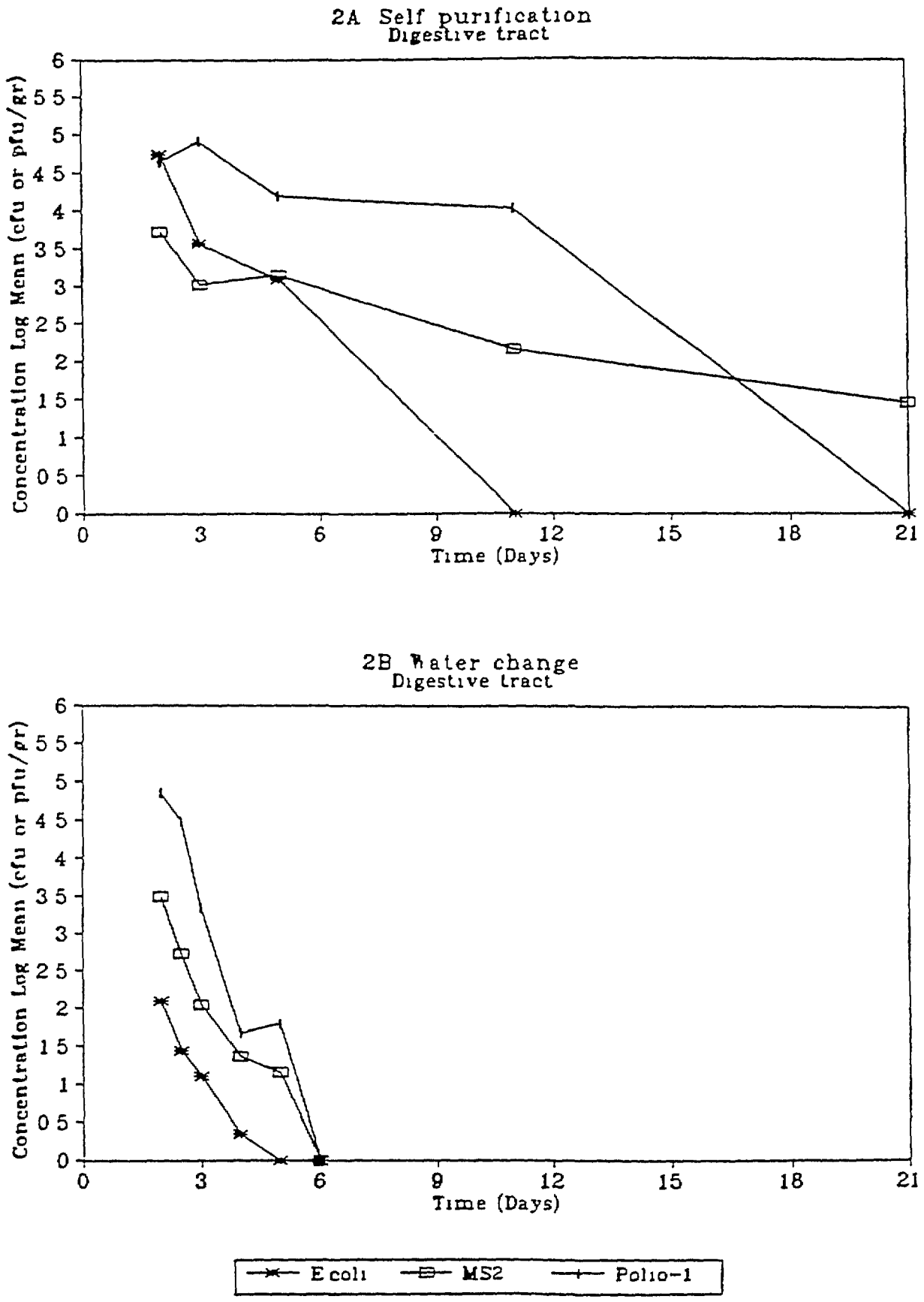


Figure 2 Residual concentration (log mean) of E. coli, MS2 and Polio 1 in the Digestive tract in self purification (A) and water changes experiments (B).

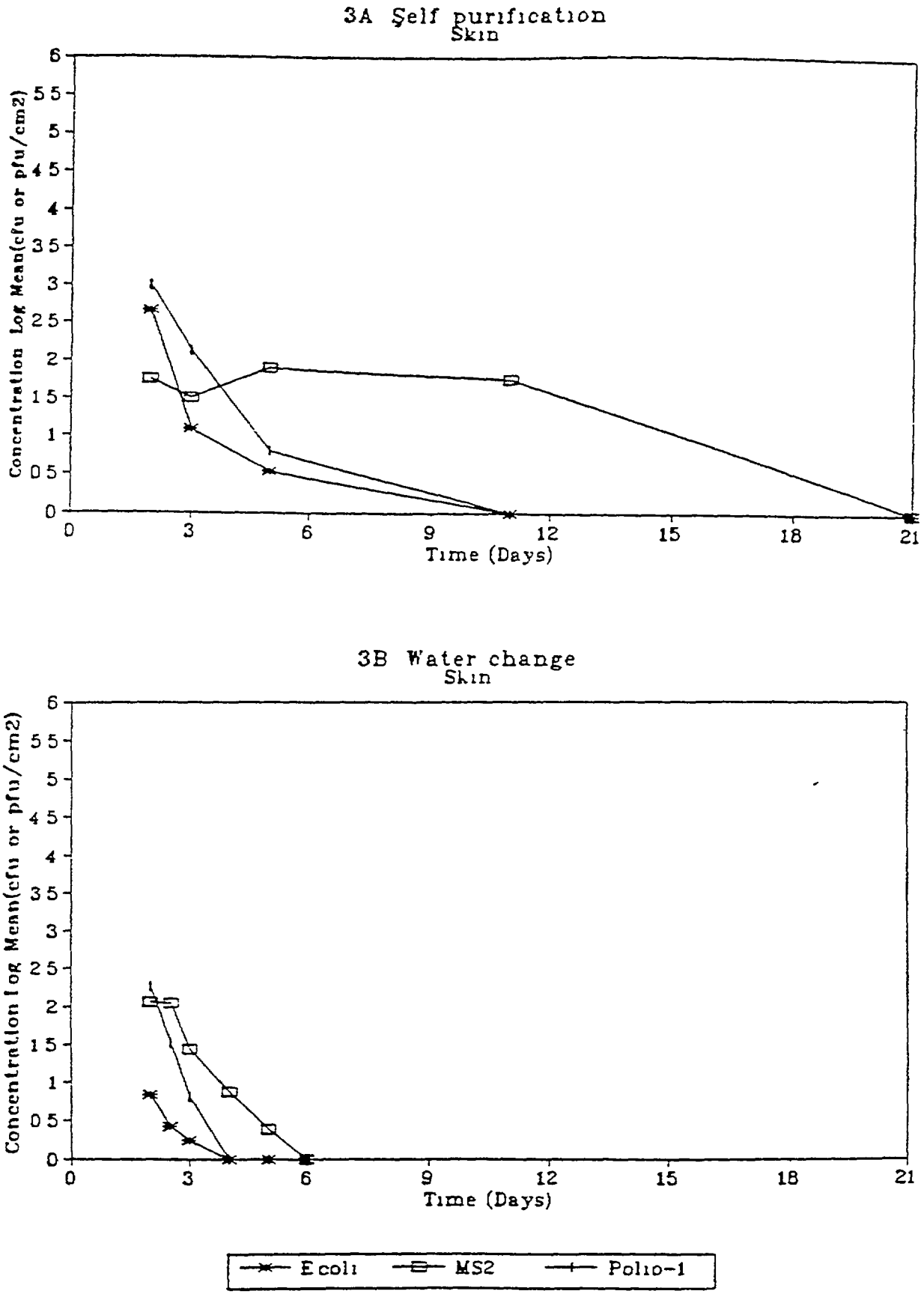


Figure 3 Residual concentration (log mean) of E coli, MS2 and Polio 1 in the skin in self purification (A) and water changes experiments (B)

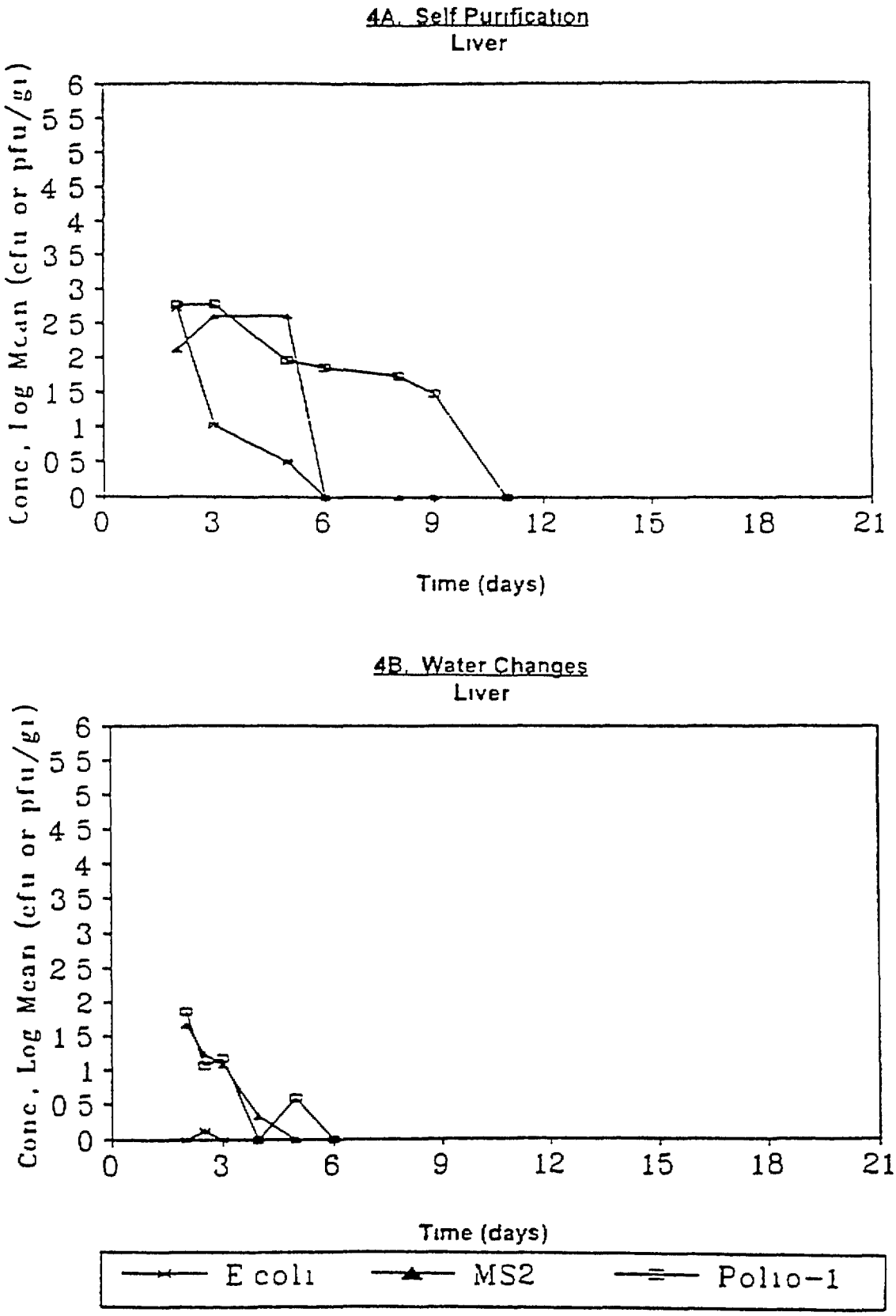
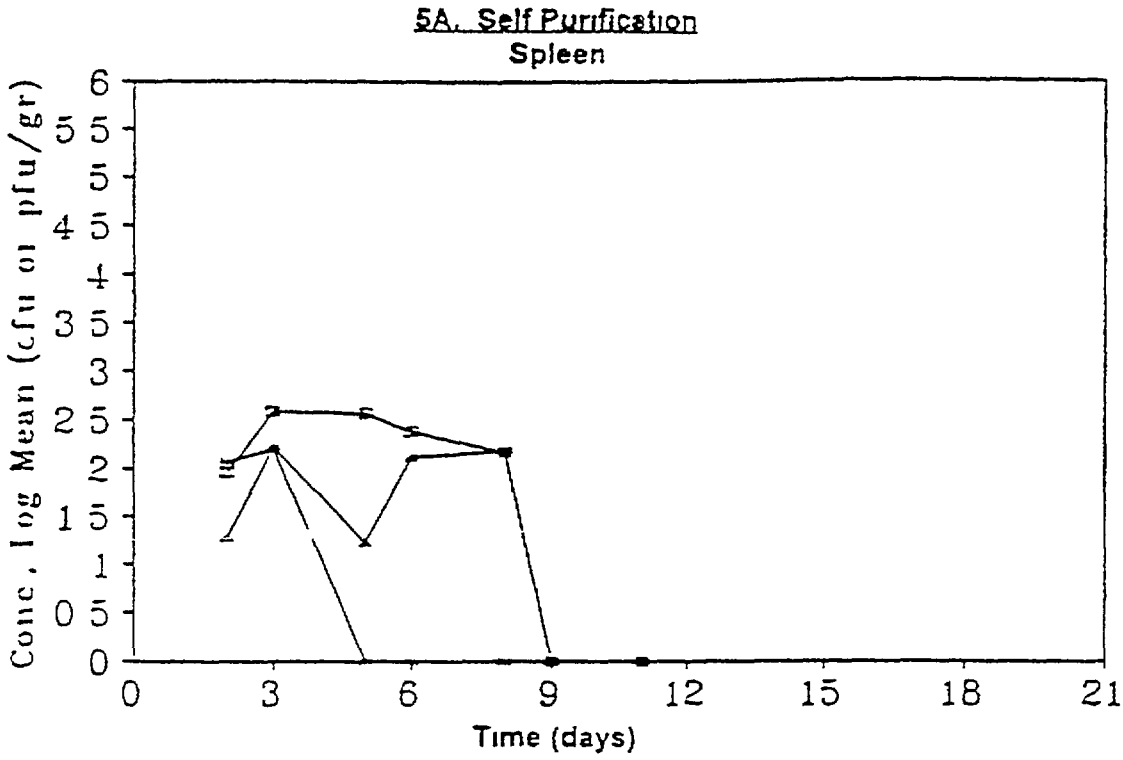


Figure 4. Residual concentration (log mean) of E coli, MS2 and polio 1 in the liver in self purification (a) and water changes experiments (b)



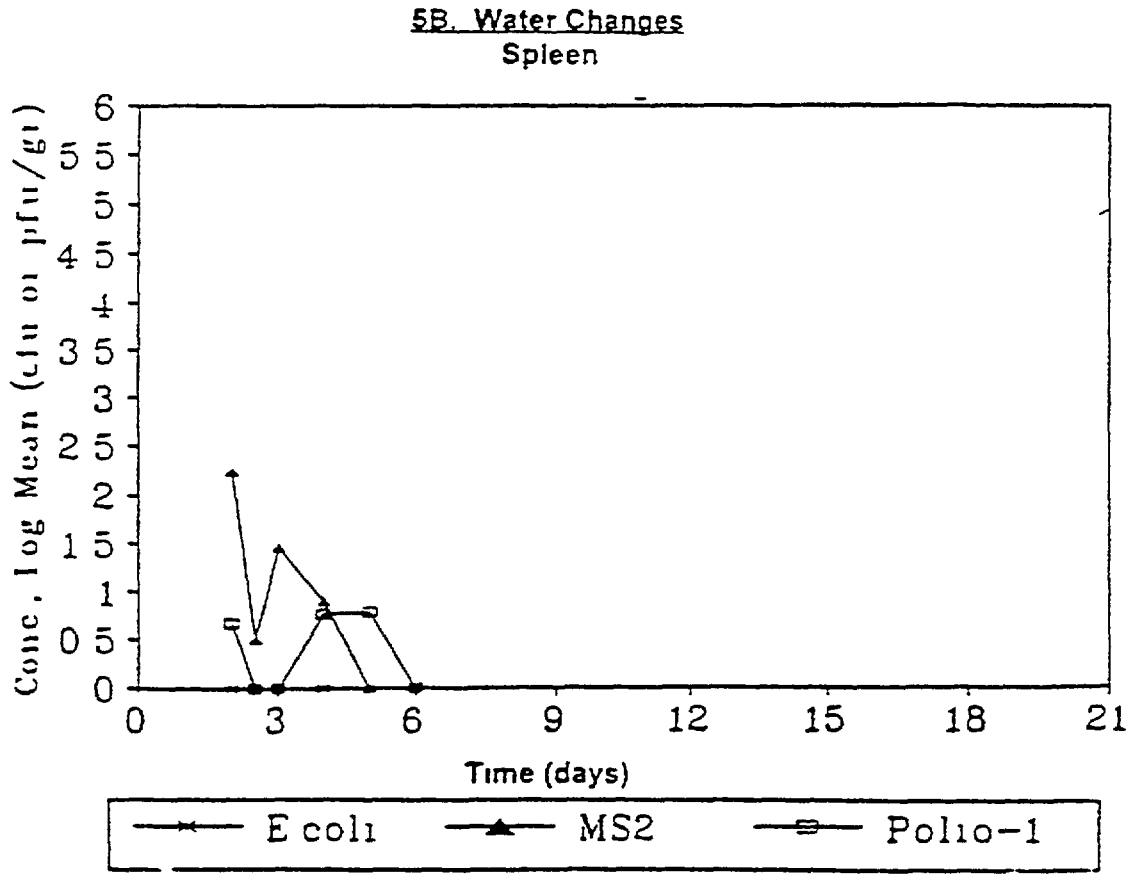


Figure 5. Residual concentration (log mean) of E coli MS2 and polio 1 in the spleen in self purification (a) and water changes experiments (b)

Conclusions of Part I

1 Contamination of fish with E coli, MS2 Phage and Polio 1 via maintenance water, causes massive contamination of the digestive tract as compared with other organs (shown in the previous report)

2 In the muscle no test microorganisms were detected even in high liter of contamination

3 Decontamination time of the digestive tract by active depuration through water changes was shortened from 21 days to 6 days and less, depending on fish organs

4 The results of this study indicate the feasibility of purifying fish by a simple technique of water changing, and this may reduce the risk of disease transmission by fish handling or consumption

5 In order to conserve water, the contaminated ambient water may be recycled through a process of filtration and disinfection

PART II

A Preliminary results of the toxicity of hydrogen peroxide (H_2O_2) and silver ions (Ag+) on fish

B Die-off microorganisms (E coli, MS2 Phages and Polio 1) by hydrogen peroxide and silver ions

Introduction

Part I of this report deals with decontamination of fish by water changes without any addition of disinfectant into the maintenance water

In part II of this report we decided to perform preliminary experiments in order to test if the efficiency of the addition of any disinfectant to the fish maintenance water will increase decontamination of fish organs from various microorganisms At the beginning we thought to use chlorine which still serves as the principal drinking water disinfectant in most water utilities due to its high inactivation efficiency towards a broad spectrum of microorganisms, its low operation and maintenance costs and its relatively simple application techniques However, the findings of Rook and Stevens in the mid-seventies (Rook, 1974, Stevens et al, 1976) that chlorination is associated with trihalomethane formation triggered intensive studies into the interaction of chlorine and other disinfectants with natural organic materials in drinking water and led to a parallel research into the adverse health effects associated with strong oxidizers and their disinfection by-products (DBPs) (Jolley et al , 1990, Bull and Kopfler, 1991, USEPA, 1993) Despite their tremendous benefits in protecting human health, most of the recent studies have led to the inevitalbe conclusion that each of the current chemical disinfectants is associated with some undesirable chemical products

Chlorination produces a plethora of halogenated DBPs including the notorious trihalomethanes and halo-acetic acids Some of these compounds were declared as probable human carcinogens (e g , chloroform, bromodichloromethane and bromoform) and there is growing apprehension that the number of regulated compounds reflects the

current, limited epidemiological knowledge rather than the real potential health risk Likewise, each of the alternatively proposed oxidants has its inherent disadvantages Chlorine dioxide produces chlorate and chlorite, ozone yields a number of low molecular weight ketones and aldehydes (e g , formaldehyde), bromate - in bromide rich water, in addition to its assimilable organic carbon (AOC) yield, monochloramine has only mild disinfection capability and in the presence of other disinfectants may form nitrite or odorous dichloramine in addition to the serious problems that may be caused to patients using home dialysis units It is notes that both France and Germany have outlawed the use of chloramines in drinking water for these reasons

It is worth noting that besides the above mentioned limitations of using Chlorine itself or its combination as a disinfectant for drinking water, it is also known that chlorine has a toxic effect on fish even at low levels

The scientific and technological efforts to replace the chlorine are accordingly directed in several research paths UV irradiation with, or without, addition of radical forming compounds (such as hydrogen peroxide) may provide alternative primary disinfection and ways to improve UV disinfection performance in turbid water are still under investigation Membranal processes may provide alternative chemical "free" pathogen removal procedure These two approaches still require use of an additional residual disinfectant Another approach is to use a combination of two or more disinfection agents, which exhibit additive or synergistic effect Thus, a combination of hydrogen peroxide and ozone is being developed by the Metropolitan Water District of Southern California (Wolf et al , 1989) and chlorine and copper or silver or hydrogen

peroxide and iron or copper were investigated (Landeen et al , 1989[a b]) Our own studies were stimulated by the development of commercial stabilized formulation of silver and hydrogen peroxide for use in the disinfection of drinking water and other antibacterial applications By combining two or more disinfection agents it is possible to lower the exposure of the water consumer to each of the components, and thus minimize the associated health risks and remain well below the Maximum Contaminant Level (MCL) of each component Such a combination can be especially attractive when the components of the combined disinfectant target different and complementary populations of microorganisms or when the combination of the two disinfectants exhibit synergistic effect

Hydrogen peroxide has a known bacteriocidal action (Gardiner et al , 1983), and is frequently used in the food and pharmacologic industries Hydrogen peroxide is a mild disinfectant but its disinfection capabilities can be improved when it is used in combination with other compounds Hydrogen peroxide produces only low levels of DBPs and it is not toxic at levels that are relevant to the water industry The relative stability of hydrogen peroxide in low pH conditions facilitates long storage and safe transportation Additionally, its low rate of decomposition (at the common pH levels of drinking water) and its mild reactivity with organic compounds assure long lasting residual disinfection Silver is a bacteriostatic/bacteriocidal agent (Woodward, 1963, Chambers et al , 1962), which is often used in point of use activated carbon based filters (Bell, 1991) The USEPA has recently declared that silver does not cause adverse health effects and set a secondary MCL for silver (90 ppb) based upon

cosmetic consideration, chronic exposure to silver may result in argyria, a blue-gray discoloration of the skin The EEC, WHO and the Israel Ministry of Health have specifically approved of the use of silver as a drinking water disinfectant at an MCL of 80 ppb The ministry of health in France has approved of the use of hydrogen peroxide for the disinfection of drinking water and a number of countries including Switzerland, Germany and Australia have given their approval for the use of the commercial formulation of silver and hydrogen peroxide as drinking water disinfectant While, both silver and hydrogen peroxide were well investigated, their combination became a serious candidate for large scale water disinfection only recently

Based on the above review we decided to test a) the efficiency of using hydrogen peroxide and silver ions as a disinfectant against various microorganisms, and b) the toxicity of this compound on fish

The preliminary objectives of this part were

1 To test the maximum concentration of hydrogen peroxide (H_20_2) and silver ions which can cause toxicity on fish

2 To test the residual of this compound in clear and maintenance water at various time intervals in order to determine the active residual concentration of this compound in the water

3 To determine the kinetic die-off for E coli, MS2 bacteriophages (phages) and poliovirus 1 at different concentrations of hydrogen peroxide and silver ions

Materials and Methods

1 Preparation of Glassware

All glassware were soaked in 5% nitric acid and then washed 8 times in double distilled water All solutions used in the experiments of this work were prepared in special bottles which were covered with aluminum foil and kept in the refrigerator until used

2 Preparation of Hydrogen Peroxide and Silver Ions Stock Solution

A stock solution contained 2500 mg/l hydrogen peroxide was mixed with 7 mg/L silver ions prepared from silver nitrate was used as one solution for all experiments Proper dilutions were made in order to prepare the appropriate concentrations for H_20_2 and Ag+ in the water for each experiment, i e 10 mg/L H_20_2 + 28 ppb Ag+, 20 mg/L H_20_2 + 56 ppb Ag+ and 30 mg/L H_20_2 + 84 ppb Ag+

3 Preparation of Neutralization Solution

Ten ml of the sample contained hydrogen peroxide and silver ions were neutralized with 0 1 ml of 15% of sodium thiosulfate and 10% thioglicolic acid stock solution This combination is often used for neutralization of chlorine and silver or copper combined disinfectant (Landeen et al , 1989) At a later stage Padahzur et al , (1994) found that this neutralization procedure falsely increased the measured inactivation level of the microorganisms They replaced this procedure with catalase-thioglycolate-pepton neutralizer, and they found that this solution passed the validation test, and the nautralized sample exhibited similar titer to the control sample and the titer remained stable for at least several hours

4 Determination of the Concentration of Hydrogen Peroxide

Two solutions were used

Solution A - $KMnO_4$ 0 002N

Solution B - 0 2367 MnO_4+2N H_2SO_4 2% to 1000 ml of double distilled water

To determine the concentration of hydrogen peroxide in 10 ml sample, 10 ml of solution B was added and titerate with solution A, till you get a stable pink color Each ml of solution A react with 3 4 mg of H_2O_2 The concentration of hydrogen peroxide in the sample was determined by multiplying the volume of solution A (in ml) by 3 4

5 Preparation of Stock Microorganisms and Assays

For preparation of stock microorganisms and assay of E coli, MS2 Bacteriophage and poliovirus see part IV of the progress report covering the period of September 29, 1992 to September 30, 1993 (See Appendix 3)

6 The Water Used for Laboratory Experiments

a Clean water - tap water without residual chlorine was filtered in activated carbon filter

b Maintenance water - "clean water" in which 5 fish were kept during a one month period At that period the fish were fed with high protein pellets at a rate of 1% body weight to allow only maintenance of the fish but no growth The fish were kept in 100 – 1 container of "clean water" whereas during the experiments the water was not filtered nor changed

7 The Fish Used for the Experiment

In all experiments we used Tilaphia-Sarotherodon aureus X S nilaticus hybrids weighing between 100 gr to 150 gr

8 Description of the Experiments

The experiments were conducted in three different ways

a To test the residual of hydrogen peroxide in different kinds of water (clean water and maintenance water) at different periods of time

These were conducted in two ways

1 In the presence of fish with airing the water

2 Without the presence of fish and without airing the water To perform this, 2 Erlenmeyer flasks were used, in the first we poured 500 ml of clean water, and in the second - 500 ml of maintenance water To each of these 2 Erlenmeyers, we added 0 5 ml of stock culture of E coli at concentrations of 10^9 cfu/ml, 0 5 ml of MS2 Phages (10^9 pfu/ml) and 0 5 ml of Polio 1 (10^9 pfu/ml) To each Erlenmeyer, hydrogen peroxide was added to reach a final concentration of 10, 20 or 30 mg/l The experiments were conducted at room temperature The water was mixed with magnetic stirrer during the time of the experiment

b To test the resistance of fish to various concentrations of hydrogen peroxide and silver ions These experiments lasted 10 days during which the concentration of hydrogen peroxide was adjusted every day in order to

reach the initial concentration at beginning of the experiment i e , 10, 20 or 30 mg/l These experiments were conducted in 20 liter containers of clean water with the presence of fish Acclimatization period of the fish in the containers was 24 hours prior to the experiment The experiments were conducted in 4 containers the first was with clean water without hydrogen peroxide (control) To the three other containers, hydrogen peroxide was added at a levels of 10, 20 and 30 mg/l respectively All 4 containers were aerated by compressed air using porous plastic tubing

Samples of water were taken every 24 hours and before the adjustment of hydrogen peroxide to the initial concentration at the beginning of the experiment In addition samples were taken during the first 18 hours at the time periods of 0, 10, 20, 40, 60, 90 and 120 minutes and also after 18 hours At each time period, 20 ml of water samples were taken to a test tube which contained 50 microlite neutralization solution Each sample was tested twice to determine the concentration of hydrogen peroxide in the water Based on the results of the titration, the concentration of hydrogen peroxide was adjusted to initial concentration which was at the beginning of the experiment

c To test the die off kinetics of E coli, MS2 phages and Polivirus1 in different concentrations levels of hydrogen peroxide and silver ions

These were performed in two stages

1 Without the adjustment of hydrogen peroxide

These experiments were conducted, using two Erlenmeyers, in the first 500 ml of clean water were poured and in the second - 500 ml maintenance water To each of these Erlenmeyer we added 0 5 ml of stock cultures of E coli, MS2, polio and hydrogen peroxide as mentioned in section 8a2 above The experiment lasted between 2 to 24 hours

2 With the adjustments of hydrogen peroxide

These experiments were conducted as follows two Erlenmeyers were used in which we poured 500 ml maintenance water of the stock cultures of E coli, MS2 and Polio1 at concentration as mentioned in Section 8a2 To the first Erlenmeyer, 30 mg/l of hydrogen peroxide was added and the second one served as control (without hydrogen peroxide) During the experimental period the water in both Erlenmeyers were mixed using magnetic stirrer, and the experiment lasted 6 hours Within that period the hydrogen peroxide was adjusted

Results

I The active residual of H_2O_2 and Ag+ ions in different kind of water

a At presence of fish and airing the water

Table 1a and b summarises the results of changing the concentration of H_2O_2 in the clean water during 10 days period Table 1a showed that the levels of H_2O_2 in the water did not change appreciably during 10 days This is true at the initial concentration of 10, 20 and 30 mg/l of H_2O_2 On the other hand it seemed that the levels of H_2O_2 were decreased significantly starting from the second day mainly at the concentrations of 10 and 20 mg/L This happened in spite of the fact that the concentration of H_2O_2 was adjusted every day to the initial calculated levels The data from Table 1 was also presented in figures 6A and 6B

b Without the presence of fish and airing the water but with the addition of stock cultures of E coli, MS2 Phages and Poliovirus 1

Table 2 presents the results of 3 different experiments, in changing of the levels of H_2O_2 from the initial concentration of 30 mg/L in clean vs maintenance water for the period of 24 hours (see also Figure 7) It can be seen

from this table that, on the average, there are relative slight decrease in the concentration of H_2O_2 in clean and maintenance water However, this was not the case in each single experiment (see exp 1 in both clean and maintenance water), where there was high decreases in H_2O_2 concentration and reaches to very low levels within 24 hours This indicates that the active concentration of H_2O_2 in the water is changing between different experiments, and therefore it is recommended to test the levels of H_2O_2 in the water at a constant time of the experiment Table 3 presents the results with the initial concentration of 10 mg/l of H_2O_2 It can be notice a decreases of over 60% within 4 hours in the maintenance water as compared with over 50% in clean water

c **Comparison between the residual of active H_2O_2 with aerated water**

Tables 4 and 5 comparing the residual concentrations of H_2O_2 in clean water with airing and with presence of fish vis a vis to mixed water without fish Table 4 presents the data for the initial concentration of 10 mg/l H_2O_2 and table 5 for 30 mg/l H_2O_2 It can be seen that the concentrations of H_2O_2 in the aerated water were more stable than in the

mixed water Figure 8 presents the same data from tables 4 and 5

II The resistance of fish to different levels of H_20_2 and Ag+ ions

As stated in Materials and Methods, (8b) four Telapia fish were kept in four containers with clean water The experiment lasted 10 days during which the concentration of hydrogen peroxide and Ag+ was adjusted everv day in order to reach the initial concentration at the beginning of the experiment, i e , 10, 20 or 30 mg/l for H_20_2 and 28, 56 or 84 ppb Ag+ respectively The fourth container served as a control with 0 H_20_2 and Ag+ It was bound that no fish mortality occur in any of the container Moreover, the fish looked very good (without performing any phiological observations) The smell of the fish in the containers with the disinfectants was also good, whereas in the control container, a typical smell of fish was noticeable In addition, the quality and the clearness of the water improved with increases of the concentration of H_20_2 and Ag+ in the water This means that H_20_2 and Ag+ at levels tested are not toxic to the fish

III The die-off kinetics of the different microorganisms in clean and maintenance water

a Die-off kinetics of E. coli

Five experiments were conducted at 9 different concentration levels of H_2O_2 and Ag+ with and without the adjustment of the H_2O_2 and Ag+ to the initial concentration at the beginning of the experiment Tables 6 to 8 and Figures 9 to 11 present the results It can be seen that the E. coli die-off in clean water and maintenance water was very low at concentrations of 10 mg/l H_2O_2 + 28 ppb Ag+ (see Table 6 and also Figures 9 to 10) Table 7(a, b and c) presents data from 3 experiments with initial concentration of 30 mg/l H_2O_2 + 84 ppb Ag+ (with no adjustment) It can be seen that the E. coli reduction was 5-6 logs within 24 hours in both kind of waters This is true only when the residual of H_2O_2 is stable in the water during the experiment period (see experiment 2 and 3, Table 7b and 7c) When the concentration of H_2O_2 low, the reduction of E. coli is also low (see experiment 1, Table 1a) Table 8 shows the E. coli die-off in maintenance water with and without the addition of hydrogen peroxide and Ag+ It can be seen that E coli

reduction is almost null in the control experiment, while in presence of H_2O_2 and Ag+ the reduction was 2 logs

b Die-off kinetics of MS2 and poliovirus 1

Five experiments were conducted, part of which with the adjustment of the concentration of H_2O_2 and Ag+ during the expeiment period The results are summaries in Tables 9 to 11 and Figures 12 to 14

Table 9 shows that at concentration of 10 mg/l H_2O_2 and 28 ppb Ag+, the MS2 phage die-off in clean water was very high (over 6 logs within 3 hours) On the other hand the die-off of MS2 in maintenance water and polio 1 in clean and maintenance water was very low

Table 10 (a, b and c) shows the die-off of MS2 and polio 1 in 3 different experiments, at a concentration of 30 mg/l H_2O_2 and 84 ppb Ag+ without the adjustment of the concentrations of these disinfectants in the water during the experiment period The results indicate that the reduction of MS2 in clean and maintenance water was high (4 to 6 logs within less than 24 hours) However, almost no die-off of polio 1 was observed It can be notice also that the data was not reproducible in the three experiments Table 11 (a and b) presents the die-off of MS2 and polio 1 in clean and

maintenance water in two different experiments at a concentration of 30 mg/l H_2O_2 and 84 ppb Ag+ with the adjustment of the concentrations of these disinfectants in the water during the experiment period to the initial calculated concentration it can be seen that in the control experiments, no die-off was observed neither for MS2 nor for polio However, no die-off was seen for polio even after the adjustment of concentrations of H_2O_2 and Ag+

Conclusion of Part II

1 At concentration of 30 mg/l H_2O_2 and 84 ppb Ag+ the fish were growing very well with no sign of toxicity

2 At the above mentioned concentration levels of these disinfectants, the die-off of E. coli and MS2 phages was very good, mainly in clean water The polio 1 die-off was very poor

3 The die-off of the tested microorganisms was not the same in the different experiments The die-off in one experiment was very high (mainly for MS2 and E coli) while for the others no die-off was observed inspite of the fact that all experiments were conducted in the same conditions

Table 1a

Changes in the concentration of hydrogen peroxide (H_2O_2) in clean water as a function of time

a During 18 hours from the beginning of the experiment (1) (2)

	Initial calculated of H_2O_2 in water (mg/l) (3)		
	30	20	10
time (min)	measured H_2O_2 (mg/l)	measured H_2O_2 (mg/l)	measured H_2O_2 (mg/l)
0	28 5	20 0	10 5
10	30 9	19 0	11 6
20	31 5	21 4	11 9
40	28 9	21 0	10 5
60	27 9	19 0	9 5
90	27 9	19 7	9 9
120	27 9	18 4	10 0
18 h	27 5	13 6	9 5

(1) During the first 18 hours the concentration of H_2O_2 was not adjusted

(2) The figure in this table express the concentrations of H_2O_2 in the water before the adjustment The concentration of H_2O_2 in the water was adjusted every day to the calculated concentration which presented in the upper part of this table

(3) The initial concentrations of Ag+ were 84 ppb for 30 mg/l H_2O_2, 56 ppb for 20 mg/l H_2O_2 and 28 ppb for 10 mg/l H_2O_2

Table 1b

Changes in the concentration of hydrogen peroxide (H_2O_2) in clean water as a function of time

b During 10 days from the beginning of the experiment (3)

	Initial calculated concentration of H_2O_2 in water (mg/l)		
	30	20	10
time (days)	measured H_2O_2 (mg/l)	measured H_2O_2 (mg/l)	measured H_2O_2 (mg/l)
0	28 5	20 0	10 5
1	28 2	13 6	9 5
2	30 9	2 7	3 4
3	26 2	2 7	2 0
4	17 0	2 4	2 0
5	2 4	2 4	2 0
8	10 9	5 4	2 4
9	17 0	9 5	2 4
10	19 0	8 8	2 0

(3) See note 2 and 3 in table 1a

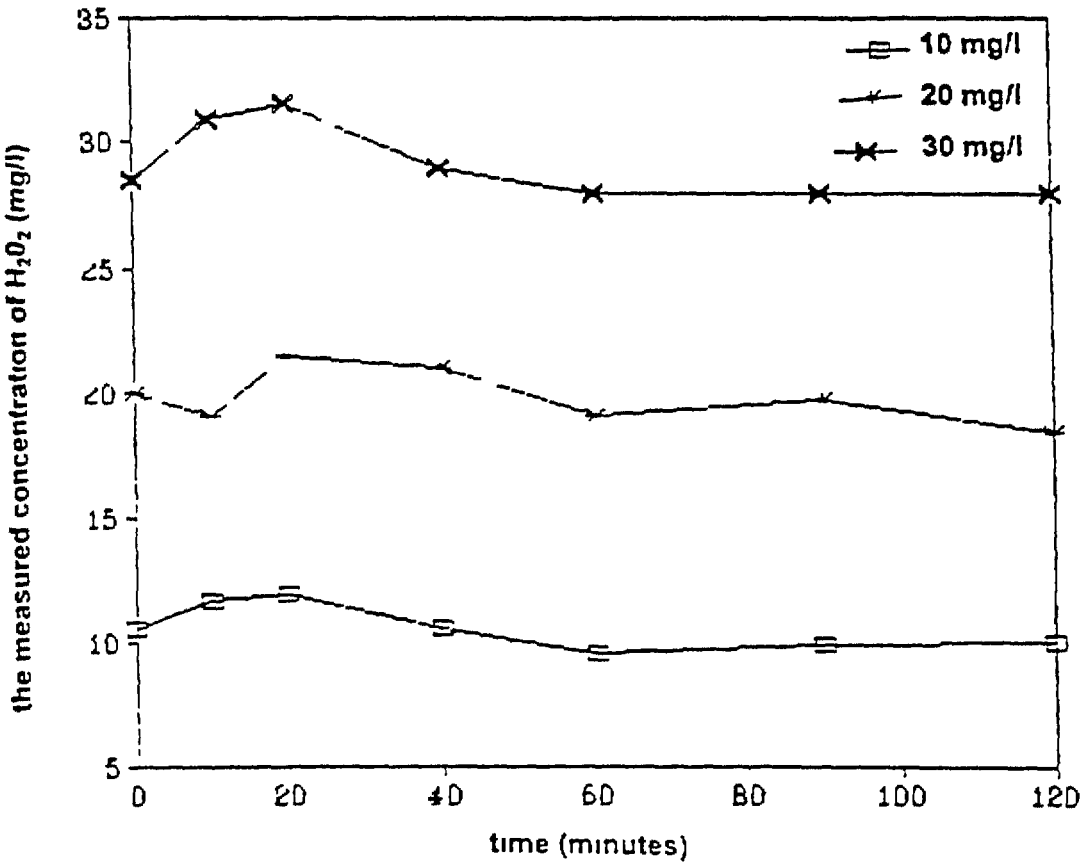


Figure 6A. Changes in the concentration of H_2O_2 in clean water during two hours without adjusting H_2O_2 or Ag+ levels (with fish and airing)

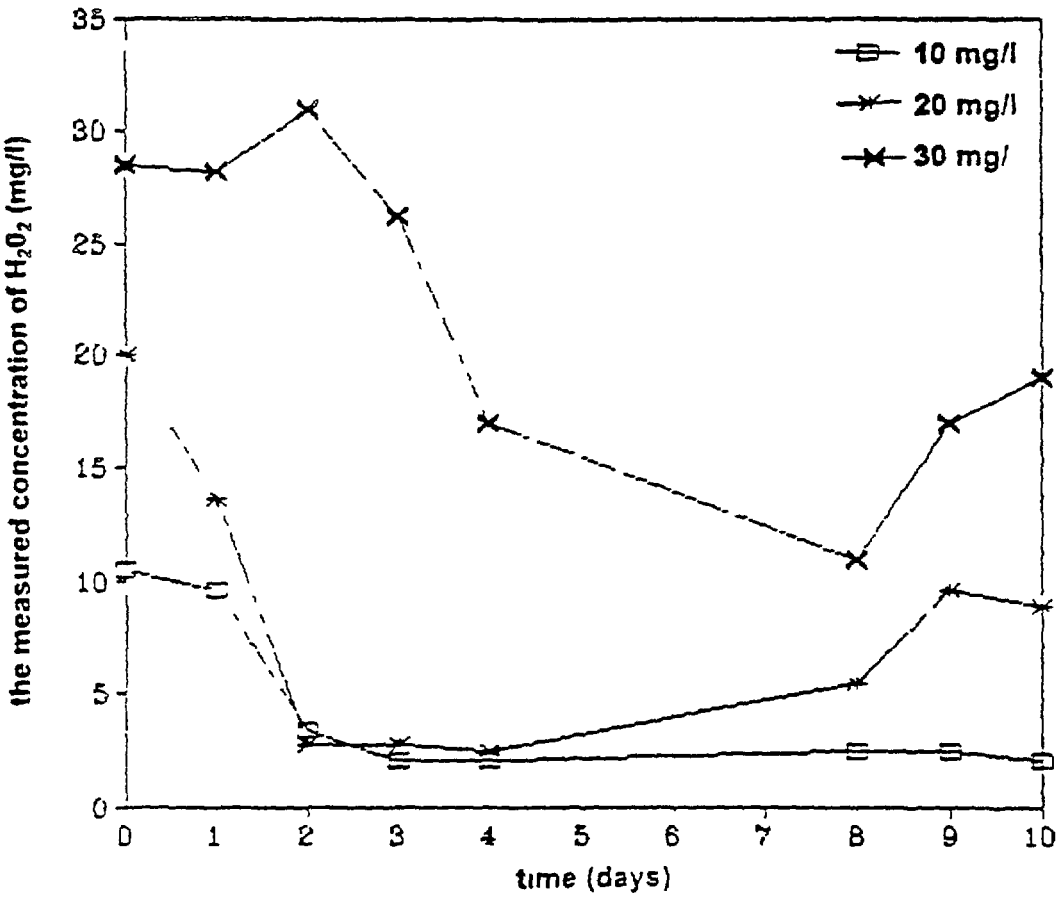


Figure 6B Changes in the concentration of H_2O_2 in clean water as a function of time (The concentration of H_2O_2 and Ag+ was adjusted every day starting from second day) (with fish and airing)

Table 2

Changes in the concentration of hydrogen peroxide (H_2O_2) in different kind of water as a function of time (The initial calculated concentration of H_2O_2 was 30 mg/l and for Ag+ was 84 ppb Without fish and airing but with addition of stocks of E coli + phages MS2 and polio 1) (1)

	Concentration of H_2O_2 (mg/l)							
	clean water				maintenance water			
time (min)	single experiment 1	2	3	mean	single experiment 1	2	3	mean
0	29 2	34 3	30 6	31 4	30 6	34 0	33 0	32 5
20	20 4	25 8	29 9	25 4	25 2	28 6	26 2	26 7
40	11 9	26 2	28 6	22 2	20 7	23 8	24 5	23 0
60	8 5	23 8	26 9	19 7	20 4	20 7	23 5	21 5
120	4 8	23 8	26 2	18 3	16 0	20 7	18 7	18 5
300	2 4	22 4	26 2	17 8	9 9	20 7	16 0	15 5
1440	1 7	20 6	23 5	15 3	5 1	20 7	14 3	13 4
24 h								

(1) During the experiment the concentration of H_2O_2 or Ag+ in the water was not adjusted

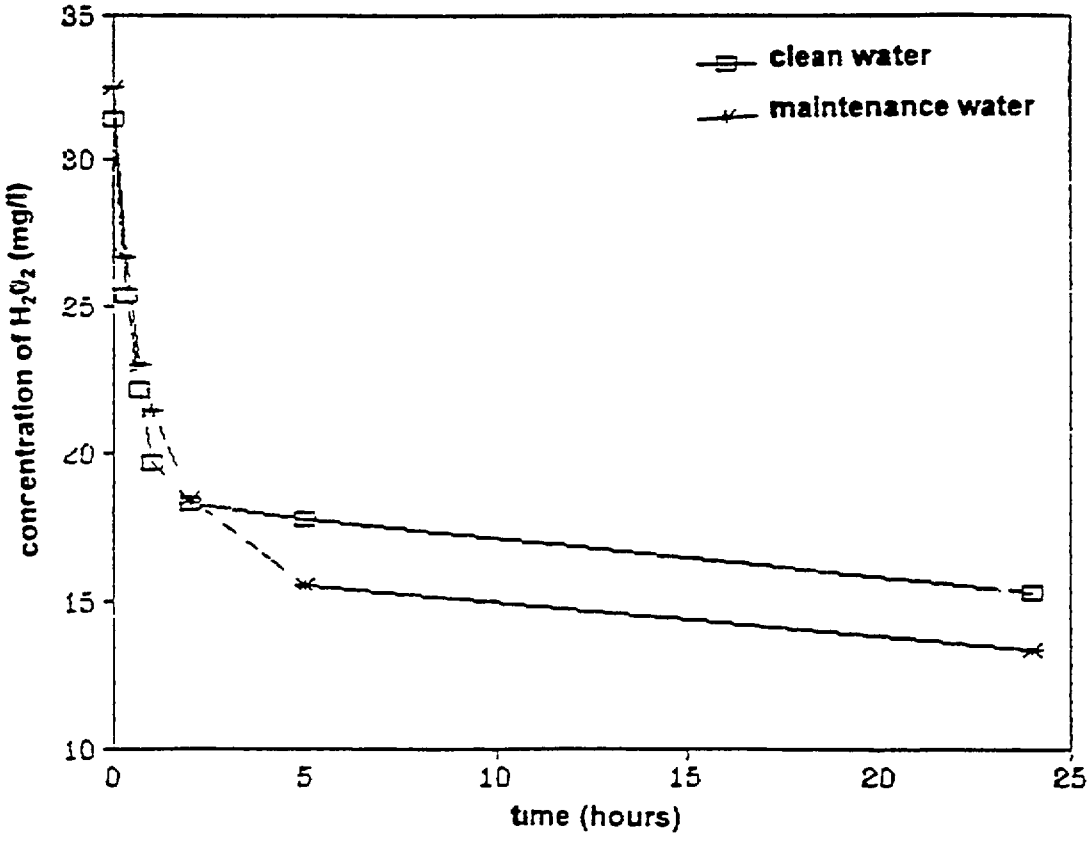


Figure 7. Mean changes in the concentration of H_2O_2 in various kinds of water as a function of time
(The initial calculated concentration of H_2O_2 was 30 mg/l and for Ag+ was 84 ppb and was not adjusted during the experiment) (Without fish and airing but with the presence of E. coli, MS2 phages and Polio 1)

Table 3

Changes in the concentration of hydrogen peroxide in different kind of water as a function of time
(the initial calculated concentration for H_20_2 was 10 mg/l and for Ag^+ was 28 ppb)
(Without fish and airing but with addition of stocks
of E coli + Phages MS2 and Polio 1) (1)

	The concentration of H_20_2 (mg/l)	
Time (min)	Clean Water	Maintenance Water
0	-	10 2
10	8 5	9 5
20	7 8	10 9
40	7 8	7 5
60	7 8	5 8
90	6 8	5 1
120	6 1	4 4
180	5 1	4
240	4 4	3 4

(1) During the experiment the concentration of H_20_2 or Ag^+ in the water was not adjusted

Table 4

Comparison of the concentration of residual H_2O_2 in clean water with airing and fish vis a vis to mixed water without fish as a function of time
(The initial calculated concentration of H_2O_2 was 10 mg/l and for Ag^+ was 28 ppb) (1) (2)

The concentration of H_2O_2 (mg/l)		
Time (min)	Aereted Water	Mixed Water
10	11 6	8 5
20	11 9	7 8
40	10 5	7 8
60	9 5	7 8
90	9 9	6 8
120	10 0	6 1

(1) During the experiment the concentration of H_2O_2 or Ag^+ in the water was not adjusted

(2) With the addition of stocks of E coli + MS2 phages and polio 1

Table 5

Comparison of the concentration of residual H_2O_2 in clean water with airing and fish vis a vis to mixed water without fish as a function of time
(The initial calculated concentration of H_2O_2 was 30 mg/l and for Ag+ was 84 ppb) 1)(2)

The concentration of H_2O_2 (mg/l)		
Time (min)	Aereted Water	Mixed Water
20	31 6	25 8
40	28 9	26 2
60	27 9	23 8
90	27 9	23 8
120	27 9	22 4
300	27 9	20 6

(1) During the experiment the concentration of H_2O_2 or Ag+ in the water was not adjusted

(2) With the addition of stocks of E coli + MS2 phages and polio 1

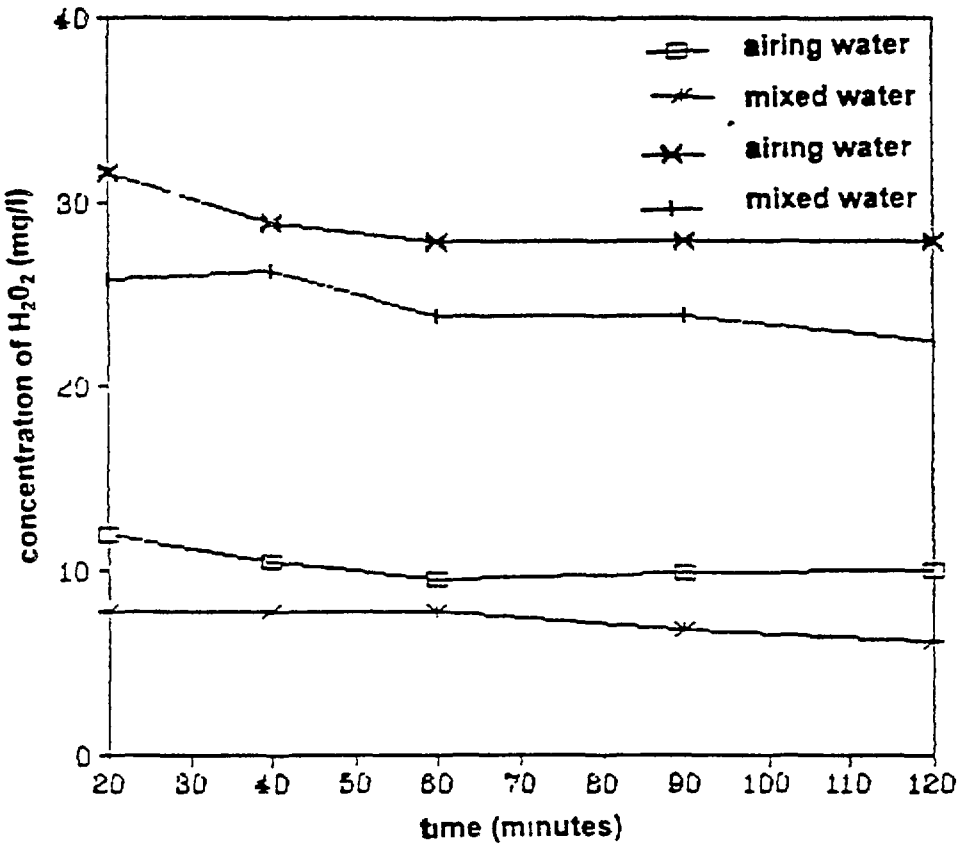


Figure 8. The concentration of H_2O_2 in airing and mixed water as a function of time

Table 6

E. coli die-off in clean and maintenance water by hydrogen peroxide (H_2O_2) and Ag+ as a function of time
(The initial calculated concentration of H_2O_2 was 10 mg/l and for Ag+ was 28 ppb) (1)

	Clean Water		Maintenance Water	
Time (min)	E. coli (cfu/ml)	H_2O_2 (mg/l)	E. coli (cfu/ml)	H_2O_2 (mg/l)
0	$1.8 * 10^6$	-	$8.6 * 10^5$	10.2
10	-	8.5	$9.4 * 10^5$	9.5
20	$8.6 * 10^5$	7.8	$9.5 * 10^5$	10.9
40	$8.0 * 10^5$	7.8	$9.0 * 10^5$	7.5
60	$7.0 * 10^5$	7.8	$8.5 * 10^5$	5.8
90	$5.0 * 10^5$	6.8	$7.0 * 10^5$	5.1
120	$3.9 * 10^5$	6.1	$6.0 * 10^5$	4.4
180	$1.4 * 10^5$	5.1	$5.7 * 10^5$	4.0

(1) During the experiment, the concentration of H_2O_2 or Ag+ in the water was not adjusted

Table 7a

E. coli die-off in clean and maintenance water by hydrogen peroxide (H_2O_2) and Ag+ as a function of time
(The initial calculated concentration for H_2O_2 was 30 mg/l and for Ag+ was 84 ppb)
(experiment no 1) (1)

	Clean Water		Maintenance Water	
Time (min)	E. coli (cfu/ml)	H_2O_2 (mg/l)	E. coli (cfu/ml)	H_2O_2 (mg/l)
0	$1.8 * 10^7$	29.2	$5.5 * 10^6$	30.6
20	$1.5 * 10^6$	20.4	$1.9 * 10^5$	25.2
40	$6.6 * 10^6$	11.9	$2.0 * 10^4$	20.7
60	$6.0 * 10^6$	8.5	$1.6 * 10^4$	20.4
90	$4.7 * 10^6$	5.8	$2.0 * 10^5$	16.3
120	$2.1 * 10^6$	4.8	$2.0 * 10^5$	16.0
180	$2.6 * 10^6$	5.8	$5.5 * 10^4$	11.6
240	$1.6 * 10^6$	2.4	$6.1 * 10^4$	10.2
300	$2.9 * 10^4$	2.4	-	9.9
420	$3.6 * 10^5$	2.0	$2.7 * 10^4$	8.2
720 12 h	$7.4 * 10^4$	2.0	$1.7 * 10^3$	8.2
1440 24 h	$9.0 * 10^3$	1.7	$5.0 * 10^3$	5.1

(1) During experiments 1, 2 and 3 (tables a, b and c) the concentration of H_2O_2 or Ag+ in the water was not adjusted

Table 7b

E. coli die-off in clean and maintenance water
by hydrogen peroxide (H_2O_2) and Ag+ as a function of time
(The initial calculated concentration for H_2O_2 was 30 mg/l and for Ag+ was 84 ppb)
(experiment no 2)

	Clean Water		Maintenance Water	
Time (min)	E. coli (cfu/ml)	H_2O_2 (mg/l)	E. coli (cfu/ml)	H_2O_2 (mg/l)
0	$5.3 * 10^5$	30.6	$7.5 * 10^5$	33.0
20	$7.8 * 10^4$	29.9	$1.5 * 10^5$	26.2
60	$1.0 * 10^3$	28.6	$1.0 * 10^5$	24.5
120	$5.5 * 10^3$	26.9	$1.8 * 10^5$	23.5
300	$1.2 * 10^2$	26.2	$1.6 * 10^4$	18.7
1440	0	23.5	$6.5 * 10^1$	14.3
24 h				

Table 7c

E. coli die-off in clean and maintenance water by hydrogen peroxide (H_2O_2) and Ag+ as a function of time
(The initial calculated concentration for H_2O_2 was 30 mg/l and for Ag+ was 84 ppb)
(experiment no 3)

	Clean Water		Maintenance Water	
Time (min)	E. coli (cfu/ml)	H_2O_2 (mg/l)	E. coli (cfu/ml)	H_2O_2 (mg/l)
0	$3.0 * 10^5$	34.3	$5.5 * 10^6$	34.0
20	$1.0 * 10^4$	25.8	$7.3 * 10^5$	28.6
40	$1.5 * 10^4$	26.2	$3.9 * 10^5$	23.8
60	$3.0 * 10^3$	23.8	$4.5 * 10^5$	20.7
120	$8.0 * 10^3$	23.8	$9.0 * 10^4$	20.7
300	0	22.4	$3.0 * 10^3$	20.7
1440	0	20.6	0	20.7
24 h				

Table 8

E. coli die-off in maintenance water with and without hydrogen peroxide (H_2O_2) and Ag+ as a function of time
(The initial calculated concentration for H_2O_2 was 30 mg/l and for Ag+ was 84 ppb)

Maintenance Water			
Without H_2O_2 or Ag+ (control)		With H_2O_2 + Ag+	
Time (min)	E. coli (cfu/ml)	E. coli (cfu/ml)	H_2O_2 (mg/l)
0	$1.0 * 10^6$	$3.5 * 10^5$	34.9
20	N.T. (1)	$2.6 * 10^5$	30.9
40	N.T.	$2.3 * 10^5$	29.2 (2)
60	$8.0 * 10^5$	$1.4 * 10^5$	31.6
90	N.T.	$2.0 * 10^5$	27.2 (2)
120	$7.2 * 10^5$	$1.8 * 10^4$	28.6 (2)
360	$6.0 * 10^5$	$9.5 * 10^3$	20.7 (2)

(1) N.T. - not tested

(2) During the experiment the concentration of H_2O_2 and Ag+ in the water at these points was adjusted

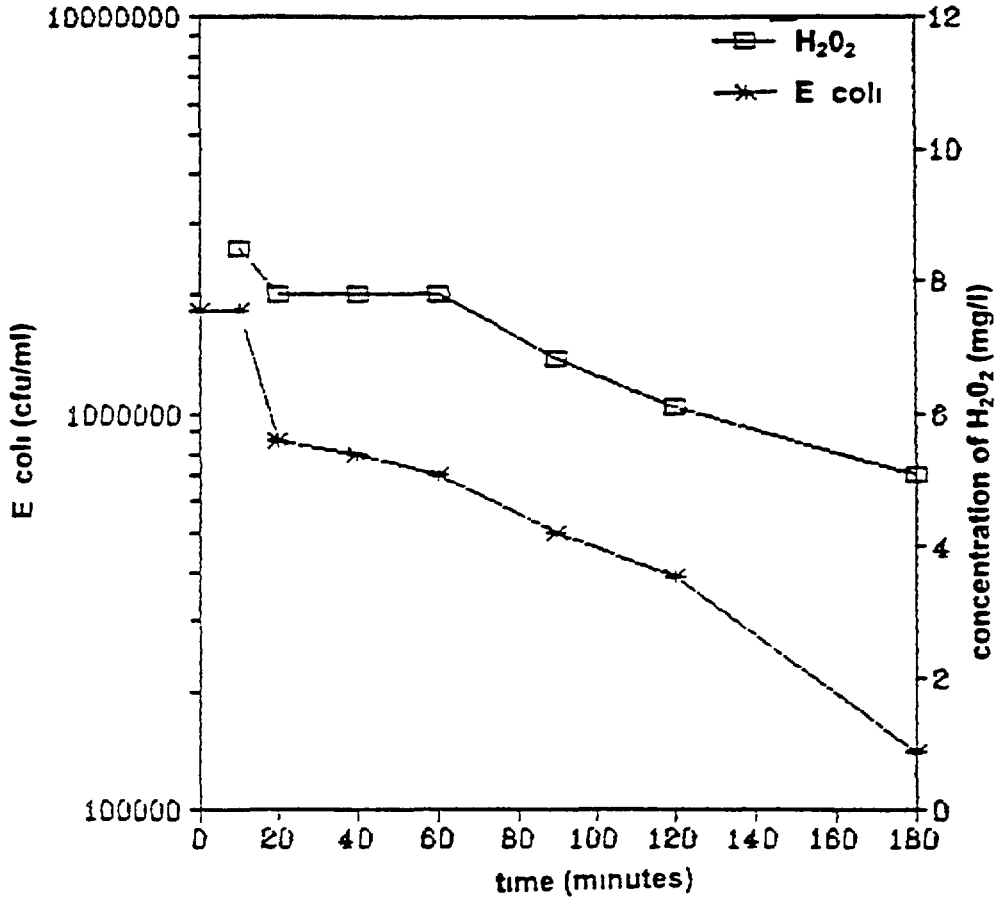


Figure 9. E. coli die-off in clean water vs the residual of H_2O_2 as a function of time (The initial calculated concentration of H_2O_2 was 10 mg/l and for Ag+ was 28 PPb)

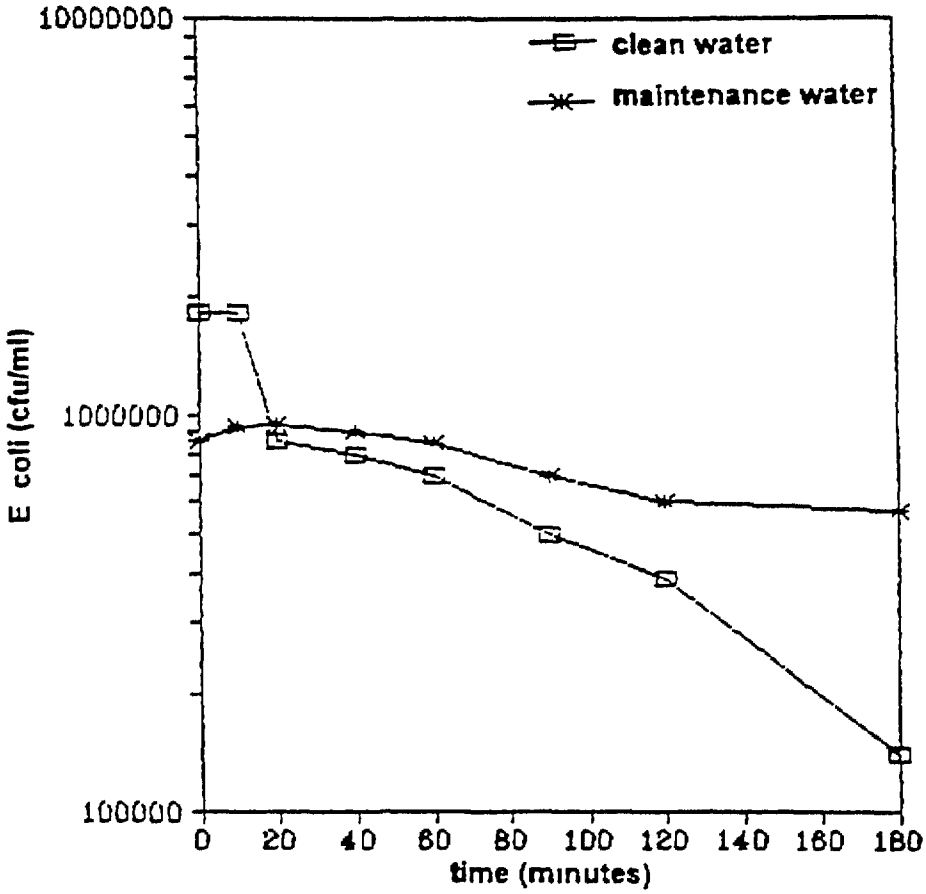


Figure 11. E. coli die-off in clean vs maintenance water as a function of time (The initial calculated concentration of H_2O_2 was 10 mg/l and for Ag+ was 28 PPb)

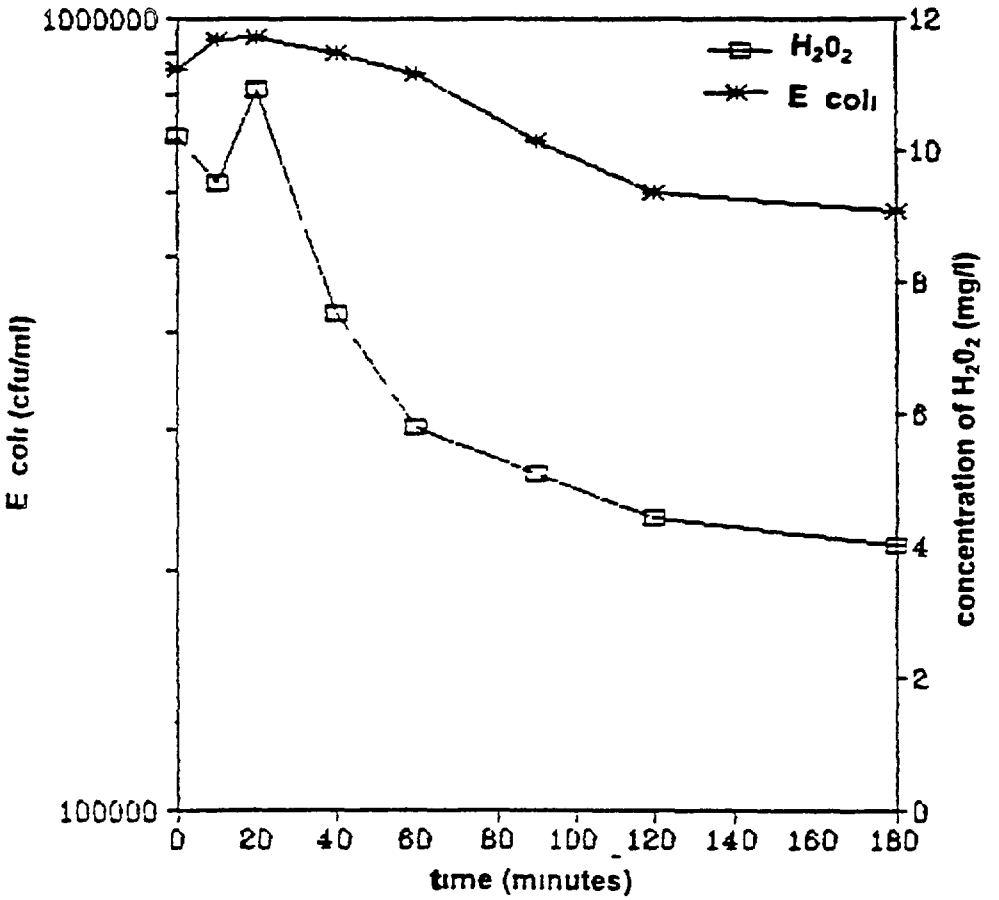


Figure 10. E. coli die-off in maintenance water vs the residual of H_2O_2 as a function of time
(The initial calculated concentration of H_2O_2 was 10 mg/l and for Ag+ was 28 PPb)

Table 9

Die-off microorganisms (MS2 phages and polio 1) in clean and maintenance water by hydrogen peroxide (H_2O_2) and Ag+ as a function of time
(The initial calculated concentration for H_2O_2 was 10 mg/l and for Ag+ was 28 ppb) (1)

	Clean Water			Maintenance Water		
Time (min)	Phage (pfu/ml)	Polio 1 (pfu/ml)	H_2O_2 (mg/l)	Phage (pfu/ml)	Polio 1 (pfu/ml)	H_2O_2 (mg/l)
0	$4.0 * 10^7$	$7.5 * 10^5$	-	$5.1 * 10^6$	$6.5 * 10^5$	10.2
20	$2.8 * 10^6$	$6.0 * 10^5$	7.8	$2.9 * 10^6$	$3.5 * 10^5$	10.9
40	$1.1 * 10^6$	$2.2 * 10^5$	7.8	$2.6 * 10^6$	$3.5 * 10^5$	7.5
60	$3.5 * 10^5$	$1.9 * 10^5$	7.8	$2.1 * 10^6$	$3.5 * 10^5$	5.8
90	$8.5 * 10^4$	$4.9 * 10^5$	6.8	$1.8 * 10^6$	$3.5 * 10^5$	5.1
120	$5.0 * 10^4$	$4.4 * 10^5$	6.1	$1.4 * 10^6$	$3.5 * 10^5$	4.4
180	$1.9 * 10^1$	$2.8 * 10^5$	5.1	$9.3 * 10^5$	$3.4 * 10^5$	4.0
240	0	$1.5 * 10^5$	4.4	$8.5 * 10^5$	$3.2 * 10^5$	3.4
300	0	$1.5 * 10^5$	4.4	$7.7 * 10^5$	$3.4 * 10^5$	3.4
5 h						

(1) During the experiment the concentration of H_2O_2 or Ag+ in the water was not adjusted

Table 10a

Die-off microorganisms (MS2 phages and polio 1) in clean and maintenance water by hydrogen peroxide (H_2O_2) and Ag+ as a function of time
(The initial calculated concentration for H_2O_2 was 30 mg/l and for Ag+ was 84 ppb) (1)
(experiment 1)

	Clean Water			Maintenance Water		
Time (min)	Phage (pfu/ml)	Polio 1 (pfu/ml)	H_2O_2 (mg/l)	Phage (pfu/ml)	Polio 1 (pfu/ml)	H_2O_2 (mg/l)
0	$4.0 * 10^4$	$6.8 * 10^6$	29.2	$6.5 * 10^5$	$3.6 * 10^6$	30.6
20	$2.0 * 10^4$	$6.8 * 10^6$	20.4	$1.0 * 10^4$	$3.0 * 10^6$	25.2
40	$2.0 * 10^4$	$6.8 * 10^6$	11.9	$2.1 * 10^4$	$3.0 * 10^6$	20.7
60	$2.0 * 10^4$	$6.4 * 10^6$	8.5	4	$3.0 * 10^6$	20.4
90	$2.0 * 10^4$	$5.9 * 10^6$	5.8	4	$3.0 * 10^6$	16.3
120	$2.0 * 10^4$	$5.9 * 10^6$	4.8	0	$3.0 * 10^6$	16.0
180	$5.0 * 10^3$	$5.9 * 10^6$	5.8	0	$3.0 * 10^6$	11.6
240	$5.0 * 10^3$	$5.9 * 10^6$	2.4	0	$3.0 * 10^6$	10.2
300	$5.0 * 10^3$	$5.9 * 10^6$	2.4	0	$3.0 * 10^6$	9.9
420	$5.0 * 10^3$	$5.0 * 10^6$	2.0	0	$3.0 * 10^6$	8.2
720	$2.1 * 10^3$	$3.8 * 10^6$	2.0	0	$2.8 * 10^6$	8.2
1440 (24h)	$1.0 * 10^1$	$2.8 * 10^6$	1.7	0	$2.8 * 10^6$	5.1

(1) During the experiment the concentration of H_2O_2 or Ag+ in the water was not adjusted

Table 10b

Die-off microorganisms (MS2 phages and polio 1) in clean and maintenance water by hydrogen peroxide (H_2O_2) as a function of time
(The initial calculated concentration for H_2O_2 was 30 mg/l and for Ag+ was 84 ppb) (1)
(experiment 2)

	Clean Water			Maintenance Water		
Time (min)	Phage (pfu/ml)	Polio 1 (pfu/ml)	H_2O_2 (mg/l)	Phage (pfu/ml)	Polio 1 (pfu/ml)	H_2O_2 (mg/l)
0	$2.3 * 10^6$	$3.1 * 10^6$	30.6	$1.8 * 10^6$	$4.6 * 10^6$	33.0
20	0	$3.1 * 10^6$	29.9	$1.3 * 10^6$	$4.2 * 10^6$	26.2
60	0	$2.0 * 10^6$	28.6	$1.3 * 10^6$	$3.0 * 10^6$	24.5
120	0	$2.0 * 10^6$	26.9	$1.3 * 10^5$	$3.0 * 10^6$	23.5
300	0	$1.9 * 10^6$	26.2	$1.0 * 10^1$	$3.0 * 10^6$	18.7
1440 (24h)	0	$1.9 * 10^6$	23.5	0	$3.0 * 10^6$	14.3

(1) During the experiment the concentration of H_2O_2 or Ag+ in the water was not adjusted

Table 10c

Die-off microorganisms (MS2 phages and polio 1) in clean and maintenance water by hydrogen peroxide (H_20_2) and Ag+ as a function of time
(The initial calculated concentration for H_20_2 was 30 mg/l and for Ag+ was 84 ppb) (1)
(experiment 3)

	Clean Water			Maintenance Water		
Time (min)	Phage (pfu/ml)	Polio 1 (pfu/ml)	H_20_2 (mg/l)	Phage (pfu/ml)	Polio 1 (pfu/ml)	H_20_2 (mg/l)
0	$7\ 3 * 10^6$	$3\ 7 * 10^6$	34 3	$7\ 0 * 10^6$	$4\ 2 * 10^6$	34 0
20	$1\ 0 * 10^3$	$3\ 3 * 10^6$	25 8	$6\ 9 * 10^6$	$4\ 0 * 10^6$	28 6
60	$8\ 5 * 10^1$	$3\ 1 * 10^6$	23 8	$3\ 8 * 10^6$	$3\ 0 * 10^6$	20 7
120	$1\ 0 * 10^1$	$2\ 0 * 10^6$	23 8	$3\ 5 * 10^6$	$2\ 4 * 10^6$	20 7
300	0	$1\ 6 * 10^6$	22 4	$3\ 0 * 10^3$	$2\ 0 * 10^6$	20 7
1440 (24h)	0	$1\ 3 * 10^6$	20 6	$1\ 5 * 10^2$	$1\ 5 * 10^6$	20 7

(1) During the experiment the concentration of H_20_2 or Ag+ in the water was not adjusted

Table 11a

Die-off microorganisms (MS2 phages and polio 1) in maintenance water with and without hydrogen peroxide (H_2O_2) and Ag+ as a function of time (The initial calculated concentration for H_2O_2 was 30 mg/l and for Ag+ was 84 ppb) (1) (experiment 1)

	Maintenance Water				
	without H_2O_2 or Ag+ (control)		with H_2O_2 + Ag+		
Time (min)	Phage (pfu/ml)	Polio 1 (pfu/ml)	Phage (pfu/ml)	Polio 1 (pfu/ml)	H_2O_2 (mg/l)
0	$5.0 * 10^6$	$5.0 * 10^6$	$8.3 * 10^6$	$4.0 * 10^6$	34.9
20	N T	N T	$7.9 * 10^6$	$3.0 * 10^6$	30.9
40	N T	N T	$4.2 * 10^6$	$2.9 * 10^6$	29.2
60	$1.6 * 10^6$	$4.5 * 10^6$	$4.0 * 10^6$	$2.9 * 10^6$	31.6
90	N T	N T	$3.3 * 10^6$	$2.9 * 10^6$	27.2
120	$1.3 * 10^6$	$1.7 * 10^6$	$2.6 * 10^6$	$2.9 * 10^6$	28.6
360	$1.1 * 10^6$	$1.2 * 10^6$	$9.6 * 10^5$	$2.9 * 10^6$	20.7

N T Not tested

(1) During the experiment the concentration of H_2O_2 and Ag+ in the water was adjusted

Table 11b

Die-off microorganisms (MS2 phages and polio 1) in maintenance water with and without hydrogen peroxide (H_2O_2) and Ag+ as a function of time (The initial calculated concentration for H_2O_2 was 30 mg/l and for Ag+ was 84 ppb) (1) (experiment 2)

	Maintenance Water				
	without H_2O_2 or Ag+ (control)		with H_2O_2 + Ag+		
Time (min)	Phage (pfu/ml)	Polio 1 (pfu/ml)	Phage (pfu/ml)	Polio 1 (pfu/ml)	H_2O_2 (mg/l)
0	$5.8 * 10^6$	$6.8 * 10^6$	$2.7 * 10^6$	$2.4 * 10^6$	31.6
20	N.T.[1]	N.T.	$6.4 * 10^3$	$2.0 * 10^6$	28.2[2]
40	N.T.	N.T.	0	$1.9 * 10^6$	27.5[2]
60	$3.0 * 10^6$	$5.5 * 10^6$	0	$1.8 * 10^6$	31.6
90	N.T.	N.T.	0	$1.5 * 10^6$	30.3
120	$3.3 * 10^6$	$5.5 * 10^6$	0	$1.5 * 10^6$	29.3[2]
360	$3.3 * 10^6$	$5.5 * 10^6$	0	$1.5 * 10^6$	29.5

(1) N.T. Not tested

(2) During the experiment the concentration of H_2O_2 and Ag+ in the water was adjusted

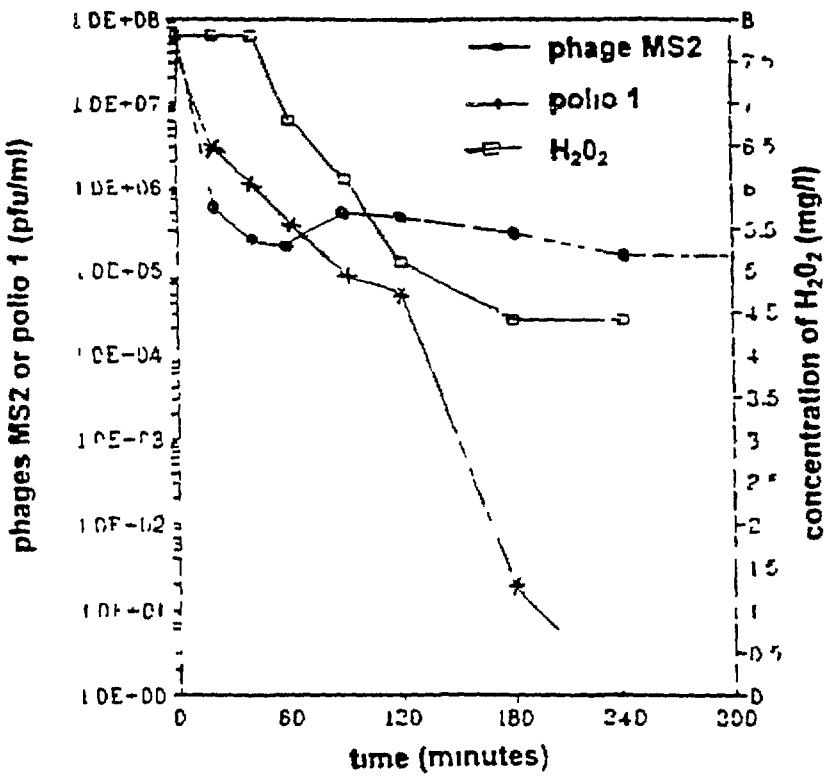


Figure 12. MS2 phages and polio 1 die-off in clean water vs the residual of H_2O_2 as a function of time
(The initial calculated concentration of H_2O_2 was 10 mg/l and for Ag+ was 28 PPb)

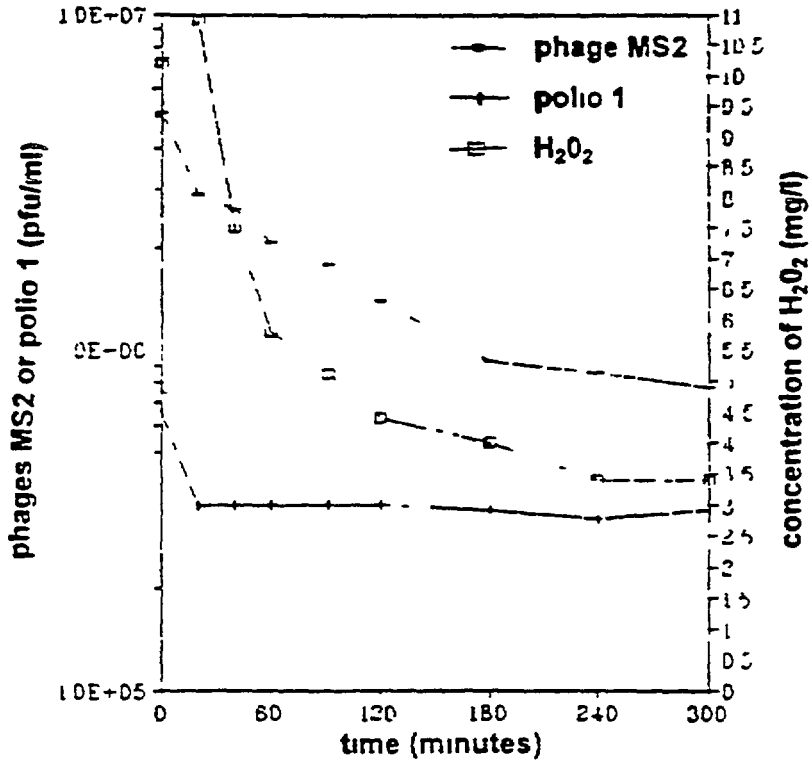


Figure 13. MS2 phages and polio 1 die-off in maintenance water vs the residual of H_2O_2 as a function of time
(The initial calculated concentration of H_2O_2 was 10 mg/l and for Ag+ was 28 PPb)

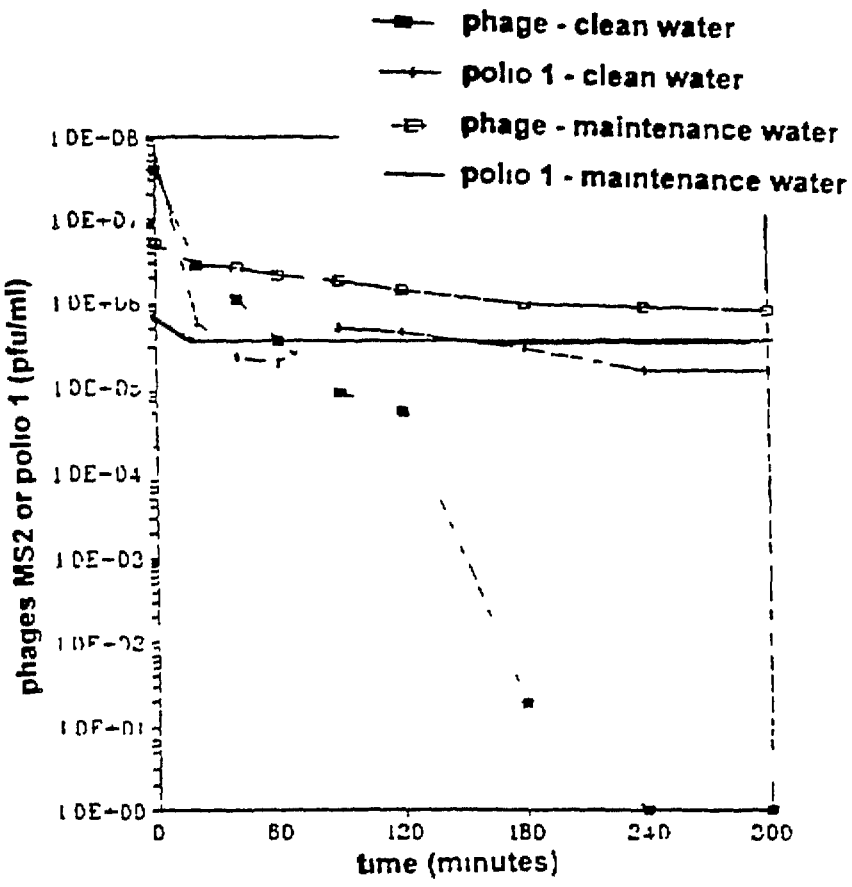


Figure 14. MS2 phages and polio 1 die-off in clean and maintenance water as a function of time
(The initial calculated concentration of H_2O_2 was 10 mg/l and for Ag+ was 28 PPb)

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Appendix 1

The concentration of E coli MS2 phages and polio 1 (cfu or pfu/cm^2 gr or ml) in different fish organs and water in the control self purification experiment. (Two experiments were conducted A and B The total number of fish tested was 25 with no water changes) (1)

Experiment A

Day No.	Ino No.	Hour Afinoc	No ch	Fish No.	SKIN E.c	SKIN Phage	SKIN Polio	LIVER E.c	LIVER Phage	LIVER Polio	SPLEEN E.c	SPLEEN Phage	SPLEEN Polio
1	0	0	0	201	0	0	0	0	0	0	0	0	0
2	1	24	0	201	260	300	4800	37	0	1800	7500	0	0
2	1	24	0	203	330	0	700	0	0	0	0	0	0
3	1	48	0	204	97	0	240	0	0	700	0	0	22000
3	1	48	0	205	230	70	270	0	0	100	0	0	0
5	1	96	0	206	30	220	0	0	200	0	0	0	0
5	1	96	0	207	0	0	0	100	930	0	0	77000	0
6	1	120	0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
7	1	144	0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
9	1	192	0	208	0	0	0	14	14	0	0	0	0
9	1	192	0	209	0	0	0	0	0	0	0	0	0
10	1	216	0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
21	1	480	0	210	0	0	0	0	0	0	0	1000	0

Day No.	Ino No.	Hour afinoc	No ch	Fish No.	DIGESTIVE TRACT E.c	DIGESTIVE TRACT Phage	DIGESTIVE TRACT Polio	WATER E.c	WATER Phage	WATER Polio
1	0	0	0	201	0	0	0	0	0	0
2	1	24	0	201	5000	230	2800	1000000	6000	10000
2	1	24	0	203	4900	620	13000	NT	NT	NT
3	1	48	0	204	4400	0	59000	560000	50	8500
3	1	48	0	205	8000	50	60000	NT	NT	NT
5	1	96	0	206	8500	14000	9000	4000	14	35
5	1	96	0	207	1800	10	10000	NT	NT	NT
6	1	120	0	NT	NT	NT	NT	200	0	13
7	1	144	0	NT	NT	NT	NT	19	0	0
9	1	192	0	208	340	0	130	3	0	5
9	1	192	0	209	220	0	29	NT	NT	NT
10	1	216	0	NT	NT	NT	NT	3	0	1000
21	1	480	0	210	4	28	0	0	0	4500

(1) In each experiment only one inoculated microorganisms was performed The concentration of the inoculated microorganisms was 10^5 cfu/ml for E. coli, 10^6 pfu/ml for MS2 phages and 10^5 pfu/ml poliovirus 1 in the water

NT = Not tested
No = number
Ino = inoculation
Ch = Water change
afinoc = after inoculation
E c = E coli
Phage = MS2 phage

Experiment B

Day No.	Ino No.	Hour Afinoc	No. ch	Fish No.	SKIN E.c	SKIN Phage	SKIN Polio	LIVER	LIVER Phage	LIVER Polio	SPLEEN E.c	SPLEEN Phage	SPLEEN Polio
1	0	0	0	50	0		0		0	0	0	0	
1	1	1	0	NT	NT	[illegible]	NT	–	NT	NT	NT	NT	NT
2	1	24	0	50	490	2	[illegible]	[illegible]	[illegible]	4000	0	24000	8[illegible]
2	1	24	0	50	1000	1	[illegible]	[illegible]	0	[illegible]200	24000	0	[illegible]
3	1	48	0	50	0		[illegible]		[illegible]80	390	67000	24000	
3	1	48	0	50	0	3	666		44000	000	9500	13000	95000
4	1	72	0	NT	NT		NT	–	NT	NT	NT	NT	NT
5	1	96	0	50	[illegible]	[illegible]	[illegible]		0	8[illegible]00	0	0	140000
5	1	96	0	50	0	28	667		0	0000	0	0	[illegible]
6	1	120	1	50	0		[illegible]		0	0	0	50000	5900
6	1	120	0	50	0		[illegible]7		0	[illegible]400	0	20000	[illegible]
8	1	168	0	5[illegible]	0		[illegible]		0	[illegible]00	0	0	[illegible]
8	1	168	0	5	0		[illegible]7		0	900	0	[illegible]000	[illegible]
9	1	192	0	5[illegible]	0	–	[illegible]		0	000	0	0	[illegible]
9	1	192	0	5	0		[illegible]		0	0	0	0	[illegible]
11	1	240	0	5[illegible]	0		[illegible]		0	0	0	0	[illegible]
11	1	240	0	5	0		[illegible]		0	0	0	0	[illegible]
18	1	432	0	NT	NT	–	NT	–	NT	NT	NT	NT	NT

Day No.	Ino No.	Hour afinoc	No ch	Fish No.	DIGESTIVE TRACT E.	DIGESTIVE TRACT Phage	DIGESTIVE TRACT Polio	WATER E.c	WATER Page	WATER Pc
1	0	1	0	501		0	00	0	0	[illegible]
1	1		0	NT	NT	NT	NT	[illegible]0000	6 E-07	1.2E-[illegible]
2	1	24	0	502	[illegible]1000	[illegible]	[illegible]	4000	1 9000	5500
2	1	24	0	503	[illegible]48000	[illegible]	[illegible]	NT	NT	NT
3	1	48	0	504	39	[illegible]	[illegible]	[illegible]70	120000	1900
3	1	48	0	505	1300	[illegible]	[illegible]	NT	NT	NT
4	1	72	0	NT	NT	NT	NT	34	39000	1700
5	1	9[illegible]	0	506	68	[illegible]	[illegible]	16	10000	1000
5	1	9[illegible]	0	507	22	[illegible]	[illegible]	NT	NT	NT
6	1	12[illegible]	0	508	21	[illegible]	[illegible]	12	[illegible]3000	8[illegible]
6	1	12	0	509	12	[illegible]	[illegible]	NT	NT	NT
3	1	16[illegible]	0	510	6	78	[illegible]	6	200	48[illegible]
8	1	16[illegible]	0	511	14	[illegible]	[illegible]	NT	NT	NT
9	1	19[illegible]	0	512	[illegible]	[illegible]	[illegible]	40	500	[illegible]
9	1	19[illegible]	0	513	[illegible]	[illegible]	[illegible]	NT	NT	NT
11	1	24	0	514		[illegible]	[illegible]	70	[illegible]00	[illegible]
11	1	24[illegible]	0	515		[illegible]	[illegible]	NT	NT	NT
18	1	4[illegible]	0	NT	NT	NT	NT	45	20	[illegible]

NT = Not tested
No = number
Ino = inoculation
Ch = Water change
afinoc = after inoculation
E c = E. coli
Phage = MS2 phage

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Appendix 2

The concentration of E coli MS2 phages and polio 1 (cfu or pfu/cm^2 gr or ml)
in different fish organs and water in decontamination experiments
(Three experiments were conducted A and B with clean water and C with turbid water (maintenance water)
The total number of fish tested was 35 with water changes) (1)

Experiment A (clean water)

Day No.	Ino No.	Hour Afinoc	No ch	Fish No.	SKIN E.c	SKIN Phage	SKIN Polio	LIVER E.c	LIVER Phage	LIVER Polio	SPLEEN E.c	SPLEEN Phage	SPLEEN Polio
1	0	0	0	401	0	0	0	0	0	0	0	0	0
1	1	1	0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
1	1	12	0	402	0	3	170	0	0	240	0	0	98000
1	1	12	0	403	0	180	100	0	9	0	0	0	0
2	1	24	0	404	30	100	100	0	0	0	0	0	0
2	1	24	0	405	27	270	300	0	0	0	0	0	0
21	36	3	406	6	80	500	0	0	0	0	0	0	
2	1	36	3	407	2	26	300	0	0	0	0	1100	0
3	1	48	8	408	0	13	0	0	0	0	0	9400	0
3	1	48	8	409	0	0	0	0	5	0	0	0	0
4	1	72	16	410	0	7	0	0	0	0	0	0	0
4	1	72	16	411	0	0	0	0	0	0	0	0	0

Day No.	Ino No.	Hour afinoc	No ch	Fish No.	DIGESTIVE TRACT E.c	DIGESTIVE TRACT Phage	DIGESTIVE TRACT Polio	WATER E.c	WATER Phage	WATER Polio
1	0	0	0	401	0	0	0	0	0	0
1	1	1	0	NT	NT	NT	NT	880000	9000000	13000
1	1	12	0	402	11	2300	96000	6600	16000	16000
1	1	12	0	403	0	180	11000	NT	NT	NT
2	1	24	0	404	0	490	280000	200	5900	20000
2	1	24	0	405	13	79	240000	NT	NT	NT
2	1	36	3	406	0	0	210000	0	0	1900
2	1	36	3	407	0	2	850000	NT	NT	NT
3	1	48	8	408	0	0	1900	0	0	23
3	1	48	8	409	0	3	2300	NT	NT	NT
4	1	72	16	410	0	3	0	0	0	0
4	1	72	16	411	0	0	0	NT	NT	NT

(1) In each experiment only one inoculated microorganisms was performed The concentration of the inoculated microorganisms was 10^5 cfu/ml for E. coli, 10^6 pfu/ml for MS2 phages and 10^5 pfu/ml poliovirus 1 in the water

NT = Not tested
No = number
Ino = inoculation
Ch = Water change
afinoc = after inoculation
E c = E coli
Phage = MS2 phage

Appendix 2 (continued)

Experiment B (clean water)

Day No.	Ino No.	Hour Afinoc	No ch	Fish No.	SKIN E.c	SKIN Phage	SKIN Polio	LIVER E.c	LIVER Phage	LIVER Polio	SPLEEN E.c	SPLEEN Phage	SPLEEN Polio
1	1	0	0	601	0	0	0	0	0	0	0	0	0
1	1	1	0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
2	1	24	0	602	10	130	170	0	0	0	0	0	0
2	1	24	0	603	13	63	330	0	0	100	0	0	10000
2	1	36	3	604	25	2E+05	94	0	3600	300	0	0	0
2	1	36	2	605	0	107	44	0	57	9000	0	0	0
3	1	48	8	606	28	700	280	0	5900	20000	0	58000	0
3	1	48	8	607	0	150	250	0	110	560	0	0	0
4	1	72	16	608	0	180	0	0	94	0	0	300000	37400
4	1	72	16	609	0	140	0	0	0	0	0	0	0
5	1	96	24	610	0	33	0	0	0	230	0	0	50000
5	1	96	24	611	0	0	0	0	0	0	0	0	0

Day No.	Ino No.	Hour afinoc	No ch	Fish No.	DIGESTIVE TRACT E.c	DIGESTIVE TRACT Phage	DIGESTIVE TRACT Polio	WATER E.c	WATER Phage	WATER Polio
1	1	0	0	601	0	0	0	0	0	0
1	1	1	0	NT	NT	NT	NT	30000	4700000	67000
2	1	24	0	602	1000	24000	83000	4500	360000	83000
2	1	24	0	603	2000	2.6E+06	413000	NT	NT	NT
2	1	36	3	604	109	270000	14400	2500	1400	400
2	1	36	2	605	1860	490000	750000	NT	NT	NT
3	1	48	8	606	153	43000	354000	0	280	350
3	1	48	8	607	570	660000	240000	NT	NT	NT
4	1	72	16	608	0	9000	400000	0	110	220
4	1	72	16	609	29	5800	25000	NT	NT	NT
5	1	96	24	610	0	160	2000	0	0	45
5	1	96	24	611	0	260	7700	NT	NT	NT

NT = Not tested
No = number
Ino = inoculation
Ch = Water change
afinoc = after inoculation
E c = E coli
Phage = MS2 phage

Appendix 2 (continued)

Experiment C (turbid or maintenance water)

Day No.	Ino No.	Hour Afinoc	No ch	Fish No.	SKIN E.c	SKIN Phage	SKIN Polio	LIVER E.c	LIVER Phage	LIVER Polio	SPLEEN E.c	SPLEEN Phage	SPLEEN Polio
1	1	0	0	301	0	0	0	0	0	0	0	0	0
1	1	1	0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
1	1	12	0	302	0	490000	30	110	730000	3500	0	16000000	24000
1	1	12	0	303	7	0	300	0	38000	0	0	10000000	8300
2	1	24	0	304	0	53	170	0	480000	3000	0	1600000	0
2	1	24	0	305	0	270	150	0	19000	2300	0	1400000	0
2	1	36	3	306	0	10	0	0	0	0	0	0	0
2	1	36	3	307	0	5	0	5	140	0	0	0	0
3	1	48	8	308	0	20	0	0	0	0	0	0	0
3	1	48	8	309	0	13	0	0	0	0	0	0	0
4	1	72	16	310	0	0	0	0	0	0	0	0	0
4	1	72	16	311	0	0	0	0	0	0	0	0	0
5	1	96	24	312	0	0	0	0	0	0	0	0	0
5	1	96	24	313	0	0	0	0	0	0	0	0	0

Day No.	Ino No.	Hour afinoc	No ch	Fish No.	DIGESTIVE TRACT E.c	DIGESTIVE TRACT Phage	DIGESTIVE TRACT Polio	WATER E.c	WATER Phage	WATER Polio
1	1	0	0	301	0	0	0	0	0	0
1	1	1	0	NT	NT	NT	NT	70000	14000000	7600
1	1	12	0	302	260	96000	60000	100	140000	1000
1	1	12	0	303	710	550000	64000	NT	NT	NT
2	1	24	0	304	28	6700	120000	70	5600	3300
2	1	24	0	305	0	59	43000	NT	NT	NT
2	1	36	3	306	18	290	880	2	2600	87
2	1	36	3	307	130	340	530	NT	NT	NT
3	1	48	8	308	0	23	0	0	0	6
3	1	48	8	309	47	0	320	NT	NT	NT
4	1	72	16	310	0	0	0	0	0	0
4	1	72	16	311	4	0	0	NT	NT	NT
5	1	96	24	312	0	0	0	0	0	0
5	1	96	24	313	0	0	0	NT	NT	NT

NT = Not tested
No = number
Ino = inoculation
Ch = Water change
afinoc = after inoculation
E c = E coli
Phage = MS2 phage

Appendix 3

Preparation of stock Microorganisms and Assays

1 Assays for different microorganisms

The following microorganisms were assayed in water and fish organs sample Poliovirus 1 MS2 bacteriphage (phage) and E coli

1 1 Viruses and Assay Methods

1 1 1 Poliovirus 1

Poliovirus 1 Lsc strain was cultivated in Buffalo green monkey kidney (BGM) cells Cytopathic effect was observed within 48 hr of infection The virus was released from infected cell cultures by three cycles of freezing and thawing and concentrated by ultracentrifugation Poliovirus was purified by chloroform extraction Enumeration of poliovirus 1 was accomplished by the plague-assay method as described by (Guttmann-Bass and Nasser (1984) Briefly, confluent monolayers of BGM cells are inculated with 0 3 ml of polio 1 samples After 1 hr incubation at 37°c, monolayers are overlayed with medium containing 1% agar, and 2% calf serum

Then the monolayers are overlayed with medium containing 1% agar, 15 ug/ml neutral red and 2% calf serum Plagues are counted 2-3 days later

1 1 2 MS2 Bacteriophage

Mixed population of MS2 phage were obtained from raw wastewater, which usually contains less than 10 pfu/ml MS2 phage was concentrationed from 5-10 liters of raw wastewater by precipitation with 12% polyethylene glycol (PEG) 8000 Precipitation was performed at 5°c for 16 hrs, followed by low speed centrifugation to pellet the virus-precipitation The pellet was then suspended in Phosphate Buffered Saline (PBS), and extracted with an equal volume of chloroform to remove impurities

Assay for MS2 phage was done on Salmonella typhimurium (WG-40) as host, as described previously by Havelaar et al , (1984) MS2 phage enumeration was done by the double agar layer Briefly, phage samples (0 3 ml) were mixed with log-phase bacterial cultures (0 2 ml) in 3 ml molted agar kept at 45°c After vortex, the samples were poured in plates containing bottom agar Plagues were

developed and counted after 4 to 12 hours of incubation in 37°c incubator

1 1 3 E.coli

E.coli resistant to Streptomycin and Nalidixic acid was cultured in 500-1000 ml nutrient broth (Difco) using a thermostatic shaker at 37°c for 18-24 hours The final concentration of the culture was 10^8 - 10^9 cfu/ml A sample of the culture was assayed for E coli with and without antibiotics in the medium Samples from water and various fish tissues were assayed to determine the levels of microbial contamination The samples were assayed for E coli and enumerated on mTEC agar (Dufour et al 1981)

The indicator system of the mTEC agar consistes of bromocresol purple and bromophenol red The pH of the medium was 7 3 ± 0 1 The medium supplemented with 50 ppb of Streptomycin and Nalidixic acid Appropriate sample volumes were passed through 0 45 um porosity memebrane filters and the filters were then placed on the medium The plates were incubated for 2 hrs at 37°c and for 20 hrs at 44 5°C The plates were removed from the incubator and all yellow colonies were considered as fecal coli

2 Sample Custody

Virus stocks and virus isolates, before and after processing, are stores at -70°c BGM cells were stored in liquid nitrogen All information is recorded in laboratory folders by dates

3 Assays for Microorganisms Used for Inoculations in the Laboratory Experiments

The penetration of microorganisms into fish tissues were studied using E coli bacteria, male-specific MS2 bacteriophage and Poliovirus 1 The stocks of the E coli and MS2 Phage were prepared in the laboratory as previously described The stock of E coli was prepared 18 to 24 hrs prior to experiment The stocks of the Phage and Polio 1 were prepared at the beginning of the study and were freezed at -30°c

The concentrations of the stocks were as follows

E. coli = 10^{10} cfu/ml

MS2 phages = 10^{11} pfu/ml

Polio 1 = 10^{10} pfu/ml

Each inoculum (in all the experiments), consist of these 3 microorganisms

PART III

Survival of Hepatitis A Virus (HAV), Poliovirus I, F+ Bacteriophages and E. coli in Phosphate-Buffered Saline (PBS), Ground Water and Wastewater as it Pertains to Seafood Safety

Introduction

Hepatitis A Virus (HAV) is a major waterborne disease agent with worldwide distribution (see Nasser 1994, a review of the literature on the prevalence and fate of HAV in water) The main transmission route of HAV is direct person-to-person contact However, an outbreak of hepatitis caused by the consumption of raw oysters was described by Jean-Claude et al (1991), and also by Portnoy et al (1975) Shellfish can filter, accumulate, and retain viruses from surrounding waters It is possible that shellfish concentrate the hepatitis virus from surrounding water in a manner similar to that observed for other enteroviruses (Goyal et al 1979) Therefore it is very important to study the die-off and the survival of HAV in waters in order to analyze the public health implication and seafood safety guidelines

Fishponds are generally supplemented with animal manure and in some farms, with wastewater However, animals and untreated human wastes can be loaded with pathogenic bacterial and viral agents and their introduction into fishponds may pose a health hazard for fish handlers and consumers The impact of microbiological loading of growth waters on the levels of contamination of various fish organs has been studied previously (Buras et al 1987 Slabbert et al 1989, and Fattal et al 1992) It can be concluded from these studies that fish grown in cotaminated waters may not be considered safe for human consumption In part 2 of this report the test of marketed fish for different microorganisms is presented

Materials and Methods

Four different water types were used Raw wastewater samples were obtained from the wastewater treatment plant of Jerusalem after primary sedimentation Sterile wastewater was prepared by autoclaving the raw wastewater for 30 min at 121^0 C Groundwater samples were obtained from a well in Tira, a small town in the central part of Israel (this well is 55m deep and is used as drinking water), PBS 0 01M, pH- 7 2 served as a control

One liter of each water type was seeded with various microorganisms to reach an initial titer of 10^6 cfu/ml E coli and 10^4 - 10^5 pfu/ml of the viruses After thorough mixing each sample was divided into 20ml portions in sterile plastic tubes The tubes were kept, in the dark at 4^{0}C, 10^{0}C 20^{0}C, 30^{0}C and 37^{0}C in covered water bath or incubator Samples were removed at (0 time) and after 1, 5, 10, 20 30, 60 and 90 days of incubation The samples for E coli were assayed on the same day and the remainder of the samples stored at -20^{0}C for later virus assay

Virus and virus-assays

Poliovirus Lsc strain was cultivated in Buffalo Green Monkey Kidney (BGMK) cultures Enumeration of poliovirus 1 was performed according to Guttmann-Bass and Nasser 1984

Hepatitis A virus (HAV), a cell culture-adapted HM-175 strain, was obtained from M D Sobsey, the University of North Carolina, Chapel Hill, USA HAV was cultivated in FRhK-4 cell Enumeration of HAV was performed according to Cromeans et al 1987

F$^+$bacteriophages were concentrated from raw wastewater by the polyethylene glycol (PEG)-precipitation method (Lewis and Metcalf 1988) A specially constructed host bacterium Salmonella typhimurium WG49, was obtained from L Havelaar Netherlands The F$^+$bacteriophages used in this study are an endogenous group which grows in Salmonella typhimurium WG49 host bacterium Enumeration of F$^+$bacteriophages was performed by the double-agar method (Adams, 1959)

E coli strain resistant to ampicillin and streptomycin was received from V Cabelli, Rhode Island University Enumeration of E coli was done by the membrane filtration method on mTEC agar containing ampicillin and streptomycin (Dufour et al 1981)

Results

The results of the survival of HAV poliovirus1 F^+ bactenophages and E coli in PBS solution, Wastewater (stenle and raw) and groundwater are summanzed in figure 1 (A & B)

Figure 1A shows the die-off of these microorganisms after 30 days and Figure 1B - after 90 days incubation penod at 10^0C 20^0C and 30^0C Figure 2 shows the die-off coefficient log Nt/No x day of tnese microorganisms in PBS groundwater and wastewater at temperatures of 4^0C 10^0C 20^0C 30^0C and 37^0C It can be seen from these two figures that the die-off of HAV polio1 and F^+bactenophage were similar at 4^0C in PBS groundwater and wastewater The die-off averaged from 0 to 1 $\log_{10}$ after 3 months of incubation The decay of E coli was greater than those of the viruses and reached 2 to 6 $\log_{10}$

In all water samples at 20^0C, the decay of F^+bactenophages was lower than that observed for poliovirus 1 and HAV The die-off of F^+bactenophages was incubation No decay of E coli was recorded at 20^0C in PBS or groundwater whereas in raw wastewater the E coli die off reached 3 $\log_{10}$

The highest decay rates of the vanous microorganisms was recorded at 30^0C and 37^0C After one month of incubation at 37^0C, HAV and poliovirus 1 were undetectable in the various water samples E coli die-off was lowest in PBS and was similar to HAV and poliovirus 1 in raw wastewater and groundwater
highest in groundwater and wastewater and reached about 2 $\log_{10}$ after 2 months of
At 20^0C the decay rates of HAV and poliovirus 1 were highest in wastewater

The results presented in this study indicate that both HAV and poliovirus 1 persist in groundwater and wastewater for several months at lower temperatures and that transmission of these pathogenic viruses dunng the winter months is highly possible This may also explain some of the groundwater outbreaks caused by HAV (Craun 1985)

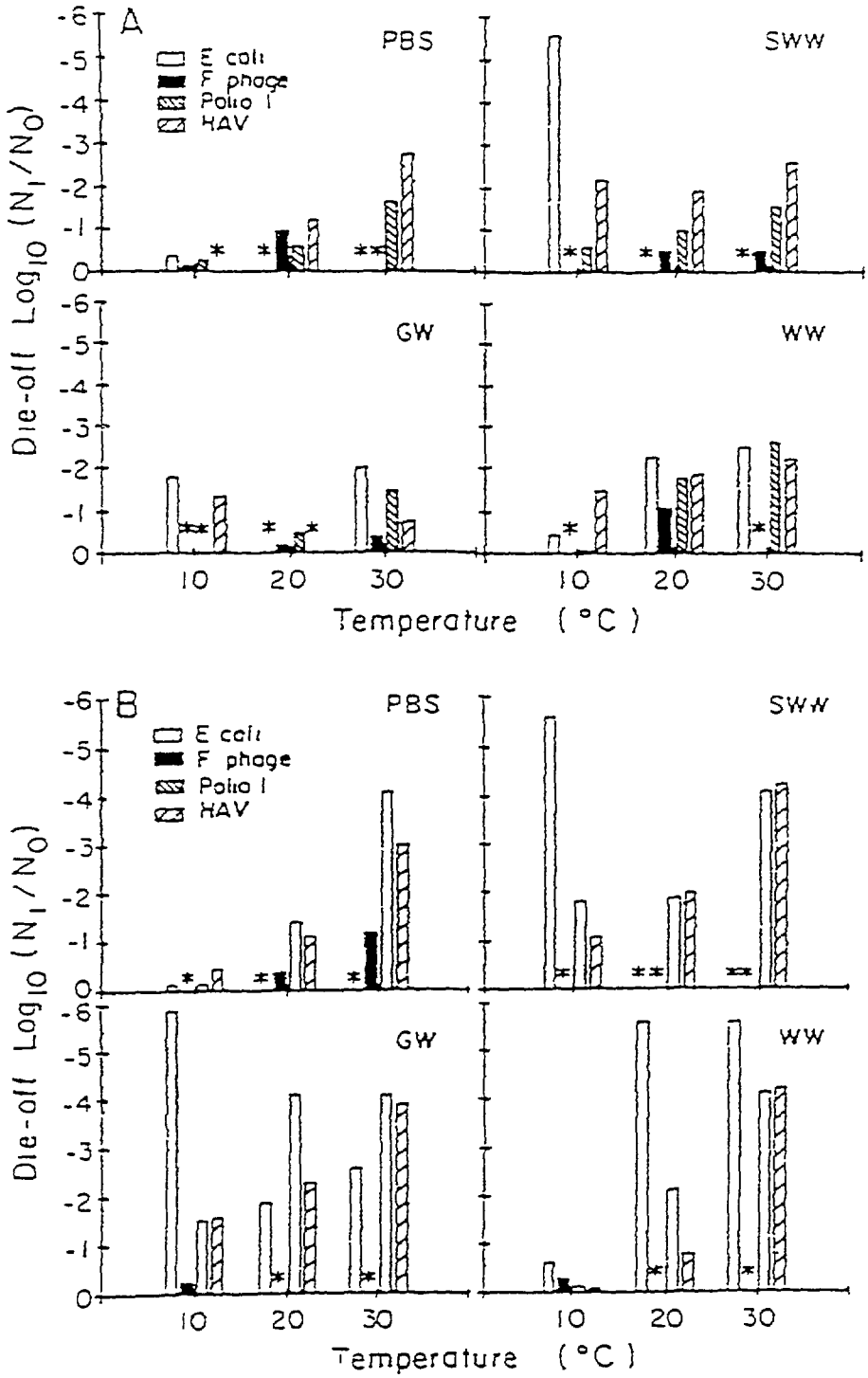

Figure 1 Die-off E coli, F⁺bacteriophages, Poliovirus 1 and Hepatitis A Virus (HAV) After 30 Days (A) and 90 Days (B) Incubation at 10°C, 20°C and 30°C in Phosphate Buffered Saline (PBS), Groundwater (GR), Sterile Wastewater (SWW)and Raw Wastewater (WW)
(The initial titer of tested microorganisms was10^6 CFU/ml for E coli and 10^4-10^5 PFU/ml for viruses, * No die-off was observed)

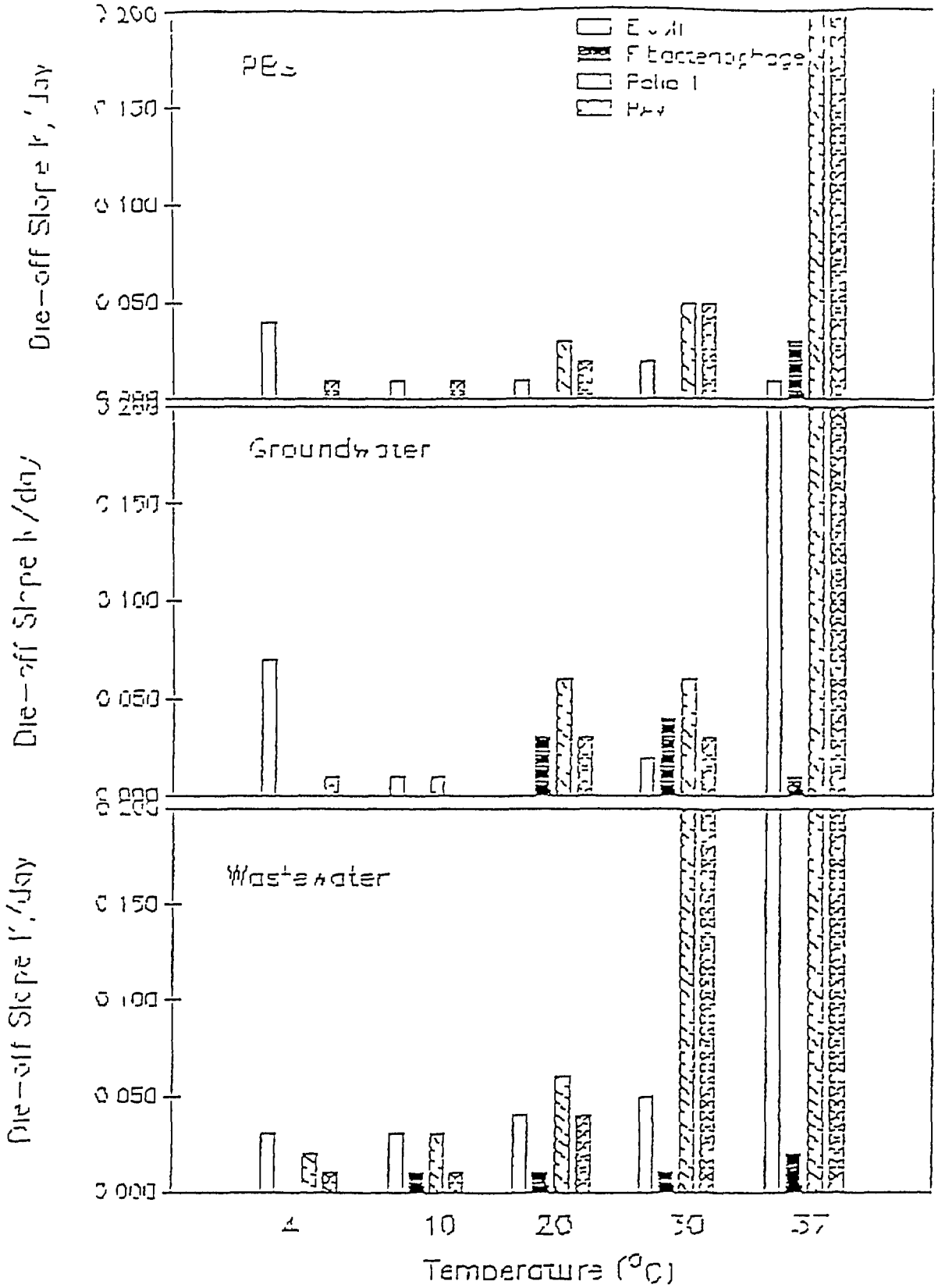

Figure 2 Die-Off coefficient Log_{10} Nt/N0 x day of Hepatitis A Virus (HAV), Poliovirus 1, F^+bacteriophages and E coli in Phosphate Buffered Saline (PBS), Groundwater (GR) and Wastewater (WW) at 4°C, 10°C, 20°C, 30°C and 37°C (The initial titer of tested microorganisms was 10^6 CFU/ml for E coli and 10^4-10^5 PFU/ml for viruses)

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PART IV

Microbe and Heavy Metals Detected in Fresh Waters Aquacultured Fish Marketed in Israel

MICROBES AND HEAVY METALS DETECTED IN FRESH WATER AQUACULTURED FISH MARKETED IN ISRAEL

ABSTRACT

Two species of fish, *Tilapia sp* and *Mugil sp* , grown in fresh water aquaculture and marketed in Israel were tested for the presence of microbes and heavy metals These species are known to tolerate heavy organic loads Samples of digestive tract (DT), liver, spleen, skin and muscle were assayed for *Aeromonas*, *Fecal coliforms*, and F+ coliphages DT and muscle were assayed also for enteroviruses The most frequent microorganism found was *Aeromonas*, followed by *Fecal coliforms*, and F+ coliphage In both fish species, the highest concentration of *Aeromonas* was present in the DT, followed by skin, liver, spleen and muscle *Fecal coliforms* were not found in any of the muscles sampled Enteroviruses were not detected in either DT or muscle Even though our results show that both microbial and heavy metal concentrations were within the acceptable Israeli ranges, we recommend that measures be taken in processing to prevent cross-contamination of edible tissue by DT and skin

INTRODUCTION

The demand for low-fat and high protein food is on the rise, leading to an increased demand for fishery products Overfishing and exploitation of most fishing grounds caused a levelling- off in marine catch in the early 1970's Aquaculture, by increasing fish production, has become a major means of closing this gap, and is recognized, in Israel and elsewhere, as an inexpensive practice, especially when fish ponds are fertilized with chicken and cattle manure and/ or domestic wastewater (Hepher and Pruginin, 1981) However, untreated animal and human wastewater is a potential source of heavy loads of pathogenic microorganisms, whose introduction into fish ponds may pose a health hazard to both fish handlers and consumers Microbial contamination of various fish organs has been studied both in Israel (Buras *et al* 1987,

Fattal *et al* 1992, Fattal *et al* 1993, Zuaretz-Peled et al 1996) and abroad (Slabbert *et al* 1989)

The aim of the present study was to determine whether the microbial level of fresh fish sold on the open market had changed following of the Israeli fish breeders association's ban on the use of wastewater in aquaculture The heavy metal content of the fish was also examined

MATERIALS AND METHODS

Samples and sample preparation

The study was conducted during 1995 A total of 22 *Tilapia sp* and 15 *Mugil* sp were purchased at Jerusalem's open market and analyzed for microbial quality The fish were transferred to the laboratory on ice, dissected aseptically within 1 hr of arrival and samples of skin, muscle, spleen, liver and digestive tract (DT) were excised After the addion of 0 01M phosphate buffered saline (PBS), pH 7, the samples were homogenized in an Omni mixer followed by centrifugation for 5 min at 1,500 rpm and immediately assayed for *Fecal coliforms* and *Aeromonas* The remainder of each sample was stored at -20°C for later assay of F+ coliphages and enteroviruses Additional specimens, 5 *Tilapia sp* and 5 *Mugil sp* , were examined for heavy metal content The fish were stored at -20°C until assayed for heavy metal concentration at the Israel Oceanographic & Limnological Research (IOLR) laboratory

Bacterial assay

filtration technique (Bisson *et al* , 1979, Dufour *et al* , 1981) Briefly, du *Aeromonas* and *Fecal coliforms* were determined by the membrane plicate samples were blended and diluted in PBS, passed through a 0 45µm Millipore filter and placed either on m-TEC agar for detection *of Fecal coliforms* or on M-*Aeromonas* agar To enhance the recovery of *Fecal coliforms*, m-TEC plates were incubated for 2 hrs at 37°C and transferred to a 44 5°C incubator M-*Aeromonas* plates were incubated at 37°C All colonies were counted after 20-24 hrs

F+ coliphage assay

A specially constructed bacterial host, *E coli* strain, resistant to ampicillin and streptomycin, developed for selective detection of F+ coliphage, was kindly supplied by V J Cabelli, RI, University F+ coliphages were detected by the double agar layer technique (Adams 1959) Briefly, 1 ml of blended tissue sample was mixed with 1 ml molten soft agar containing the bacterial host and the mixture was then poured onto a suitable bottom agar (Debartolomeis and Cabelli , 1991) Plates were incubated for 16 hrs at 37°C and the plaques were counted

Isolation of entero viruses

Only DT and muscle samples were assayed for enteroviruses Blended samples were centrifuged at low speed (1500 rpm) for 10 min and seeded on confluent monolayers of Buffalo Green Monkey Kidney (BGMK) After incubation for 1 hr at 37°C to allow adsorption, the supernatant was aspirated to prevent cytotoxicity RPMI-1640 medium (5 ml) supplemented with 2% fetal calf serum was then added and the plates were incubated at 37°C and observed daily for any cytopathogenic effect

Heavy metal detection

Duplicate muscle tissue samples were digested with concentrated nitric acid in high pressure decomposition vessels and analysed for Hg, Cd, Cu, and Zn by atomic absorption Mercury was measured by cold vapor atomic absorption spectrophotometry as previously described (Roth and Hornung 1977, Hornung *et al*, 1989) Concentrations are expressed on a wet weight basis The actual work was carried out by H Hornung from the ILR

RESULTS

The concentration of the tested microorganisms found in the various fish organs is presented in Tables 1, 2 and in Figs 1, 2 *Aeromonas* was the most prevalent, *Fecal coliforms* ranking second and F+ coliphage-third The *Aeromonas* concentration was highest in the DT, followed by skin, liver, spleen and muscle In the *Tilapia sp*, the greatest number of *Aeromonas* was found in the DT and liver, in the *Mugil sp*, the highest level was present in spleen, skin and muscle *Fecal coliforms* were not detected in any of the muscle samples assayed In *Mugil sp*, the spleen was also free of *Fecal coliforms* F+ coliphage were found in all the *Tilapia sp* and *Mugil sp* tissue samples In 20 fish, enteroviruses were not present in either DT or muscle

The concentrations of heavy metals (Table 3) were low, and within the acceptable Israeli range For more details see Appendix A

Presence of *Aeromonas*, *Fecal coliforms*, and F+ coliphages in various fish organs

Table 1 *Tilapia sp*

Micro-organism	parameters	Tissue				
		DT/ gr	Liver/ gr	Spleen/ gr	Skin/ cm2	Muscle/ gr
	Log mean	5 59	2 50	0 99	3 10	-0 25
Aeromonas	STD±	0 85	1 18	2 28	0 62	0 89
(cfu)	Min	3 78	BDL	BDL	1 60	BDL
	Max	7 10	4 51	4 60	4 19	2 40
Total n=22	Positive n	22/22	19/20	11/22	22/22	5/22
	% positive	100	95	50	100	23
	Log mean	1 91	0 25	-0 66	0 12	BDL
Fecal	STD±	2 35	1 31	1 11	1 04	0
coliforms	Min	BDL	BDL	BDL	BDL	BDL
(cfu)	Max	5 48	2 18	1 78	2 34	BDL
Total n=11	Positive n	7/14	5/11	2/11	5/11	0/11
	% positive	67	45	18	45	0
	Log mean	0 05	-0 20	-0 55	0 26	0 22
F+	STD±	1 10	0 98	1 18	1 03	1 34
coliphages	Min	BDL	BDL	BDL	BDL	BDL
(pfu)	Max	3 20	1 75	2 18	1 70	4 00
Total n=22	Positive n	9/22	7/22	5/22	11/22	9/22
	% positive	41	32	23	50	41

DT- Digestive Tract, cfu- colony forming units, pfu- plaque forming units

BDL-Below Detectable Level

Presence of *Aeromonas*, *Fecal coliforms*, and F+ coliphages in various fish organs

Table 2 *Mugil sl.p*

Micro-organism	parameters	Tissue				
		DT/ gr	Liver/ gr	Spleen/ gr	Skin/ cm2	Muscle/ gr
Aeromonas (cfu) Total n=12	Log mean	4 71	2.09	1 61	3 44	0.32
	STD±	0 61	2.02	1.57	1 10	1.35
	Min	3.54	BDL	BDL	1 45	BDL
	Max	5.70	4 62	3 40	5.02	2.35
	Positive n	12/12	9/12	9/12	12/12	5/12
	% positive	100	75	75	100	42
Fecal coliforms (cfu) Total n=9	Log mean	0 59	-0 54	-1 15*	1 24	BDL
	STD±	1 26	0 84	0	1 26	0
	Min	BDL	BDL	-	BDL	BDL
	Max	2 10	1 69	-	2 92	BDL
	Positive n	5/9	1/9	0/9	7/9	0/9
	% positive	56	11	0	78	0
F+ coliphages (pfu) Total n=15	Log mean	-0 61	-0 51	-0 51	-0 23	-0 07
	STD±	0 34	0 66	1 12	0 73	1 19
	Min	BDL	BDL	BDL	BDL	BDL
	Max	0 60	0 84	1 70	1 30	2 74
	Positive n	1/15	3/15	4/15	5/15	4/15
	% positive	7	20	27	33	27

DT- Digestive Tract, cfu- colony forming units, pfu- plaque forming units

BDL-Below Detectable Level

ble 3 Trace metal concentration in muscle tissue

(n=5 for both species)

Fish species	Elements (range) (µg*g-1 wet wt)			
	Hg	Cd	Cu	Zn
Tilapia sp	BDL-0 007	0 015-0 027	0 19-0 44	3 43-6 52
Mugil sp	BDL-0 006	0 015-0 024	0 18-0 42	2 47-4 58

BDL-Below Detectable Level

Detection levels for mercury - 0 005µg/g wet wt ;

and cadmium-0 015µg/g wet wt

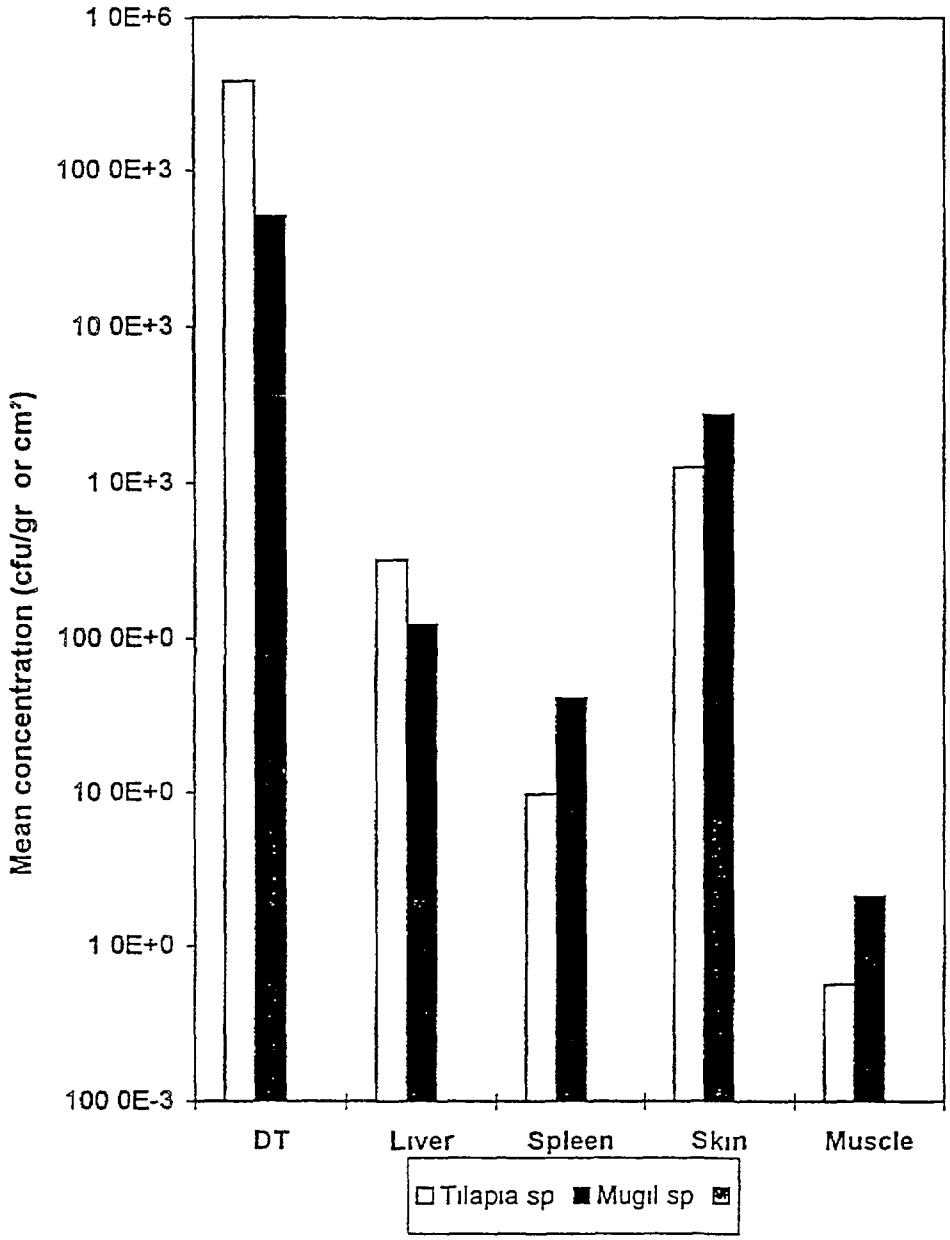

Fig 1 Mean *Aeromonas* concentration

(*Tilapia sp* - n=22, *Mugil sp* -n=12)

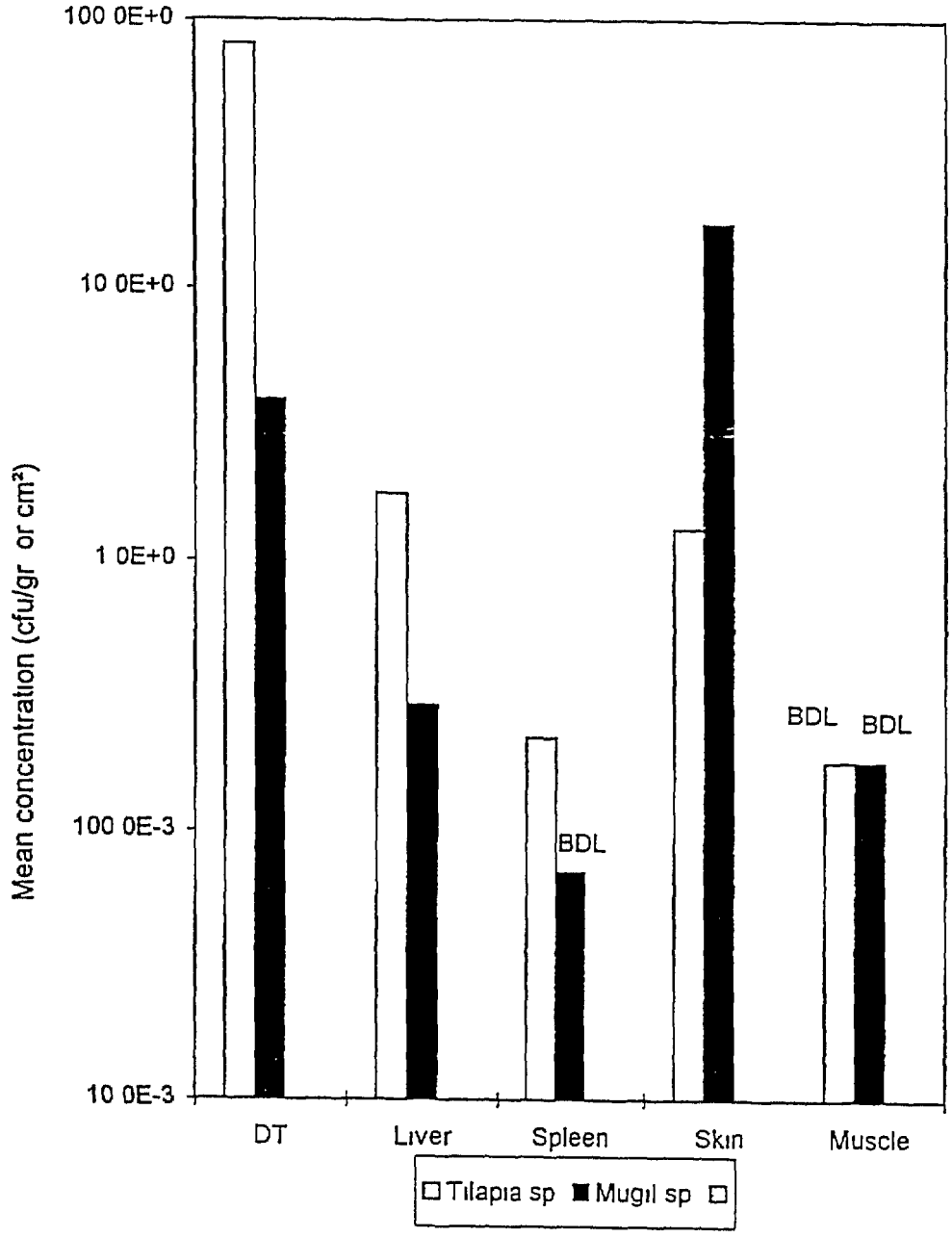

Fig 2: Mean *Fecal coliform* concentration

(Tilapia sp - n=11, Mugil sp - n=9)

* **BDL**-Below Detectable Level

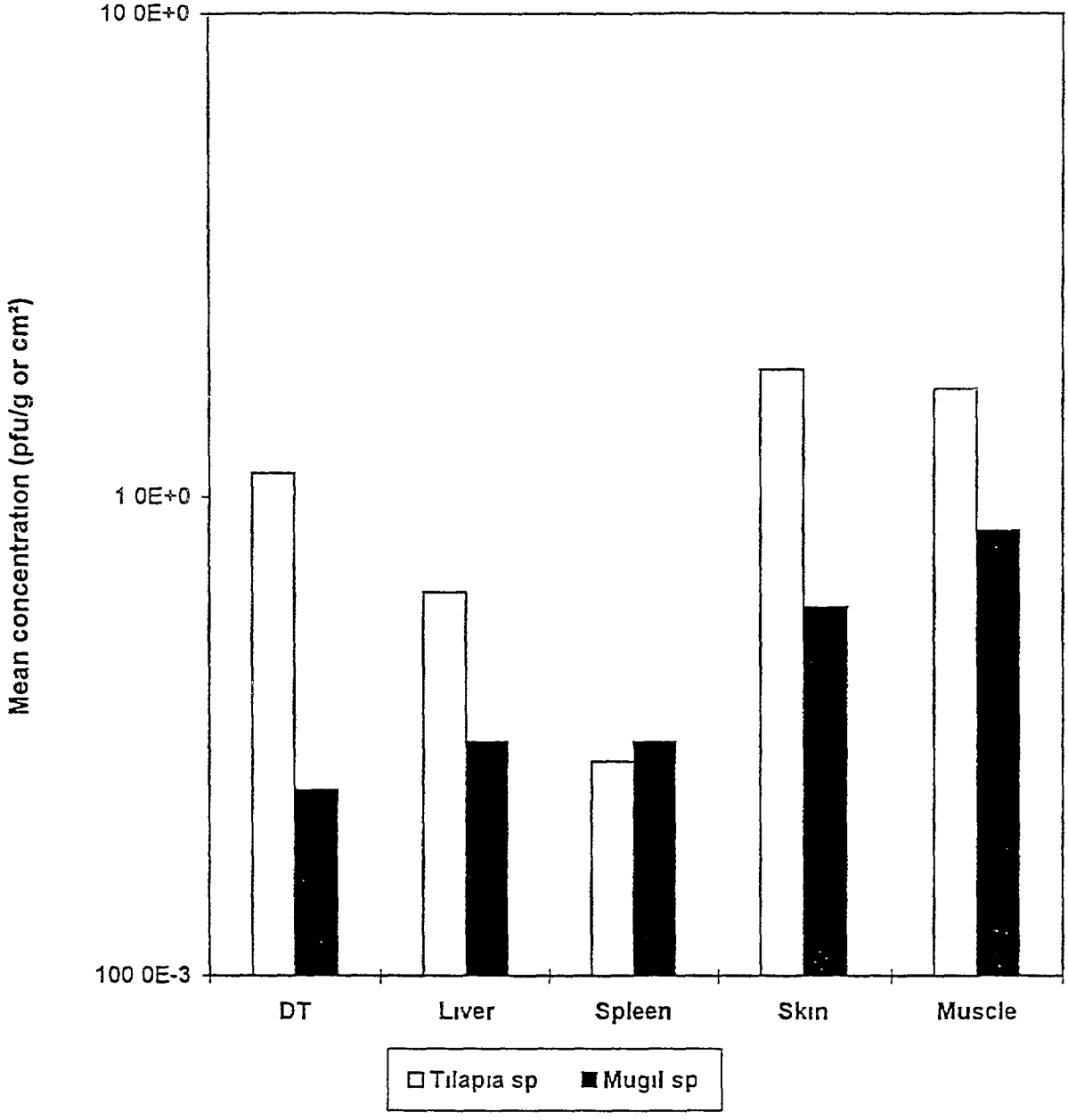

Fig 3 Mean F+ coliphage concentration

(Tilapia sp - n=22, Mugil sp - n=15)

* BDL-Below Detectable Level

DISCUSSION

This study was conducted to determine the microbial and heavy metal levels of fish cultivated and marketed in Israel *Aeromonas* are commonly found in soil and surface waters, where their concentration reflects the trophic state of this environment High concentrations of *Aeromonas* indicate a heavy organic load in the water *Aeromonas* is considered an opportunistic pathogen of fish and humans, causing wound infections, septicemia, cellulitis and diarrhea (MacCracken and Barkely 1972, Ketover et al 1973, Janda et al 1983, Trust 1986)) Of all the microorganisms tested in our study, *Aeromonas* was present in the highest concentration in all fish organs All *Tilapia* and *Mugil* muscle samples in the present study, as in the previous one (Nasser *et al* 1992), were free of *Fecal coliforms* and fit for human consumption, according to the newly recommended Israeli standard for microbial contamination of fish (Israeli Ministry of Health, 1995) The recommended level is 10^3 cfu *Total coliforms*/gr muscle tissue Three common carp used as reference values contained a less than 200 cfu/gr concentration of *Fecal coliforms* Since the ratio between *Fecal coliforms* and *Total coliforms* is < 1 7 (Shuval *et al* 1971) 20 cfu *Fecal coliforms*/gr tissue is equivalent to < 140 *Total coliforms*/gr tissue, i e , did not exceed the permissible edible tissue standard In the present study the highest levels of *Fecal coliforms* were found in the DT Therefore, great caution must be practised when handling and gutting fish either commercially or domestically to avoid cross-contamination of the edible tissue Our results also indicate that the microbial contamination of the edible tissue was within safe limits, and essentially similar to the concentrations obtained in our previous study of ponds supplemented with wastewater

Due to vast world-wide industrialization, heavy metal pollution has become a major public health concern Studies have been conducted on the health hazards posed by heavy metals This problem may be more acute when dealing with sea food, due to the following a) Since most industrial pollutant effluents are generally run through the sewage system, they finally reach natural aquatic ecosystems including rivers and seas, b) Aquatic organisms such as tuna (Peterson *et al* 1973) and molluscs accumulate heavy metals (Bernhard and Andreae 1984) In our study we checked the level of four major heavy metals Hg, Cd, Cu and Zn in fish muscle tissue The concentrations found were low and within the newly recommended Israeli standards, and US, French, Canadian, Danish and German regulations The results indicate that there is an advantage in growing fish in controlled and monitored aquaculture ponds

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