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BIOSTIMULATION AND ALGAL GROWTH KINETICS OF WASTEWATER

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In recent years the consistent occurrence of algal blooms has forced man to inquire seriously into the degree of enrichment of lakes and reservoirs. Algal blooms have caused nuisances, aroused public indignation, and increased the costs of treating water. Public agencies responsible for water quality standards intend to preclude the discharge of growth-stimulating factors into receiving waters.

Municipal and industrial wastewaters are obvious causes of water degradation. Until recently, the biochemical oxygen demand (BOD) of effluents has been a criterion of water quality, and wastewater treatment processes have become more sophisticated only in their ability to oxidize organic matter. Unfortunately, the oxidized forms of nitrogen and phosphorus are also significant stimulants. These materials, coupled with biosynthesized vitamins, amino acids, and growth factors found in biologically treated wastewater, raise serious questions about the suitability of conventional waste treatment. Thus the requirement that effluents be more highly treated has caught the engineering profession without established technology that is of known economic dimension or performance for removal of biostimulants. Furthermore, it has not been demonstrated that wastewater that has been stripped of nitrogen and phosphorus still does not contain factors capable of stimulating algal blooms in receiving water already relatively rich in nitrogen and phosphorus.

The increasing problem of eutrophication and attempts to assess its effects have brought attention to the urgent need of assaying growth-stimulating characteristics of nutrient materials. Parameters are needed for all types of materials that will allow one to predict the effects of these materials on a lake or reservoir.

Limited success has been achieved in quantitatively defining the term eutrophication. As a qualitative concept, it signifies increasing fertility and biological productivity in lakes, reservoirs, sluggish streams, and estuaries, as enrichment of such water advances. But if solutions to the problems associated with productivity are ever to be developed, a quantitative description of degrees of eutrophication must be provided, as well as a capability to predict rates of eutrophication.

Bioassays related to the possible productivity or biostimulatory effects represent a reasonable approach to quantifying the degrees and rates of eutrophication. It is well known that nutrients such as nitrogen and phos-

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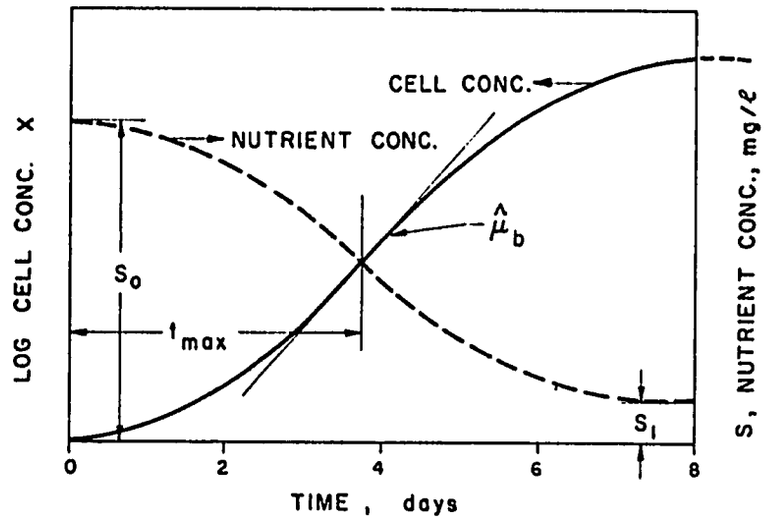

FIGURE 1.—Batch microbial growth curve.

phorus are required for algal growth. However, the question remains: Do nitrogen and phosphorus, either alone or in concert, usually or frequently regulate the growth of aquatic organisms in most receiving waters? To reduce effectively the rate of eutrophication of natural waters, this question must be answered. It is not enough to remove nitrogen and phosphorus from wastewater and hope that this alone will reduce the growth rate of plankton and attached plants.

To develop a realistic waste management program for a body of water excessively enriched by such chemical elements as phosphorus or nitrogen or other biostimulatory materials, it must be possible to accomplish the following objectives:

1. To estimate with reasonable accuracy the background or new level of algae that will be supported by a specific increase in concentration of nutrient.

2. To estimate the concentration of a rate-limiting nutrient that will produce an algal bloom, defined as an increase in specific growth rate.

3. To determine whether more than one factor is operating simultaneously in the processes of cell growth and division, even though one factor appears to be rate-limiting.

These were the objectives of a systematic study initiated at Lake Tahoe under the financial support of FWQA. The initial phase of the study involved the assessment of the biostimulatory potential of wastewater effluents and surface runoff in Lake Tahoe water by the batch assay method. It is the purpose of this paper to evaluate the results of these batch assays of wastewater effluents.

Theory and Rationale

The batch growth culture method follows the concentration of cells in an inoculated flask starting with an initial substrate with all constituents of concern (energy sources, nutrients, and growth factors) in excess except the one that is considered to be growth limiting. Figure 1 is a graph of a typical microbiological growth curve for cells (X) with the concentration-time curve for the rate-limiting nutrient (S) also shown.

There are a variety of ways to describe the characteristics of the batch culture growth curve. One characteristic of the curve that has been suggested is the maximum cell con-

centration, X_t, reached by a particular organism in a specific time with an initial substrate concentration, S_o.

If the concern about nutrient concentrations present in the environment is their effect on growth rate (or pulse) of specific algae, then a better parameter of the curve might be the maximum growth rate obtained with the test substrate. This characteristic is shown in Figure 1 as $\hat{\mu}_b$ (maximum specific batch growth rate). However, it has been established by Michaelis and Menten,[1] Monod,[2] Caperon,[3] Maddux,[4] Williams,[5] Jannasch and Vaccaro,[6] Dugdale,[7] Pearson,[8] and others that the specific growth rate of microorganisms is either a first order or first order-zero order (Michaelis and Menten[1]) function of the rate-limiting nutrient concentration. The equation is

$$\mu = kS \quad \text{or} \quad \mu = \frac{\hat{\mu}S}{K_s + S}$$

where

k = first order rate constant, time^{-1},
μ = specific growth rate $\left[\frac{\text{g cells produced}}{\text{g cells-day}}\right]$, time^{-1},
$\hat{\mu}$ = maximum specific growth rate,
K_s = nutrient concentration at one-half the maximum specific growth rate, and
S = rate-limiting nutrient concentration.

Figure 1 shows that one can determine a maximum growth rate for the batch culture, but that this maximum growth rate occurs at some undetermined but lesser concentration of the rate-limiting nutrient than was in the original sample, S_o. This maximum growth rate should indicate the maximum rate at which the culture can utilize the nutrient level present in the sample. However, it does not define the precise relationship between growth rate and substrate concentration, and an estimate of this is needed. It would be better to assess the biostimulant response under low nutrient conditions. Possibly, the maximum batch growth rate and the time to reach the maximum growth rate can be correlated with the substrate concentration; however, this has yet to be done, and, for a transient system such as the batch culture, it is not a simple task.

From Figure 1 it can be seen that the maximum growth rate is a simple function of the cell concentration, X. This relationship may be expressed as

$$\frac{dX}{dt} = \hat{\mu}_b X$$

where

$\hat{\mu}_b$ = maximum specific growth rate (batch), day^{-1},
X = cell concentration, mg/l, and
t = time in days.

Therefore,

$$\hat{\mu}_b = \frac{\ln (X/X_o)}{t}.$$

Similarly the Michaelis and Menten[1] equation may be written for the batch assay as follows:

$$\hat{\mu}_b = \frac{\hat{\mu}S_o}{K_{s_o} + S_o}$$

where the subscript o refers to the initial nutrient concentration.

Procedure

All samples assayed by the flask culture method, including Lake Tahoe water used for dilution, were sterilized by aseptic filtration through millipore filters (0.45-μ pore size). Glassware employed in the assay was sterilized with dry heat to avoid bacterial contamination.

In making an assay of any nutrient source, the filtered sample first was diluted to the desired concentration with filtered Lake Tahoe water. Then 150 ml of this dilution was placed in each of five sterile 250-ml Erlenmeyer flasks. Cells of *Selenastrum gracile* Reinsch in good physiological condition were centrifuged and resuspended twice

in Lake Tahoe water to minimize nutrient carry-over from the stock culture to the test flasks. The concentration of *S. gracile* in the final suspension was calculated either by measurement of absorbance at 370 mμ or, in later experiments, by counting under a microscope. An equal volume of the suspended cells was then added to each test flask so that the concentration of cells in the 150 ml of liquid was approximately 50 cells/cu mm.

The test alga, *S. gracile*, has many qualities that make it especially suitable for bioassays, one of which is that it has very simple requirements for growth, requiring only the minimum amounts of inorganic ions. As a result, assays with this alga probably do not detect effects of vitamins and other biostimulants.

The flasks were then closed with foam plugs. However, although these plugs had good air diffusion properties, the foam decayed with time and shed debris into the cultures. Therefore, the method was abandoned in favor of loose-fitting plastic beakers inverted over the tops of the flasks.

The inoculated test flasks were placed in a 20°C constant temperature room and incubated on a gently moving (30 cycles/min) shaker table for 5 days. Illumination of approximately 170-ft-c (1,830-lux) intensity was provided by a pair of 30-w fluorescent lamps, 3 ft (0.9 m) in length.

The cell concentration in the test flasks was determined by cell counts at the end of 1, 3, and 5 days during a 5-day period of incubation. After the final counts were completed, a suspended solids (SS) measurement was made on a composite of the liquid in the five replicate flasks.

At the time specified for counting cells, a 10-ml aliquot was taken from each flask and centrifuged for 10 to 15 min at 2,000 rpm. After centrifugation, 8 to 9 ml of the supernatant were removed with a Pasteur pipette and the pellet of cells resuspended in the remaining liquid medium. A drop of the suspension was then put on a hemacytometer for counting under the microscope. Duplicate counts of at least 100 cells were made for each flask, and five replicates were performed for each concentration; thus, 30 counts were made for each concentration of sample tested.

Duplicate counts for each flask were averaged, and the resulting values were then averaged to obtain a mean count for the five replicates constituting the assay. By plotting these data as in Figure 1 and observing the maximum slope of the curve, values of the mean maximum growth rate, $\hat{\mu}_b$, (hereinafter designated as "maximum growth rate") were obtained for each of the test flasks, as well as for the mean of the five replicates. A second value of the maximum growth rate, designated as $\hat{\mu}_{bl}$, was obtained for each assay by determining the log growth equation of best fit to the 1-, 3-, and 5-day cell count data by the method of least squares. In each assay the maximum number of cells attained at the end of the growth period, $\hat{X}_5$, was observed. Then the growth response of *S. gracile* was expressed according to three different parameters: $\hat{\mu}_b$, $\hat{\mu}_{bl}$, and $\hat{X}_5$.

Growth rates, $\hat{\mu}_b$, $\hat{\mu}_{bl}$, and cell concentrations, $\hat{X}_5$, for the first five of the six sources shown in the table were in turn compared statistically, analyzed for seasonal variation, and interpreted in terms of nutrient concentration vs. growth response.

Results and Discussion

For the batch assays reported here, samples were collected only from the South Tahoe Public Utilities District (STPUD) Waste Water Reclamation Plant. The decision to limit sampling to this particular installation was based on the unique opportunity the plant afforded through year-round operation of a multistage treatment process. Thus it was intended to obtain comparative data in relation to seasonal

TABLE I.—Maximum Growth Rates, $\hat{\mu}_b$

Source of Sample	Conc. of Sample (%)	Sample 1S 5/21/68		Sample 2S 6/11/68		Sample 3S 6/25/68		Sample 4S 8/6/68		Sample 5S 10/23/68		Sample 6S 1/18/69	
		Max.* Rate (day^{-1})	Coef. Var. (%)	Max.* Rate (day^{-1})	Coef Var. (%)	Max.* Rate (day^{-1})	Coef. Var. (%)	Max.† Rate (day^{-1})	Coef. Var. (%)	Max.† Rate (day^{-1})	Coef. Var. (%)	Max.† Rate (day^{-1})	Coef. Var. (%)
Raw wastewater	0.1	—		—		—		0.79	52.[illegible]	0.33	42.5	0.34	25.1
	1.0	—		—		0.53	56.4	0.88	14.2	0.67	20.7	0.44	26.6
	10.0	—		—		1.02	0	0.73	18.2	0.92	16.9	0.88	10.5
	50.0	—		—		1.09	26.3	—		—		—	
Primary effluent	0.1	—		—		—		0.76	43.6	0.33	48.8	0.35	24.5
	1.0	—		—		0.44	75.2	0.90	19.5	0.64	13.5	0.50	24.0
	10.0	—		—		1.59	16.0	1.08	21.9	0.86	9.8	0.87	18.1
	50.0	—		—		1.41	60.0	—		—		—	
Secondary effluent	0.1	—		—		—		0.59	16.6	0.15	67.5	0.47	25.1
	1.0	0.42	10.7	0.91	22.1	0.60	19.0	0.77	21.1	0.65	17.1	0.43	26.1
	10.0	0.57	17.4	0.84	35.1	0.87	29.1	0.87	53.6	0.94	9.1	0.98	4.1
	50.0	0.57	56.0	0.64	4.9	0.55	12.8	—		—		—	
Tertiary effluent	0.1	—		—		—		0.55	26.8	0.25	64.1	0.36	31.7
	1.0	0.48	0	0.75	5.9	0.78	54.3	0.81	11.0	0.61	20.2	0.38	23.8
	10.0	0.45	0	0.76	70.6	0.71	19.1	0.19	57.8	1.02	19.5	1.09	5.6
	50.0	0.10	53.5	0.45	92.0	0.49	11.2	—		—		—	

* Based on two replicate assays of grab samples.
† Based on five replicate assays of 24-hr composite samples.

TABLE II.—Maximum Growth Rates, $\hat{\mu}_{bl}$, and Correlation Coefficients

Source of Sample	Conc. of Sample (%)	Sample 4S 8/6/68		Sample 5S 10/23/68		Sample 6S 1/18/69	
		Maximum Rate (day^{-1})	Corr. Coef.	Maximum Rate (day^{-1})	Corr. Coef.	Maximum Rate (day^{-1})	Corr. Coef.
Raw wastewater	0.1	0.380	0.8194	0.215	0.8915	0.182	0.8041
	1.0	0.507	0.9291	0.451	0.9693	0.278	0.8895
	10.0	0.457	0.9648	0.657	0.9814	0.815	0.9947
Primary effluent	0.1	0.419	0.8920	0.223	0.8535	0.166	0.7811
	1.0	0.536	0.9527	0.476	0.9810	0.315	0.9291
	10.0	0.604	0.9367	0.703	0.9765	0.723	0.9804
Secondary effluent	0.1	0.418	0.9592	0.131	0.8274	0.278	0.8750
	1.0	0.519	0.9447	0.451	0.9665	0.307	0.9319
	10.0	0.582	0.9649	0.756	0.9872	0.912	0.9963
Tertiary effluent	0.1	0.392	0.9482	0.131	0.7234	0.199	0.8456
	1.0	0.604	0.9704	0.425	0.9760	0.247	0.9051
	10.0	0.068	0.5759	0.667	0.9716	0.599	0.8969

variations and degree of treatment of wastewater.

The results of 60 separate flask assays of STPUD samples are reported in Tables I, II, and III. The maximum growth rates, $\hat{\mu}_b$ and $\hat{\mu}_{bl}$, and cell concentration, $\hat{X}_5$, attained at the end of 5 days, together with the coefficient of variation for $\hat{\mu}_b$ and $\hat{X}_5$ and the correlation coefficient for $\hat{\mu}_{bl}$, are reported for each of the sample concentrations assayed.

The assessment of growth response using the cell concentration data, $\hat{X}_5$, seems to be more precise than that measured by $\hat{\mu}_b$. The coefficients of variation for assay results in which five replicate assays for each concentration were performed (Sample 4S, 5S, and 6S) were smaller or equivalent for the $\hat{X}_5$ data in 32 of 36 assays.

Highly significant (99 percent confidence level) correlation coefficients were obtained for the least squares fit of the cell concentration data to the log growth equation, the exception being the 10 percent tertiary effluent sample collected on August 6, 1968, which produced a value of 0.5759. This deviation could be attributable to a failure to dechlorinate the sample before introducing it into the assay flask. However, later results have shown that the toxic effect remains after dechlorinating. Data from assays with 0.1 percent sample concentrations, in general, showed the lowest correlation coefficients.

Statistical Comparisons

A split-plot design was used to analyze the results of wastewater assays. As noted above, all values ($\hat{\mu}_b$, $\hat{\mu}_{bl}$, and $\hat{X}_5$) used to express the growth response are directly related, but the reproducibility of each method of calculation is not the same. Tables IV through XI were prepared to show the variation within individual samples and between various concentrations of sample for two of the three methods of expressing growth response. The results for $\hat{\mu}_b$ were not shown because more precise results were obtained with $\hat{\mu}_{bl}$ and $\hat{X}_5$. Theoretically, the differences in response for various samples would depend on varying nutrient concentrations and toxic materials in the sample. These statistical analyses are designed to show whether differences exist between effluents and whether dilution in Lake Tahoe water

TABLE III.—Maximum Cell Concentrations, $\hat{X}_5$, after 5 Days

Source of Sample	Conc. of Sample (%)	Sample 1S 5/21/68		Sample 2S 6/11/68		Sample 3S 6/25/68		Sample 4S 8/6/68		Sample 5S 10/23/68		Sample 6S 1/15/69	
		Cell* Conc. (cells/cu mm)	Coef. Var. (%)	Cell* Conc. (cells/cu mm)	Coef. Var. (%)	Cell* Conc. (cells/cu mm)	Coef. Var. (%)	Cell† Conc. (cells/cu mm)	Coef. Var. (%)	Cell† Conc. (cells/cu mm)	Coef. Var. (%)	Cell† Conc. (cells/cu mm)	Coef. Var. (%)
Raw wastewater	0.1	—		—		—		302	26.5	141	29.1	80	17.4
	1.0	—		—		467	71.5	454	12.5	409	17.1	196	20.5
	10.0	—		—		780	29.9	514	16.2	977	24.8	1,067	16.5
	50.0	—		—		616	53.0	—		—		—	
Primary effluent	0.1	—		—		—		325	13.2	147	41.3	86	17.9
	1.0	—		—		428	76.0	455	10.2	473	6.7	199	11.7
	10.0	—		—		1,350	20.9	1,074	23.1	828	4.9	950	20.8
	50.0	—		—		472	42.4	—		—		—	
Secondary effluent	0.1	—		—		—		366	21.4	103	17.1	131	23.0
	1.0	185	0	410	8.5	413	18.8	436	6.5	408	26.3	182	13.7
	10.0	322	50.4	901	18.5	1,045	12.7	1,005	34.8	1,348	14.2	1,435	6.1
	50.0	454	22.2	689	63.5	330	7.1	—		—		—	
Tertiary effluent	0.1	—		—		—		338	29.9	108	35.1	81	16.2
	1.0	280	5.2	362	3.2	462	14.6	784	51.2	358	11.1	129	19.4
	10.0	357	2.4	670	8.9	1,057	47.0	79	28.2	701	15.3	322	19.8
	50.0	55	12.9	182	37.4	338	12.4	—		—		—	

* Based on two replicate assays of grab samples.
† Based on five replicate assays of 24-hr composite samples.

affects these differences. The results for two samples may not differ because they have the same characteristics or the test is not sensitive enough to determine differences.

Tables showing the detailed statistical comparisons are given only for Sample 4S because of space limitations; however, all results are discussed herein, and tables showing comparisons for Samples 5S and 6S are available.[9] Samples 1S, 2S, and 3S were not discussed because only two replicate assays were performed on these samples, and it was impractical to make a detailed statistical analysis of such limited data.

Table IV presents a summary and statistical comparison of ranked values of the maximum cell concentration, $\hat{X}_5$, observed at the three concentrations (0.1, 1.0, and 10 percent) for the wastewater sample designated Sample 4S in Table I. The underlines bracket the cell concentrations and the percentage concentrations that are not significantly different at a 95 percent confidence limit. The 10 percent tertiary effluent assay shows the apparent effect of not dechlorinating the sample. The $\hat{X}_5$ values tend to show differences in growth response that were not detectable by the $\hat{\mu}_b$ values.

Table V evaluates the $\hat{X}_5$ data of Table IV in relation to concentration of the sample assayed. When compared with the results for $\hat{\mu}_b$, $\hat{X}_5$ is a somewhat more discriminatory measure. Discounting the low value of $\hat{X}_5$ for tertiary effluent (78.8 cells/cu mm), on the basis of chlorine toxicity, Table V shows that at 1 percent concentration tertiary effluent had a significantly greater ability to support algal growth than did either raw, primary, or secondary effluents, and that the latter three do not differ significantly from each other. Furthermore, at the 10 percent concentration, secondary and primary effluents are equally capable of stimulating algal growth that is significantly greater than that of raw wastewater.

Table VI presents a summary and statistical significance comparison of ranked maximum growth rates, $\hat{\mu}_{bt}$, observed at three sample concentrations (0.1, 1.0, and 10 percent) for Sample 4S. The table was prepared by comparing the slopes of the least squares regression analyses at the 95 percent confidence level to determine significant differences. When com-

TABLE IV.—Maximum Cell Concentrations, $\hat{X}_5$, for Sample 4S

Source of Sample	$\hat{X}_5$ (cells/cu mm), Sample Concentration (%), and Statistical Significance. (Underlines Indicate No Significant Difference at 95% Confidence Level.)		
Raw wastewater	302.4 (0.1%)	453.6 (1%)	514.0 (10%)
Primary effluent	324.8 (0.1%)	455.8 (1%)	1,074.4 (10%)
Secondary effluent	366.4 (0.1%)	436.2 (1%)	1,004.8 (10%)
Tertiary effluent	78.8 (10%)	338.0 (0.1%)	783.6 (1%)

TABLE V.—Maximum Cell Concentrations, $\hat{X}_5$, for Sample 4S

Sample Concentration (%)	$\hat{X}_5$ (cells/cu mm), Source of Sample, and Statistical Significance. (Underlines Indicate No Significant Difference at 90% Confidence Level.)			
0.1	302.4*	324.8†	338.0§	366.4‡
1	436.2‡	453.6*	455.8†	783.6§
10	78.8§	514.0*	1,004.8‡	1,074.4†

* Raw wastewater.
† Primary effluent.
‡ Secondary effluent.
§ Tertiary effluent.

TABLE VI.—Maximum Growth Rate, $\hat{\mu}_{bt}$, for Sample 4S

Source of Sample	$\hat{\mu}_{bt}$ (day^{-1}), Sample Concentration (%), and Statistical Significance. (Underlines Indicate No Significant Difference at 95% Confidence Level.)		
Raw wastewater	0.380 (0.1%)	0.457 (10%)	0.507 (1.0%)
Primary effluent	0.419 (0.1%)	0.536 (1.0%)	0.601 (10%)
Secondary effluent	0.418 (0.1%)	0.519 (1.0%)	0.582 (10%)
Tertiary effluent	0.068 (10%)	0.392 (0.1%)	0.601 (1.0%)

TABLE VII.—Maximum Growth Rate, $\hat{\mu}_{bl}$, for Sample 4S

Sample Concentration (%)	$\hat{\mu}_{bl}$ (day⁻¹), Source of Sample, and Statistical Significance. (Underlines Indicate No Significant Difference at 95% Confidence Level.)			
0.1	0.380*	0.392§	0.418‡	0.419†
1	0.507*	0.519‡	0.536†	0.601§
10	0.068§	0.157*	0.582‡	0.601†

* Raw wastewater.
† Primary effluent.
‡ Secondary effluent.
§ Tertiary effluent.

pared with $\hat{\mu}_b$ and $\hat{X}_5$, it was evident that the growth of *S. gracile* as measured by $\hat{\mu}_b$ and $\hat{\mu}_{bl}$ was quite similar, with $\hat{X}_5$ seeming to be the most sensitive of the three methods in assessing differences in growth response.

Table VII presents a summary of the $\hat{\mu}_{bl}$ values similar to Table VI. Inspection of the table reveals that only the 10 percent concentration assays for raw wastewater and tertiary effluent show a significant difference in biostimulatory response. Again, the comparative inability of raw wastewater at 10 percent concentration to support growth is revealed.

A similar comparison, including a statistical assessment, was made of maximum growth rates, $\hat{\mu}_b$ and $\hat{\mu}_{bl}$, and cell concentrations, $\hat{X}_5$, attained at the end of 5 days in flask culture, observed at three sample concentrations (0.1, 1.0, and 10 percent) for each effluent sample collected on October 23, 1968 (Sample 5S). Comparison of the three sample concentrations tested (0.1, 1.0, and 10 percent) for the same wastewater samples revealed that all three methods of expressing growth response show a significant difference in all three sample concentrations for the four effluent samples, which was not observed in Sample 4S (Tables IV and VI). It is likely that these differences were detectable because of improvements in laboratory technique gained from experience in performing assays.

At the 0.1 percent and 1.0 percent sample concentrations for Sample 5S, there was no significant difference in the biostimulatory properties of any of the four types of wastewater effluents. At the 10 percent sample concentrations, there was no significant difference between any of the four types of effluents when measured by the maximum growth rate, $\hat{\mu}_b$. When the growth response was measured by the maximum growth rate, $\hat{\mu}_{bl}$, however, the rate for the 10 percent sample concentration of secondary effluent was significantly greater than the growth rate for 10 percent raw and tertiary effluents but did not differ with the primary effluent response. Differences in biostimulatory response were not detectable for the 10 percent sample concentrations of raw, primary, and tertiary effluents.

Expressing the biostimulatory properties for Sample 5S in terms of maximum cell concentrations, $\hat{X}_5$, did not reveal significant differences in response in the 0.1 percent and 1.0 percent sample concentrations of any of the four types of wastewater effluent samples. At the 10 percent sample concentrations, $\hat{X}_5$ showed the secondary effluent as yielding a significantly different and higher response than any of the other assays at this concentration. Response in the tertiary and primary 10 percent assays did not differ, nor did primary and raw when expressed as $\hat{X}_5$.

The analysis of Sample 6S showed that only the primary effluent assays yielded significantly different growth rates ($\hat{\mu}_b$) at all three sample concentrations. The growth rates, $\hat{\mu}_b$, for raw wastewater, and secondary and tertiary effluents at 0.1 percent and 1.0 percent sample concentrations did not differ statistically. When the growth response for Sample 6S was expressed as the maximum cell concentration, $\hat{X}_5$, and growth rate, $\hat{\mu}_{bl}$, both methods of expressing growth response show the same statistical results for all three sample concentrations for the various samples. Only the 0.1 percent and 1.0 percent sample concentrations of

secondary and tertiary effluents did not differ significantly when the growth response was expressed as $\hat{X}_5$ and $\hat{\mu}_{bl}$.

The statistical comparison of the three samples assayed for each wastewater effluent revealed that of the 10 percent concentrations only the 10 percent tertiary effluent assay showed a different growth rate when the response was expressed as $\hat{\mu}_b$, and this difference was marginal. The comparison for $\hat{X}_5$ values showed only the 10 percent sample concentration assays of secondary and tertiary effluents differing significantly, whereas the comparison of $\hat{\mu}_{bl}$ values shows that at a 10 percent sample concentration, all four wastewater samples gave a significantly different growth response.

A collective examination of the assays for all three sampling dates reveals that statistically significant differences are detected somewhat more easily with the flask assay when the growth response is expressed as $\hat{X}_5$ or $\hat{\mu}_{bl}$. It seems that both methods are about equal in showing differences in growth response, and both should be used in data interpretation. The fact that it is possible to detect differences in growth at various sample concentrations for the four types of samples, and yet difficult to detect differences in response for the four types of samples (raw wastewater and primary, secondary, and tertiary effluent) apparently indicates that a toxic factor is present in effluents regardless of the degree of treatment. This hypothesis is supported by the apparent lack of direct increase in growth rate with increasing sample (nutrient) concentrations as would be expected (at concentrations less than the nutrient excess level) according to theory. Furthermore, an approximately equal growth response was obtained for all types of samples. This is contrary to the theory that response increases with the nutrient concentration in the nutrient-limiting range. As the degree of treatment is increased, a reduction in toxicity would be expected; therefore, a greater response could be obtained in the tertiary effluent assays than for the less refined effluents even though the nutrient concentration in the tertiary assay flasks would be much less than that in the other flasks. However, the possibility of the increased release of bound forms of nutrients plus the production of vitamins and growth factors (biostimulants in general) with increasing degrees of biological treatment cannot be neglected as a factor in increasing the apparent biostimulatory properties of treated effluents.

Pearson *et al.*[10] have shown a toxic effect for domestic wastewater and treatment plant effluents in fish bioassay data. Several thousand bioassays were performed on wastewater samples collected throughout the San Francisco Bay area, and the toxic effect of wastewater was quite pronounced. The assays included samples containing only domestic wastewater and a mixture of domestic and industrial wastes; the toxicity was detectable in all assays. Therefore, the fact that STPUD wastewater is principally domestic in origin would seem to have little bearing on the hypothesis that wastewater could exhibit a toxic effect in the algal assay procedure.

Seasonal Variation

With only three assays to compare, it is difficult to make an analysis of seasonal variation in the wastewater samples; however, several interesting comparisons are presented here for each type of wastewater assayed. Detailed comparisons of the assay results are shown only for the secondary and tertiary effluents because of the greater interest in biostimulatory properties of effluents likely to be discharged to streams and lakes. However, the results for the raw and primary samples are discussed, and detailed comparisons are available.[9]

Secondary Effluent:—A comparison and statistical assessment of maximum

growth rate and cell concentrations observed at all three sample concentrations for the three secondary effluent samples assayed are presented in Tables VIII and IX. Individual comparisons of the growth parameters are also shown in these tables.

As shown for all previous samples, the values of $\hat{\mu}_{bl}$ and $\hat{X}_5$ show more distinct differences in growth response. All $\hat{\mu}_{bl}$ values differed at all concentrations of sample except for the 1.0 percent concentration of the samples collected on August 6 and October 23.

Wastewater having undergone biological treatment processes (secondary effluent) might be expected to show less seasonal variation than raw or primary effluents, provided the treatment plant is designed properly and not greatly underloaded or overloaded during any particular season. The STPUD plant is loaded more heavily during the summer because of the heavy influx of tourists and vacationers. However, the growth response expressed in terms of either of the three parameters does not seem to be related to the season of the year unless it is assumed that an increase in organic loading causes an increase in toxicity of the effluent. Such an effect could contribute to the growth response obtained for the August 6 sample, which was shown to be significantly less than the values for the other two samples at the 10 percent sample concentration when expressed as $\hat{X}_5$ or $\hat{\mu}_{bl}$, but gave the highest response at the 1.0 percent concentration.

Tertiary Effluent:—Tables X and XI show collective and individual comparisons and statistical assessment of the maximum growth rates and cell concentrations observed at the three sample concentrations for the three tertiary effluent samples assayed.

The collective comparisons of $\hat{\mu}_b$ and $\hat{X}_5$ show approximately the same number of values differing; however, the ranking of the various samples differs more in the tertiary effluent assays than any of the effluents previously discussed. In general, the sample concentration and sampling

TABLE VIII.—Maximum Growth Rates, $\hat{\mu}_{bl}$, Corresponding Sampling Date, and Statistical Significance for Secondary Effluent

Sample Concentrations Compared Collectively

Sampling date	10/23/68	1/18/69	1/18/69	8/6/68	10/23/68	8/6/68	8/6/68	10/23/68	1/18/69
Sample concentration (%)	0.1	0.1	1.0	0.1	1.0	1.0	10.0	10.0	10.0
$\hat{\mu}_{bl}$	0.131	0.278	0.307	0.418	0.451	0.519	0.582	0.756	0.912
Underlines show no significant difference at P = 95%									

Sample Concentrations Compared Individually

Sample concentration (%)	0.1			1.0			10.0		
Sampling date	10/23/68	1/18/69	8/6/68	1/18/69	10/23/68	8/6/68	8/6/68	10/23/68	1/18/69
$\hat{\mu}_{bl}$	0.131	0.278	0.418	0.307	0.451	0.519	0.582	0.756	0.912
Underline shows no significant difference at P = 95%									

TABLE IX.—Maximum Cell Concentration, $\hat{X}_b$, Corresponding Sampling Date, and Statistical Significance for Secondary Effluent

Sample Concentrations Compared Collectively

Sampling date	10/23/68	1/18/69	1/18/69	8/6/68	10/23/68	8/6/68	8/6/68	10/23/68	1/18/69
Sample concentration (%)	0.1	0.1	1.0	0.1	1.0	1.0	10.0	10.0	10.0
X_b	102.6	131.0	181.6	366.4	408.0	436.2	1,004.8	1,348.0	1,435.4
Underlines show no significant difference at P = 95%									

Sample Concentrations Compared Individually

Sample concentration (%)	0.1			1.0			10.0		
Sampling date	10/23/68	1/18/69	8/6/68	1/18/69	10/23/68	8/6/68	8/6/68	10/23/68	1/18/69
X_b	102.6	131.0	366.4	181.6	408.0	436.2	1,004.8	1,348.0	1,435.4
Underlines show no significant difference at P = 95%									

date relationships in raw, primary, and secondary effluents followed the same ranking, particularly in values of $\hat{X}_b$ and $\hat{\mu}_b$, but for the tertiary effluent samples the order in which the values appear varied for all three parameters.

Wastewater:—When the values of $\hat{\mu}_b$ for the three raw wastewater samples (August 6, 1968, October 23, 1968, and January 18, 1969) for all sample concentrations were compared collectively, the variation in the values of $\hat{\mu}_b$ within the five replicate assays were of such magnitude that statistically different response rates were difficult to detect. When values of $\hat{\mu}_b$ were compared individually, the assays at the 0.1 percent sample concentration seemed to differ only in the sample collected on August 6, 1968. However, all three samples differed statistically at the 1.0 percent sample concentration, but neither was found to give statistically different growth rates at the 10 percent sample concentration. These results may be explained in part by the fact that the variances for the replicate assays differ considerably from one sample concentration to another and result in different statistical comparison levels. The decreasing growth rate with concentration increase (1.0 to 10 percent) for the sample of August 6, 1968, may also be explained by the apparent toxic effect.

Differences in growth response for $\hat{\mu}_{bl}$ and $\hat{X}_b$ at the various sample concentrations are shown readily by both parameters, and both show essentially the same pattern for division. A ranking of the values of $\hat{\mu}_b$, $\hat{\mu}_{bl}$, and $\hat{X}_b$ revealed that $\hat{\mu}_{bl}$ and $\hat{X}_b$ are in the same order by date and concentration, an exception being the reversal of the values for the 1.0 percent and 10 percent sample concentrations of the August 6, 1968, sample. Also, the ranking of the values of $\hat{\mu}_b$ differ from both $\hat{\mu}_{bl}$ and $\hat{X}_b$. This observation further supports the recommendation that $\hat{\mu}_{bl}$ and $\hat{X}_b$ be used to express the growth response in flask assays.

Primary Effluent:—The collective comparisons of all concentrations again

show that $\hat{\mu}_{bl}$ and $\hat{X}_5$ are superior to $\hat{\mu}_b$ in showing statistically significant differences in growth response. The $\hat{\mu}_{bl}$ values showed a better division in the ranked array than $\hat{X}_5$. The individual comparisons of growth parameters obtained at the three sample concentrations show that only the August 6, 1968, sample differed statistically at the 0.1 percent concentration regardless of the method used to express the growth response. At the 1.0 percent sample concentration, $\hat{\mu}_{bl}$ and $\hat{X}_5$ yielded the same results, showing that samples collected on August 6, and October 23, 1968, did not differ statistically at the 95 percent confidence level, but that the January 18, 1969, sample response for primary effluent differs from the other samples. The $\hat{\mu}_b$ values showed that the October 10 and January 18 samples yielded results that do not differ at the 95 percent significance level.

Comparisons of $\hat{\mu}_b$ and $\hat{\mu}_{bl}$

Because of the different methods of calculating the growth rates, it was necessary to evaluate differences between the two rates on the basis of mean values obtained from assays of samples collected from the same source. Five values of $\hat{\mu}_b$ were obtained for each assay, whereas only one value of $\hat{\mu}_{bl}$ was obtained. This makes it difficult to compare the individual growth rates obtained for each sample. However, statistically significant differences between the mean values of $\hat{\mu}_b$ and $\hat{\mu}_{bl}$ for a series of samples from the same sampling point should illustrate whether or not differences exist between the two values of the growth rate.

A comparison of the mean values of $\hat{\mu}_b$ (0.47 day^{-1}) and $\hat{\mu}_{bl}$ (0.27 day^{-1}) obtained in eight assays of Lake Tahoe water collected at mid-lake yielded a t value of 2.47, which indicates that a significant difference exists between the mean values at the 95 percent probability level. An equivalent number of

TABLE X.—Maximum Growth Rates, $\hat{\mu}_{bl}$, Sampling Date, and Statistical Significance for Tertiary Effluent

Sample Concentrations Compared Collectively

Sampling date	8/6/68	10/23/68	1/18/69	1/18/69	8/6/68	10/23/68	1/18/69	8/6/68	10/23/68
Sample concentration (%)	10.0	0.1	0.1	1.0	0.1	1.0	10.0	1.0	10.0
$\hat{\mu}_{bl}$	0.068	0.131	0.199	0.217	0.392	0.425	0.599	0.601	0.667
Underlines show no significant difference at P = 95%									

Sample Concentrations Compared Individually

Sample concentration (%)	0.1			1.0			10.0		
Sampling date	10/23/68	1/18/69	8/6/68	1/18/69	10/23/68	8/6/68	8/6/68	1/18/69	10/23/68
$\hat{\mu}_{bl}$	0.131	0.199	0.392	0.217	0.425	0.601	0.068	0.599	0.667
Underlines show no significant difference at P = 95%									

TABLE XI.—Maximum Cell Concentration, $\hat{X}_b$, Sampling Date, and Statistical Significance for Tertiary Effluent

Sample Concentrations Compared Collectively

Sampling date	8/6/68	1/18/69	10/23/68	1/18/69	1/18/69	8/6/68	10/23/68	10/23/68	8/6/68
Sample concentration (%)	10.0	0.1	0.1	1.0	10.0	0.1	1.0	10.0	1.0
$\hat{X}_b$	78.8	81.4	107.6	120.2	322.0	338.0	358.4	700.8	783.6
Underlines show no significant difference at P = 95%									

Sample Concentrations Compared Individually

Sample concentration (%)	0.1			1.0			10.0		
Sampling date	1/18/69	10/23/68	8/6/68	1/18/69	10/23/68	8/6/68	8/6/68	1/18/69	10/23/68
$\hat{X}_b$	81.4	107.6	338.0	120.2	358.4	783.6	78.8	322.0	700.8
Underline shows no significant difference at P = 95%									

shore samples also showed a significant difference between the mean values of $\hat{\mu}_b$ (0.43 day^{-1}) and $\hat{\mu}_{bl}$ (0.25 day^{-1}) at the 95 percent probability level.

A comparison of the mean values of $\hat{\mu}_b$ and $\hat{\mu}_{bl}$ obtained in 36 assays of the wastewater and wastewater treatment plant effluent samples ($\hat{\mu}_b = 0.663$ day^{-1}; $\hat{\mu}_{bl} = 0.450$ day^{-1}) showed a significant difference between the means at the 99 percent probability level.

The difference between the two growth rates may be attributed to the averaging effect of the experimental error by the least squares fit of the data and the influence of the 5-day cell counts, which were generally lower than predicted by the growth rate obtained during the first and third days of culture. Both factors tend to decrease the slope of the growth curve making $\hat{\mu}_{bl}$ consistently smaller than $\hat{\mu}_b$. The decline in growth rate during the third and fifth days of culture is discussed in a following section.

Although greater precision in the assay results is obtained when $\hat{\mu}_{bl}$ and $\hat{X}_b$ are used to interpret the data, this does not mean necessarily that more accuracy or sensitivity is attained. It is possible that the wide fluctuation in the response when measured by $\hat{\mu}_b$ indicates the occurrence of an adaptation to an environmental change.

Evaluation of Wastewater Assays

In reviewing the results shown for all types of wastewater treatment plant effluents, it is difficult to arrive at conclusions about seasonal variations when only three samples are available. However, from the limited data available it seems that $\hat{\mu}_{bl}$ and $\hat{X}_b$ yield more discernable results with which to compare algal growth response measurements. All types of wastewater effluents apparently are toxic to algal growth in an assay such as that described in this paper. Similar effects for wastewater effluents have been reported in fish bioassay work. Further evidence of an apparent toxic effect in wastewater effluents is shown in Tables

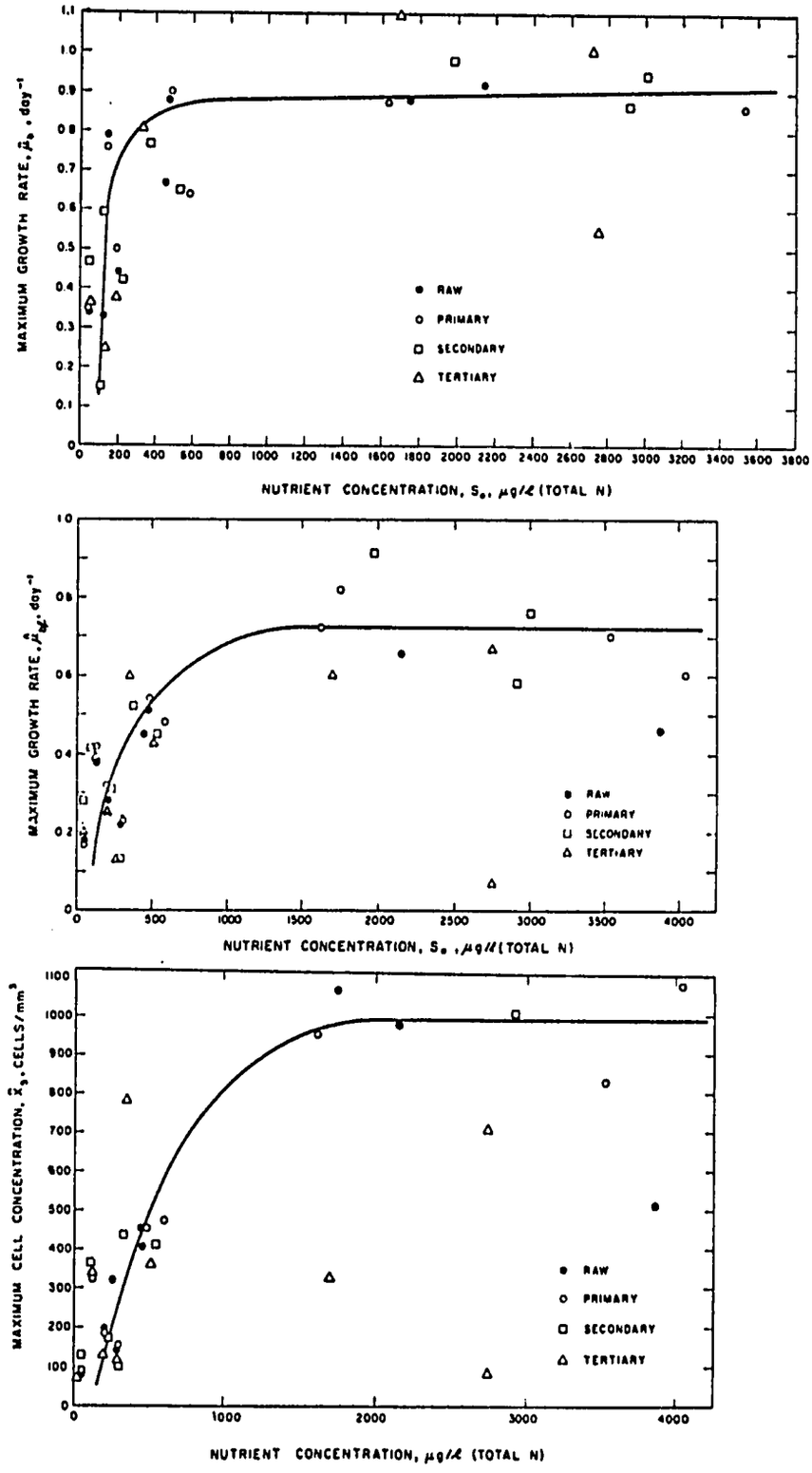

FIGURE 2.—Variation of maximum growth rates and cell concentrations with total nitrogen.

I to III, where response values for 50 percent sample concentration assay are reported for three samples other than the three discussed in detail above. There is a definite decrease in growth response for all three samples of secondary and tertiary effluents when the sample concentration is increased to 50 percent. Only one sample was assayed at the 50 percent concentration for raw wastewater and primary effluent, and there was no decrease in response in these samples. However, the secondary and tertiary effluent assays exhibited significant reductions in response when the sample concentration was increased from 10 to 50 percent. Although the raw and primary effluent assays did not show a decrease in response with an increase in sample concentration, a significant increase in growth rate did not accompany a substantial increase in nutrients.

Nutrient Concentration and Growth Response

Figures 2 and 3 show the observed variation in growth response ($\hat{\mu}_b$, $\hat{\mu}_{bl}$, and $\hat{X}_5$) in relation to the initial concentration (S_o) of total nitrogen and total phosphorus, respectively, using raw wastewater and effluent samples from STPUD in Lake Tahoe water as the nutrient sources.

In Figure 2 the values of $\hat{\mu}_b$ and $\hat{\mu}_{bl}$ seem to increase linearly with the initial concentration of total nitrogen up to about 300 μg N/l. Using the analogy of the Michaelis-Menten kinetics, the nutrient concentration at half the maximum growth rate would be the saturating concentration or the saturation constant (K_{s_0}). It seems that a reasonable estimate of K_{s_0} would be about 150 μg/l of total nitrogen. A similar estimate is obtained from the plot of $\hat{\mu}_{bl}$. An estimation of the yield (cells produced per nitrogen utilized) was not attempted because it was not possible to decide which factor was limiting. It must be remembered that nitrogen and phosphorus concentrations in the different samples do not vary independently. Also, the $\hat{X}_5$ values obtained at higher nutrient concentrations (greater than 1,000 μg N/l) were apparently light- or CO_2-limited, and hence the amount of cells attained was less than expected on the basis of nitrogen concentration.

From Figure 3 it seems that in these wastewater effluents, the initial concentration of phosphorus beyond which *S. gracile* approaches a maximum growth rate is approximately 50 μg/l of total phosphorus. Calculating the saturation coefficient for total phosphorus reveals that K_{s_0} is about 10 μg/l total phosphorus.

The variation of growth response ($\hat{\mu}_b$, $\hat{\mu}_{bl}$, and $\hat{X}_5$) was also observed in relation to the reactive iron concentration. An estimate of K_{s_0} for reactive iron of less than 2 μg Fe/l was obtained. Because the concentration of iron was usually considerably higher, iron was probably not the rate-limiting nutrient in these assays.

These results show that there is a relationship between growth rate and nutrient concentration. However, the relationship is expressed only below certain critical concentrations, approximately 300 μg N/l and 50 μg P/l. Once nutrient concentrations above these levels were attained, the test alga grew at a rate independent of variation in the nutrient concentrations. The estimate of K_{s_0}, 150 μg N/l and 10 μg P/l, gives a relation that allows calculation of the growth response of algae to the presence of the nutrients, nitrogen and phosphorus. Deviations from the expected relationships could be caused by the presence of toxicants as discussed above or such biostimulants as organic materials, vitamin B_{12}, or trace elements.

The flask assay method has been criticized on a theoretical basis because the organisms grow in a continuously changing environment with cells of varying ages that use different amounts of nutrient in different ways as the environment changes. The question

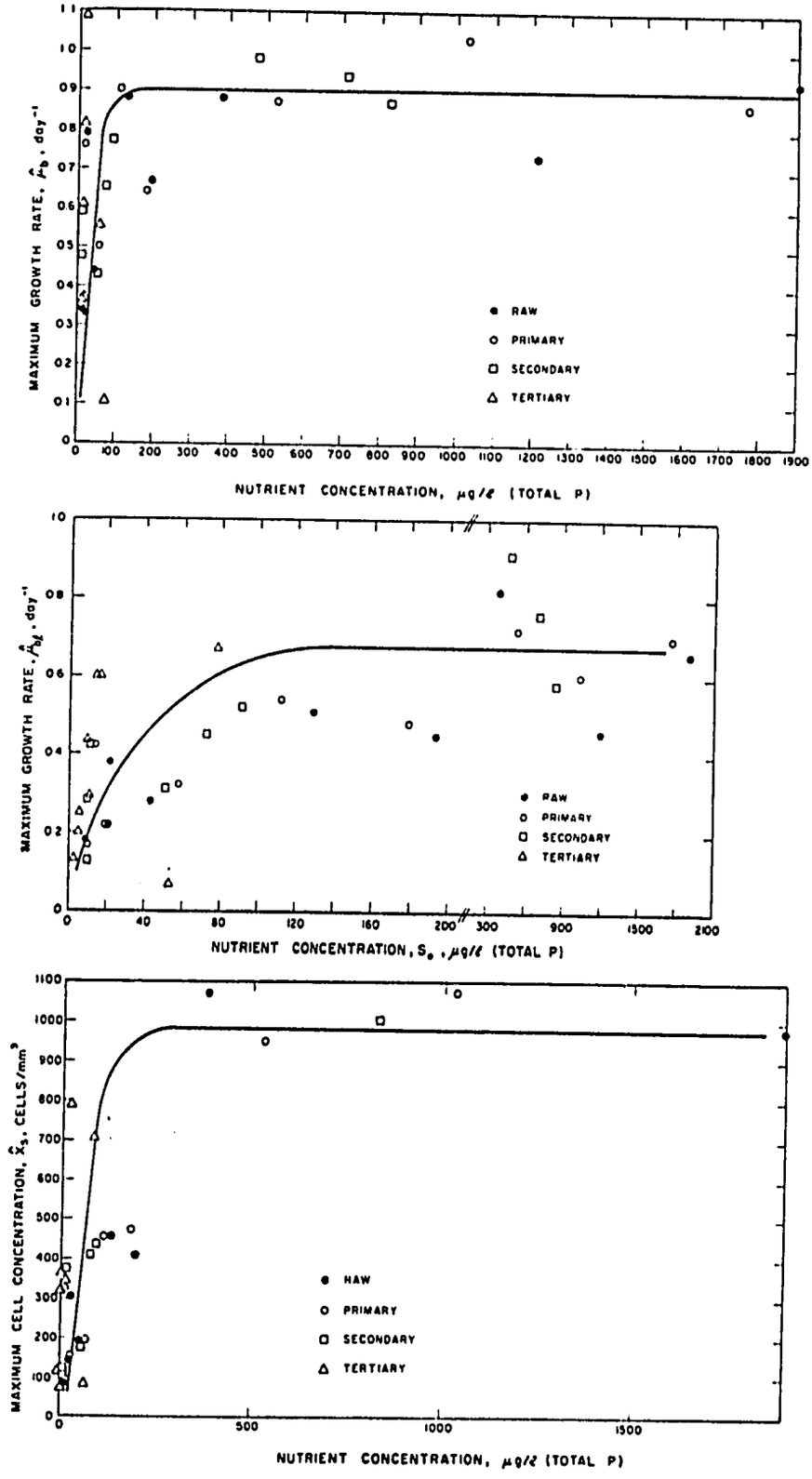

FIGURE 3.—Variation of maximum growth rates and cell concentrations with total phosphorus.

then becomes one of interpretation of results, and it is possible to make statistically valid measurements of growth rates and final cell concentration ($\hat{\mu}_b$, $\hat{\mu}_{bl}$, and $\hat{X}_5$) provided carbon- and light-limiting conditions are not encountered.

Figure 4 shows the effects of carbon-limiting conditions on cell concentration experienced after 3 days of flask culture at low nutrient concentration where small quantities of carbon were available initially. The decline in cell reproduction after the third day could, of course, be attributed partly to nutrient exhaustion. However, in all cases where such a decline occurred, pH values greater than 9.0 occurred in the assay flask, indicating that carbon-limiting conditions contributed to the decline in growth response. Thus it is evident that some method of ventilation to insure a free exchange of air containing CO_2 is essential in flask assays. A similar decline in growth rate would occur in a culture containing high concentrations of nutrients when the cell concentration became great enough to limit light transmission.

From a practical viewpoint the flask assay must depend on the culture of a single species, or some standard mixture of species, of algae. Thus it seems inevitable that the results can never be interpreted directly in terms of outdoor ecosystems involved in eutrophication. Nevertheless, experience with a standard method should, as in the case of BOD, become interpretable in practice.

Applied to the assay of possible inputs to Lake Tahoe, the flask culture method did consistently show that some sources could certainly be expected to increase the productivity of Lake Tahoe water. It also revealed evidence that wastewater contains some factor that reduces the growth response to a level less than might be achieved with nutrients alone as the rate-limiting factor.

The flask assays also showed that the maximum cell concentrations, $\hat{X}_5$, and

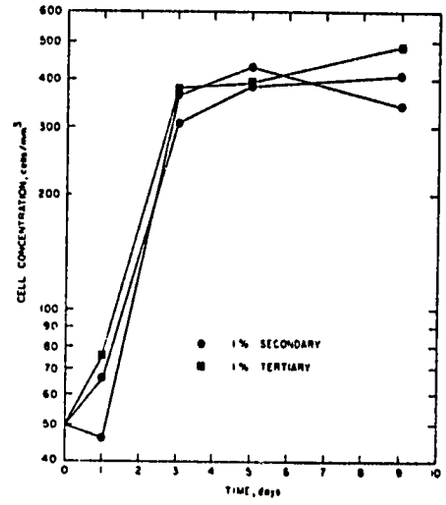

FIGURE 4.—Effects of carbon-limiting conditions.

the growth rate determined by least squares curve fitting, $\hat{\mu}_{bl}$, are more reliable for evaluating growth response than the observed maximum growth rate, $\hat{\mu}_b$, at least at the frequency of cell counting (1, 3, and 5 days) used in the study.

The principal weakness of the method for the purposes of the project was the experimental error inherent in the cell counting technique. This reduced the statistical validity of data to rather broad ranges and therefore limited the sensitivity of the method to changes in growth response. Some cell counting procedure is needed that will limit the variance in data more nearly to that inherent in the response of individual organisms to a given environment. The use of a Coulter counter is planned for future assays at Lake Tahoe. By increasing both the accuracy of single counts, as well as the number of replicate counts, the variance in counts of cell concentration should be greatly reduced.

Conclusions

At the present stage of these investigations the following conclusions may be drawn from results:

Nutrient Relationships

1. All types of wastewater effluents apparently are toxic to algal growth in assays such as those described in this paper.

2. Both growth rates ($\hat{\mu}_b$ and $\hat{\mu}_{bl}$) seem to increase linearly with the initial concentration of total nitrogen, up to approximately 300 μg N/l.

3. The saturation coefficient (K_{s_0}) for nitrogen of approximately 150 μg/l of total nitrogen was obtained using both growth rate parameters, $\hat{\mu}_b$ and $\hat{\mu}_{bl}$.

4. The initial concentration of phosphorus beyond which *S. gracile* approaches a maximum growth rate ($\hat{\mu}_b$ or $\hat{\mu}_{bl}$) is approximately 50 μg/l of total phosphorus.

5. The saturation coefficient (K_{s_0}) for total phosphorus was found to be approximately 10 μg/l of total phosphorus.

6. A K_{s_0} value for reactive iron was found to be less than 2 μg Fe/l.

7. Deviations from the expected growth response predicted by nitrogen and phosphorus concentrations could be caused by the presence of toxicants or of biostimulants such as organic materials, vitamin B_{12}, and trace elements.

8. Assays of possible inputs to Lake Tahoe consistently showed that all sources could be expected to increase the productivity of Lake Tahoe water.

Assay Technique

1. The least squares maximum specific growth rate ($\hat{\mu}_{bl}$) and the maximum cell concentration ($\hat{X}_b$) yield more discernable results with which to compare algal growth response measurements.

2. It is possible to make statistically valid measurements of growth response. Therefore, the usefulness of the assay technique becomes a question of interpretation of the results.

3. Because of the practical necessity of using a single species, or some standard mixture of species, it seems inevitable that direct interpretation in terms of outdoor ecosystems is unattainable. Nevertheless, experience with a standard method should become interpretable in practice.

4. Maximum cell concentrations obtained at higher nutrient concentrations (greater than 1,000 μg N/l) were either light- or CO_2-limited, and the quantity of cells attained was less than expected on the basis of nitrogen concentration.

5. A cell enumerating procedure is needed that will limit the variance in data more nearly to that inherent in the response of individual organism to the assay environment.

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