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THE EFFECTS OF OUTBOARD MOTOR SUBSURFACE EXHAUSTS ON THE GROWTH CHARACTERISTICS OF TWO COMMON FRESHWATER ALGAL SPECIES

Lawrence N. Kuzminski
Charles Fredette

Progress Report for Division of Water Pollution Control,
Massachusetts Water Resources Commission.
Contract Number 15-51451.

ENVIRONMENTAL ENGINEERING
DEPARTMENT OF CIVIL ENGINEERING
UNIVERSITY OF MASSACHUSETTS
AMHERST, MASSACHUSETTS

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Environmental Engineering Program

Department of Civil Engineering

University of Massachusetts

Amherst, Massachusetts

PREFACE

This progress report is the seventh in a series of detailed progress reports prepared for the Division of Water Pollution Control, Massachusetts Water Resources Commission, Contract Number 15-51451, "Effect of Outboard Motor Exhausts on Water Quality and Associated Biota of Small Lakes".

This report focuses on the effects of acute concentrations of exhausts from a 7.5 horsepower outboard engine on two freshwater algal species, Selenastrum capricornutum and Anabaena Flos-aqae. It represents a portion of the research activities by the authors during the period from August 1972 to October 1973. The authors are, respectively, assistant professor and graduate research assistant, Department of Civil Engineering, University of Massachusetts at Amherst.

This report will be brought to the attention of various agencies, organizations, companies, industries, and individuals interested in the preservation of our natural resources.

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ABSTRACT

ABSTRACT

Little is known about the effects of outboard motor subsurface emissions on phytoplankton productivity. The Algal Assay Procedure Bottle Test was used to assess the effects of selected acute initial concentrations of outboard motor subsurface exhaust (OMSE) water on the growth characteristics of pure cultures of two common freshwater phytoplankton organisms, Selenastrum capricornutum and Anabaena flas-aquae.

The inhibition of growth at OMSE concentrations ranging from 1/1600 (one part fuel combusted to 1600 parts culture medium) to 1/13,333 was observed for Selenastrum capricornutum. At concentrations of 1/1600 and 1/3333, death kinetics were observed during the early stages of incubation. However, the inhibitory effects at these concentrations declined with time and culture growth was nearly normal thereafter. At concentrations of 1/11,429, 1/12,308, and 1/13,333 the inhibitory effects did not significantly alter the growth characteristics of Selenastrum.

The inhibition of growth at OMSE concentrations of 1/888, 1/8888, and 1/10,000 was observed for Anabaena flos-aquae. However, the growth characteristics were not significantly altered at these concentrations. No undisputable stimulatory effects were observed with either test alga.

It was concluded, based on the OMSE concentrations used in the assay, that outboard motor usage in the field probably would no inhibit the growth of phytoplankton organisms.

INTRODUCTION

INTRODUCTION

In recent years recreational boating pursuits have attracted the interest of increasing numbers of Americans. Estimates from the Boating Industry Association and the National Association of Engine and Boat Manufacturers document the high and increasing level of boating activities on a national scale (1)*. In 1970, an estimated 44.1 million persons participated in recreational boating. An estimated 7.6 million boats generally powered by outboard motors were in use. These boats comprised over 85 percent of all recreational boats in use that year. An estimated 7.2 million outboard motors were in use, of which 98 percent were of the two-stroke cycle design. These motors consumed an estimated 1.05 billion gallons of gasoline. Furthermore, a comparison of 1968 and 1970 estimates revealed increases in each of the aforementioned categories.

The high level of outboard motor usage has prompted conservationists to express concern over the impact of outboard motor emissions on the inland aquatic environment (2). In response to this concern, substantial research has been directed toward assessing the effects of these emissions on water quality and on the aquatic biota (3). Aspects of water quality which have been examined are tastes, odors, turbidity, and oily surface films. Studies concerned with effects on the aquatic biota have focused on fish, benthic invertebrates, and plankton. However, more information is needed in several vital areas before an assessment of the effects of outboard motor emissions on the inland freshwater environment will be complete.

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*Numbers in parentheses refer to equivalent referenced article.

One particular area which requires further investigation is the consideration of effects on the growth of phytoplankton organisms. The phytoplankton group is a key component of the aquatic biota. These organisms are the most important producer organisms in lentic freshwater ecosystems (4). The productivity of the phytoplankton thus to a large degree governs the potential overall biological productivity of the ecosystem. In addition, the metabolic activities of these organisms indirectly moderate two important chemical characteristics of the water, dissolved oxygen and buffering capacity (4,5). Any alteration in the growth of phytoplankton organisms by outboard motor usage would thus affect both the flow of energy through the aquatic biota and the chemistry of the aquatic environment.

As of January 1, 1974, no published study had provided any substantial information on this topic. In a related study, Lagler et al. (6) considered the effects of outboard motor usage on plankton development. The study was limited in scope and did not differentiate between phytoplankton and zooplankton. In another study, Environmental Engineering, Inc. (1970) compared phytoplankton samples from a lake subjected to long-term outboard motor usage to phytoplankton samples from an undisturbed lake (7). However, only a few samples were collected and the sample analyses were cursory. A current field study sponsored jointly by the U.S. Environmental Protection Agency and the Boating Industry Association is examining the effects of outboard motor usage on phytoplankton and phytoplankton productivity (8). The results of this study are scheduled to be released in the spring of 1974 (9).

The lack of adequate information about this subject can be attributed to difficulties inherent in the study of phytoplankton organisms. Many individual species comprise the phytoplankton population, the species composition varies spatially within a lake and periodically with time, and the species composition varies naturally from one lake to another (10,4). Therefore, in order to characterize the polluted and unpolluted conditions, a field study would entail the collection and analysis of numerous plankton samples. This would require a substantial investment of manpower, facilities, and time. The associated financial commitment would preclude the field study approach for most investigators.

A laboratory study of the problem would entail the culture of phytoplankton organisms under suitable rigorously controlled growth conditions. This approach has been hampered by the lack of standard assay procedures (11). An investigation would thus be required to develop laboratory conditions and assay procedures which would be amenable to the particular organism(s) being studied and the particular problem being examined. This would preclude the laboratory approach for most investigators.

In August of 1971, the National Eutrophication Research Program of the U.S. Environmental Protection Agency released for routine use the Algal Assay Procedure: Bottle Test (AAPBT), (12). This test is a comparative algal growth assay procedure which is useful for determining the differences in the growth characteristics of pure algal cultures under different culture medium conditions but the same constant and controlled environmental conditions. The assay is based on Leibig's law of the minimum, which states that growth is limited by the growth requirement that is present in minimal quantity with respect to the needs of the organism. Although the assay was intended primarily

for eutrophication-related studies, it can readily be adapted for use in a number of other general situations. In particular, the AAPBT can be used to determine the potential effects of materials on algal growth in receiving waters. It is also useful for determining whether or not substances are toxic or inhibitory to algae. It was felt, therefore, that a meaningful laboratory study of the effects of outboard motor subsurface emissions on the growth of phytoplankton organisms would be feasible through the application of the techniques presented in the AAPBT.

The objectives of this study were:

1. To adapt the AAPBT to the problem of evaluating the effects of outboard motor subsurface emissions on the growth characteristics of representative freshwater phytoplankton organisms.

2. To determine, using the adapted AAPBT, whether outboard motor subsurface emissions are stimulatory or inhibitory to the growth of representatives of the dominant types of freshwater phytoplankton organisms which prevail during the months of peak outboard motor usage.

3. To establish the lowest concentration of outboard motor subsurface emissions at which the stimulatory or inhibitory effects become statistically significant with respect to quantitative growth parameters.

4. To relate the aforementioned concentrations to those concentrations which have been established as TL_{50} concentrations for fish and benthic invertebrates; and

5. To predict the effects of outboard motor usage on the growth of phytoplankton organisms in the field through the application of the laboratory results.

LITERATURE REVIEW

LITERATURE REVIEW

Jackivicz and Kuzminski (1) reviewed the factors associated with the operation of two-stroke cycle outboard motors which have raised concern about the impact of outboard motor emissions on the inland aquatic environment. The combustion process is inefficient, and raw fuel as well as its combustion products are emitted to the receiving waters. Also, with many pre-1972 outboard motors, crankcase liquids consisting of compounds present in the raw fuel are drained into the receiving waters. Emissions are expected to contain gases (water vapor, oxides of carbon, oxides of nitrogen, and oxides of sulfur), the hydrocarbons and lead compounds of the unburned fuel mixture, complexed particular lead compounds, hydrocarbons derived from rearrangement, and partial oxidation products. Volatile oil, non-volatile oil, lead, and phenols have been identified as components of outboard motor subsurface exhaust (OMSE) water.

Two-stroke cycle outboard motors operate on a fuel mixture of gasoline and lubricating oil, usually in a ratio of one part oil to fifty parts gasoline. Gasoline consists primarily of hydrocarbons in the 6 carbon to 10 carbon range. Among the more than 100 gasoline components which have been identified are normal alkanes, branched alkanes, cycloalkanes, and alkylbenzenes. Gasoline additives include tetraethyl lead, ethylene dibromide, and ethylene dichloride.

Lubricant oils contain organic compounds which vary in carbon number from 26 to 38. These oils also contain biodegradable organic detergents, unspecified additives, and elements such as zinc, sulfur, and phosphorus. Hobson (1973) reported a popular brand of outboard motor oil, Quicksilver Formula 50, to be essentially free of metal and phosphorus (13).

Jackivicz (10) identified 45 major hydrocarbon compounds in the raw outboard motor fuel mixture. Most of the compounds were in the 6 carbon to 12 carbon range. There were approximately the same number of alkylbenzene compounds as aliphatic compounds. In the same study, the major hydrocarbon compounds identified in OMSE water were in the 7 carbon to 12 carbon range. Similarities between the raw fuel and the OMSE water suggested that the hydrocarbons of the OMSE water were derived from the passage of a portion of unburned fuel through the outboard engine and into the receiving waters.

Bancroft (14) provided information related to the fate of the organic emission compounds in the eceiving waters. After extremely heavy outboard motor usage which produced a fuel to dilution water ratio of 1/6420, a field test site required 12 days to return to initial Total Organic Carbon (TOC) levels. Surface (1/4 inch depth) TOC values were generally greater than subsurface values. This suggested that the rapid disappearance of the organic emission compounds may have been due to the fact that they are relatively insoluble and less dense than water.

Jackivicz and Kuzminski (3) reviewed the effects of outboard motor emissions on the aquatic environment. Several studies have assessed the effects on water quality. These studies have investigated tastes, odors, turbidity, and oily surface films. Other studies have assessed effects on the aquatic biota. These studies have focused on fish, benthic invertebrates, and plankton.

Kuzminski et al. (15) established OMSE toxicity levels for fathead minnows (Pimephales promelas) and bluegills (Lepomis macrochirus) based on static bioassays. The 24-hour, 48-hour, and 96-hour TL_{50} (tolerance limit

for 50 percent survival) OMSE concentrations for fatheat minnows were, respectively, 1/2150, 1/2640, and 1/3130. The corresponding values for bluegills were 1/2260, 1/2260, and 1/2260. The suggested safe OMSE concentrations for fathead minnows and bluegills were, respectively, 1/62,500 and 1/45,500.

Jackivicz (16) established OMSE toxicity levels for two benthic invertebrates based on static bioassays. The average 24-hour, 48-hour, and 96-hour TL_{50} values for adult scuds (Crangonyx gracilis) were, respectively, 1/3340, 1/3400, and 1/3400. The corresponding values for spring-collected dragonfly nymphs (Tetragoneuria cynosura) were 1/540, 1/610, and 1/635.

Lagler et al. (6) compared the plankton development in a pond subjected to outboard motor usage to that of a control pond free from motor usage. The summary of this study is as follows:

> Plankton Production. Plankton samples were collected at weekly intervals from ponds 19 (bass, control) and 22 (bass, motor use) to detect gross differences. It was thought that motor use might affect the production of free-swimming micro-organisms. Each sample consisted of a concentrate from 40 gallons of water strained through a plankton net (Material: Standard Grade Silk bolting cloth No. 20). The water was dipped from near the center of each pond after skimming away any evident surface concentration. The concentrate, plus a few drops of formalin, was allowed to settle for about 18 hours in a centrifuge tube graduated to tenths of a c.c. The volume was read, estimating to the nearest 0.05 c.c. (Table 1).
>
> Although plankton samples are few and have limitations because of the method of collection, it is evident that outboard motor use did not prohibit plankton development and probably did not even inhibit it in any way. At any rate the observed difference of more plankton in the motor use pond can hardly be ascribed to motor use on the basis of those analyses which we made.

Environmental Engineering, Inc. (7) studied the effects of power boat fuel exhausts on the phytoplankton population of a Florida lake. Six phytoplankton samples were analyzed, four from a lake subjected to long-term

TABLE 1

SAMPLES OF THE PLANKTON POPULATIONS IN EXPERIMENTAL PONDS (EXCERPTED FROM LAGLER ET AL., 1950)

Sampling Date 1949	Control Pond 19 Plankton in 40 gals. of water, cc.	Motor Use Pond 22 Plankton in 40 gals. of water, cc.
June 29	.25	.30
July 6	.35	.25
July 13	.30	.35
July 20	.40	.55
July 27	.15	.40
Average	.29	.27

outboard motor usage and two from an undisturbed lake. The phytoplankton organisms in each sample were listed according to taxonomic class, order, or family. The numbers per ml of each taxon was reported. Particular attention was given to two classes of algae which were "reported to be indicative of clean, unpolluted water." These were the Chryptophyceae (olive-green Flagellates) and the Chrysophyceae (yellow-green Flagellates). These organisms were equally abundant in the samples from the stressed lake and the samples from the undisturbed lake. A detailed discussion of the data was not presented. It was concluded that phytoplankton were not affected by exhaust water hydrocarbons.

Weitzel (8) reported that the effects of outboard motor emissions on phytoplankton are being studied extensively in a current investigation sponsored jointly by the U.S. Environmental Protection Agency and the Boating Industry Association. The study entails both field studies and laboratory analyses. The field studies consist of monitoring the phytoplankton populations of stressed lakes and control lakes under natural conditions. The stress level has been set at 1.4 gallons of fuel consumed per one million gallons of lake water per day. This stress level is considered to be three times the maximum outboard engine stress of normal boating activities. Replicate composite phytoplankton samples are being collected as often as twice weekly from three sites in each pond. The species compositions of the samples are being characterized. Data is being composed with diversity indices based on the species compositions. The similarity between stressed and control lake populations is being determined with Jaccard and Sorenson similarity coefficients. The lakes are also being sampled as often as once weekly for chlorophyll (acetone extraction/spectrophotometric method), dry

weight, and C^{14} productivity analyses. In addition, studies on the effects of engine operation on cell structure are being conducted. McCarthy (9) reported that the results of the study will not be released until the spring of 1974.

MATERIALS AND METHODS

MATERIALS AND METHODS

Test Microorganisms

Pure cultures of two test algae were used in the study. These were a green alga, Selenastrum capricornutum, and a blue-green alga, Anabaena Flos-aquae (Appendix A).

Algal samples for the preparation of stock cultures were obtained from:

National Eutrophication Research Program
Pacific Northwest Water Laboratory
Environmental Protection Agency
200 S.W. 35th Street
Corvallis, Oregon 97330

Pure stock cultures of the test algae were maintained in culture flasks containing the AAPBT nutrient medium. Cells from dense stock cultures were inoculated into fresh medium routinely in order to maintain viable cultures. Transfers were usually made at three week intervals.

Growth Conditions

Nutrient Medium

The AAPBT synthetic algal nutrient medium was used during the study. The Macronutrient and Micronutrient compositions of this medium are listed in Table 2. $MgCl_2$ was not available, so 12.17042 mg/l $MgCl_2 \cdot 6H_2O$ was substituted for 5.700 mg/l $MgCl_2$.

Stock solutions of the individual Macronutrient salts were made up at one thousand times the final nutrient medium concentration. The compositions of the six one-liter Macronutrient Salt Stock Solutions are listed in Appendix B, Table B-1. All weighings were done on a single pan Mettler Analytical Balance.

A one-liter Micronutrient Salt Stock Solution was prepared by combining the micronutrient salts at a concentration one thousand times the final

nutrient medium concentration. The final concentration of several micronutrients was in the order of 10^{-3} μ g/l, or 10^{-9} gm/l (Table 2). The preparation of a one-liter stock solution at one thousand times this concentration required the weighing of chemicals in the order of 10^{-6} grams. Since this was not possible, a one-liter stock solution at ten thousand times the final nutrient medium concentration was prepared. The preparation of this solution required weighings in the order of 10^{-5} grams. The desired one-liter Micronutrient Salt Stock Solution was prepared from this solution by dilution. The compositions of these two solutions are listed in Appendix B, Table B-2.

The 6 Macronutrient Salt Stock Solutions and the Micronutrient Salt Stock Solution were stored in clean one-liter glass bottles in a closed wooden cupboard at room temperature. The bottles were stoppered with ground glass tops. Aluminum foil was wrapped around the glass tops and the bottle necks.

Single strength (1x) nutrient medium wsa prepared by pipetting one ml of each of the 7 stock solutions into a one-liter volumetric flask. The flask was then filled to the mark with distilled water. THe nutrient medium was then autoclaved at 15 psi (248°F, 121°C) for 30 minutes. Experimental design during the study necessitated the preparation of double strength (2x) nutrient medium. This was prepared by pipetting two ml of each stock solution into a one-liter volumetric flask and proceeding similarly.

Environmental Factors

The critical environmental factors which needed to be controlled were aeration and pH, temperature, and illumination.

Culture flasks were maintained on two variable-speed Eberbach reciprocating shakers (#6000). The shakers operated continuously at 100

TABLE 2

SYNTHETIC ALGAL NUTRIENT MEDIUM (REAGENT GRADE CHEMICALS IN DISTILLED WATER)

Macronutrients				Micronutrients			
Compound	Concentration (mg/l)	Element	Concentration (mg/l)	Compound	Concentration (µg/l)	Element	Concentration (µg/l)
N_aNO_3	25.500	N	4.200	H_3BO_3	185.520	B	32.460
K_2HPO_4	1.044	P	0.186	$MnCl_2$	264.264	Mn	115.374
$MgCl_2$	5.700	Mg	2.904	$ZnCl_2$	32.709	Zn	15.691
($MgCl_2 \cdot 6H_2O$)	(12.17042)	S	1.911	$CoCl_2$	0.780	Co	0.354
$MgSO_4 \cdot 7H_2O$	14.700	C	2.143	$CuCl_2$	0.009	Cu	0.004
$CaCl_2 \cdot 2H_2O$	4.410	Ca	1.202	$Na_2MoO_4 \cdot 2H_2O$	7.260	Mo	2.878
$NaHCO_3$	15.000	Na	11.001	$FeCl_3$	96.000	Fe	33.051
		K	0.469	$Na_2EDTA \cdot 2H_2O$	300.000		

oscillations per minute. Loose-fitting aluminum foil served as culture closures. This setup facilitated a free gas exchange at the air-water interface. This maintained the pH of the culture media below 8.5 and insured that an adequate supply of CO_2 would be continuously available to the algal cells (12).

Culture flasks were incubated at a temperature of 24 ± 2°C. Temperature control was achieved by conducting the assays in a constant temperature incubation room. A piece of wallboard insulation was installed underneath the flask carrier tray of each Eberbach shaker to insulate the culture flasks from the heat of the shaker motors. At a room temperature of 18°C, an acceptable temperature distribution was achieved. Spot checks of the temperature of culture flasks at random shaker sites during the assays indicated that the temperature distribution remained stable.

Illumination was provided by a ceiling lamp equipped with four 48-inch cool white fluorescent bulbs (ITT F/40 CW). The lamp was suspended from the ceiling of the incubation room and centered over the shaker platform. The light intensity was adjusted by varying the distance between the lamp and the platform. Continuous illumination was provided at 400 foot-candles ± 10 percent for the culture of Selenastrum capricornutum and at 200 foot-candles ± 10 percent for the culture of Anabaena flos-aquae. The light intensities were measured adjacent to the flasks at liquid level with a Sekonic Studio Deluxe Exposure Meter.

Quantitative Growth Parameters

Maximum Specific Growth Rate

The maximum specific growth rate (μ_{max}) is defined to be the highest specific growth rate which is attained during the incubation of a culture.

The specific growth rate is calculated from the following expression:

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1}$$

where μ = specific growth rate

t_1 = initial sampling time

t_2 = final sampling time

x_1 = algal biomass at t_1

x_2 = algal biomass at t_2

The specific growth rate can be calculated from indirect measurements of biomass. The maximum specific growth rate is related to the concentration of the growth-limiting nutrient (12).

Maximum Standing Crop

The maximum standing crop is a measure of the maximum algal biomass concentration which is achieved during the incubation of a culture. For practical purposes, it is achieved when the increase in biomass is less than five percent per day. The maximum standing crop is proportional to the initial amount of the growth-limiting nutrient.

Qualitative Assessments of Growth

Algal Growth Curves

Growth curves were used to qualitatively evaluate and compare changes in the algal cell concentrations of cultures during incubation. An algal growth curve is a plot of the logarithm of cell concentration versus time of incubation.

Time of Achievement of Quantitative Growth Parameters

The incubation times at which cultures attained their μ_{max} and maximum

standing crops were used to qualitatively evaluate and compare the growth characteristics of cultures.

Growth Monitoring Techniques

Growth was monitored during assays in order to give data to allow for the construction of growth curves and the determination of the quantitative growth parameters. The following AAPBT techniques were used:

Gravimetric Analysis

A suitable aliquot of an algal culture was filtered through a tared Millipore type AA filter (0.80 μ pore size) under a vacuum of 0.5 atmospheres. The filters were than rinsed with 50 ml of distilled water, placed in individual paper folders, and dried for several hours at 90°C. The filters were then cooled in a desiccator and weighed. During the filtration process, the filters themselves lose weight. To correct for this loss, two tared blank filters were washed 50 ml of distilled water under a vacuum of 0.5 atmospheres, dried, cooled in a desiccator, and weighed. The average weight loss of the blank filters was added to the final culture filtration weights. All weighings were done on a single pan Mettler Analytical Balance.

Cell Counting

Cell counts during the study were obtained by techniques presented in Standard Methods (17). Sedgwick-Rafter counting chambers and a conventional compound microscope were used. A Whipple micrometer disk was installed in the eyepiece of the microscope. The counting chambers were filled with one ml culture aliquots by the standard technique. The samples were allowed to settle 15 minutes before counting. Neither Selenastrum cells nor Anabaena

cells were observed to rise to the underside of the cover slip during the settling period. Thus, after settling, all cells were at approximately the same focal distance at the bottom of the counting cell.

All counts were obtained at a magnification of 100X using a 10X objective lens and a 10X ocular lens. At this magnification, the area delineated by the entire Whipple grid was one mm^2. The depth of the Sedgwick-Rafter counting cell was one mm. Thus, the plankton sample volume observed in the microscopic field within the boundaries of the Whipple disk was one mm^3, or 10^{-3} ml (17).

Field counting, as opposed to strip counting, was employed. At low cell densities, the entire Whipple grid was used as a field. As culture densities increased during incubation, the field size was reduced to 1/2, 1/4, 1/10, 1/20, and 1/100 of the entire grid. A field size was chosen which would produce a representative count while at the same time allow the count to be conducted within a reasonable amount of time. The time necessary for counting one slide after settling was usually between 10 and 20 minutes.

In most cases, counts of cultures were obtained from one slide and were based on 10 random field counts. In order to obtain representative counts of Anabaena cultures it was sometimes necessary to count more than 10 fields. Such cases occurred when the variability among individual field counts was greater than that normally observed. In all cases, the total number of cells counted was divided by the total volume of culture observed. This value was converted to cells per ml by the appropriate factor.

Fluorometry

A Turner Model 111 fluorometer was used to measure in vivo chlorophyll

fluorescence. The fluorometer was equipped for this purpose with a blue fluorescent lamp (F4T5), a blue primary monochromator (Corning CS 5-60), a red secondary monochromator (Corning CS 2-64), and a red sensitive photomultiplier tube (R-136).

In vivo chlorophyll fluorescence was measured by the AAPBT method. The fluorometer was zeroed with a blank before each sample reading and after each change in sensitivity setting. The culture flask was swirled to insure a homogeneous suspension of algal cells. A 5 ml sample of the culture was then poured into a cuvette. The cuvette was placed in the fluorometer and the fluorescence was read to the nearest 0.5 units. If the reading was less than 15 units, a higher sensitivity setting was used. If the reading was greater than 90 units, a lower sensitivity setting was used. The fluorescence readings were converted to relative fluorescence values based on a factor relating the four sensitivity settings (1X, 3X, 10X, and 30X) to the 30X setting. Thus, readings from the 1X setting were multiplied by 30, readings from the 3X setting were multiplied by 10, and readings from the 10X setting were multiplied by three.

Relative in vivo chlorophyll fluorescence had been demonstrated to be linearly related to both cell per ml and dry weight for Selenastrum capricornutum. The AAPBT presented data which demonstrated an excellent correlation between relative fluorescence and cells per ml during the logarithmic phase of growth. It was recognized that the chlorophyll a content of algal cells is dependent on their physiological condition. It was concluded that, with due consideration to this factor, fluorescence measurements could aid in the evaluation of valid increases in cell populations.[1] Results from the Interlaboratory Precision Test demonstrated a very high

correlation between relative fluorescence and cell mass for Selenastrum (18). The correlation coefficient was 0.964. It was concluded that fluorescence could be used for the indirect determination of cell mass with an accuracy of more than 95 percent.

Culture Flasks

Five hundred ml Erlenmeyer flasks were used. Each flask was numbered and retained its numerical designation throughout the study. Aluminum foil was used for culture closures.

Prior to each assay, the flasks were randomized with respect to the culture medium to be contained in each flask and the shaker site on which each flask would be incubated. Three piles of tags were made, one with flask numbers, a second with shaker site numbers, and a third with culture media. A shaker site and culture medium type were assigned to each flask by chance draws.

Preparation of Glassware

Flasks, bottles, centrifuge tubes, graduated cylinders, and other glassware were washed with detergent and rinsed thoroughly with tap water. The glassware was then washed with a 10 percent solution of hydrochloric acid. The acid wash was followed by five rinses with tap water and five rinses with distilled water. The clean glassware was dried at 105°C. It was then stored on open shelves with the tops covered with aluminum foil. Before use, the glassware was autoclaved at 15 psi for 15 minutes.

Pipettes were allowed to stand in a 10 percent HCl solution for 12 hours or longer. They were than rinsed at least 10 times with tap water in an automatic pipette washer. These rinses were followed by a distilled water rinse. The pipettes were than sterilized overnight in an oven at 170°C.

Preparation of OMSE Water

The outboard motor subsurface exhaust water (OMSE water) was generated in a 500 gallon stainless steel tank (69.5" L x 47" W x 47" D). The tank was filled with a volume of tap water appropriate to the needs of each particular assay (200 gallons or 400 gallons). A 7.5 horsepower 1970 model Kiekhaefer Mercury outboard motor was used to generate the exhaust. For each assay, onegallon of fuel was burned at 1700 ± 100 RPM (trolling speed). The fuel was a mixture of one part Quicksilver Formula 50 lubricating oil to fifty parts Gulf Regular Leaded gasoline. The OMSE water was generated immediately prior to the inoculation of an assay. It was assumed that any chlorine in the tap water would be driven off as gas by the violent agitation caused by the outboard motor propeller.

Preparation of Tap Water

Assay design usually necessitated the use of tap water as a constituent of the Control culture medium. The tap water was collected in an Erlenmeyer flask when the OMSE water tank was filled. The water was dechlorinated by shining ultraviolet light on the flask for a minimum of three hours.

Preparation of Inoculum

Cells from a stock culture were centrifuged and the supernatant was discarded. The sedimented cells were resuspended in distilled water containing 15 mg/l $NaHCO_3$. The cells were again centrifuged and resuspended in fresh $NaHCO_3$ solution. Cells in the resuspension were counted and used for the inoculum.

The initial cell concentration for assay cultures of Selenastrum capricornutum was 10^3 cells per ml. The initial cell concentration for

assay cultures of Anabaena flos-aquae was 50 x 10^3 cells per ml.

Statistical Analyses

Linear regressions were done with a computer program which is in the Statistics file of the public catalog on the UMass time-sharing system. The program, named LinReg X, computes the slope, y-intercept, the correlation coefficient of the regression line.

The student's t-Test was used to compare the quantitative growth parameters of two sets of assay cultures (19). The t-Test analyses were done with two computer programs which are in the statistics file of the public catalog on the UMass time-sharing system. The programs, named TTEST A and TTEST B, compute the means, variances, standard deviations, and t-ratio for two samples. TTEST A was designed for two groups of unpaired data with equal variances. TTEST B was designed for two groups of unpaired data with unequal variances. THe F Test of Variance was used to determine the quality of the variances of two samples (20). The 95 percent level of significance was used in all analyses.

Studies with Selenastrum Capricornutum

Exploratory Assay

The objective of the assay was to provide introductory information about the effects of outboard motor subsurface emissions on the growth characteristics of Selenastrum capricornutum. TL_{50} concentrations for bluegills and fathead minnows were consistently between 1/2000 and 1/3000 (15). It was considered appropriate to utilize a range of OMSE concentrations in the exploratory assay which would include these concentrations. Thus, five Tap Water Control cultures, three cultures with an acute initial OMSE concentration

of 1/6667, three cultures with an acute initial OMSE concentration of 1/3333, and three cultures with an acute initial OMSE concentration of 1/1600 were incubated.

Each culture had the same initial cell concentration and the same concentration of AAPBT nutrients (Table 3). The OMSE water was generated in 200 gallons of tap water immediately prior to the inoculation. The culture medium in each flask also was made up from its constituents immediately prior to the inoculation. One hundred ml of autoclaved double strength (2X) AAPBT nutrient medium were added to each flask with a 100 ml volumetric pipette. The appropriate volumes of OMSE water and dechlorinated tap water were added with TD graduated pipettes. The appropriate volumes of autoclaved distilled water were measured with a 100 ml graduated cylinder. The inoculum was added with a one ml TD pipette graduated at 0.1 ml intervals.

Growth was monitored daily in each culture by the Sedgwick-Rafter cell counting method (the fluorometer was not available). The times at which the cell counts were recorded. The μ_{max}'s were computed directly from the cell count data. The growth curves were also constructed from the cell count data. The maximum standing crops were not measured. It was assumed that acceptable dry weight measurements of the maximum standing crops of cultures in subsequent assays would be obtained since cultures at this stage would have high population densities.

Full-Scale Assays

A series of three full-scale assays were conducted with Selenastrum capricornutum. The objective of the first full-scale assay was to evaluate

TABLE 3

FLASK COMPOSITIONS FOR THE EXPLORATORY ASSAY OF *SELENASTRUM CAPRICORNUTUM*

Flask	Culture Medium	Inoculum	2X Nutrient Medium	Autoclaved Distilled Water	Dechlorinated Tap Water	OMSE Water	Total Volume
2	Tap Water Control	0.3 ml	100 ml	74.7 ml	25 ml	0 ml	200 ml
9	"	"	"	"	"	"	"
10	"	"	"	"	"	"	"
14	"	"	"	"	"	"	"
19	"	"	"	"	"	"	"
1	1/1600 OMSE	0.3 ml	100 ml	74.7 ml	0 ml	25 ml	200 ml
12	"	"	"	"	"	"	"
23	"	"	"	"	"	"	"
7	1/3333 OMSE	0.3 ml	100 ml	87.7 ml	0 ml	12 ml	200 ml
17	"	"	"	"	"	"	"
22	"	"	"	"	"	"	"
5	1/6667 OMSE	0.3 ml	100 ml	93.7 ml	0 ml	6 ml	200 ml
20	"	"	"	"	"	"	"
24	"	"	"	"	"	"	"

the effects of the acute initial OMSE concentrations used in the Exploratory Assay on the growth characteristics of Selenastrum. The objective of the second and third full-scale assays was to determine the highest acute initial OMSE concentrations at which the inhibitory effects of the OMSE water do not significantly alter the growth characteristics of Selenastrum.

The same basic approaches and methods were used during all three full-scale assays. These were as follows:

Five replicates of each culture type were incubated. The OMSE water was generated immediately prior to the inoculation. The culture medium in each flask also was made up from its constituents immediately prior to the inoculation. Each culture received the same volume of inoculum, 100 ml of 2X AAPBT nutrient medium, and had an initial total volume of 200 ml. Thus, every culture had the same initial cell concentration and a culture medium of the same AAPBT nutrient strength. The nutrients were added to each flask with a 100 ml volumetric pipette. The appropriate volumes of OMSE water and dechlorinated tap water were added with TD graduated pipettes. The appropriate volumes of autoclaved distilled water were measured with a 100 ml graduated cylinder. The inoculum was added last, with a one ml TD pipette graduated at 0.1 ml intervals.

Growth was monitored daily in each culture by the measurement of in vivo chlorophyll fluorescence. After the selection of the appropriate fluorometer intensity setting and prior to the measurement of the fluorescence of a sample, the fluorometer was zeroed with a blank which contained a solution identical to the culture medium of the sample. When the fluorescence of a sample exceeded the reliable range of the instrument (i.e., greater than 90 on the 1X intensity setting), an appropriate dilution of the sample was made

with distilled water. Depending on the culture density, a 1/2, 1/5, 1/10, or 1/50 dilution was prepared using TD graduated pipettes. A 1/2 dilution was prepared from 2.5 ml of culture and 2.5 ml of distilled water. A 1/5 dilution was prepared from one ml of culture and four ml of diluent. A 1/10 dilution was prepared from 0.5 ml of culture and 4.5 ml of diluent. A 1/50 dilution was prepared from 0.1 ml of culture and 4.9 ml of diluent.

For each reading, the time of the measurement, the fluorometer intensity setting, the fluorescence value, and the dilution factor (if any) were recorded on a daily data sheet. A sample daily data sheet is presented in Table 4. The fluorescence reading was converted to a relative fluorescence value by the appropriate factor.

For each full-scale assay, the relationship between relative in vivo chlorophyll fluorescence and cell counts was established by counting selected cultures during the assay. Counts of OMSE treatment cultures as well as Control cultures were incorporated into the relationship. Thus, any significant difference in the pigment characteristics of the cells in OMSE treatment cultures would be demonstrated by a distortion of the linearity of the relationship.

Relative fluorescence values were converted to indirect cell counts with the linear regression relationship between the two parameters. The indirect cell counts were used to compute the μ_{max}'s and to construct the growth curves. One growth curve was constructed for each treatment based on the daily averages of that treatment. The growth curve was discontinued when at least one of the five cultures attained its maximum standing crop. The maximum standing crop was sampled when the indirect cell counts increased less than five percent per day. The crop was determined both as the final

TABLE 4

A SAMPLE DAILY DATA SHEET (FROM THE FIRST FULL-SCALE ASSAY OF *SELENASTRUM CAPRICORNUTUM*)

Flask	Nutrient Medium	Time (pm)	Intensity	Reading	Relative Fluoresence	Cells per Ml from Curve	Dilution	Cells per Ml Considering Dilution Factor	Average Cells per Ml for Treatment
2	Tap Control	2:13	1X	43.5	1,305	81,400	1/10	814,000	
5	"	2:18	1X	60.0	1,800	113,000	1/10	1,130,000	
13	"	2:22	1X	39.5	1,185	74,000	1/10	740,000	902,600
14	"	2:28	1X	41.5	1,245	77,400	1/10	774,000	
19	"	2:34	1X	56.0	1,680	105,500	1/10	1,055,000	
1	1/6667 OMSE	2:40	3X	48.0	480	29,900	1/10	299,000	
3	"	2:43	3X	71.5	715	44,800	1/10	448,000	
8	"	2:48	3X	47.5	475	29,600	1/10	296,000	393,000
9	"	2:53	3X	35.5	1,065	66,400	1/10	664,000	
10	"	2:57	3X	41.5	415	25,800	1/10	258,000	

TABLE 4 -- Continued

Flask	Nutrient Medium	Time (pm)	Intensity	Reading	Relative Fluorescence	Cells per Ml from Curve	Dilution	Cells per Ml Considering Dilution Factor	Average Cells per Ml for Treatment
4	1/333 OMSE	3:00	1X	49.5	1,485	93,000	--	93,000	
11	"	3:02	1X	44.5	1,335	83,400	--	83,400	
12	"	3:04	1X	34.0	1,020	63,500	--	63,500	151,980
16	"	3:06	1X	51.0	1,530	96,000	1/2	192,000	
18	"	3:09	1X	87.0	2,610	164,000	1/2	328,800	
6	1/1600 OMSE	3:12	30X	1.5	1.5	62	--		
7	"	3:18	30X	1.5	1.5	62	--		
15	"	3:22	30X	3.0	3.0	155	--		180
17	"	3:25	30X	4.0	4.0	218	--		
20	"	3:29	30X	6.0	6.5	404	--		

indirect cell count and from a dry weight measurement. Dry weights were based on the filtration of two separate 20 ml aliquots.

The days of achievement of the μ_{max} and the maximum standing crop were recorded and compared. T-Test comparisons were done between treatments with respect to the quantitative growth parameters.

Specific details of the methodology of each full-scale assay were as follows:

First full-scale assay. Five replicates each of Tap Water Control cultures, 1/1600 OMSE cultures, 1/3333 OMSE cultures, and 1/6667 OMSE cultures were assayed. The flask compositions and fluorometer blank compositions are presented in Table 5. The Tap Water Control cultures received 25 ml of dechlorinated tap water. Growth in the Control cultures would thus account fot the effects of the highest volume of tap water that OMSE treatment cultures would receive as OMSE water (25 ml in the 1/1600 OMSE cultures). The OMSE water was generated in 200 gallons of tap water.

Second full-scale assay. Five replicates each of Tap Water Control cultures, 1/8000 OMSE cultures, 1/8888 OMSE cultures, and 1/10,000 OMSE cultures were assayed. The OMSE concentrations were acute initial concentrations. The flask compositions and fluorometer blank compositions are presented in Table 6. The Tap Water Control cultures received 10 ml of dechorinated tap water because the culture medium with the highest OMSE concentration in the assay, 1/8000 OMSE, received 10 ml of OMSE water. The OMSE water was generated in 400 gallons of tap water.

Third full-scale assay. Five replicates each of Tap Water Control cultures, 1/11,429 OMSE cultures, 1/12,308 OMSE cultures, and 1/13,333 OMSE

TABLE 5

FLASK COMPOSITIONS AND FLUROMETER BLANK COMPOSITIONS FOR THE FIRST FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM

Flask	Culture Medium	Inoculum	2X Nutrient Medium	Autoclaved Distilled Water	Declorinated Tap Water	OMSE Water	Total Volume
2	Tap Water Control	0.3 ml	100 ml	74.7 ml	25 ml	0 ml	200 ml
5	"	"	"	"	"	"	"
13	"	"	"	"	"	"	"
14	"	"	"	"	"	"	"
19	"	"	"	"	"	"	"
6	1/1600 OMSE	0.3 ml	100 ml	74.7 ml	0 ml	25 ml	200 ml
7	"	"	"	"	"	"	"
15	"	"	"	"	"	"	"
17	"	"	"	"	"	"	"
20	"	"	"	"	"	"	"

TABLE 5 -- Continued

Flask	Culture Medium	Inoculum	2X Nutrient Medium	Autoclaved Distilled Water	Dechlorinated Tap Water	OMSE Water	Total Volume
4	1/3333 OMSE	0.3 ml	100 ml	87.7 ml	0 ml	12 ml	200 ml
11	"	"	"	"	"	"	"
12	"	"	"	"	"	"	"
16	"	"	"	"	"	"	"
18	"	"	"	"	"	"	"
1	1/6667 OMSE	0.3 ml	100 ml	93.7 ml	0 ml	6 ml	200 ml
3	"	"	"	"	"	"	"
8	"	"	"	"	"	"	"
9	"	"	"	"	"	"	"
10	"	"	"	"	"	"	"
TAP	Fluorometer Blank	0.0 ml	100 ml	75.0 ml	25 ml	0 ml	200 ml
1/1600	"	"	"	75.0 ml	0 ml	25 ml	"
1/3333	"	"	"	88.0 ml	"	12 ml	"
1/6667	"	"	"	94.0 ml	"	6 ml	"

TABLE 6

FLASK COMPOSITIONS FOR THE SECOND FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM

Flask	Culture Medium	Inoculum	2X Nutrient Medium	Autoclaved Distilled Water	Dechlorinated Tap Water	OMSE Water	Total Volume
8	Tap Water Control	0.4 ml	100 ml	89.6 ml	10 ml	0 ml	200 ml
11	"	"	"	"	"	"	"
15	"	"	"	"	"	"	"
17	"	"	"	"	"	"	"
18	"	"	"	"	"	"	"
2	1/8000 OMSE	0.4 ml	100 ml	89.6 ml	0 ml	10 ml	200 ml
12	"	"	"	"	"	"	"
16	"	"	"	"	"	"	"
20	"	"	"	"	"	"	"
21	"	"	"	"	"	"	"

TABLE 6 -- Continued

Flask	Culture Medium	Inoculum	2X Nutrient Medium	Autoclaved Distilled Water	Dechlorinated Tap Water	OMSE Water	Total Volume
3	1/8888 OMSE	0.4 ml	100 ml	90.6 ml	0 ml	9 ml	200 ml
5	"	"	"	"	"	"	"
9	"	"	"	"	"	"	"
10	"	"	"	"	"	"	"
19	"	"	"	"	"	"	"
1	1/10,000 OMSE	0.4 ml	100 ml	91.6 ml	0 ml	8 ml	200 ml
4	"	"	"	"	"	"	"
6	"	"	"	"	"	"	"
13	"	"	"	"	"	"	"
14	"	"	"	"	"	"	"
TAP	Fluorometer Blank	0.0 ml	50 ml	45.0 ml	5 ml	0 ml	100 ml
1/8000	"	"	"	45.0 ml	0 ml	5 ml	"
1/8888	"	"	"	45.5 ml	"	4.5 ml	"
1/10,000	"	"	"	46.0 ml	"	4 ml	"

cultures were assayed. The OMSE concentrations were acute initial concentrati
The flask compositions and fluorometer blank compositions are presented in Table 7. The Tap Water Control cultures received 7 ml of dechlorinated tap water because the culture medium with the highest OMSE concentration in the assay, 1/11,429 OMSE, received 7 ml of OMSE water. The OMSE water was generated in 400 gallons of tap water.

Studies with Anabaena flos-aquae

Exploratory Assay

The objectives of this assay were to evaluate the feasibility of applying the approaches and methods used in the study of Selenastrum to the study of Anabaena and to provide introductory information about the effects of a range of OMSE concentrations on the growth characteristics of Anabaena flos-aquae.

It was not known if Anabaena was more or less sensitive to OMSE water than Selenastrum. Therefore it was decided that an appropriate range of acute initial OMSE concentrations would be the middle range used in the study of Selenastrum. Five replicates each of Tap Water Control cultures, 1/8000 OMSE cultures, 1/8888 OMSE cultures, and 1/10,000 OMSE cultures were assayed.

The flask compositions and fluorometer blank compositions are listed in Table 8. The OMSE water was generated in 400 gallons of tap water. The initial total volume of the cultures was 100 ml instead of the 200 ml which had been used in Selenastrum assays. It was felt that the increased agitation in the culture flasks due to this lower initial total volume would hinder the formation of mats and clumps, thereby increasing cell dispersal in the cultures. Consequently, the 2X AAPBT nutrient medium was added to the culture flasks with a 50 ml TD volumetric pipette. The other culture media constituents were

TABLE 7

FLASK COMPOSITIONS FOR THE THIRD FULL-SCALE ASSAY OF *SELENASTRUM CAPRICORNUTUM*

Flask	Culture Medium	Inoculum	2X Nutrient Medium	Autoclaved Distilled Water	Dechlorinated Tap Water	OMSE Water	Total Volume
1	Tap Water Control	0.2 ml	100 ml	92.8 ml	7 ml	0 ml	200 ml
4	"	"	"	"	"	"	"
6	"	"	"	"	"	"	"
18	"	"	"	"	"	"	"
20	"	"	"	"	"	"	"
9	1/11,429 OMSE	0.2 ml	100 ml	92.8 ml	0 ml	7 ml	200 ml
11	"	"	"	"	"	"	"
14	"	"	"	"	"	"	"
15	"	"	"	"	"	"	"
19	"	"	"	"	"	"	"

TABLE 7 -- Continued

Flask	Culture Medium	Inoculum	2X Nutrient Medium	Autoclaved Distilled Water	Dechlorinated Tap Water	OMSE Water	Total Volume
5	1/12,308 OMSE	0.2 ml	100 ml	93.30 ml	0.0 ml	6.50 ml	200 ml
7	"	"	"	"	"	"	"
8	"	"	"	"	"	"	"
10	"	"	"	"	"	"	"
13	"	"	"	"	"	"	"
2	1/13,333 OMSE	0.2 ml	100 ml	93.80 ml	0.0 ml	6.00 ml	200 ml
3	"	"	"	"	"	"	"
12	"	"	"	"	"	"	"
16	"	"	"	"	"	"	"
17	"	"	"	"	"	"	"
TAP	Flurometer Blank	0.0 ml	50 ml	46.50 ml	3.5 ml	0.00 ml	100 ml
1/11,429	"	"	"	46.50 ml	0.0 ml	3.50 ml	"
1/12,308	"	"	"	46.75 ml	"	3.25 ml	"
1/13,333	"	"	"	47.00 ml	"	3.00 ml	"

added by the same techniques used in the Selenastrum studies.

The inoculation procedure was modified. A matted pellet formed during the centrifugations of the conventional AAPBT inoculation procedure. The resuspension of this pellet was difficult, and the degree of cell dispersal in the resuspension was unacceptable. In order to maximize the dispersal of cells in the inoculation stock, the centrifugations and washings were eliminated. A stock culture was counted and, based on this count, the rppropriate volume was pipetted directly from the stock culture to the assay flasks.

It was recognized that valid cell counts would be more difficult to obtain for Anabaena than they had been for Selenastrum. The average cell size was smaller, and the enumeration of individual cells at a magnification of 100X required close observation. Also, due to the formation of filaments and filament aggregates, the cells were not evenly dispersed in the Sedgwick-Rafter counting chamber. In order to obtain valid estimates, cell counts were based on 10 to 20 microscopic fields depending on the density of the culture and variability of field counts.

Growth was monitored daily in each flask by the measurement of in vivo chlorophyll fluorescence. The relationship between relative fluorescence and cell counts was determined by counting selected cultures during the assay. Counts from both Control cultures and OMSE treatment cultures were used to determine the regression line. The μ_{max}'s and the growth curves were to be determined from indirect cell counts.

Evaluation of the Relationship Between Maximum in vivo Chlorophyll Fluorescence and Culture Dry Weight

An experiment was performed to determine if the measurement of in vivo

TABLE 8

FLASK COMPOSITIONS OF THE EXPLORATORY ASSAY OF *ANABAENA FLOS-AQUAE*

Flask	Culture Medium	Inoculum	2X Nutrient Medium	Autoclaved Distilled Water	Dechlorinated Tap Water	OMSE Water	Total Volume
1	Tap Water Control	1.1 ml	50 ml	53.9 ml	5 ml	0.0 ml	100 ml
6	"	"	"	"	"	"	"
9	"	"	"	"	"	"	"
11	"	"	"	"	"	"	"
12	"	"	"	"	"	"	"
2	1/8000 OMSE	1.1 ml	50 ml	43.9 ml	0 ml	5.0 ml	100 ml
5	"	"	"	"	"	"	"
7	"	"	"	"	"	"	"
8	"	"	"	"	"	"	"
21	"	"	"	"	"	"	"

TABLE 8 -- Continued

Flask	Culture Medium	Inoculum	2X Nutrient Medium	Autoclaved Distilled Water	Dechlorinated Tap Water	OMSE Water	Total Volume
3	1/8888 OMSE	1.1 ml	50 ml	44.4 ml	0 ml	4.5 ml	100 ml
4	"	"	"	"	"	"	"
13	"	"	"	"	"	"	"
16	"	"	"	"	"	"	"
20	"	"	"	"	"	"	"
10	1/10,000 OMSE	1.1 ml	50 ml	44.9 ml	0 ml	4.0 ml	100 ml
14	"	"	"	"	"	"	"
15	"	"	"	"	"	"	"
18	"	"	"	"	"	"	"
19	"	"	"	"	"	"	"
Fluoro-meter Blank	Tap Water Control	0.0 ml	50 ml	45.0 ml	5 ml	0.0 ml	100 ml
"	1/8000 OMSE	"	"	45.0 ml	0 ml	5.0 ml	"
"	1/8888 OMSE	"	"	45.5 ml	"	4.5 ml	"
"	1/10,000 OMSE	"	"	46.0 ml	"	4.0 ml	"

chlorophyll fluorescence could be used to monitor the occurrence of the maximum standing crop in cultures of Anabaena. The experiment was designed to determine if, and to what extent, cell growth continued as cultures approached, attained, and surpassed their maximum fluorescences.

Two Tap Water Control cultures, one 1/10,000 OMSE culture, one 1/8888 OMSE culture, and one 1/8000 OMSE culture were incubated. Growth was monitored daily in each culture by the measurement of *in vivo* chlorophyll fluorescence. When the relative fluorescence of each culture approached, attained, and surpassed the maximum value, the dry weight was measured. Dry weights were determined by the Millipore filter method based on the filtration of two 10 ml aliquots.

Full-Scale Assay

The objective of this assay was to assess the effects of the acute initial OMSE concentrations of the Exploratory Assay on the growth characteristics of Anabaena.

Five replicates each of Tap Water Control cultures, 1/8000 OMSE cultures, 1/888 OMSE cultures, and 1/10,000 OMSE cultures were assayed. The flask compositions (Table 9) were essentially the same as those of the Exploratory Assay (Table 8).

Growth was monitored daily by the measurement of *in vivo* chlorophyll fluorescence. The relationship between fluorescence and cell counts was determined for fluorescence values of 500 or less. The Exploratory Assay had indicated that the relationship was linear within this fluorescence range. It had also indicated that the μ_{max}'s of cultures occurred when the fluorescence values were within this range. Counts from both Control cultures

TABLE 9

FLASK COMPOSITIONS AND FLUOROMETER BLANK COMPOSITIONS
FOR THE FULL-SCALE ASSAY OF ANABAENA FLOS-AQUAE

Flask	Culture Medium	2X Nutrients	Inoculum	OMSE Water	Declorinated Tap Water	Autoclaved Distilled Water	Total Volume
1	Tap Control	50 ml	1.0 ml	0.0 ml	5 ml	44.0 ml	100 ml
6	"	"	"	"	"	"	"
9	"	"	"	"	"	"	"
11	"	"	"	"	"	"	"
12	"	"	"	"	"	"	"
10	1/10,000 OMSE	50 ml	1.0 ml	4.0 ml	0 ml	45.0 ml	100 ml
14	"	"	"	"	"	"	"
15	"	"	"	"	"	"	"
18	"	"	"	"	"	"	"
19	"	"	"	"	"	"	"
3	1/8888 OMSE	50 ml	1.0 ml	4.5 ml	0 ml	44.5 ml	100 ml
4	"	"	"	"	"	"	"
13	"	"	"	"	"	"	"
16	"	"	"	"	"	"	"
20	"	"	"	"	"	"	"

TABLE 9 -- Continued

Flask	Culture Medium	2X Nutrients	Inoculum	OMSE Water	Dechlorinated Tap Water	Autoclaved Distilled Water	Total Volume
2	1/8000 OMSE	50 ml	1.0 ml	5.0 ml	0 ml	44.0 ml	100 ml
5	"	"	"	"	"	"	"
7	"	"	"	"	"	"	"
8	"	"	"	"	"	"	"
21	"	"	"	"	"	"	"
Control Blank	--	50 ml	--	0.0 ml	5 ml	45.0 ml	100 ml
1/10,000 Blank	--	"	--	4.0 ml	0 ml	46.0 ml	"
1/8888 Blank	--	"	--	4.5 ml	"	45.5 ml	"
1/8000 Blank	--	"	--	5.0 ml	"	45.0 ml	"

and OMSE treatment cultures were used in the relationship. The linear regression equation was used to convert relative fluorescence values to indirect cell counts.

The μ_{max}'s were computed from indirect cell counts. The growth curves were constructed from the relative fluorescence measurements. The curves were not constructed from indirect cell counts because the relationship with fluorescence was valid only for low fluorescence values. One curve was constructed for each treatment based on the daily averages of the five fluorescence valves. The curve was terminated when at least one of the five cultures attained its maximum fluorescence.

The dry weight was determined for each culture on the day of maximum fluorescence. Dry weights were measured by the Millipore filter method based on the filtration of two separate 10 ml aliquots. Cell counts of a few random cultures were taken on their days of maximum fluorescence. The cell counts were used to compare the growth of the cultures to the typical Anabaena growth pattern presented by the AAPBT. The comparison indicated that all assay cultures would be at their maximum standing crops on day XIII of the assay. The dry weight of each culture was measured on day XIII of the assay. The Millipore filter method was used based on the filtrationof two separate 10 ml aliquots.

T-Test comparisons were done between treatments with respect to the quantitative growth parameters.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Studies with Selenastrum Capricornutum

Exploratory Assay

A plot of the growth curves of each culture produced a crowding of lines which interfered with curve comparisons. However, the growth curves within each treatment exhibited similar trends. It was therefore decided that one representative growth curve could be constructed for each treatment by averaging the daily measurements for that treatment (Figure 1). This approach would also be used in subsequent assays.

The Tap Water Control curve exhibited the characteristic algal growth pattern. The curve was similar to the Control curves of the preliminary studies. The growth curves of the three OMSE treatments demonstrated growth inhibition. Inhibition was manifested by a lengthening of the lag periods and apparent death kinetics during the lag periods. The degree of inhibition increased with the OMSE concentration. The inhibitory effects declined with time and the curves demonstrated an essentially normal growth pattern thereafter. The decline of the inhibitory effects was probably due to one or more of several factors, including the volatilization of the inhibitory compounds, bacterial decomposition of the inhibitory compounds, and acclimatization of the algal cells to the inhibitory compounds.

The inhibition of growth was also manifested by a later attainment of the μ_{max} in the OMSE treatment cultures (Table 10). The μ_{max}'s were attained between days II and III in the Control cultures with one exception. In contrast, the μ_{max}'s were attained between days IV and V in the 1/6667 OMSE cultures, between days V and VI on the average for the 1/3333 OMSE cultures, and after day VIII for the 1/1600 OMSE cultures. These comparisons again

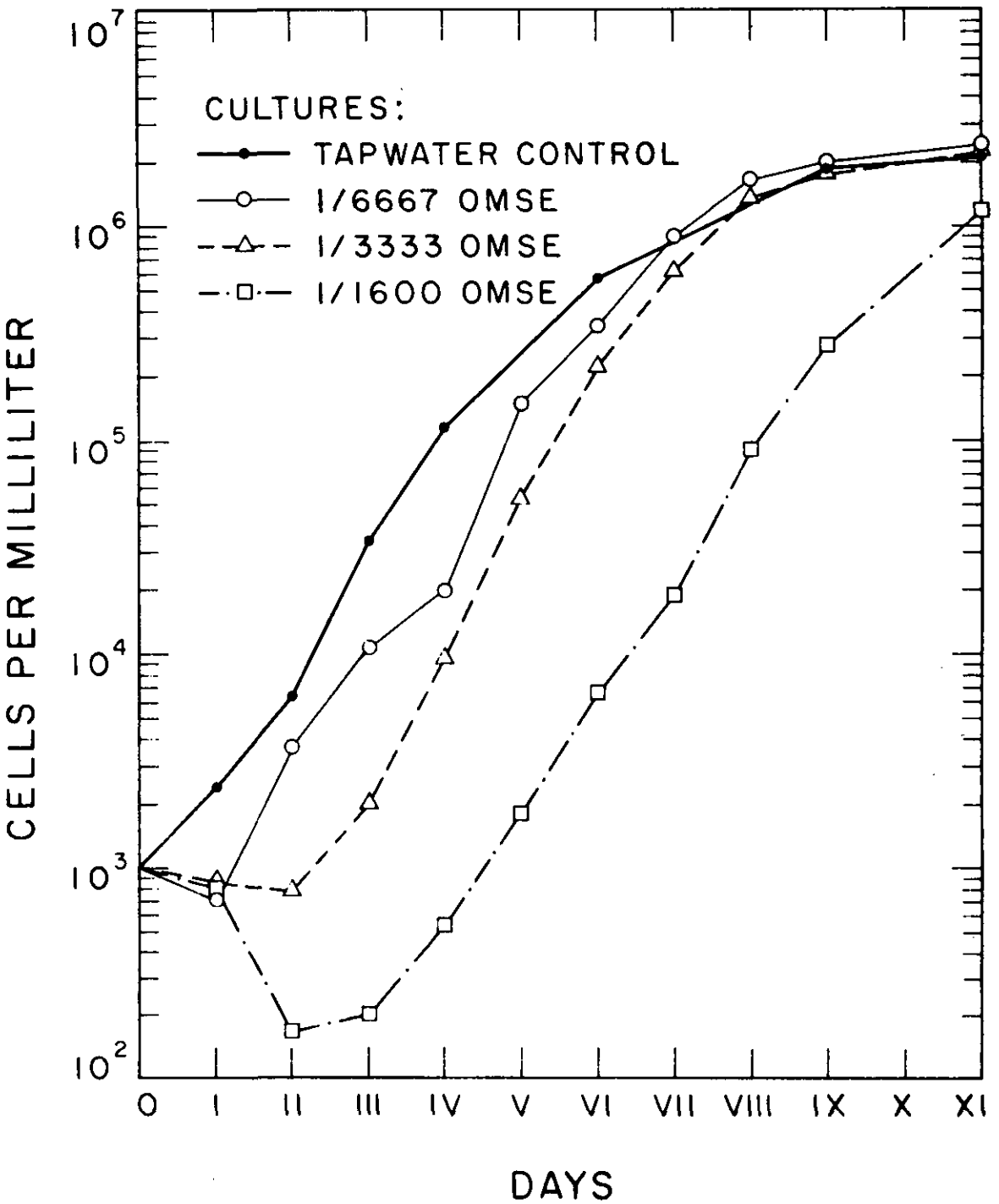

FIGURE 1. GROWTH CURVES FOR THE EXPLORATORY ASSAY OF SELENASTRUM CAPRICORNUTUM

TABLE 10

μ_{MAX} DATA FOR EXPLORATORY BIOASSAY

OF SELENASTRUM CAPRICORNUTUM

Flask	Culture Medium	μ_{max} Days	μ_{max} Counts (Cells/Ml)	μ_{max} ($Days^{-1}$)	Mean μ_{max}
2	Tap Water Control	III IV	19,100 105,600	1.693	
9	"	II III	7,200 36,100	1.648	
10	"	II III	7,900 46,800	1.800	1.712
14	"	II III	6,600 40,400	1.864	
19	"	II III	5,600 25,000	1.554	
1	1/1600 OMSE	VII VIII	7,100 30,700	1.496	
12	"	VII VIII	47,200 321,600	1.958	1.598
23	"	IX X	16,800 78,000	1.341	
7	1/3333 OMSE	VI VII	34,600 114,000	1.251	
17	"	IV V	21,900 134,400	1.763	1.628
22	"	V VI	13,000 82,400	1.871	
5	1/6667 OMSE	IV V	18,300 128,800	1.895	
20	"	IV V	13,300 79,200	1.748	1.904
24	"	IV V	26,500 218,800	2.070	

indicated that the degree of inhibition increased with the OMSE concentration.

The μ_{max}'s of the 1/1600 OMSE cultures and the 1/3333 OMSE cultures were lower than the Control μ_{max}'s on the average (Table 10). Thus, the inhibitory effects apparently remained significant during the exponential phase of growth in these OMSE treatment cultures. The μ_{max}'s of the 1/6667 OMSE cultures were higher on the average than the Control culture μ_{max}'s. The inhibitory effects had apparently worn off completely by the exponential phase in these OMSE treatment cultures. The increased magnitude of the μ_{max}'s of these cultures was possibly due to a stimulatory effect from the OMSE water which had been masked by the inhibitory effects in the 1/1600 and 1/3333 OMSE cultures. However, it was also observed that the general trend of the 1/6667 OMSE culture growth curve was distorted by unusually low cell counts on day IV (Figure 1). Similar distortions were not observed in the other growth curves. Thus, it was likely that these low counts were due to experimental counting error. Day IV marked the beginning of growth at the μ_{max} in the 1/6667 OMSE cultures (Table 10). Since these counts were used in the calculation of the μ_{max}, it was also possible that the μ_{max}'s had been artificially increased through experimental error.

It was decided to repeat these acute initial OMSE concentrations in a full-scale assay employing five replicates of each culture type. Valid statistical comparisons between the quantitative growth parameters would then be possible. These comparisons would permit a more meaningful evluation of the effects of these OMSE concentrations on the growth characteristics of Selenastrum.

Full-Scale Assays

First full-scale assay. The relationship between cell counts and fluorescence is presented in Figure 2. The linear regression had a high

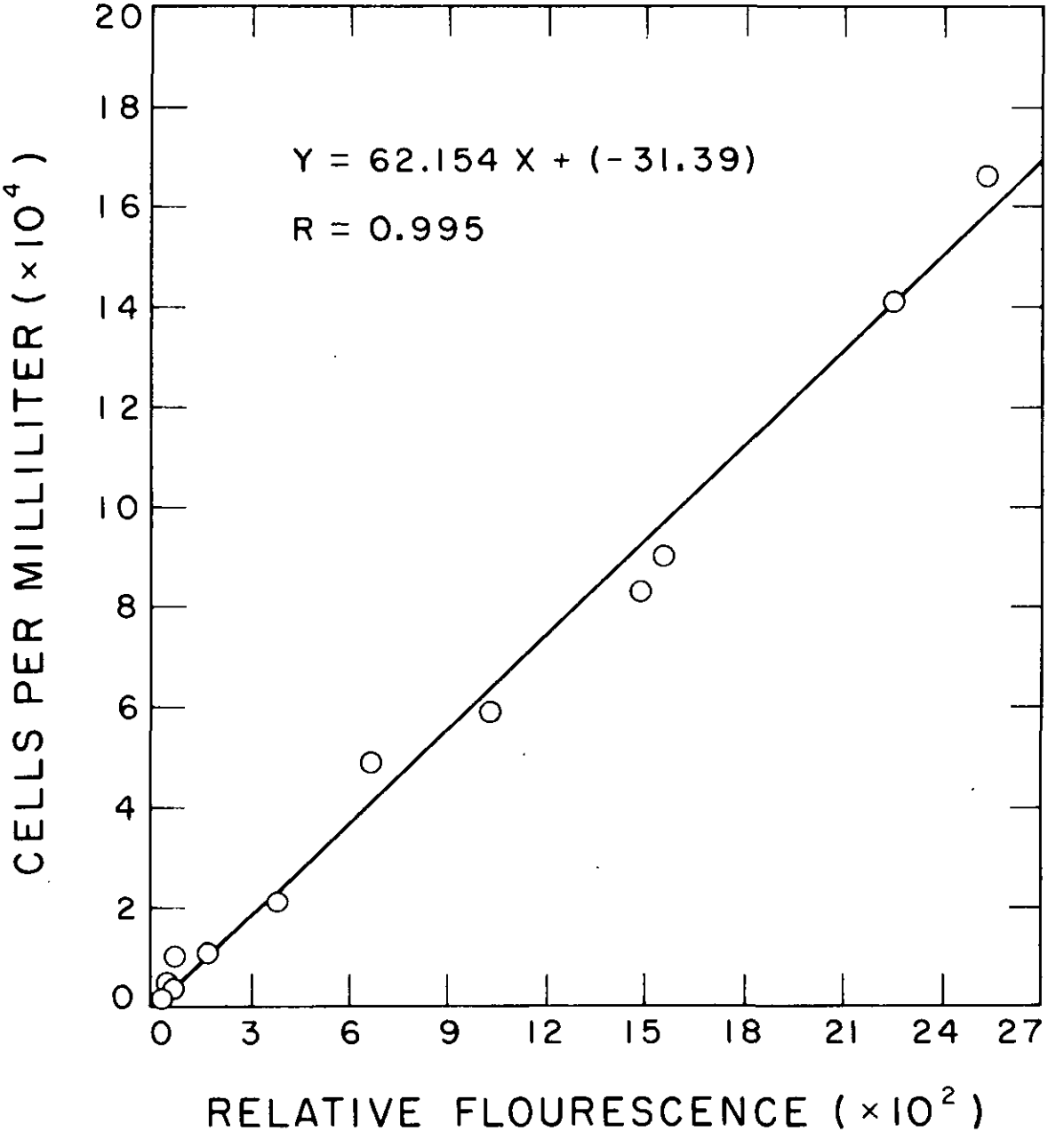

FIGURE 2. RELATIONSHIP BETWEEN CELL COUNTS AND RELATIVE IN VIVO CHLOROPHYLL FLOURESCENCE FOR THE FIRST FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM

correlation coefficient, 0.995. The y-intercept, at -31 cells per ml, was sufficiently close to the origin to give reasonable cell counts at the lowest fluorescence values obtained during the assay. Information about the data points used in the linear regression is presented in Table 11. No positive or negative trend in deviation was apparent for either the OMSE treatment cultures of the Tap Water Control cultures. The deviations were therefore probably due to random variations in the error of the cell count estimates and not to differences in the pigment characteristics of cells in the OMSE treatment cultures.

The growth curves are presented in Figure 3. The Tap Water Control curve exhibited the usual pattern of growth. The lag period was short, lasting only one day beyond the time of inoculation. The steepest slope during the exponential phase was between days II and III, indicating that the μ_{max}'s of these cultures occurred during this time period. This was also apparent from data presented in Table 12. The μ_{max} occurred between days II and III in four of the Control cultures and between days I and II in the fifth culture.

The growth curve for the 1/6667 OMSE cultures also had a one day lag period (Figure 3). However, there was a slower transition to the exponential phase of growth. This was apparent from the relatively mild slope between days II and III. The steepest slope occurred between days III and IV, one day later than it did in the Control curve. It can be seen from Table 12 that the μ_{max} of each of the 1/6667 OMSE cultures occurred between days III and IV. The curve also attained its maximum stationary phase later than the Control curve. This was also apparent from data in Table 12.

TABLE 11

DATA POINTS USED TO DETERMINE THE RELATIONSHIP BETWEEN CELL COUNTS AND RELATIVE *IN VIVO* CHLOROPHYLL FLUORESCENCE FOR THE FIRST FULL SCALE ASSAY OF *SELENASTRUM CAPRICORNUTUM*

Flask	Nutrient Medium	Day	Fluorometer Intensity Setting	Fluorescence Reading	Relative Fluorescence	Cells per Milliliter
12	1/3333 OMSE	II	30X	14.5	14.5	1,000
9	1/6667 OMSE	II	30X	48.5	48.5	4,200
18	1/3333 OMSE	III	30X	54.5	54.5	3,700
2	Tap Control	II	30X	67.5	67.5	9,300
3	1/6667 OMSE	III	10X	33.5	166.5	9,900
2	Tap Control	III	3X	39.0	390.0	20,400
5	Tap Control	VIII	1X	22.0	660.0*	49,200*
12	1/3333 OMSE	VI	1X	34.0	1,020	58,800
2	Tap Control	IV	1X	49.5	1,485	82,400
9	1/6667 OMSE	VII	1X	51.5	1,545**	89,600**
5	Tap Control	IV	1X	75.0	2,250	140,800
19	Tap Control	IV	1X	84.0	2,520	165,600

* 1/50 dilution

** 1/10 dilution

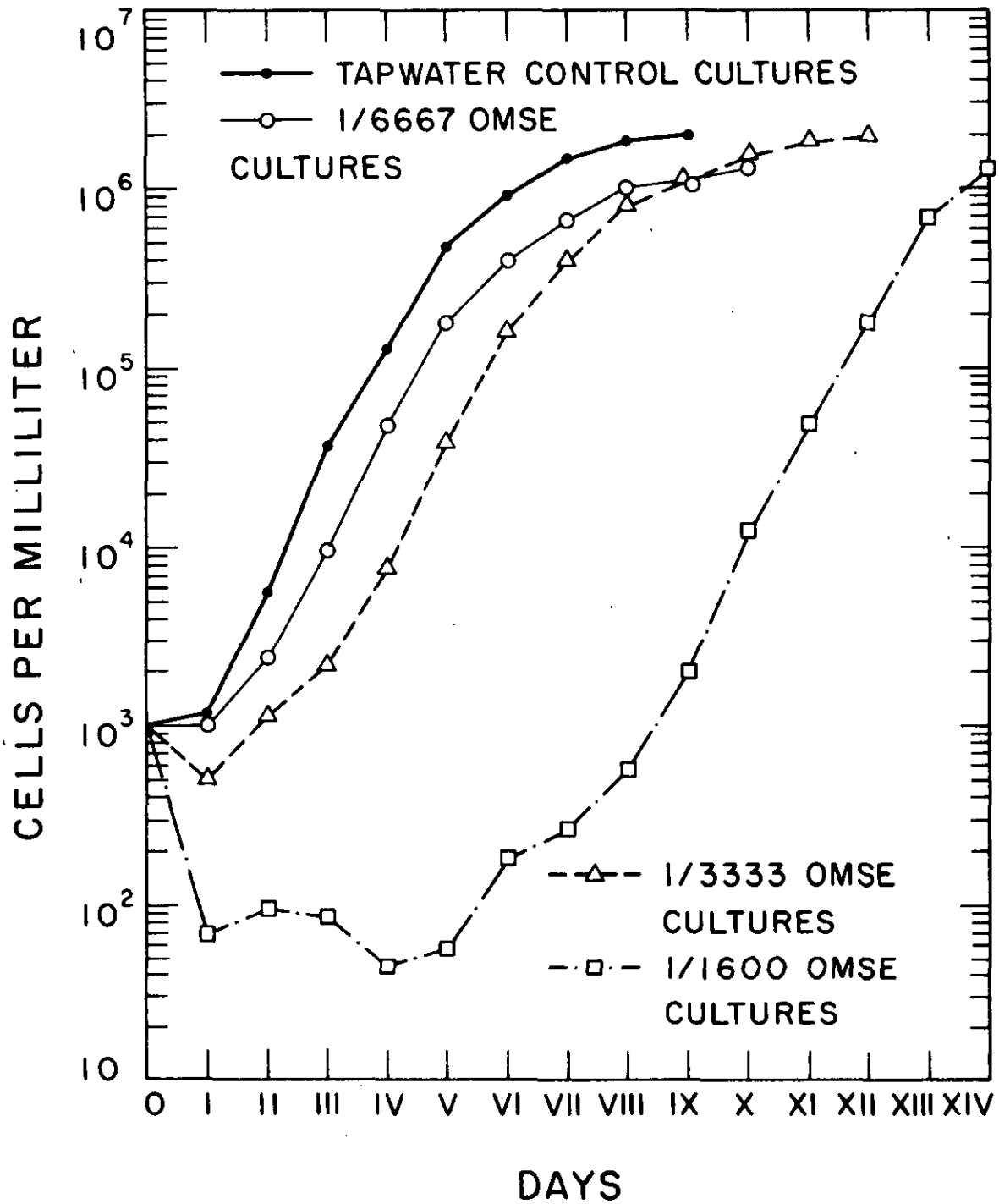

FIGURE 3. GROWTH CURVES FOR THE FIRST FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM

TABLE 12

μ_{MAX} AND MAXIMUM STANDING CROP DATA SUMMARY

FOR THE FIRST FULL-SCALE ASSAY

OF SELENASTRUM CAPRICORNUTUM

Flask	Culture Medium	μ_{max} Days of Occurrence	μ_{max} ($days^{-1}$)	μ_{max} Statistics
2	Tap Control	II-III	1.744	$\bar{X}$ = 1.826
5	"	I-II	1.837	S = 0.099
13	"	II-III	1.903	
14	"	II-III	1.709	95% CL =
19	"	II-III	1.940	1.826 ± 0.198
1	1/6667 OMSE	III-IV	1.453	$\bar{X}$ = 1.614
3	"	III-IV	1.730	S = 0.117
8	"	III-IV	1.673	
9	"	III-IV	1.687	95% CL =
10	"	III-IV	1.529	1.614 ± 0.234
4	1/3333 OMSE	V-VI	1.366	$\bar{X}$ = 1.507
11	"	IV-V	1.459	S = 0.142
12	"	IV-V	1.402	
16	"	IV-V	1.608	95% CL =
18	"	IV-V	1.701	1.507 ± 0.284
6	1/1600 OMSE	X-XI	1.659	$\bar{X}$ = 1.673
7	"	XI-XII	1.534	S = 0.097
15	"	IX-X	1.720	
17	"	IX-X	1.653	95% CL =
20	"	XII-XIII	1.800	1.673 ± 0.194

TABLE 12 -- Continued

Max Crop Days of Occurrence	Max Crop Weight (mg/l)	Max Crop Weight Statistics	Max Crop Counts (10^6 Cells/Ml)	Max Crop Count Statistics
IX	61.5	$\overline{X}$ = 77.80	1.920	$\overline{X}$ = 2.015
IX	83.5	S = 9.28	2.110	S = 0.160
IX	83.0		1.875	
X	82.0	95% CL =	2.250	95% CL =
VIII	79.0	77.8 $\pm$ 18.56	1.920	2.015 + 0.32
X	90.5	$\overline{X}$ = 83.12	1.075	$\overline{X}$ = 1.4754
XII	81.6	S = 15.64	1.525	S = 0.661675
IX	85.5		1.730	
XIV	100.0	95% CL =	2.395	95% CL =
X	58.0	83.12 $\pm$ 31.28	0.652	1.4754 $\pm$ 1.32335
XIV	109.5	$\overline{X}$ = 96.20	2.660	$\overline{X}$ = 2.125
XII	89.5	S = 10.09	2.140	S = 0.495227
XIII	83.0		1.370	
XIII	101.0	95% CL =	2.455	95% CL =
XI	97.0	96.2 + 20.18	2.000	2.125 $\pm$ 0.990454
XVIII	140.5	$\overline{X}$ = 107.60	2.885	$\overline{X}$ = 2.520
XVIII	65.0	S = 29.75	2.165	S = 0.2822
XVI	99.5		2.380	
XVII	102.0	95% CL =	2.460	95% CL =
XVIII	131.0	107.6 $\pm$ 59.5	2.710	2.52 $\pm$ 0.5644

The Control cultures attained their maximum standing crops on day IX on the average whereas the 1/6667 OMSE cultures attained their crops on day XI on the average. The delaying of the exponential phase, the later attainment of the μ_{max}'s, and the later achievement of the maximum standing crops indicated that growth was inhibited by compounds in the OMSE water of the 1/6667 OMSE cultures.

The curve for the 1/3333 OMSE cultures exhibited death kinetics during the first day of incubation (Figure 3). This indicated that at this acute initial concentration, compounds in the OMSE water had a lethal toxic effect on the algal cells during this time period. The cultures quickly recovered from this effect and entered into the exponential phase. The transition to the exponential phase was again more gradual than it had been in the Control cultures. The steepest slope occurred between days IV and V, two days later than the Control cultures and one day later than the 1/6667 OMSE cultures. It can be seen from Table 12 that the μ_{max}'s of four of the 1/333 OMSE cultures occurred between days IV and V. The curve reached its maximum stationary phase later than the Control and 1/6667 OMSE curves. This was again supported by the data in Table 12. The 1/333 OMSE cultures attained their maximum standing crops on the average on day XIII, two days later than the 1/6667 OMSE cultures and four days later than the Control cultures. These observations indicated that the inhibitory effects of the OMSE compounds in the 1/3333 OMSE cultures were similar to but more pronounced than those observed in the 1/6667 OMSE cultures, particularly during the first two days of incubation.

The curve for the 1/1600 OMSE cultures exhibited an even more pronounced lethal toxicity during the early days of the assay (Figure 3). Although the average culture density increased slightly between days I and II, it decreased

between days II and III and again between days III and IV. This indicated that at an acute initial concentration of 1/1600, the OMSE compounds exerted a lethal toxic effect during the first four days of incubation. After day IV, the cultures recovered and gradually entered the exponential phase. The graduate four day transition to the exponential phase indicated that the inhibitory effects were still significant between the fourth and eighth days of incubation.

The steepest slope occurred betwwen days IX and X. The μ_{max}'s actually occurred a day or so later on the average (Table 12), 6 days later than the 1/3333 OMSE cultures, 7 days later than the 1/6667 OMSE cultures, and 8 days later than the Control cultures. The 1/1600 OMSE curve also attained the maximum stationary phase later than the other three curves. The maximum standing crops of these cultures were achieved on the average on day XVII (Table 12). This was four days later than the 1/3333 OMSE cultures, 6 days later than the 1/6667 OMSE cultures, and 8 days later than the Control cultures.

Clearly, this OMSE concentration (1/1600) produced the most severe lethal toxicity and the strongest growth inhibition during the assay. However, these effects declined and the cultures then grew in a pattern that was not drastically different from the growth pattern of the Tap Water Control cultures. Thus, even at an acute initial OMSE concentration as high as 1/1600, the inhibition of the growth of Selenastrum was of finite duration and cells which survived the initial toxicity apparently were not permanently altered in a manner that would be detrimental to their growth. The decline in the inhibitory effects was probably due to one or more of several factors, including the volatilization of the inhibitory compounds, bacterial decomposition of the inhibitory compounds and acclimatization of the algal cells to the inhibitory compounds.

A further evaluation of the duration of the inhibitory effects and the possible alteration of the nutrient medium strength in OMSE treatment cultures was acquired from a quantitative analysis of the μ_{max}'s and the maximum standing crops. A summary of the data is included in Table 12. The t-Test comparisons are presented in Table 13 and a summary of the t-Test conclusions is presented in Table 14.

The average μ_{max} for each of the three OMSE treatments were lower in magnitude than the average μ_{max} of the Tap Water Control cultures. The t-Test comparisons indicated that the μ_{max}'s of each of the OMSE treatments were significantly different from the Control μ_{max}'s. It was apparent that the inhibitory effects of the OMSE compounds were exerted during the exponential phase in each of the three OMSE culture media and contributed to a lowering of the maximum specific growth rates.

The μ_{max}'s of the 1/3333 OMSE cultures were lower than but not significantly different than the μ_{max}'s of the 1/6667 OMSE cultures. The μ_{max}'s of the 1/1600 OMSE cultures, on the other hand, were higher than those of both the 1/6667 OMSE cultures and the 1/3333 OMSE cultures and were significantly different from those of the latter treatment. It was apparent that the difference in the degree of inhibition of two OMSE treatments was not always reflected in a comparison of the relative magnitudes of their μ_{max}'s. This was probably due to the fact that much of the inhibition had declined by the time the μ_{max}'s were achieved. The degree of inhibition during this phase of incubation was therefore probably similar for even widely varying acute initial concentrations. A difference in the magnitudes of the μ_{max}'s of two OMSE treatments was probably due to interactions between the cells and the remaining inhibitory compounds as well as to slight differences in illumination and temperature conditions.

TABLE 13

μ_{MAX} AND MAXIMUM STANDING CROP T-TEST COMPARISONS FOR THE FIRST FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM (95% LEVEL OF SIGNIFICANCE)

Parameter	Treatments Compared	T Ratio	Degrees of Freedom	Critical T Value	Conclusion
μ_{max}	Control, 1/6667	3.081	8	1.860	Different
	Control, 1/3333	4.115	8	1.860	Different
	Control, 1/1600	2.461	8	1.860	Different
	1/6667, 1/3333	1.298	8	1.860	Same
	1/6667, 1/1600	-0.859	8	1.860	Same
	1/3333, 1/1600	-2.149	8	1.860	Different
Maximum Standing Crop Cell Count	Control, 1/6667	1.773	4.46	2.132-2.015	Same
	Control, 1/3333	-0.473	4.82	2.132-2.015	Same
	Control, 1/1600	-3.483	8	1.860	Different
	1/6667, 1/3333	1.758	8	1.860	Same
	1/6667, 1/1600	-3.247	8	1.860	Different
	1/3333, 1/1600	-1.550	8	1.860	Same
Maximum Standing Crop Dry Weight	Control, 1/6667	-0.654	8	1.860	Same
	Control, 1/3333	-3.002	8	1.860	Different
	Control, 1/1600	-2.138	4.77	2.132-2.015	Different
	1/6667, 1/3333	1.571	8	1.860	Same
	1/6667, 1/1600	-1.628	8	1.860	Same
	1/3333, 1/1600	-0.811	4.91	2.132-2.015	Same

TABLE 14

μ_{MAX} AND MAXIMUM STANDING CROP T-TEST COMPARISON CONCLUSIONS FOR THE FIRST FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM (95% LEVEL OF SIGNIFICANCE)

Treatments Compared	μ_{max}	Max Crop Cell Count	Max Crop Dry Weight
Control, 1/6667	Different	Same	Same
Control, 1/3333	Different	Same	Different
Control, 1/1600	Different	Different	Different
1/6667, 1/3333	Same	Same	Same
1/6667, 1/1600	Same	Different	Same
1/3333, 1/1600	Different	Same	Same

The maximum standing crop was measured both as a dry weight and as an indirect cell count. The crop dry weights of the OMSE treatment cultures were greater than those of the Control cultures and increased as the OMSE concentration increased. The crop weights of the 1/6667 OMSE cultures, although greater, were not significantly different from the crop weights of the Control cultures. The crop weights of the 1/3333 OMSE cultures and the 1/1600 OMSE cultures were greater than and significantly different from those of the control cultures. This indicated that a contribution from the OMSE water increased the growth potential of the culture medium. As the acture initial OMSE concentration and hence its stimulatory contribution increased, the growth potential increased. At concentrations of 1/3333 and 1/1600, the potential increased to the point where it was significantly different than that of the Controls. However, it was recognized that the dry weight measurements were determined by a technique that had proven to be insensitive with cultures of lower densities during the preliminary studies. Thus, the confidence necessary for strong conclusions could not be placed in this data.

A slightly different trend was exhibited by the maximum standing crop cell count data. The crop counts of the 1/6667 OMSE cultures, although not significantly different from those of the Control cultures, were lower. This suggested a decrease in the growth potential of the treated flasks. The crop counts for the 1/3333 OMSE cultures were greater than but not significantly different from those of the Control cultures. This indicated a possible increase in the growth potential of the treated cultures, although not as strongly as the dry weight data had. The crop counts of the 1/1600 OMSE cultures were greater than and significantly different from those of the

Control cultures. This indicated that a contribution from the OMSE water had significantly increased the growth potential of the culture medium. The same effect had been suggested by the dry weight data.

It was recognized that the method by which the maximum standing crop cell counts were determined was open to error. The dense cultures were sampled and a dilution, usually 1/50, was prepared from the sample and analyzed fluorometrically. The fluorescence of the sample was then converted to a cell count by the established relationship (Figure 2). The maximum standing crop of the culture was then computed by multiplying the cell count by the initial dilution factor. The dilution error was thus incorporated into the measurement.

In summary, the inhibition of the growth of Selenastrum was observed at acute initial OMSE concentrations of 1/6667, 1/3333, and 1/1600. Lethal toxicity was exerted for one day at a concentration of 1/3333 and for four days at a concentration of 1/1600. The inhibitory effects declined with time in all three OMSE culture media, and the cells subsequently grow in an apparently normal pattern. The inhigibory compounds exerted an effect during the exponential phase, however, and the μ_{max}'s of the treated cultures were lower than and significantly different from the μ_{max}'s of the Control cultures. The maximum standing crop data suggested that the growth potential of the treated cultures, particularly at an OMSE concentration of 1/1600 was increased by a contribution from the OMSE water.

Both the inhibitory effect and its lowering of the μ_{max} and the stimulatory effect as evidenced by the raising of the maximum standing crop warranted further investigation. It was speculated that the stimulatory effect, if valid, was due to the presence of a non-volatile algal nutrient

in the OMSE water which supplemented the limiting nutrient of the AAPBT medium. Its influence would have been exerted during the exponential phase and probably was masked by the inhibitory effects. If the presence of an algal nutrient in the OMSE water were to be confirmed, it would indicate that outboard motor usage would contribute to an increase in the phytoplankton of natural waters. Although an acute OMSE concentration would not be experienced in the field, chronic lower concentrations due to regular outboard motor usage might presumably result in a concentration of the postulated nutrient equivalent to that present in the 1/1600 OMSE cultures.

It was recognized that a continuous flow chemostat assay would be more appropriate for examining this problem than the AAPBT. It was also recognized that the use of the AAPBT would dictate the preliminary development of a reliable technique for measuring the maximum standing crop. Based on these considerations, it was decided not to examine this aspect of the problem.

The inhibitory effects of the OMSE compounds also warranted further investigation. If significant inhibitory effects were observed at concentrations that could be regularly achieved in the field, outboard motor usage would contribute to a decrease in the phytoplankton of natural waters. It was decided to determine the acute initial OMSE concentrations at which the inhibitory effects were reduced to the point that they did not significantly alter the growth characteristics of Selenastrum. In accordance with this objective, it was decided to assess the effects of acute initial OMSE concentrations approximating 1/8000, 1/9000, and 1/10,000 on the growth of Selenastrum in a full-scale assay.

Second full-scale assay. The relationship between cell counts and fluorescence is presented in Figure 4. It was similar to the relationship of the First Full-Scale Assay (Figure 2). The correlation coefficient, 0.992, again was high. The intercept, at 250 cells per ml, was sufficiently close to the origin to produce reasonable cell counts at the lowest fluorescence values obtained during the assay. The data points used in the regression are presented in Table 15. Again, deviations from the regression line were random for both OMSE treatment and Control cultures and probably were due to cell count estimate errors.

The growth curves are presented in Figure 5. The Tap Water Control curve exhibited the characteristic pattern. The lag period lasted one day. The steepest slope during the exponential phase occurred between days II and III. All five Control cultures attained their μ_{max}'s between days II and III (Table 16).

The growth curves of the OMSE treatment cultures exhibited growth inhibition which was similar to but not as pronounced as the inhibition exhibited by the OMSE treatment cultures of the First Full-Scale Assay. No death kinetics were apparent during the lag phases. This was consistent with the fact that death kinetics were not observed at an acute initial OMSE concentration of 1/6667 during the First Full-Scale Assay. The curve for the 1/8888 OMSE cultures and the curve for the 1/8888 OMSE cultures were similar. The lag phases were not lengthened. The transitions from the lag phase to the exponential phase were more gradual than the transition exhibited by the Control curve. The steepest slope for both curves occurred between days III and IV, one day later than the Control curve. The μ_{max} occurred between days III and IV on the average for both these culture media (Table 16).

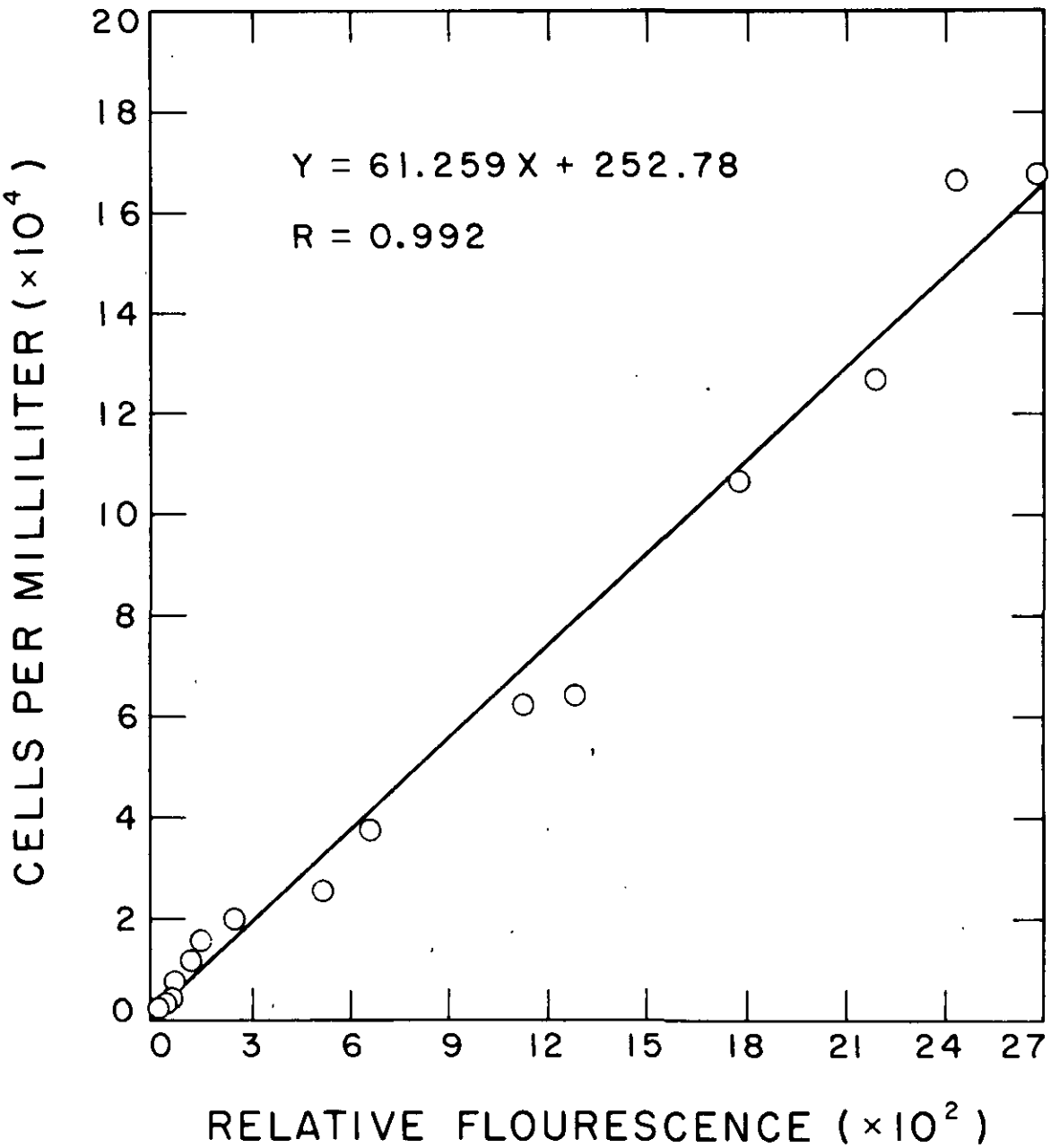

FIGURE 4. RELATIONSHIP BETWEEN CELL COUNTS AND RELATIVE IN VIVO CHLOROPHYLL FLOURESCENCE FOR THE SECOND FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM

TABLE 15

DATA POINTS FOR THE DETERMINATION OF THE RELATIONSHIP BETWEEN CELL COUNTS AND RELATIVE IN VIVO CHLOROPHYLL FLUORESCENCE FOR THE SECOND FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM

Flask	Nutrient Medium	Day	Flurometer Intensity Setting	Fluorescence Reading	Relative Fluorescence	Cells per Milliliter
8	Tap Control	I	30X	18.5	18.5	2,300
15	Tap Control	I	30X	29.5	29.5	3,400
9	1/8888 OMSE	II	30X	44.5	44.5	4,200
8	Tap Control	II	30X	56.0	56.0	7,533
15	Tap Control	II	10X	38.5	115.5	11,600
3	1/8888 OMSE	III	10X	50.0	150.0	15,720
17	Tap Control	III	3X	25.0	250.0	19,700
10	1/8888 OMSE	V	3X	52.0	520.0	25,100
20	1/8000 OMSE	V	1X	22.0	660.0	37,600
16	1/8000 OMSE	IV	1X	38.5	1,155	62,400
10	1/8000 OMSE	VI	1X	42.5	1,275	64,000
21	1/8000 OMSE	VI	1X	59.0	1,770	106,400
12	1/8000 OMSE	VI	1X	70.5	2,175	126,400
20	1/8000 OMSE	VI	1X	80.5	2,145	166,400
15	Tap Control	IV	1X	89.5	2,685	167,200

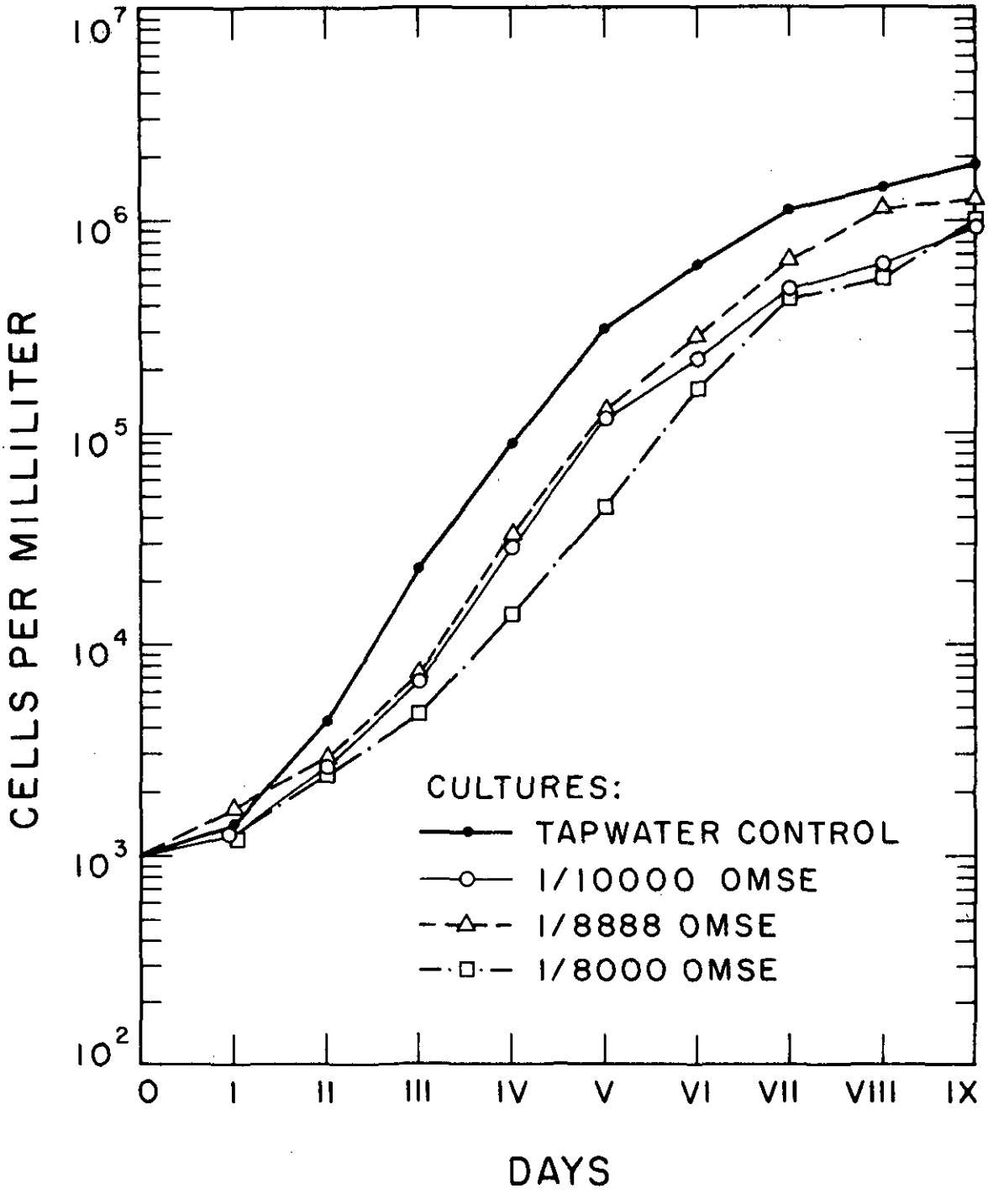

FIGURE 5. GROWTH CURVES FOR THE SECOND FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM

TABLE 16

μ_{MAX} AND MAXIMUM STANDING CROP DATA SUMMARY
FOR THE SECOND FULL-SCALE ASSAY
OF SELENASTRUM CAPRICORNUTUM

Flask	Culture Medium	μ_{max} Days of Occurrence	μ_{max} (days^{-1})	μ_{max} Statistics
8	Tap Control	II-III	1.776	$\bar{X}$ = 1.672
11	"	II-III	1.638	S = 0.063
15	"	II-III	1.690	
17	"	II-III	1.632	95% CL =
18	"	II-III	1.623	1.672 ± 0.126
1	1/1,000 OMSE	III-IV	1.599	$\bar{X}$ = 1.422
4	"	III-IV	1.532	S = 0.223
6	"	III-IV	1.619	
13	"	III-IV	1.156	95% CL =
14	"	V-VI	1.206	1.422 ± 0.446
3	1/8888 OMSE	III-IV	1.678	$\bar{X}$ = 1.443
5	"	III-IV	1.140	S = 0.275
9	"	III-IV	1.552	
10	"	V-VI	1.156	95% CL =
19	"	III-IV	1.691	1.443 ± 0.550
2	1/8000 OMSE	IV-V	1.228	$\bar{X}$ = 1.265
12	"	V-VI	1.270	S = 0.082
16	"	V-VI	1.389	
20	"	V-VI	1.273	95% CL =
21	"	V-VI	1.164	1.265 ± 0.164

TABLE 16 -- Continued

Max Crop Days of Occurrence	Max Crop Weight (mg/l)	Max Crop Weight Statistics	Max Crop Counts (10^6 Cells/Ml)	Max Crop Count Statistics
X	105.5	$\bar{X}$ - 116.8	2.098	$\bar{X}$ = 2.2236
XII	125.5	S = 9.14	2.619	S = 0.222189
X	117.0		2.159	
XII	110.0	95% CL =	2.129	95% CL =
X	126.0	116.8 ± 18.28	2.113	2.2236 ± .444378
XI	82.0	$\bar{X}$ = 91.4	1.272	$\bar{X}$ = 1.5072
XII	131.5	S = 23.54	1.930	S = 0.353995
XI	86.0		1.516	
XII	88.0	95% CL =	1.761	95% CL =
XI	69.5	91.4 ± 47.08	1.057	1.5072 ± 0.70799
XI	90.0	$\bar{X}$ = 99.5	1.868	$\bar{X}$ = 1.8746
XIV	100.5	S = 10.31	2.129	S = 0.450207
XII	110.5		2.435	
XII	88.0	95% CL =	1.241	95% CL =
XII	108.5	99.5 ± 20.62	1.700	1.8746 ± 900414
XIII	128.0	$\bar{X}$ = 123.0	1.991	$\bar{X}$ = 1.8134
XIII	134.0	S = 15.73	2.037	S = 0.419665
XI	95.5		1.225	
XIII	132.0	95% CL =	1.547	95% CL =
XIII	125.5	123.0 ± 31.46	2.267	1.8134 ± .839330

The maximum standing crops also tended to occur later in these OMSE treatment cultures than in the Control cultures. The Control cultures achieved their crops on days X and XII whereas the 1/10,000 OMSE cultures achieved their crops on days XI and XII and the 1/8888 OMSE cultures achieved their crops on days XI, XII, and XIV.

Greater inhibition was exhibited by the 1/8000 OMSE cultures. The lag phase was again only one day, but the transition from the lag phase to the exponential phase was more gradual than it had been the other two OMSE treatments. The steepest slope occurred between days V and VI, one day later than the 1/8888 OMSE cultures and the 1/10,000 OMSE cultures and two days later than Control cultures. From data in Table 16 it can be seen that the μ_{max} in four of the five 1/8000 OMSE cultures occurred between days V and VI. The maximum standing crops were achieved on day XIII (with one exception), later on the average than in any of the other culture media.

The t-Test comparisons are presented in Table 17 and a summary of the t-Test conclusions is presented in Table 18. The μ_{max}'s for each of the three OMSE treatments were lower than and significantly different from the μ_{max}'s of the Control cultures. Thus, the inhibitory effects at acute initial OMSE concentrations of 1/8000, 1/8888, and 1/10,000 were exerted during the exponential phase of growth and the μ_{max}'s were consequently lowered. The μ_{max}'s of the 1/10,000 OMSE cultures and the μ_{max}'s of the 1/8888 OMSE cultures were similar in magnitude. The μ_{max}'s of the 1/8000 OMSE cultures, in comparison, were slightly lower on the average. However, there was no significant difference between the μ_{max}'s of any two of these three OMSE treatments. This indicated that the inhibitory effects on the μ_{max} by all three OMSE concentrations was similar.

TABLE 17

μ_{MAX} AND MAXIMUM STANDING CROP T-TEST COMPARISONS FOR THE SECOND FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM (95% LEVEL OF SIGNIFICANCE)

Parameter	Treatments Compared	T Ratio	Degrees of Freedom	Critical T Value	Conclusion
μ_{max}	Control, 1/10,000	2.400	4.65	2.132-2.015	Different
	Control, 1/8888	1.808	4.43	2.132-2.015	Different
	Control, 1/8000	8.744	8	1.860	Different
	1/10,000, 1/8888	-0.132	8	1.860	Same
	1/10,000, 1/8000	1.480	5.06	2.015-1.943	Same
	1/8888, 1/8000	1.391	4.71	2.132-2.015	Same
Maximum Standing Crop Cell Count	Control, 1/10,000	3.833	8	1.860	Different
	Control, 1/8888	1.554	8	1.860	Same
	Control, 1/8000	1.932	8	1.860	Different
	1/10,000, 1/8888	1.434	8	1.860	Same
	1/10,000, 1/8000	-1.247	8	1.860	Same
	1/8888, 1/8000	0.222	8	1.860	Same
Maximum Standing Crop Dry Weight	Control, 1/10,000	2.249	5.18	2.015-1.943	Different
	Control, 1/8888	2.807	8	1.860	Different
	Control, 1/8000	-0.762	8	1.860	Same
	1/10,000, 1/8888	0.705	8	1.860	Same
	1/10,000, 1/8000	-2.496	8	1.860	Different
	1/8888, 1/8000	-2.794	8	1.860	Different

TABLE 18

μ_{MAX} AND MAXIMUM STANDING CROP T-TEST COMPARISON CONCLUSIONS FOR THE SECOND FULL-SCALE ASSAY OF *SELENASTRUM CAPRICORNUTUM* (95% LEVEL OF SIGNIFICANCE)

Treatments Compared	μ_{max}	Max Crop Cell Count	Max Crop Dry Weight
Control, 1/10,000	Different	Different	Different
Control, 1/8888	Different	Same	Different
Control, 1/8000	Different	Different	Same
1/10,000, 1/8888	Same	Same	Same
1/10,000, 1/8000	Same	Same	Different
1/8888, 1/8000	Same	Same	Different

The maximum standing crop dry weights of the 1/10,000 OMSE cultures and the 1/8888 OMSE cultures were lower than and significantly different from those of the Control cultures. This suggested that the inhibitory effects had exerted an influence during the maximum stationary phase. This was in contradiction to the stimulatory effect that was suggested by the maximum standing crop data of the First Full-Scale Assay. The maximum standing crop dry weights of the 1/8000 OMSE cultures were higher than but not significantly different from the Control crop weights. This indicated that the OMSE water had no effect during the maximum stationary phase. The crop weight data analysis thus raised questions about the sensitivity of the dry weight measurement technique.

A somewhat different trend was indicated by the maximum standing crop cell count data. All three OMSE treatments had lower crop counts than the Controls. The crop cell counts of the 1/10,000 OMSE cultures and the 1/8000 OMSE cultures were significantly different from those of the Control cultures. This suggested that the OMSE water exerted an inhibitory effect during the maximum stationary phase of these OMSE treatment cultures. This was contradictory to the conclusion that was reached for the 1/8000 OMSE cultures based on the crop weight data. The crop counts of the 1/8888 OMSE cultures were not significantly from those of the Controls, indicating that the OMSE water had no effect during the maximum stationary phase of these cultures. This contradicted the conclusion reached for the 1/8888 OMSE cultures based on the crop weight data. The crop count data in general gave no indication of the stimulatory effect that was apparent in the First Full-Scale Assay. Thus, the validity of the maximum standing crop cell count measurements was questioned.

In summary, the inhibition of the growth of Selenastrum was exhibited at acute initial OMSE concentrations of 1/10,000, 1/8888, and 1/8000. Although death kinetics were not observed and the lag periods were not lengthened, the transitions to the exponential phase were more gradual for the OMSE treatment cultures. The maximum specific growth rate was significantly lowered by all three OMSE concentrations. The maximum standing crops in general were also lower in the OMSE treatment cultures. This was contradictory to the observ ations of the First Full-Scale Assay, raising questions about the validity of the maximum standing crop measurements. The inhibition was not as pronounced as it had been at the OMSE concentrations of the First Full-Scale Assay, and the individual OMSE culture curves were not as distinctly different from one another as they had been in the First Assay. It was decided that a third full-scale assay should be conducted to assess the effects of three acute initial OMSE concentrations approximating 1/11,000, 1/12,000, and 1/13,000 on the growth characteristics of Selenastrum.

Third full-scale assay. The relationship between cell counts and fluorescence is presented in Figure 6. It was similar to the curves of the previous two assays. The correlation coefficient, 0.998, was again high. The y-intercept, at 1900 cells per ml, was high. Consequently, relatively high but reasonable cell counts were obtained from the relationship at the lowest fluorescence values observed during the assay. The intercept was acceptable when viewed with respect to the data points near the origin (Figure 6). The data points are listed in Table 19. Deviations from the regression line were random for both OMSE treatment cultures and Control cultures.

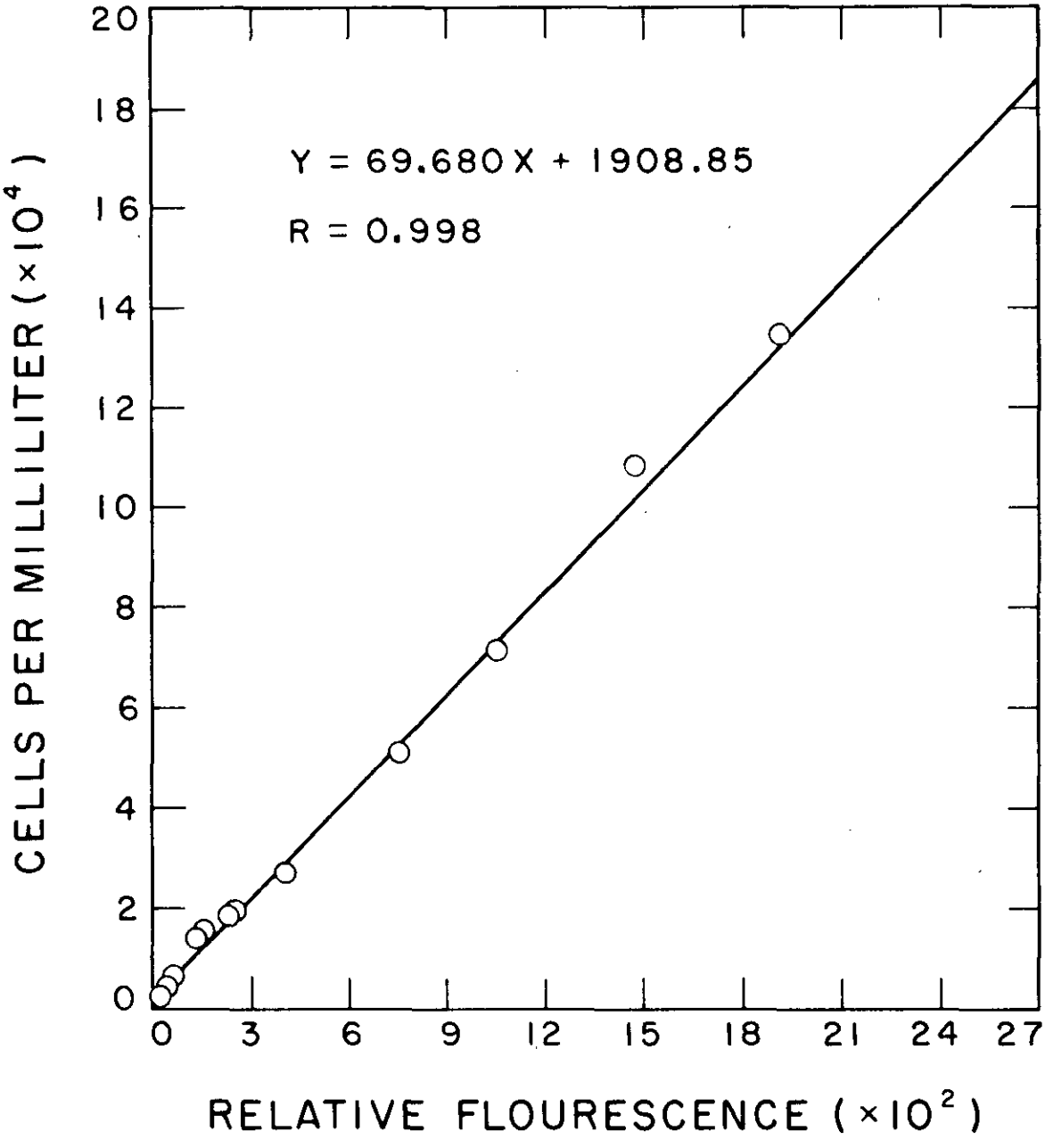

FIGURE 6. RELATIONSHIP BETWEEN CELL COUNTS AND RELATIVE IN VIVO CHLOROPHYLL FLOURESCENCE FOR THE THIRD FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM

TABLE 19

DATA POINTS USED TO DETERMINE THE RELATIONSHIP BETWEEN CELL COUNTS AND RELATIVE *IN VIVO* CHLOROPHYLL FLUORESCENCE FOR THE THIRD FULL-SCALE ASSAY OF *SELENASTRUM CAPRICORNUTUM*

Flask	Nutrient Medium	Day	Fluorometer Intensity Setting	Fluorescence Reading	Relative Fluorescence	Cells per Milliliter
1	Tap Control	I	30X	13.5	13.5	2,400
3	1/13,333 OMSE	II	30X	38.0	38.0	4,300
1	Tap Control	II	30X	62.5	62.5	6,200
20	Tap Control	II	10X	41.0	123.0	14,000
17	1/13,333 OMSE	III	10X	50.0	150.0	15,100
9	1/11,429 OMSE	III	10X	76.0	228.0	18,900
2	1/13,333 OMSE	III	3X	25.5	255.0	19,300
6	Tap Control	III	3X	40.0	400.0	27,900
7	1/12,308 OMSE	IV	3X	75.0	750.0	51,400
9	1/11,429 OMSE	IV	1X	35.0	1,050	71,400
6	Tap Control	IV	1X	49.0	1,470	108,800
15	1/11,429 OMSE	IV	1X	63.5	1,905	134,200

The growth curves are presented in Figure 7. The initial sharp increase in cell density from day 0 to day I in all four curves was probably partly due to the high intercept of the cell count-fluorescence relationship (Figure 6). It was probably also due partly to an inoculation density that was higher than the desired inoculation density of 1,000 cells per ml. Since a one day lag period had been customary in previous assays, the inoculation density was probably approximately 3,000 cells per ml.

The curve of the Tap Water Control cultures and the curve of the 1/11,429 OMSE cultures (the highest acute initial concentration of this assay) exhibited nearly identical patterns of growth. The pattern was that normally exhibited by Tap Water Control cultures. The lag periods lasted one day, and the steepest slopes during the exponential phase occurred between days II and III. It can be seen from data in Table 20 that the μ_{max}'s for these cultures were achieved between days II and III (with one exception). The maximum standing crops of the 1/11,429 OMSE cultures were actually achieved earlier than those of the Control cultures. The crops occurred on the average on day IX in the treated cultures and on day XI in the Control cultures. Thus, a qualitative analysis did not reveal any growth inhibition in the 1/11,429 OMSE cultures.

The growth curve of the 1/12,308 OMSE cultures and the growth curve of the 1/13,333 OMSE cultures exhibited growth patterns which were nearly identical. This pattern was only slightly different from that exhibited by the 1/11,429 OMSE cultures and the Control cultures. The lag periods lasted one day. The transitions between the lags and the exponential phases were slightly more gradual than that of the Control curve. The steepest slope occurred between days III and IV for both curves, one day later than

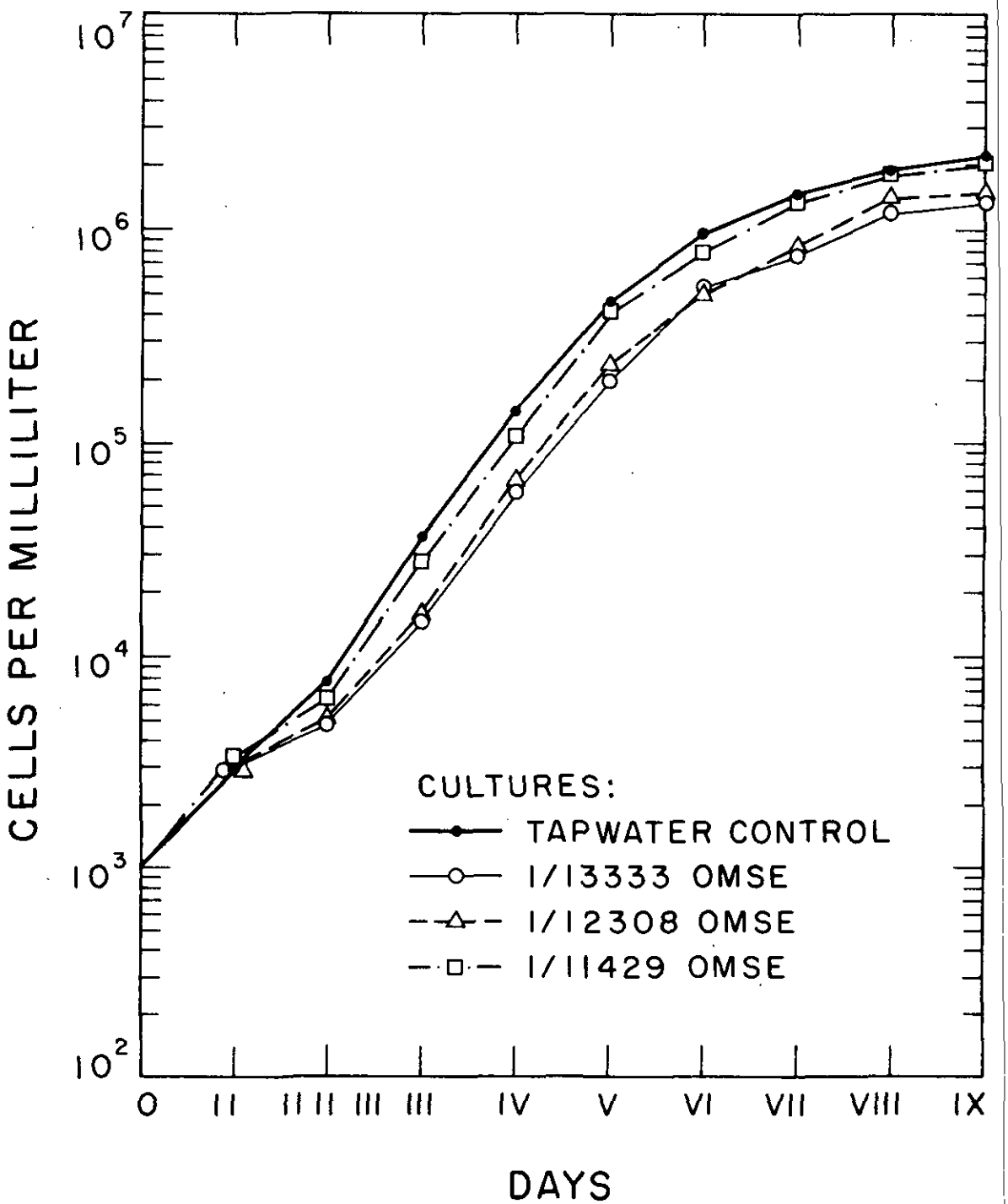

FIGURE 7. GROWTH CURVES FOR THE THIRD FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM

TABLE 20

μ_{MAX} AND MAXIMUM STANDING CROP DATA SUMMARY
FOR THE THIRD FULL-SCALE ASSAY
OF SELENASTRUM CAPRICORNUTUM

Flask	Culture Medium	μ_{max} Days of Occurrence	μ_{max} (days^{-1})	μ_{max} Statistics
1	Tap Control	II-III	1.576	$\bar{X}$ = 1.548
4	"	II-III	1.606	S = 0.112
6	"	II-III	1.473	
18	"	II-III	1.401	95% CL =
20	"	II-III	1.685	1.548 ± 0.224
2	1/13,333 OMSE	II-III	1.406	$\bar{X}$ = 1.403
3	"	III-IV	1.386	S = 0.072
12	"	III-IV	1.294	
16	"	III-IV	1.488	95% CL =
17	"	III-IV	1.441	1.403 ± 0.144
5	1/12,308 OMSE	III-IV	1.364	$\bar{X}$ = 1.440
7	"	III-IV	1.493	S = 0.120
8	"	IV-V	1.276	
10	"	II-III	1.580	95% CL =
13	"	III-IV	1.489	1.440 ± 0.240
9	1/11,429 OMSE	III-IV	1.498	$\bar{X}$ = 1.503
11	"	II-III	1.554	S = 0.061
14	"	II-III	1.447	
15	"	II-III	1.576	95% CL =
19	"	II-III	1.440	1.503 ± 0.122

TABLE 20 -- Continued

Max Crop Days of Occurrence	Max Crop Weight (mg/l)	Max Crop Weight Statistics	Max Crop Counts (10^6 Cells/Ml)	Max Crop Count Statistics
XII	138.0	$\bar{X}$ = 108.3	2.796	$\bar{X}$ = 2.5134
IX	106.0	S = 18.96	2.586	S = 0.433681
XII	111.5		3.005	
X	87.0	95% CL =	2.273	95% CL =
IX	99.0	108.3 ± 37.92	1.907	2.5134 ± .87362
VIII	100.0	$\bar{X}$ = 83.7	2.012	$\bar{X}$ = 1.5972
X	66.0	S = 18.71	1.419	S = 0.257450
XII	93.0		1.611	
X	61.0	95% CL =	1.350	95% CL =
XII	98.5	83.7 ± 37.42	1.594	1.5972 ± 0.51490
XIII	85.0	$\bar{X}$ = 81.0	2.256	$\bar{X}$ = 2.0294
VIII	83.5	S = 5.51	1.472	S = 0.339811
XIII	81.0		2.012	
XIII	71.5	95% CL =	2.064	95% CL =
XIII	84.0	81.0 ± 11.02	2.343	2.0294 ± .678622
IX	83.5	$\bar{X}$ = 89.0	1.977	$\bar{X}$ = 2.0884
IX	106.0	S = 9.70	2.656	S = 0.331023
VIII	87.5		1.785	
IX	85.5	95% CL =	2.012	95% CL =
IX	82.5	89.0 ± 19.40	2.012	2.0084 ± .662046

it had occurred in the Control curve. From data in Table 20 it can be seen that the μ_{max} of these cultures occurred on the average between days III and IV. However, one one culture flask for both media the μ_{max} occurred between days II and III, as early as the Control cultures. The maximum standing crops of the 1/13,333 OMSE cultures were achieved at about the same time as they were in the Control cultures. The maximum standing crops of the 1/12,308 OMSE cultures were achieved approximately a day later. Thus, a qualitative analysis of the growth in these two OMSE media revealed only slight growth inhibition.

The t-Test comparisons are presented in Table 21 and a summary of the t-Test conclusions is presented in Table 22. The μ_{max}'s of the OMSE treatment cultures were lower than the μ_{max}'s of the Tap Water Control cultures (Table 20). However, only in the mildest OMSE treatment, 1/13,333, was the difference significant. The inhibitory effects of the OMSE compounds in the 1/13,333 OMSE cultures thus exerted enough influence during the exponential phase of growth to significantly lower the maximum specific growth rate. The inhibitory effects of the OMSE compounds in the 1/12,308 OMSE cultures and the 1/11,429 OMSE cultures, on the other hand, did not exert enough influence during the exponential phase to produce this effect.

It was clear that within the range of acute initial OMSE concentrations of this assay, the inhibitory effects of the OMSE compounds during the exponential phase of growth became insignificant. It remained to be explained why the inhibition was significant at a concentration of 1/13,333 but was not significant at two higher concentrations, 1/12,308 and 1/11,429. One possible explanation was that the t-Test for the 1/13,333 OMSE cultures produced an incorrect decision. A more probably explanation was that during the randomizations for the assay, the 1/13,333 OMSE culture flasks were

TABLE 21

μ_{MAX} AND MAXIMUM STANDING CROP T-TEST COMPARISONS FOR THE THIRD FULL-SCALE ASSAY OF SELENASTRUM CAPRICORNUTUM (95% LEVEL OF SIGNIFICANCE)

Parameter	Treatments Compared	T Ratio	Degrees of Freedom	Critical T Value	Conclusion
μ_{max}	Control, 1/13,333	2.437	8	1.860	Different
	Control, 1/12,308	1.469	8	1.860	Same
	Control, 1/11,429	0.792	8	1.860	Same
	1/13,333, 1/12,308	-0.598	8	1.860	Same
	1/13,333, 1/11,429	-2.360	8	1.860	Different
	1/12,308, 1/11,429	-1.039	8	1.860	Same
Maximum Standing Crop Cell Count	Control, 1/13,333	-4.062	6.508	1.943-1.895	Different
	Control, 1/12,308	1.964	8	1.860	Different
	Control, 1/11,429	1.742	8	1.860	Different
	1/13,333, 1/12,308	-2.267	7.454	1.895-1.860	Different
	1/13,333, 1/11,429	-2.619	7.543	1.895-1.860	Different
	1/12,308, 1/11,429	-0.278	8	1.860	Same
Maximum Standing Crop Dry Weight	Control, 1/13,333	2.065	8	1.860	Different
	Control, 1/12,308	-3.092	4.671	2.132-2.015	Different
	Control, 1/11,429	2.027	2.027	1.860	Different
	1/13,333, 1/12,308	-0.310	-0.310	2.132-2.015	Same
	1/13,333, 1/11,429	-0.560	-0.562	1.860	Same
	1/12,308, 1/11,429	1.604	1.604	1.860	Same

TABLE 22

μ_{MAX} AND MAXIMUM STANDING CROP T-TEST COMPARISON CONCLUSIONS FOR THE THIRD FULL-SCALE ASSAY OF *SELENASTRUM CAPRICORNUTUM* (95% LEVEL OF SIGNIFICANCE)

Treatments Compared	μ_{max}	Max Crop Cell Count	Max Crop Dry Weight
Control, 1/13,333	Different	Different	Different
Control, 1/12,308	Same	Different	Different
Control, 1/11,429	Same	Different	Different
1/13,333, 1/12,308	Same	Different	Same
1/13,333, 1/11,429	Different	Different	Same
1/12,308, 1/11,429	Same	Same	Same

assigned to comparatively unfavorable incubation sites whereas the 1/11,429 OMSE cultures were assigned to comparatively favorable incubation sites (Table 23). The light intensities and temperatures of the incubation sites occupied by the 1/13,333 OMSE culture flasks were, on the average, lower than the light intensities and temperatures of the sites occupied by the Tap Water Control culture flasks. On the other hand, the light intensities and temperatures of the 1/11,429 OMSE culture sites were higher than those of the Control cultures. The incubation conditions of the 1/12,308 OMSE cultures were essentially the same as those of the Controls. Thus, the incubation conditions of the 1/13,333 OMSE cultures were less favorable than those of the Control cultures and much less favorable than those of the 1/11,429 cultures. The discrepancy in the μ_{max} t-Test analysis was thus probably due to the fact that inhibition in the 1/13,333 OMSE cultures was supplemented by relatively unfavorable incubation conditions whereas inhibition in the 1/11,429 OMSE cultures was offset by relatively more favorable incubation conditions.

The maximum standing crop dry weights and the maximum standing crop cell counts for all three OMSE treatments were lower than and significantly different from those of the Control cultures. This indicated that the growth potential of the treated flasks was lowered by the presence of the OMSE compounds. However, in two of the treatments (1/11,429 and 1/12,308) the μ_{max} had not been significantly lowered. In the absence of inhibition the μ_{max} and the maximum standing crop are both determined by the concentration of the growth limiting nutrient. Since the inhibitory effects had been observed to decline with time, the maximum standing crops of the 1/11,429 and 1/12,308 OMSE cultures should not have been lowered. This discrepancy was attributed to the questionable validity of the maximum standing crop measurement techniques.

TABLE 23

FLASK SITE INCUBATION CONDITIONS DURING THE THIRD FULL-SCALE ASSAY OF *SELENASTRUM CAPRICORNUTUM*

Flask	Culture Medium	Incubation Site	Light Intensity (Foot-candles)	Average Light Intensity (Foot-candles)	Temperature °C	Average Temperature °C
1	Tap Control	L-9	385-385		24	
4	"	R-8	420-420		24	
6	"	L-5	365-375	386.5	24	24.4
18	"	L-7	370-385		24	
20	"	R-2	380-380		26	
2	1/13,333 OMSE	L-4	380-380		23	
3	"	R-9	360-360		24	
12	"	L-15	375-375	375.5	23	23.2
16	"	L-10	375-380		24	
17	"	L-14	385-385		22	
5	1/12,308 OMSE	L-13	380-380		22	
7	"	L-8	385-400		23	
8	"	R-13	380-390	393.5	26	23.2
10	"	R-6	400-380		22	
13	"	R-12	420-420		23	
9	1/11,457 OMSE	L-3	375-375		24	
11	"	R-1	380-375		26	
14	"	R-11	415-380	391.6	25	25.0
15	"	R-3	375-375		25	
19	"	R-7	430-430		25	

In summary, only slight inhibition was exhibited at acute initial OMSE concentrations of 1/13,333, 1/12,308, and 1/11,429. The four growth curves were similar. Within this range of concentrations, inhibition during the exponential phase became insignificant. The maximum standing crops were significantly lower in the treated cultures. However, the validity of the maximum standing crop measurements was questionable. It was concluded that this assay accomplished the objective of determining the highest acute initial OMSE concentration at which the inhibitory effects of the OMSE water did not significantly alter the growth characteristics of Selenastrum.

Studies with Anabaena Flos-Aquae

Exploratory Assay

The relationship between cell counts and relative *in vivo* chlorophyll fluorescence is presented in Figure 8. The data points are presented in Table 24. The points did not define a distinct linear relationship. Individual points deviated significantly from the regression line, particularly at high fluorescence values. There appeared to be no consistent relationship between cell counts and fluorescence at fluorescence values higher than 1100. For example, two points with fluorescence values of 1655 and 1665 had cell counts which differed by more than 1.6 million cells per ml.

The lack of a consistent relationship at high fluorescence values was probably due to factors associated with the presence of filament aggregates in dense cultures. The cells were not evenly dispersed in the Sedgwick-Rafter counting cell, and individual field counts varied considerably in magnitude. Also, the individual cells in fields which contained filament aggregates were difficult to enumerate due to layering. Thus, the cell count estimate error was high. The fluorescence readings also probably did not

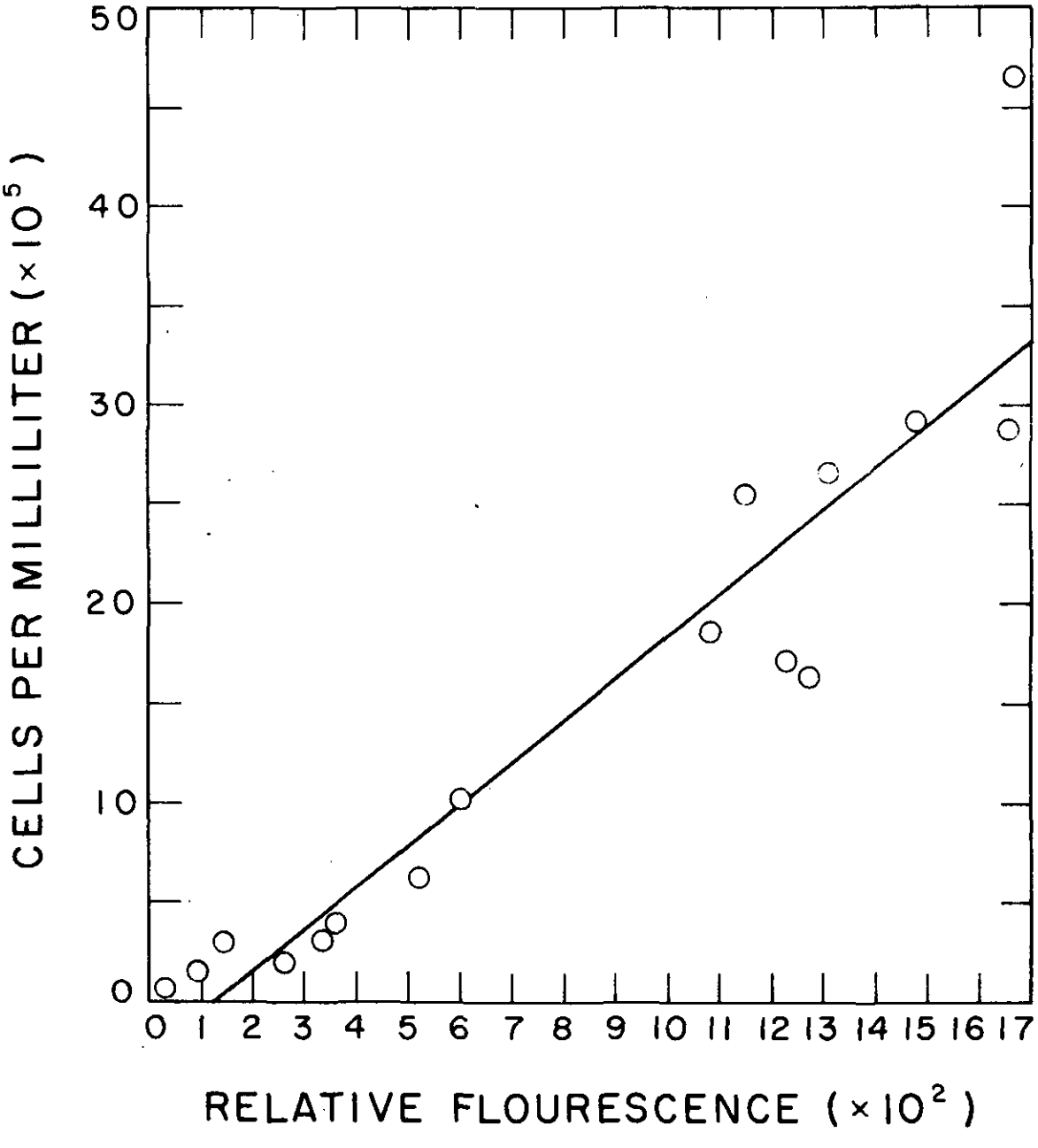

FIGURE 8. RELATIONSHIP BETWEEN CELL COUNTS AND RELATIVE IN VIVO CHLOROPHYLL FLOURESCENCE FOR THE EXPLORATORY ASSAY OF ANABAENA FLOS-AQUAE

TABLE 24

DATA POINTS USED TO DETERMINE THE RELATIONSHIP BETWEEN CELL COUNTS AND RELATIVE *IN VIVO* CHLOROPHYLL FLUORESCENCE FOR THE EXPLORATORY ASSAY OF *ANABAENA FLOS-AQUAE*

Flask	Nutrient Medium	Day	Fluorometer Intensity Setting	Fluorescence Reading	Relative Fluorescence	Million Cells per Milliliter
5	Tap Control	I	30X	24.5	24.5	0.0602
14	1/10,000 OMSE	III	30X	93.0	93.0	0.164
18	1/10,000 OMSE	II	10X	49.0	147.0	0.2956
13	1/8888 OMSE	III	10X	88.0	264.0	0.207
1	1/10,000 OMSE	IV	3X	33.5	335.0	0.3024
6	Tap Control	III	3X	35.5	355.0	0.394
15	1/10,000 OMSE	IV	3X	51.5	515.0	0.612
18	1/10,000 OMSE	III	3X	60.0	600.0	1.009
5	1/8000 OMSE	IV	1X	36.0	1080.0	1.865
8	1/8000 OMSE	VI	1X	38.5	1155.0	2.447
1	Tap Control	VI	1X	41.0	1230.0	1.720
10	1/10,000 OMSE	VI	1X	42.5	1275.0	1.640
9	Tap Control	VI	1X	43.5	1305.0	2.560
18	1/10,000 OMSE	IV	1X	49.0	1470.0	2.910
11	Tap Control	VI	1X	48.5	1655.0	2.880
19	1/10,000 OMSE	VI	1X	55.5	1665.0	4.653

account for the fluorescence of the internal cells of the filament aggregates. The inconsistency of the relationship was not due to differences in the pigment characteristics of the algal cells in OMSE treatment cultures and Control cultures since deviations from the regression line were random in both cases.

It was concluded that the relationship between cell counts and fluorescence could not be used for the construction of valid growth curves and the determination of valid μ_{max}'s. Growth curves were not constructed and the maximum standing crops were not determined. The μ_{max}'s were calculated from the relationship in order to determine in general when they occurred and at what relative fluorescence values. In all cultures the μ_{max}'s were achieved between days II and III. The fluorescence values for cultures on day III were consistently less than 400.

It was noted that the data points with fluorescence values lower than 1100 suggested a linear relationship. Since this range of fluorescence values would easily include the fluorescence values of assay cultures during the achievement of the μ_{max}, it was decided that the relationship between cell counts and fluorescence over this range could be used to determine the μ_{max}. This relationship could not be used to construct growth curves because it would not necessarily apply to cultures with higher fluorescence values. It was concluded that growth curves would have to be constructed directly from the relative fluorescence values.

During the assay the maximum fluorescence of every culture was attained early, usually on day VI or VII. Since the relationship between cell density and fluorescence was unknown during this period, it could not be assumed that the achievement of the maximum standing crop was correlated with the

attainment of maximum fluorescence. Due to the formation of filament aggregates, it was possible for cell growth to continue in a culture without an appreciable increase in fluorescence. It was known, however, that cultures at their maximum fluorescence had cell densities in the ficinity of three million cells per ml (Figure 8). It was also known, from the Anabaena growth pattern presented by the AAPBT, that cultures of this density could be expected to achieve their maximum standing crops approximately two days later at a density of approximately 7 million cells per ml. It was decided that an experiment should be performed to determine if growth in assay cultures was significant after the maximum fluorescence had been achieved.

Evaluation of the Relationship Between Maximum in vivo Chlorophyll Fluorescence and Culture Dry Weight

The results of the experiment are presented in Table 25. Only two of the cultures were sampled before and after the maximum fluorescence was achieved. These cultures were the second Tap Water Control culture and the 1/8000 OMSE culture. In both cultures the dry weight increased significantly as the fluorescence decreased from its maximum value. The volumes of the other three cultures were depleted by dry weight sampling before a fluorescence peak was observed. However, in several instances relatively low increases in fluorescence were accompanied by relatively high increases in dry weight in these cultures. This was observed between days VII and VIII for the first Tap Water Control culture, the 1/10,000 OMSE culture, and the 1/8888 OMSE culture. Although the data from the experiment was incomplete, it seemed to indicate that the maximum standing crop was achieved after the maximum fluorescence was attained.

TABLE 25

CELL GROWTH IN CULTURES OF ANABAENA FLOS-AQUAE
DURING THE ATTAINMENT OF MAXIMUM FLUORESCENCE

Nutrient Medium	Day	Relative Fluorescence	Dry Weight (mg/l)
Tap Control	VI	1,155	45
	VII	1,260	43
	VIII	1,275	74
	IX	1,350	90
Tap Control	V	1,125	50
	VI	1,170	44
	VII	1,095	84
1/10,000 OMSE	VI	1,515	59
	VII	1,710	78
	VIII	1,740	98
	IX	1,965	130
1/8888 OMSE	VI	1,440	58
	VII	1,545	78
	VIII	1,590	92
	IX	1,755	120
1/8000 OMSE	VI	1,515	59
	VII	1,545	77
	VIII	1,310	97
	IX	1,470	111

It was known that cultures of Anabaena at their maximum fluorescence had cell densities of approximately three million cells per ml (Figure 8). The Anabaena growth pattern presented by the AAPBT indicated that cultures of this density would achieve their maximum standing crops within two days and would remain at the maximum stationary phase for a period of about 10 days. It was concluded that the maximum standing crop of a culture could be sampled from two to 12 days after the culture attained its maximum fluorescence.

Full-Scale Assay

The relationship between cell counts and fluorescence is presented in Figure 9. The data points are presented in Table 26. The correlation coefficient, 0.998, was high. Individual points did not deviate greatly from the regression line. The intercept, at -3.070 cells per ml, was marginally acceptable. At the lowest fluorescence values observed during the assay, the indirect cell counts were between 21,000 and 25,000 cells per ml. These counts were lower than the inoculation concentration of 50,000 cells per ml. Although the inoculation procedure could have produced low initial concentrations due to an error in the stock culture cell count, it was also likely that the indirect cell counts based on the relationship were lower than the actual cell counts. However, since all μ_{max}'s would be computed on the basis of this relationship, the same error would be incorporated into all computations. It was concluded that the relationship would provide an acceptable common basis from which to compute and compare μ_{max}'s.

The reported maximum standing crop dry weights were obtained on day XIII for each culture (Table 27). The day on which the maximum fluorescence was

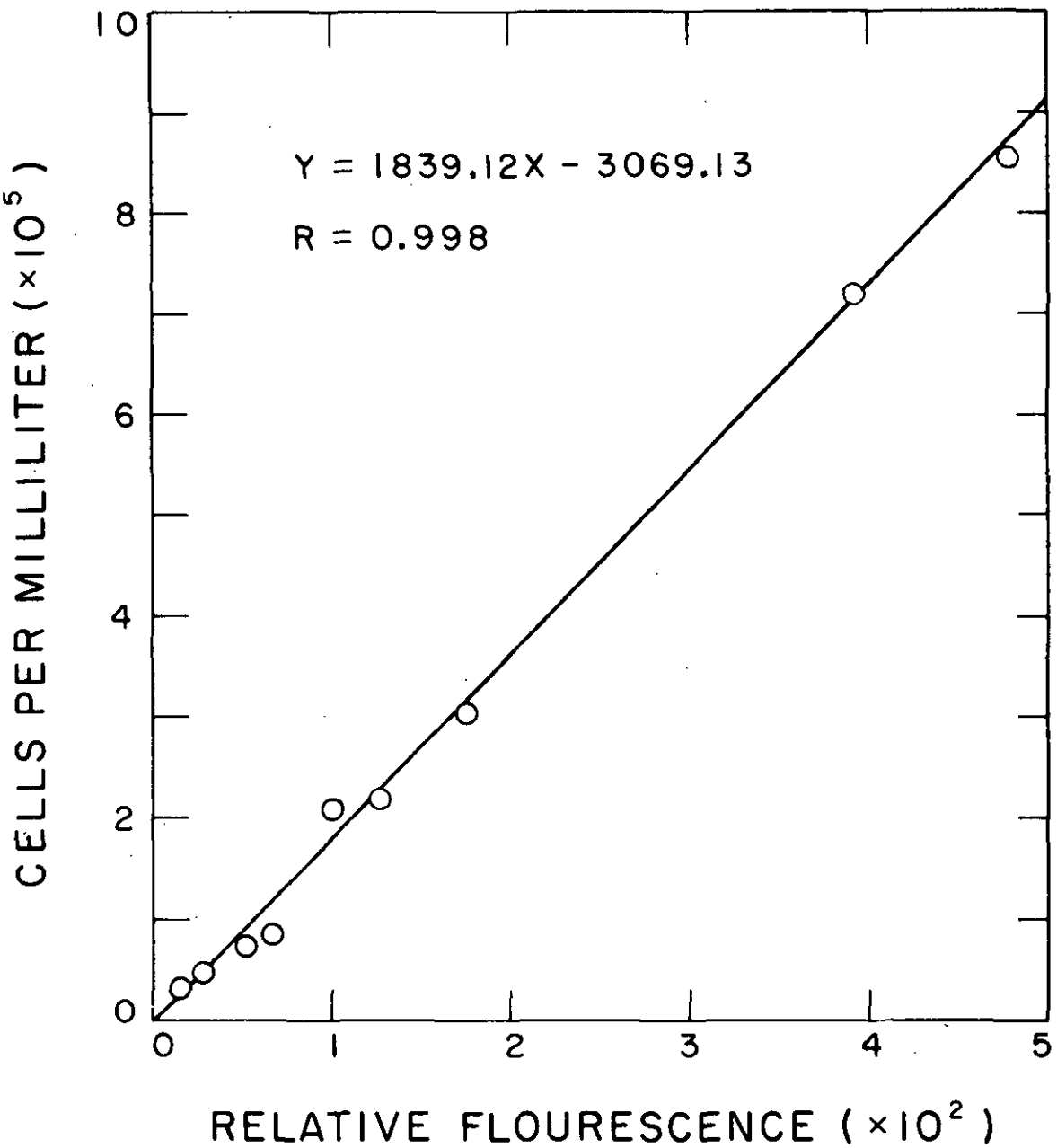

FIGURE 9. RELATIONSHIP BETWEEN CELL COUNTS AND RELATIVE IN VIVO CHLOROPHYLL FLOURESCENCE FOR THE FULL-SCALE ASSAY OF ANABAENA FLOS-AQUAE

TABLE 26

DATA POINTS USED TO DETERMINE THE RELATIONSHIP BETWEEN CELL COUNTS AND RELATIVE *IN VIVO* CHLOROPHYLL FLUORESCENCE FOR THE FULL-SCALE ASSAY OF *ANABAENA FLOS-AQUAE*

Flask	Nutrient Medium	Day	Fluorometer Intensity Setting	Fluorescence Reading	Relative Fluorescence	Cells per Milliliter
1	Tap Control	I	30X	11.0	11.0	30,200
18	1/10,000 OMSE	I	30X	24.5	24.5	45,300
5	1/8000 OMSE	II	30X	48.0	48.0	73,800
6	Tap Control	II	30X	63.0	63.0	82,200
18	1/10,000 OMSE	II	10X	32.5	97.5	210,000
13	1/8888 OMSE	III	10X	40.5	121.5	217,000
6	Tap Control	III	10X	47.5	172.5	304,000
18	1/10,000 OMSE	III	3X	39.0	390.0	740,000
12	Tap Control	IV	3X	47.5	475.0	852,000

attained in individual cultures varied between days V and IX. Thus the day on which the crop dry weight was sampled varied between four and 8 days after the day of maximum fluorescence. Since the maximum stationary phase occurred from two to 12 days after the maximum fluorescence, it was assumed that each culture was sampled while it supported its maximum standing crop. The dry weight of each culture was also sampled on the day of maximum fluorescence. These weights, however, were lower on the average than the day XIII weights. Thus, the weights obtained on day XIII were used.

The growth curves are presented in Figure 10. It was assumed, due to the low fluorescence values for all cultures on day I, that the lag period of each of the four curves occurred between days 0 and I. The steepest slope of the Tap Water Control curve occurred between days I and II. It can be seen from data in Table 27 that two Control cultures achieved their μ_{max}'s between days I and II and three Control cultures attained their μ_{max}'s between days II and III. The transition from the lag phase to the exponential phase was more gradual in each of the OMSE treatment curves than it was in the Control growth curve. This indicated that growth was inhibited in the OMSE treatment cultures.

The steepest slope for the 1/10,000 OMSE cultures curve occurred between days II and III, one day later than it did for the Control curve. It can be seen from data in Table 27 that the 1/10,000 OMSE cultures attained their μ_{max}'s either between days II and III or between days III and IV, later than the Control μ_{max}'s. The steepest slopes for the 1/8888 OMSE cultures and the 1/8000 OMSE cultures occurred between days III and IV, two days later than the Control cultures and one day later than the 1/10,000

TABLE 27

μ_{MAX} AND MAXIMUM STANDING CROP DATA SUMMARY FOR THE FULL-SCALE ASSAY OF ANABAENA FLOS-AQUAE

Flask	Culture Medium	μ_{max} Days of Occurrence	μ_{max} ($days^{-1}$)	μ_{max} Statistics	Max Crop Days of Occurrence	Max Crop Weight (mg/l)	Max Crop Weight Statistics
1	Tap Control	II-III	0.978	$\bar{X}$ = 1.213	XIII	121.5	$\bar{X}$ = 105.8
6	"	I-II	1.498	S = 0.249	"	94.0	S = 16.89
9	"	II-III	1.178		"	112.0	
11	"	II-III	0.971	95% CL =	"	119.0	95% CL =
12	"	I-II	1.440	1.213 ± 0.498	"	82.5	105.8 ± 33.78
10	1/10,000 OMSE	III-IV	1.070	$\bar{X}$ = 1.203	XIII	118.0	$\bar{X}$ = 109.6
14	"	III-IV	1.103	S = 0.151	"	116.0	S = 11.91
15	"	II-III	1.116		"	117.5	
18	"	II-III	1.415	95% CL =	"	106.5	95% CL =
19	"	III-IV	1.310	1.203 ± 0.302	"	90.0	109.6 ± 23.82
3	1/8888 OMSE	IV-V	1.131	$\bar{X}$ = 1.188	XIII	149.5	$\bar{X}$ = 113.8
4	"	III-IV	1.212	S = 0.144	"	119.5	S = 22.22
13	"	III-IV	1.378		"	92.0	
16	"	IV-V	0.985	95% CL =	"	104.0	95% CL =
20	"	III-IV	1.234	1.188 ± 0.288	"	104.0	113.8 ± 44.44
2	1/8000 OMSE	III-IV	1.468	$\bar{X}$ = 1.249	XIII	73.0	$\bar{X}$ = 99.1
5	"	II-III	1.382	S = 0.194	"	115.5	S = 17.65
7	"	III-IV	1.082		"	99.5	
8	"	V-VI	1.015	95% CL =	"	115.0	95% CL =
21	"	II-III	1.298	1.249 ± 0.388	"	92.5	99.1 ± 35.30

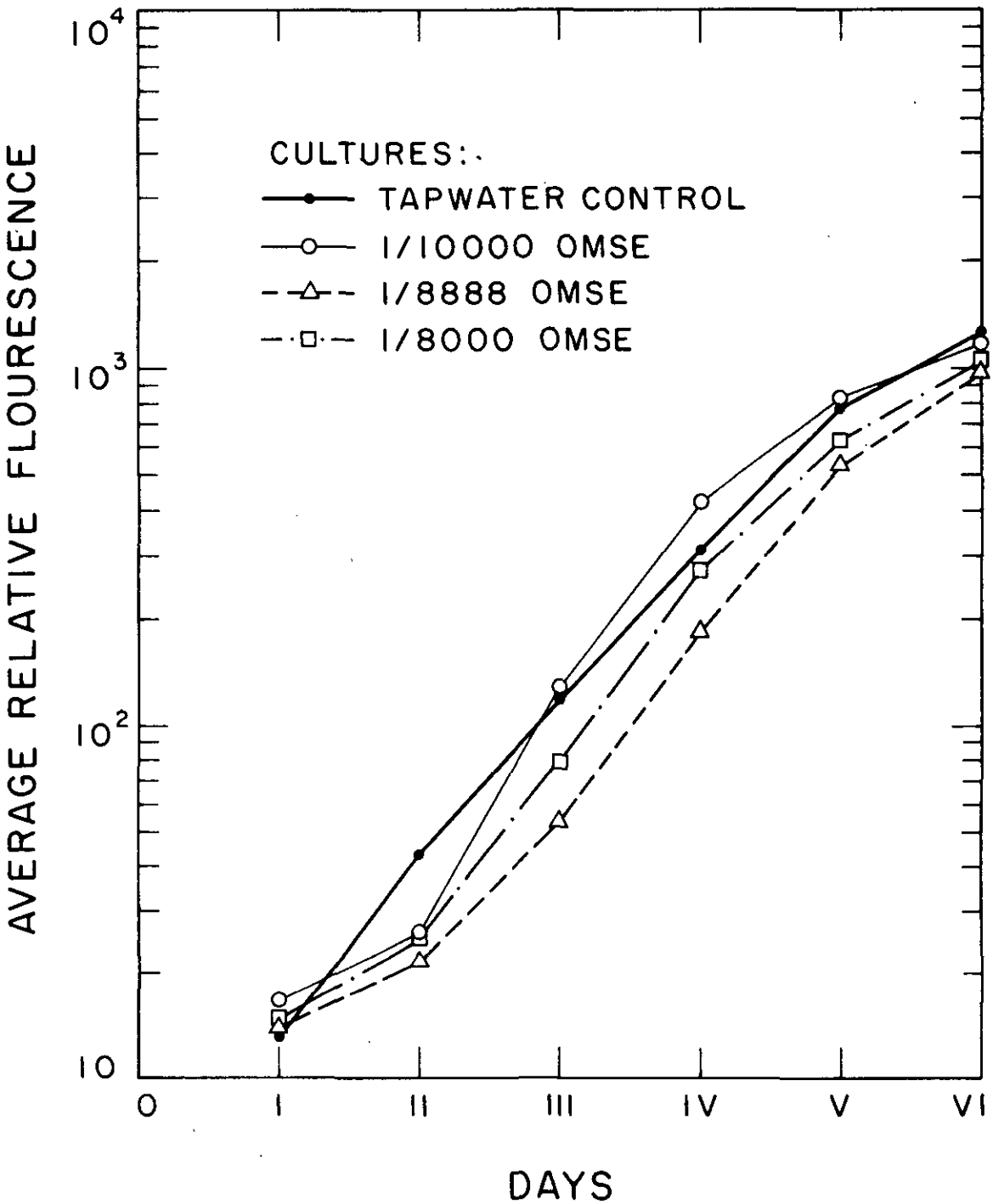

FIGURE 10. GROWTH CURVES FOR THE FULL-SCALE ASSAY OF ANABAENA FLOS-AQUAE

OMSE cultures. SImilarly, the μ_{max}'s were achieved later in these cultures (Table 27). The 1/8888 OMSE cultures attained their μ_{max}'s either between days III and IV or between days IV and V. The 1/8000 OMSE cultures attained their μ_{max}'s between days II and III, III and IV, and V and VI. These observations indicated that growth was inhibited in the OMSE treatment cultures and that the degree of inhibition increased as the OMSE concentration increased.

The μ_{max}'s of the Control cultures and the OMSE treatment cultures were all similar in magnitude (Table 27). The average μ_{max}'s of the 1/10,000 OMSE cultures and the 1/8888 OMSE cultures were lower than the average μ_{max} of the Control cultures. The average μ_{max} of the 1/8000 OMSE cultures was higher than that of the Control cultures. The maximum standing crop dry weights of the four types of cultures were also similar in magnitude. The average crops of the 1/10,000 OMSE cultures and the 1/8888 OMSE cultures were higher than the average crop of the Control cultures. The average crop of the 1/8000 OMSE cultures, on the other hand, was lower than that of the Control cultures.

The μ_{max} and maximum standing crop t-Test analyses are presented in Table 28. A summary of the t-Test conclusions is presented in Table 29. There was no significant difference between the μ_{max}'s of the Control cultures and the μ_{max}'s of each of the OMSE treatment cultures. There was also no significant difference between the μ_{max}'s of any two OMSE treatment cultures. Similarly, there was no significant difference between the maximum standing crops of the Control cultures and the crops of each of the OMSE treatment cultures. There was no significant difference between the maximum standing crops of any two OMSE treatments. THese results indicated that

TABLE 28

μ_{MAX} AND MAXIMUM STANDING CROP T-TEST COMPARISONS FOR THE FULL-SCALE ASSAY OF ANABAENA FLOS-AQUAE

Parameter	Treatments Compared	T Ratio	Degrees of Freedom	Critical T Value	Conclusion
μ_{max}	Control, 1/10,000	0.078	8	1.860	Same
	Control, 1/8888	0.194	8	1.860	Same
	Control, 1/8000	-0.255	8	1.860	Same
	1/10,000, 1/8888	-0.158	8	1.860	Same
	1/10,000, 1/8000	-0.420	8	1.860	Same
	1/8888, 1/8000	-0.564	8	1.860	Same
Maximum Standing Crop Dry Weight	Control, 1/10,000	-0.411	8	1.860	Same
	Control, 1/8888	-0.641	8	1.860	Same
	Control, 1/8000	-0.613	8	1.860	Same
	1/10,000, 1/8888	-0.373	8	1.860	Same
	1/10,000, 1/8000	1.102	8	1.860	Same
	1/8888, 1/8000	1.158	8	1.860	Same

TABLE 29

μ_{MAX} AND MAXIMUM STANDING CROP T-TEST COMPARISON CONCLUSIONS FOR THE FULL-SCALE ASSAY OF ANABAENA FLOS-AQUAE

Treatments Compared	μ_{max}	Max Crop Dry Weight
Control, 1/10,000	Same	Same
Control, 1/8888	Same	Same
Control, 1/8000	Same	Same
1/10,000, 1/8888	Same	Same
1/10,000, 1/8000	Same	Same
1/8888, 1/8000	Same	Same

acute initial OMSE concentrations as high as 1/8000 had no significant inhibitory or stimulatory effect on either the maximum specific growth rate or the maximum standing crop of cultures of Anabaena flos-aquae.

It was concluded that the inhibition of the growth of Anabaena at acute initial OMSE concentrations of 1/10,000, 1/8888, and 1/8000 was insignificant. It was also concluded that the OMSE water had no stimulatory effects at these concentrations. Since these concentrations would not be experienced in the field, it was decided that not to evaluate the effects of other acute initial OMSE concentrations on the growth characteristics of Anabaena.

GENERAL DISCUSSION

Discussion of Related Studies

The plankton study conducted by Lagler et al was part of a much broader investigation of the effects of outboard motor usage on the aquatic environment (6). Several limitations restricted the value of the plankton study. The number of samples analyzed, 10, was barely adequate. Plankton productivity was measured on a volume basis. SInce the relationship between volume and biomass could have varied with changes in plankton composition, the volume comparisons were not necessarily true productivity comparisons. The lack of background data for the plankton productivity of either pond was another notable limitation. It therefore could not be determined how the plankton samples from either pond, but particularly those from the motor use pond, compared to the norm for that pond. The observed higher plankton productivity in the motor use pond could have been due either to the higher innate productivity of that pond or to the stimulatory effects of motor usage. It was also possible that the plankton samples from the motor use pond were lower than those that would normally be obtained from that pond in the absence of motor usage. If this were the case, the inhibition of plankton productivity would have been evident.

The study by Lagler et al. (6) did not differentiate between zooplankton and phytoplankton. It is recognized that the development of the two are closely related. However, it is possible thatsome samples contained a normal balance of phytoplankton and zooplankton while others did not. For example, the stimulation of phytoplankton productivity coupled with the inhibition of zooplankton productivity in the motor use pond would not necessarily have been detected by a volume measurement of plankton productivity.

Thus, although the results of the plankton study suggested that outboard motor usage has no drastic effects on plankton development, it could not be determined if motor usage inhibited or stimulated plankton development. Also, no specific conclusions about the effects of outboard motor usage on the growth of phytoplankton organisms were possible from this study.

The phytoplankton study conducted by Environmental Engineering Inc. was also part of a broader investigation of the effects of outboard motor usage on the aquatic environment (7). Although the study was concerned specifically with phytoplankton, it was too limited in scope to provide substantial information. The number of samples was, at best, barely adequate. A total of 6 samples were collected and analyzed, four from the stressed lake and two from the undisturbed lake. SInce the samples were all collected on the same day, the study did not provide any information about the effects of motor usage on seasonal phytoplankton population trends. The sample analyses did not identify organisms to a taxon lower than family. More intensive sampling and identification to the generic and specie levels would have allowed for a more meaningful analysis. Thus, the study at best provided superficial information about the effects of outboard motor usage on phytoplankton productivity.

Various concentrations of outboard motor subsurface emissions were demonstrated to be toxic to fish and benthic invertebrates (15,16). It was therefore conceivable that these emissions would also be toxic to phytoplankton organisms. The inhibition of phytoplankton productivity by outboard motor usage would be followed by changes in biological productivity and water chemistry that would be detrimental to the recreational value of a lake. It was also possible, though not probable, that outboard emissions would stimulate algal growth. Nitrogen and phosphorous are common eutro-

phicants, and iron, zinc, sulfur, and magnesium can stimulate algal growth (10). These elements are present in outboard motor oil, although in trace amounts (13). The organic compounds in the emissions (1,16) are chemically different from the organic compounds which have been demonstrated to be heterotrophically assimilated by algae (21). However, since it has been observed that a variety of algal types proliferate in oil field sump ponds (22), they might also flourish in the presence of outboard motor emissions. Thus, the stimulation of algal growth by outboard motor usage was a possibility. In moderation, the associated changes in biological productivity and water chemistry might contribute to the recreational value of a lake. However, the stimulation of phytoplankton productivity could also result in the undesirable conditions associated with accelerated eutrophication. Thus, more information about the effects of outboard motor usage on phytoplankton productivity is needed.

In response to the need for more information, a current study sponsored by the EPA and the BIA is assessing the effects of one level of outboard motor usage on phytoplankton population compositions and seasonal phytoplankton population trends under field conditions (8). In contrast with this approach, the research contained herein has assessed the effects of varying concentrations of outboard motor subsurface emissions on the growth characteristics of two individual test species under laboratory conditions. This study should supplement the EPA-BIA study and contribute to an overall assessment of the effects of outboard motor usage on freshwater phytoplankton productivity.

Limitations of the AAPBT

When the AAPBT was selected for use in the study, it was recognized

that the growth of pure (unialgal) cultures under laboratory conditions would not account for numerous ecological interactions which occur in natural waters. The Provisional Algal Assay Procedure Bottle Test (the predecessor to the AAPBT) was evaluated and found to lack the capability of "delineating the interactions between the ecosystem and the material being assessed, as well as between the various algal species making up the natural community (23). However, the evaluation also found the procedure to be "an excellent analytical tool for assessing the physiological effects of various materials upon algae" (23). With due consideration to this evaluation, it was decided that the AAPBT could be used to provide basic information about the effects of outboard motor subsurface emissions on the growth characteristics of phytoplankton organisms.

It was recognized that a stimulatory effect observed in the lab might be increased in natural waters by the microbial decomposition of emission compounds and subsequent release of algal nutrients. Similarly, it was recognized that the absence of any effects in the laboratory would indicate the lack of toxicants but would not account for nutrients which might be released in the field by decomposition. It was also recognized that an inhibitory effect demonstrated by an AAPBT study might be reduced in natural waters by microbial decomposition of the toxicants.

Discussion of Assay Results

In each assay, the effects of outboard motor subsurface emissions on the growth of the test algae were evaluated by comparing the growth curves, maximum specific growth rates, and maximum standing crops of OMSE treatment cultures to those of Control cultures. Each culture flask in an assay contained

the same total volume and had a growth medium of the same AAPBT nutrient strength. THus, the same (unknown) nutrient limited growth in the basic medium of each culture. SInce the magnitude of the maximum standing crop is related to the initial amount of the growth-limiting nutrient and the magnitude of the μ_{max} is related to the concentration of that nutrient, the potential magnitudes of the quantitative growth parameters of all cultures in an assay were the same.

The inclusion of OMSE water in the growth media of OMSE treatment cultures constituted the addition of a wide variety of chemical substances to the basic growth medium. Toxic substances would inhibit growth even though sufficient nutrients were provided by the AAPBT growth medium. Thus, the presence of toxicants would be manifested by growth curve distortions and a lowering of the quantitative growth parameters.

A nutrient contribution from the OMSE water would be apparent only if the growth-limiting nutrient of the AAPBT medium was supplemented. In addition, the effects would be apparent only in the absence of toxicants. The stimulatory effects of the nutrients would be manifested by growth curve distortions and a raising of the quantitative growth parameters.

A nutrient contribution from the OMSE water would be apparent only if the growth-limiting nutrient of the AAPBT medium was supplemented. In addition, the effects would be apparent only in the absence of toxicants. The stimulatory effects of the nutrients would be manifested by growth curve distortions and a raising of the quantitative growth parameters. The presence of organic compounds in the OMSE water which could be assimilated heterotrophically by the test algae probably would not stimulate growth because carbon was not limiting under the assay conditions.

Thus, under the conditions of the assays, lower quantitative growth parameters in the OMSE treatment cultures would indicate the presence of toxicants inthe OMSE water. No information about the presence or absence of nutrients would be provided from this growth response. Higher quantitative growth parameters in the OMSE treatment cultures would indicate the lack of toxicants and nutrient supplementation by the OMSE water. Equivalent parameters in the OMSE treatment and Control cultures would indicate the lack of toxicants but would not necessarily indicate the lack of nutrients in the OMSE water.

A series of three full-scale assays were conducted which assessed the effects of 9 acute initial OMSE concentrations on the growth characteristics of Selenastrum capricornutum. These concentrations were 1/1600, 1/333, 1/6667, 1/8000, 1/8888, 1/10,000, 1/11,429, 1/12,308, and 1/13,333. The growth curve comparisons demonstrated growth inhibition which decreased and became insignificant as the OMSE concentration decreased. Lethal toxicity was observed at the two highest concentrations. However, the inhibitory effect declined with time and the cultures subsequently demonstrated a nearly normal growth pattern. As the OMSE concentration decreased from 1/3333, lethal toxicity was not observed, the lag periods were shortened, and the growth patterns became similar to the pattern of the Control cultures.

The μ_{max} comparisons indicated that growth was significantly inhibited during the exponential phase of growth at the 6 highest OMSE concentrations. At concentrations of 1/11,429, 1/12,308, and 1/13,333 this inhibition became insignificant. The maximum standing crop comparisons indicated that growth was stimulated during the maximum stationary phase at the four highest OMSE

concentrations. This suggested that the inhibitory effects had become insignificant at this stage of incubation and that the OMSE water supplemented the growth-limiting nutrient of the AAPBT medium. The maximum standing crop comparisons also indicated that growth was significantly inhibited during the maximum stationary phase at the 5 lower OMSE concentrations. Since the inhibitory effects had not produced lower maximum standing crops at the four highest concentrations, they should not have produced this effect at these five lower concentrations. Also, if the stimulatory effects at the highest OMSE concentrations had been due to nutrients in the OMSE water, a similar but milder effect should have been observed at the lower OMSE concentrations. The inconsistency of the maximum standing crop data was attributed to the poor sensitivity of the maximum standing crop measurement techniques. It was concluded that the stimulation of growth was not demonstrated during the assays.

One full-scale assay was conducted which assessed the effects of three acute initial OMSE concentrations - 1/8000, 1/8888, and 1/10,000 - on the growth characteristics of Anabaena flos-aquae. The inhibitory effects of the OMSE water were apparent, but they did not significantly alter the growth characteristics. The growth curves of the OMSE treatment cultures were similar to the Control curve. The μ_{max}'s and the maximum standing crops of the OMSE treatment cultures were not significantly different from those of the Control cultures. The stimulation of growth was not observed. At equivalent OMSE concentrations, the growth of Anabaena was inhibited less than the growth of Selenastrum.

The 24, 48, and 96-hour TL_{50} OMSE concentrations for bluegills, fathead minnows, and scuds were in the range of 1/2150 to 1/3400 (15,16). At these concentrations the growth of Selenastrum would be significantly inhibited and the growth of Anabaene would probably be significantly inhibited.

Cultures of Selenastrum demonstrated the ability to survive acute OMSE concentrations in this range and to recover and grow normally. The 24, 48, and 96-hour TL_{50} OMSE concentrations for dragonfly nymphs were in the range of 1/540 to 1/635 (15). The growth of Selenastrum would be significantly inhibited and the growth of Anabaena would probably be significantly inhibited at these concentrations. It is not known if cultures would recover from these acute dosages.

The suggested safe OMSE concentrations for bluegills and fathead minnows were, respectively, 1/45,500 and 1/62,500 (3). The growth of both Selenastrum and Anabaena would not be significantly inhibited by acute OMSE concentrations at these levels.

Relation of Acute OMSE Concentrations of the Laboratory Study to Outboard Motor Usage in the Field

The inhibitory effects of OMSE water did not significantly alter the growth characteristics of Selenastrum capricornutum at acute concentrations ranging from 1/11,429 to 1/13,333. It was also demonstrated that acute concentrations as high as 1/8000 did not significantly alter the growth characteristics of Anabaena flos-aquae. If these concentrations could be exceeded by normal outboard motor usage in the field, the inhibition of phytoplankton development would be a probably consequence. Since levels of outboard motor usage could most readily be visualized for a small pond, the volume of fuel consumption which would produce these concentrations on a small pond was calculated.

Factory Hollow Pond (Puffer's Pond) in Amherst, Massachusetts was chosen as an example of a small pond. The pond has a surface area of 8 acres and

an average depth of 6 feet (24). The surface area is thus 348,480 square feet and the volume is 2,090,880 cubic feet. This volume is equivalent to 1,303,541 gallons, or approximately 1.3 million gallons. For the purposes of simplification, 1/13,000 was chosen as the lowest concentration at which there would be no significant inhibitory effect on the growth of either of the two test algae. In order to achieve this concentration, 100 gallons of fuel would have to be consumed on the pond. (For a 50 acre lake with an average depth of 10 feet, a comparable fuel consumption level would be 1,041 gallons.) The consumption of 100 gallons of fuel would constitute over 300 hours of operation at trolling speeds. Outboard operation at one-tenth of this level would be unlikely. Thus, the calculation indicated that normal outboard motor usage would not produce acute OMSE concentrations which are one-tenth as high as the critical inhibitory concentrations.

It was concluded that the growth of the two test algae, Selenastrum capricornutum and Anabaena flos-aquae, would not be inhibited by normal outboard motor usage in the field. The growth of other phytoplankton species would be inhibited only if they are at least 10 times more sensitive than the two test algae. It was therefore also concluded that the growth of other phytoplankton species would probably not be inhibited by normal outboard motor usage. This conclusion was consistent with the conclusions of Lagler et al. and Environmental Engineering Incorporated (6,7). Lagler et al. (6) concluded that the outboard motor usage did not prohibit the development of plankton. Environmental Engineering Inc. concluded that outboard motor usage did not alter the phytoplankton population.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

1. The measurement of *in vivo* chlorophyll fluorescence was found to be useful for monitoring growth in assay cultures due to its sensitivity to changes in population density and practicality of laboratory execution.

2. Outboard Motor Subsurface Exhaust water (OMSE water) inhibited the growth of the green alga *Selenastrum capricornutum* and the blue-green alga *Anabaena flos-aquae*.

3. OMSE water was not observed to stimulate the growth of the two test algae.

4. At acute initial OMSE concentrations of 1/1600 and 1/3333, lethal toxicity was exerted on cultures of *Selenastrum capricornutum*. However, the inhibitory effects declined with time and the algal cultures grew in an apparently normal pattern thereafter.

5. The growth characteristics of *Selenastrum capricornutum* were not significantly altered at acute initial OMSE concentrations of 1/11,429, 1/12,308, and 1/13,333.

6. The growth characteristics of *Anabaena flos-aquae* were not significantly altered at acute initial OMSE concentrations of 1/8000, 1/8888, and 1/10,000.

7. The growth of the two test algae would not be inhibited at the suggested safe OMSE concentrations for fathead minnows and bluegills.

8. Outboard motor usage in the field would produce OMSE concentrations which would not inhibit the growth of the two test algae and which probably would not inhibit the growth of other freshwater phytoplankton species.

9. At acute initial OMSE concentrations of 1/8000, 1/8888, and 1/10,000 the growth characteristics of *Selenastrum capricornutum* were altered to a greater degree than were the growth characteristics of *Anabaena flos-aquae*.

RECOMMENDATIONS

1. Assays should be conducted to evaluate the effects of various acute initial Outboard Motor Subsurface Exhaust (OMSE) concentrations on the growth characteristics of other common freshwater phytoplankton organisms.
2. If field observations correlate increases in algal growth with increased outboard motor usage, studies should be conducted to evaluate the potential of OMSE water to stimulate the growth of freshwater phytoplankton organisms.

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LITERATURE CITED

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APPENDIX A

CHARACTERISTICS OF TEST ALGAE

Selenastrum capricornutum

This alga is a green alga, Division Chlorophyta, Class Chlorophyceae, Order Chlorococcales. Selenastrum is an important genus of the planktonic Chlorococcales and is most likely to be found in the plankton of ponds or shallow, fertile lakes. These organisms possess chlorophylls a and b only. A brief description was presented in the AAPBT. Algae of the Order Chlorococcales are characterized by their unicellular or colonial habit. Cells are non-motile for at least the greater part of their life. The genus Selenastrum is characterized by cells which are distinctly crescent-shaped. Cells may occur individually or may aggregate into groups of two to 12 cells. Several groups may aggregate to form masses of 50 or more cells. ALthough frequently found in the field, these larger cell masses usually do not occur in actively agitated cultures. Individual cells vary in length from 10μ to 48μ and in breadth from three μ to 9μ. Reproduction usually occur as normal eucaryotic cell division.

Anabaena flos-aquae

This alga is a blue-green alga, Division Cyanophyta, Class Cyanophyceae, Order Nostocales. Massive blooms of Anabaena tend to develop in temperate regions in productive lakes, and such blooms are indicators of eutrophy. These algae contain chlorophyll a only. The characteristic blue-green color is attributed to accessory biloprotein pigments. The AAPBT presented a brief description of these organisms. The Order is characterized by cells which are associated in unbranched filaments. THe filaments contain two types of specialized cells, heterocysts and akinetes. Heterocysts appear to be sites of nitrogen fixation. Akinetes are resting states. Individual cells are

spherical or slightly elongate, varying in length from four μ to 10μ and in breadth from four μ to 7μ. The filaments are straight or slightly curved and rigid. Occasionally, the filaments aggregate to form free-floating masses. Although these masses are rare in actively agitated cultures, Anabaena masses may attain a size of one centimeter in diameter in the field. Little is known about the reproduction of Anabaena.

APPENDIX B

NUTRIENT MEDIA TABLES

TABLE B-1

ONE-LITER MACRONUTRIENT SALT STOCK SOLUTIONS
(REAGENT GRADE CHEMICALS IN DISTILLED WATER)

Macronutrient	Concentration (gm/l)
$NaNO_3$	25.500
K_2HPO_4	1.044
$MgCl_2 \cdot 6H_2O$	12.17042
$MgSO_4 \cdot 7H_2O$	14.700
$CaCl_2 \cdot 2H_2O$	4.410
$NaHCO_3$	15.000

TABLE B-2

ONE-LITER MICRONUTRIENT SALT STOCK SOLUTION X10 AND
ONE-LITER MICRONUTRIENT SALT STOCK SOLUTION X1
(REAGENT GRADE CHEMICALS IN DISTILLED WATER)

Micronutrient	Stock X10 Concentration (gm/l)	Stock X1 Concentration (mg/l)
H_3BO_3	1.85520	185.520
$MnCl_2$	2.64264	264.264
$ZnCl_2$	0.32709	32.709
$CoCl_2$	0.00780	0.780
$CuCl_2$	0.00009	0.009
$Na_2MoO_4 \cdot 2H_2O$	0.07260	7.260
$FeCl_3$	0.96000	96.000
$Na_2EDTA \cdot 2H_2O$	3.00000	300.000