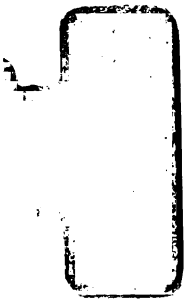
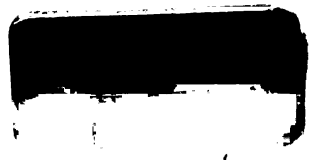
IDENTIFYING AND CONTROLLING SOME SOURCES OF ERROR FOR A STATIC ALGAL BIOASSAY WITH APPLICATIONS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
THOMAS D. FORSYTHE
1973





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ABSTRACT

IDENTIFYING AND CONTROLLING SOME SOURCES OF ERROR FOR A STATIC ALGAL BIOASSAY WITH APPLICATIONS

By

Thomas D. Forsythe

This study was for self-training in the methods necessary to conduct precise static algal bioassays. Some systematic errors were identified, investigated, and controlled when Selenastrum capricornutum cells were cultured using the Algal Assay Procedure: Bottle Test.

The growth parameters used to judge bioassay precision were Maximum Specific Growth Rates, Maximum Standing Crops, and "Cell Health" determined by cell counts and dry weights. Attempts to measure daily growth by optical density and in vivo chlorophyll fluorescence on cell suspensions were unsuccessful.

The precision in cell counting was improved by eliminating an error due to counting chamber preparation. The distribution of cell counts was Poisson and more apparent for low counts.

The first attempt at conducting a bioassay of the effects of four low levels of phosphorus on Selenastrum growth gave high Coefficients of Variation (above 15%). The algae responded to the lowest level of 0.01 mg P/l.

Light intensity, initial cell concentration, and nutrient medium freshness, when uncontrolled, were found to effect

algal growth and result in undue error for bioassays.

Methods for insuring carbon availability were tested and yielded less precision in growth parameter determinations than no insurances. The "Cell Health" parameter (dry weight per million cells) showed cells cultured under carbon stress responded by increasing cellular surface to volume ratios as noted from decreases in cell sizes and increases in cell numbers. A high correlation existed between increasing available carbon and "Cell Health".

In applications of the bioassay, three Michigan lake samples (Torch, Deep, and Lansing) were used after membrane filtration to dilute the nutrient stocks for a medium compared to the nutrient stocks diluted with distilled water. The latter treatment gave lower Maximum Specific Growth Rates, lower Maximum Standing Crops in dry weights, poorer "Cell Health", and higher Maximum Standing Crops in cell numbers. The responses were attributed to less carbon stress when lake waters were the diluent due to added carbonate alkalinity.

The same three lakes were bioassayed to determine the nutrient limiting algal production. In all three cases phosphorus stimulated algal growth while nitrogen did not.

For all six experiments applying the bioassay, the precision for the growth parameters was high according to reported "acceptable" precision levels.

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IDENTIFYING AND CONTROLLING SOME SOURCES OF ERROR FOR A STATIC ALGAL BIOASSAY WITH APPLICATIONS

By

Thomas D. Forsythe

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Fisheries and Wildlife

1973

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ACKNOWLEDGEMENTS

I wish to thank my major professor, Dr. Niles R. Kevern for criticizing and evaluating the manuscript.

Appreciation is given to my mother and my wife's parents for their encouragement and support during my years as a student.

Special thanks is expressed to my wife for her perseverance through the neglect she received while I conducted this study. I am indebted to my four-year-old son for the liberty of using his room as a study and for his taking to the sofa many evenings when a noisy typewriter was too much to sleep by.

My studies as a graduate student were made possible through a Traineeship from the U.S. Environmental Protection Agency. (EPA Training Grant No. T-900331).

Finally, I dedicate this thesis to the memory of my father. Much inspiration was gained by reflecting upon the qualities that made him outstanding.

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INTRODUCTION

The main purpose of this study is to investigate, identify, and solve the technical problems associated with a static algal bioassay procedure so that systematic variance will be minimized. Thus, by mastering the techniques, a maximum amount of confidence can be placed in results from subsequent practical applications of the bioassay procedure.

For this study bioassay is defined as biological assessments of measurable responses caused by a controlled treatment to a test organism. Some would say the word "controlled" be excluded from the definition. Their reason might be an example where caged fish are placed in a potentially polluting water with responses measured. I would term such an example as inclined more toward biomonitoring than bioassaying the water. Controlled variables are an important condition for proper bioassays.

Bioassays have been used for many years in many areas of biology since most types of treatment-response experiments are bioassays. If it were not for treatment-response studies, most agricultural, microbiological, pharmacological, and medical research would be in a primitive state. Aquatic biology compared to these areas is in a relatively less advanced state.

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With water pollution making demands of aquatic biology, there is a need for the aquatic biologist to apply fewer of the traditional descriptive investigations in proportion to treatment-response investigations.

This study is concerned with static algal bioassay as a tool for the assessment of the enrichment of aquatic environments (often termed as eutrophication). Weber (1907) first used the terms "oligotrophic" and "eutrophic" respectively as synonyms for "poor in nutrients" and "rich in nutrients". Later, Naumann (1917) used Weber's terms in his dissertation thesis and thus the words were adopted into the field of limnology. Eutrophication may be natural or man-made (cultural). Wetzel (1966) would term very extreme types of eutrophication as "hypereutrophication". Hutchinson (1969) would question whether hypereutrophication and noxious blue-green algal blooms can ever be natural phenomena and are likely always man-made.

At the 1967 International Symposium on Eutrophication (see Introduction to Eutrophication: Causes, Consequences, Correctives; Rohlich, Chairman, 1969) it was stated,

> "Many participants called for a more thorough understanding of algal physiology and ecology. More algal culture studies, coupled with experiments in chemical alteration of lake waters, are needed to better understand the interaction between organisms and nutrients. Such studies require more sensitive analytical techniques that distinguish between available and nonavailable forms of the elements."

The algal bioassay is such a technique holding considerable promise. It can distinguish between available and nonavailable

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nutrients and if performed properly can be very sensitive. Maloney and Bartsch (1969) stated,

> "In many cases these bioassays appear to be more sensitive than standard chemical determinations. Such assays, however, would have much more value with respect to comparing data amoung laboratories and geographic areas if they were standardized."

Recognizing the need for standardization, the Joint Industry/Government Task Force on Eutrophication sponsored the development of the Provisional Algal Assay Procedure (PAAP) (Bueltman, 1969). The procedure consisted of three tests which were (1) a static bottle test, (2) a continuous-flow chemostat test, and (3) an in situ test. The PAAP static bottle test was further developed by an eight-laboratory evaluation team and the subsequent detailed publication was the Inter-Laboratory Precision Test, National Eutrophication Research Program (NERP), U.S. Environmental Protection Agency (Maloney, 1971). The refined static bottle test was the Algal Assay Procedure: Bottle Test (AAP) (Bartsch, 1971).

It is the Algal Assay Procedure: Bottle Test (AAP) that is hoped was mastered in this study. The AAP has already become widely used (Brown, 1972; Brown and Porcella, 1972; Fitzgerald, 1970a,b, 1971, and 1972a; Francisco and Weiss, 1973; Lee, 1973; Maloney et al., 1972; Malueg et al., 1973; Powers et al., 1972; Toerien et al., 1971; and Tunzi, 1971 and 1972). All of these authors used Selenastrum capricornutum, the species used in this study.

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a bioassay test species outside the AAP (Berge, 1969; Chamberlain and Shapiro, 1969; Eyster, 1958; Fitzgerald, 1969; Fitzgerald and Lee, 1971; Pearson et al., 1969; and Skulberg, 1970 and 1972). Since capricornutum is the most commonly used Selenastrum species for bioassay, it will be referred to in this study as just Selenastrum as it was in Murray et al. (1971).

The Algal Assay Procedure: Bottle Test will indeed be recognized as a "standard" if and when it is placed in a new edition of Standard Methods, APHA. Maloney (1972), Chief, National Eutrophication Research Program, U.S. Environmental Protection Agency, stated in a personal communication that it is anticipated the AAP will appear in the 14th Edition of Standard Methods, APHA. He also recommended the AAP be followed as closely as possible for this study.

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TYPES OF ALGAL BIOASSAY

There are three parts to algal bioassay as outlined.

1. Variable test procedure, e.g.
   a. static bottle test,
   b. continuous-flow chemostat test,
   c. *in situ* test, and
   d. dialysis test.
2. Variable test organism, e.g.
   a. *Selenastrum capricornutum* Printz (For AAP),
   b. *Microcystis aeruginosa* Kutz. emend Elenkin (*Anacystis cyanea* Drouet and Daily) (For AAP),
   c. *Anabaena flos-aquae* (Lyngb.) De Brebisson (For AAP),
   d. other commonly used outside the AAP are *Scenedesmus* sp., *Chlorella* sp., and diatoms, and
   e. species indigenous to the test waters.
3. Variable test substance, e.g.
   a. toxicants,
   b. stimulants, and
   c. unknowns.

The choice of a test procedure depends upon the type of data desired, time considerations and economic factors, and the aquatic environment under study.

The static bottle test used in this study is preferred when rapid information on algal response is needed, when many samples are processed, and when many possibilities in experimental design are desired.

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Chemostat tests are more suitable for long-term studies when time and facilities are ample. The chemostat test has not been standardized as has the static bottle test. However, the National Eutrophication Research Center, U.S. Environmental Protection Agency, Corvallis, Oregon, is developing a procedure. Toerien et al. (1971) gives a procedure for a chemostat test. When chemostat testing is standardized, it will likely be more applicable for natural situations than the static test.

The in situ test has not been standardized. This test will probably be used with the most confidence someday. However, the static bottle test will always be useful because of the advantages mentioned previously.

The test organism selected for this study was Selenastrum capricornutum. Unlike many other species, Selenastrum multiplies without clumping and remains at reasonable uniform size throughout its life cycle. In fact, electronic particle counters have been used to determine cell numbers successfully for this species (Tunzi, 1972).

The test substances studied in many of the later experiments in this study were nitrogen and phosphorus since it has been shown they are "key elements" in eutrophication (e.g., Shapiro, 1970).

Although the bottle test requires simple set-up and common procedures, one can get very sophisticated in experimental design. For instance, bottle tests need not be limited to assaying one substance at a time. Factorial experiments

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can be used to look for interactions between two or more test substances. Geisy (personal communication) is using the AAP static algal bioassay to look at various algal-nutrient-humic acid interactions.

Biological measurements even more so than chemical measurements require meticulous technique. Because of the complexity of organisms, many variables enter into biological experimentation. It is desirable to become thoroughly familiar with bioassay by looking for common sources of systematic error before actually applying it to research areas. This is the primary purpose of this study, that is, to look for those areas in the procedure which are most likely to yield high variances and to keep these "problem" variances at a minimum.

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PRINCIPLES OF ALGAL BIOASSAY

In order to grow and reproduce an organism must have essential materials. Liebig (1840) expressed one of the first ecological principles. He said,

> "Growth of a plant is dependent on the amount of foodstuff which is presented to it in minimum quantities."

This has become known as Liebig's "Law of the Minimum". In his paper, Liebig defined "foodstuff" as "nutrients". Shelford (1913) introduced the "Law of Tolerance" which included growth limitations due to maximum as well as minimum factors. Taylor (1934) and many others have expanded the "Law of the Minimum" to include factors other than nutrients, such as pH, light, and temperature. In regard to this, Odum (1971) states,

> "To avoid confusion it seems best to restrict the concept of the minimum to chemical materials necessary for physiological growth and reproduction, as was originally intended, and to include other factors and the limiting effect of the maximum in the "Law of Tolerance"..... Extensive work since the time of Liebig has shown that two subsidiary principles must be added to the concept if it is to be useful in practice. The first is a constraint that Liebig's law is strictly applicable only under steady-state conditions, that is, when inflows balance outflows of energy and materials..... The second important consideration is factor interaction. Thus, high concentration or availability of some substance, or the action of some factor other than the minimum one may

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modify the rate of utilization of the latter." Odum points out that pristine, balanced ecosystems operate under "steady-state" conditions, however, man has caused a highly "unsteady" state. Regarding water bodies and the "transient state" due to man, Odum states the "Law of the Minimum" is of no theoretical basis under such conditions. The "limiting nutrient" is likely irrelevant according to Odum, because phosphorus, nitrogen, carbon dioxide, and many other nutrients may rapidly replace one another as limiting factor.

Others have stated the complexity of organisms alone makes the "Law of the Minimum" an oversimplifaction (e.g., Goldman, 1963).

The "Law of the Minimum" was in so very much dispute, a symposium by the American Society of Limnology and Oceanography entitled Nutrients and Eutrophication: The Limiting Nutrient Controversy (Likens, ed., 1972) was devoted to the dispute (see Preface).

While the "Law of the Minimum" may be an oversimplification for natural situations, it is easily realized for the static algal bottle tests. If one takes a bottle containing a medium having all essential nutrients and inoculates it with algae, growth will ensue until some nutrient becomes limiting to growth. At that time a steady-state condition exists although maybe for only a short period of time. (For steady-state conditions for extended periods of time one would use continuous-flow chemostat tests). At first the

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algae will grow at an increasing rate. Then growth, although increasing, will increase at a decreasing rate. The point at which the growth changes from an increasing to a decreasing rate is termed the Maximum Specific Growth Rate (μ_{max}).

Although the experiments in this study were not designed specifically to show the correctness of the "Law of the Minimum" as it applies to static bioassays, some experiments provide such evidence. Some may say if the "Law of the Minimum" does not hold for natural situations, then whatever conclusions are reached in a static bioassay are not applicable outside the laboratory. A more pragmatic approach to bioassay is used for this study. This approach is; if the algal bioassay is used to make an assessment or prediction about a natural situation and it assesses or predicts correctly, then continue using the assay. For instance, Malueg et al. (1973) used the AAP to make predictions about advanced wastewater treatment for phosphorus removal in the Shagawa Lake Restoration Project, Ely, Minnesota. This U.S. Environmental Protection Agency project will continue until at least 1976, at which time the value of the algal bioassay can be assessed. Preliminary investigations with pilot plant treatment efficiencies determined by bioassay are promising. The objective of the Shagawa Lake Project is to demonstrate the restoration of a man-made eutrophic lake by removing the critical growth promoting nutrient, phosphorus, by advanced treatment of municipal wastewater while permitting such treated effluent to continue to flow into the lake. If the

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project is successful, the algal bioassay will have helped solved a technological problem. Biology is much in need of methods and techniques that can more easily be applied to the technological problems in water pollution control. Algal bioassay shows promise as such a tool.

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Compound	Cor
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$CaCl_2 \cdot 2HOH$	
$NaHCO_3$	

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ALGAL BIOASSAY PROCEDURE

The AAP was followed closely for this study. Some modifications were necessary and are noted here.

Table 1. Synthetic algal nutrient medium macronutrients given as final concentrations in milligrams per liter.

Compound	Concentration (mg/l)	Element	Concentration (mg/l)
$NaNO_3$	25.500	N	4.200
K_2HPO_4	1.044	P	0.186
$MgCl_2 \cdot 6HOH$	12.143[a]	Mg	2.904
$MgSO_4 \cdot 7HOH$	14.143	S	1.911
$CaCl_2 \cdot 2HOH$	4.410	C	2.143
$NaHCO_3$	15.000	Ca	1.202
		Na	11.001
		K	0.469

[a] $MgCl_2 \cdot 6HOH$ is substituted for AAP's 5.700 mg $MgCl_2$/l.

Stock solutions of individual macronutrient salts were made up in 1000 times the final concentration. A six-place balance was used for weighing and appropriate dilutions were made. A four-place balance used for a stock preparation required more dilutions, but was not judged as a loss in accuracy of the final nutrient concentrations.

Micronutrient trace metals and EDTA (ethylenediaminetetraacetic acid) were combined into a single stock mix at

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$CoCl_2$	
$CuCl_2$	
$Na_2MoO_4 \cdot 2HO$	
$FeCl_3 \cdot 6HOH$	
$Na_2EDTA \cdot 2HO$	
[a] $MnCl_2 \cdot 4HOH$	
[b] $FeCl_3 \cdot 6HO$	

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1000 times the final concentrations. The EDTA chelator was necessary to insure the biological availability of certain trace elements, primarily the Fe-EDTA complex.

Table 2. Synthetic algal nutrient medium micronutrients given as final concentrations in micrograms per liter.

Compound	Concentration (µg/l)	Element	Concentration (µg/l)
H_3BO_3	185.520	B	32.460
$MnCl_2 \cdot 4HOH$	415.379[a]	Mn	115.374
$ZnCl_2$	32.709	Zn	15.691
$CoCl_2$	0.780	Co	0.354
$CuCl_2$	0.009	Cu	0.004
$Na_2MoO_4 \cdot 2HOH$	7.260	Mo	2.878
$FeCl_3 \cdot 6HOH$	159.881[b]	Fe	33.051
$Na_2EDTA \cdot 2HOH$	300.000		

[a] $MnCl_2 4HOH$ was substituted for AAP's 264.264 g $MnCl_2$/l.
[b] $FeCl_3 \cdot 6HOH$ was substituted for AAP's 96.000 g $FeCl_3$/l.

The dilution water was distilled water passed through a mixed-bed resin ion-exchange column. This water will hereafter be referred to as just distilled water.

One milliliter of each stock solution was added to distilled water to give a final volume of one liter of AAP medium. Murray et al. (1971) found the order in which the nutrients were added and the freshness of the medium changed nutrient concentrations. I did not initially realize this since the AAP does not stress the importance of medium preparation and freshness. It was initially noted the nutrients formed a precipitate when directly combined and then

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diluted. Therefore, the stocks were added to a volumetric flask half filled with distilled water and brought up to volume. It was believed this made the kinetic chances for precipitation less favorable. The AAP recommends the trace metal-EDTA stock be added after filtration. This was done with a 0.47μ membrane filter.

The nutrient stocks were autoclaved to minimize bacterial contamination. Since the algae used were not obtained in an axenic condition, sterilizing culture medium is not necessary. An experiment was conducted to determine if phosphate was lost due to autoclaving. No significant loss was detected. The AAP tested for losses in Ca, Na, K, Mg, Cl, SO_4, and NO_3 following autoclaving, membrane filtration, and centrifugation. No significant decreases were noted for these nutrients. However, autoclaving caused an increase in soluble silica concentrations, possibly from the glass containers. If diatoms are used as a test organism, this should be noted.

Reilly (1972) compared growth obtained by autoclaving and membrane filtering the medium and found no difference. He also found Microcystis aeruginosa grew better with its "symbiotic" bacteria than in an axenic state. There is a need for more studies comparing axenic with non-axenic bioassays, however, the AAP as developed is intended for non-axenic bioassays.

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was obtained from the National Eutrophication Research Center, Pacific Northwest Water Laboratory, U.S. Environmental Protection Agency, Corvallis, Oregon. When the algae were received, a transfer was made into AAP medium. Aseptic technique was used in handling the stocks to avoid unnecessary contamination. It was found if this was not done the stocks soon became cloudy with bacteria. A weekly routine stock transfer schedule was followed. Transfers were made by centrifuging and washing the cells twice with 15 mg $NaHCO_3$/l. All glassware used for stock cultures and transfers was autoclaved.

Cleaning of glassware was done according to the Handbook for Analytical Quality Control in Water and Wastewater Laboratories (U.S. Government, 1972). Glassware was washed with sodium carbonate, soaked in ten percent reagent hydrochloric acid, and rinsed several times with distilled water. Detergents were not used to avoid phosphorus contamination.

The culture flasks were 125-ml Pyrex Erlenmeyer flasks. The flasks were washed, autoclaved, and stoppered with foam plugs. Only fifty ml of sample or medium was placed in the flasks for optimum surface to volume ratios to aid in carbon dioxide diffusion into the culture solutions. Justice et al., (1972) found certain foam plugs were toxic to Selenastrum. All foam plugs were compared to foil coverings and none were significantly different.

Inoculum was prepared so nutrient carryover was minimized and all flasks got as closely as possible the same number of

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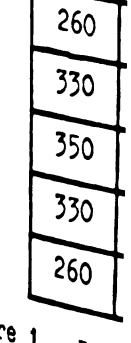

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algal cells. The AAP suggests counting the inoculum to determine how much to deliver to the flasks. The starting cell concentration should be about 10^3 cells per ml. I found delivering the inoculum with a calibrated pipet to be in excessive error. The final method I adopted was to centrifuge a portion of the stock culture, wash it twice with a solution of 15 mg $NaHCO_3$/l, and resuspend the cells in the bicarbonate solution. Inoculum was delivered with a long-stem capillary dropper. With experience, I was able to judge the number of drops needed to give an initial cell concentration of about 10^3 cells per ml. One flask was counted immediately after inoculation and this was recorded as the initial cell concentration.

Inoculated flasks were stoppered with the foam plugs and incubated at 24°C under cool-white fluorescent illumination. Squares were drawn on a white assay platform, each to accommodate one culture flask. The light intensity reaching each square was determined with a light meter and varied according to Figure 1.

260	305	330	350	350	330	305	260
330	390	420	450	450	420	390	330
350	420	450	475	475	450	420	350
330	390	420	450	450	420	390	330
260	305	330	350	350	330	305	260

Figure 1. Light intensity in foot-candles reaching each square on the assay platform.

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Figure 1 depicts the light intensity ranges from 260 ft-c to 475 ft-c. The AAP states the optimum intensity for Selenastrum is 400 ± 50 ft-c. The assay platform had twenty-two of its forty squares in this optimum range. Flasks were alloted to these squares whenever possible. Murray et al. (1971) found light intensities of 500 ft-c reduced growth rates when compared to 350 ft-c. Therefore, 500 ft-c was not exceeded in this study.

The AAP recommends the pH in a culture should be kept within the range of 8.0 to 8.5 to insure carbon availability. Methods suggested by the AAP for controlling pH were studied in Experiment 7. The approach I took to pH rises in this study followed that of Fitzgerald (1972b), who stated in a recorded discussion,

> "It doesn't make any difference what pH it is if the algae grow. I don't care if my algae grow in pH 10 or 11. If they grow, that's a successful culture".

No measures were taken in this study to control pH other than using optimum surface to volume ratios for the medium and daily hand swirling of the flasks. The AAP also suggests shaker tables, ventilation of air or carbon dioxide enriched air, and bubbling of air or carbon dioxide enriched air. The results of Experiment 7 plus the findings of the Inter-Laboratory Precision Test (Maloney, 1971) that daily hand swirling was equivalent to shaker table results led to the dismissal of venting or bubbling measures.

Two parameters are used by the AAP to describe algal

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growth: (1) the Maximum Specific Growth Rate and (2) the Maximum Standing Crops. The use of these parameters depends on the bioassay objectives.

Detailed information on the kinetics of algal growth is given by Myers (1962), Pearson et al. (1969), and Tamiya (1951). Algal growth under theoretical conditions of unlimited materials would result in a J-shaped growth curve. Because all environments have a definite carrying capacity (Odum, 1971), the S-shaped or sigmoid curve is the growth pattern in nature for most living populations. The same is true for static bioassays. The equation for the growth of a population is:

$$dX/dt = \mu X,$$

where X is some measurable reflection of growth, t is time, and μ is the specific growth rate coefficient. The symbol, μ, has been substituted by the symbols r (Odum, 1971) and k (Myer, 1962) in other literature. The specific growth rate gives an intrinsic measure of the rate of total metabolism leading to cell synthesis (Myers, 1962). Calculus manipulation of the above equation gives the following formula from which growth rate calculations are easily made:

$$\mu = \frac{\ln(X_2/X_1)}{(t_2-t_1)},$$

where X is measured directly in cell numbers or biomass. X can also be measured indirectly by calibrating optical density, *in vivo* chlorophyll fluorescence, chlorophyll

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The Maximum Specific Growth Rate (μ_{max}) is reached when the culture conditions, changing with time, are optimum for growth. For algal bioassay, μ_{max} provides a criterion of the adequacy of inorganic nutrition (Myers, 1962). It is also a function of other factors such as light intensity or temperature. For this reason, all factors affecting μ_{max} other than the factor of concern in the bioassay should be held constant and frequently checked.

The growth parameter of Maximum Standing Crop represents the maximum quantity of cells or cell biomass achievable in a bioassay. According to the AAP, it may be assumed the Maximum Standing Crop has been reached when the increase in cell numbers or biomass is less than five percent per day. This value is often not the absolute maximum. The Maximum Standing Crops are denoted as cell numbers or biomass by the same indirect methods discussed previously. The parameter is expressed as cell numbers per ml or dry weight per ml. By determining both expressions for the parameter and reporting the two as a quotient, one comes up with dry weight per cell. This is often expressed as dry weight per million cells and is descriptive of not only the nutrients assayed, but also the physiological condition ("cell health") of the cells at the time of measurement.

Devices commonly used to determine cell numbers are Sedgwick-Rafter chambers, hemacytometers, Palmer chambers, and electronic particle counters such as the Coulter counter.

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I found the Sedgwick-Rafter chamber had a volume too large (1000 mm^3) to give accurate cell counts. Cells counted after settling to the bottom of the chamber were too dense for counting. The Palmer chamber's volume (100 mm^3) gave acceptable counts according to Experiment 1. The μ_{max} usually occurred between days 0-3 and the Maximum Standing Crops between days 5-10. Therefore, cell counts were made on a daily basis.

For this study, gravimetric determinations were always dry weights of the algal cells. This method is especially useful for assessing the growth of joined or filamentous species (e.g., Anabaena, Cladophora, or Scenedesmus) since it is often difficult to obtain accurate cell counts. I found gravimetric determinations to be very precise for the Maximum Standing Crop parameter, but not suitable for the μ_{max} parameter. This is because the maximum growth rate is achieved during cell densities too low for precise dry weight determinations. Gravimetric determinations might be able to monitor daily growth for large cultures of a liter or more where large subsamples can be taken for weighing.

I used the aluminum pan method to determine dry weight. A portion of algal suspension was centrifuged, cells washed twice with distilled water, cells resuspended to the same volume as the original portion, cell suspension transferred to tared aluminum pans, cells dried overnight in a

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hot air oven at 105°C, and dried cells weighed on a six-place balance. The AAP describes a membrane filtration method which it states to be less sensitive than the aluminum pan method.

Indirect measurements are easier and faster to determine than the direct methods. Optical density or absorbance as defined by Beer's Law, can be used to determine the concentration of cells in suspension (Myers, 1962). One is not only measuring light absorption, but also light scattering caused by reflection and refraction. The amount of scattering depends upon the instrument quality. Turbidimetry is the technical name for light scattering measurements. Nephelometry is the term when a fluorometer is used instead of a photometer (Skoog and West, 1971). Absorbance is a function of cellular pigmentation and extracellular products. Turbidity is afunction of cell shape, size, and volume. I found absorbance to be useful for determining Maximum Standing Crops but not μ_{max} for the same reasons as gravimetric measurements. This was investigated in Experiment 2.

Indirect measurements of extracted chlorophyll concentrations determined by spectrophotometry are reported to be sensitive enough to monitor daily growth (Strickland and Parsons, 1968 and Yentsch and Menzel, 1963). However, the method is time consuming for routine determinations.

In vivo chlorophyll fluorescence of unextracted algal suspensions is a relatively new technique which is rapid and very sensitive. It can be used to determine growth rates.

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Lorenzen (1966) used the in vivo method on a fluorometer with a red sensitive photomultiplier which he reported to increase the sensitivity by a factor of ten. I investigated his procedure in Experiment 2, but with a fluorometer unequipped with a red sensitive photomultiplier. For details one is directed to the AAP, Lorenzen (1966), and Strickland (1968). Strickland adds a precautionary note to Lorenzen's method stating light scattering, as in optical density measurements, can add to the recorded fluorescence values. Chlorophyll can be determined using fluorometry with the extraction procedure discussed previously and is more sensitive than the spectrophotometric method.

ATP (adenosine triphosphate) concentration determinations has not seen use in algal bioassays. It is rapidly becoming used for aquatic bacterial biomass determinations since bacteria are so closely associated with detritus, making direct biomass determination almost impossible. See Sorokin and Kodota (1972) for details. Briefly, it involves the principle that one photon of light is emitted for each ATP molecule hydrolysed. The concentration of ATP in any sample can be obtained by measuring the intensity of the light emission when the sample is mixed with the proper reactants and enzyme. The reactant, luciferin, and the enzyme, luciferase, are obtained from firefly lantern extracts. The advantage of the ATP-bioluminescent method is that it measures only living material. In advanced stages of an algal bioassay one is unsure if all cells are viable.

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The ATP method is sensitive for a work range of 10^{-3} to 10^{-7} micrograms of ATP and can be calibrated to total cell carbon or biomass. This method could be used to measure daily growth and would be especially useful for toxicity bioassays where viable and nonliving cell ratios may be rapidly changing. Algae have been used to bioassay various pesticides (Sweeney, 1970).

Total carbon determinations, although very sensitive, have not found extensive applications for algal bioassay since most laboratories are not equipped with a carbon analyzer.

Goldman (1963 and 1968) uses radiocarbon procedures for algal bioassay. This method deserves more extensive use since it is a direct measure of the metabolic activity of the algae. Recently, Kerr (1972) suggested for non-axenic bioassays it is open to great error due to dilution of specific activity by carbon dioxide produced by bacteria.

King (1972) uses perhaps the simplest technique to follow daily growth. He monitors daily pH changes in closed cultures. By knowing the initial alkalinity, he can determine carbonate alkalinity decreases from the pH measurements. From this, growth rates can be calculated.

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EXPERIMENTS INVESTIGATING SOME SOURCES OF ERROR IN THE ALGAL BIOASSAY PROCEDURES

The experiments are presented in the order performed. The data and growth curves are not presented in the most concise fashion. For instance, a growth curve for each treatment replication is depicted in the figures. Normally, the treatment replication data would be averaged and figures would depict one mean growth curve for each treatment all in one figure. For this study, it was hoped a visual comparison of replication precision would be achieved by giving growth curves for each treatment replication.

The number of statistical tests has been kept to a minimum. To compare means the Student's t test (hereafter shortened to t test) was used exclusively. Other less commonly used tests, such as Duncan's Multiple Range test, Student-Newman-Keuls test, and Least Significant Difference testing might have been used. The statistic used as the indicator of experimental precision was the Coefficient of Variation (C_v) and is simply one standard deviation (SD) divided by the mean usually expressed in percent. C_v of 15 percent and below has been suggested as the acceptable level of precision for bioassay (Toerien et al., 1971).

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the difference is reported as not significant (ns) only when the confidence level is below 50%. Otherwise, the difference confidence level will be reported if above 50% and the reader is to decide on the significance.

The first eight experiments are attempts to minimize systematic variances in the procedure. The last six experiments are applications of the procedure.

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Experiment 1
Cell Counting Error

Purpose

This experiment attempted to locate, study, and eliminate sources of variability in counting algal cells. Five Palmer chambers were compared. The manufacturer gives only approximate dimensions of chamber volumes with no error estimation factor (volume given as 0.1 ml or 100 mm^3). An analysis of variance is called for.

Design

A Nested Analysis of Variance is used. The statistical notations are those used by Sokal and Rohlf (1969). The variance components tested were

$$Y_{ijk} = u + C_i + R_{ij} + M_{ijk}$$

where Y_{ijk} = sample mean (observed mean),

u = population mean (true mean),

C_i = effects of counting chambers,

R_{ij} = effects of samples within chambers,

and M_{ijk} = effects of counts within samples.

Five chambers were used for the analysis. Counting consisted of two samples per chamber from a single Selenastrum culture with five countings per sample. A "count" is a cell number determination under a Whipple micrometer grid at 100x

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$SS_{(RC)} = Y^2_{ijk}$

magnification. The cells per grid were not transformed to cells per ml since transformations do not affect variances. So the mean is:

Y_{ijk}

- k → counts (measurement) $m = 5$ as $k = 1,2,3,4,5$
- j → sampling (run) $r = 2$ as $j = 1,2$
- i → chamber $c = 5$ as $i = 1,2,3,4,5$

The nesting of the data is shown in Table 3.

Table 3. Cell counts for a Nested Analysis of Variance show-how the data is nested.

Chamber	Runs within Chamber	Measurements within Run (cells/grid)	$Y_{ij.}$	$Y_{i..}$	$Y_{...}$
1	1	96, 97,110,121,104	528		
	2	136,116,112,118,119	601	1129	
2	1	123,174,150,139,159	745		
	2	103, 95,110,116,128	552	1297	
3	1	93,116,122,127,135	593		
	2	108,130,140,128,121	627	1220	
4	1	143,143,137,134,143	700		
	2	93,120,121,112,126	572	1272	
5	1	132,122,125,153,131	663		
	2	107, 99,139,105,104	554	1217	6135

$Y^2_{ijk} = 768473.0$

$Y^2_{ij.}/m = 3808161/5 = 761632.2$

$Y^2_{i..}/mr = 7544323/10 = 754432.3$

correction term (CT) $= Y^2_{...}/mrc = 37638225/50 = 752764.5$

$SS_{total} = Y^2_{ijk} - CT = 768473.0 - 752764.5 = 15708.5$

$SS_C = Y^2_{i..}/mr - CT = 754432.3 - 752764.5 = 1667.8$

$SS_{R(C)} = Y^2_{ij.}/m - CT - SS_C = 761632.2 - 752764.5 - 1667.8 = 7199.9$

$SS_{M(RC)} = Y^2_{ijk} - CT - SS_C - SS_{R(C)} = 768473.0 - 752764.5 - 1667.8 - 7199.9 = 6840.8$

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Chamber	SS_c
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Table 4b.

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Meas.	6
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Results

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Table 4a. Symbolic Nested Analysis of Variance to study the variance in cell counting by defining the effects due to counting chambers, samples within chambers, and grid cell counts within samples.

SOURCE	SS	df	MS	EMS
Chamber	SS_C	(c-1)	$SS_C/(c-1)$	$\sigma_M^2 + m\sigma_R^2 + mr\sigma_C^2$
Run	SS_R	c(r-1)	$SS_R/c(r-1)$	$\sigma_M^2 + m\sigma_R^2$
Meas.	SS_M	cr(m-1)	$SS_M/cr(m-1)$	σ_M^2
Total	SS_{Total}	(N-1)		

Table 4b. Numerical Nested Analysis of Variance to study the variance in cell counting by defining the effects due to counting chambers, samples within chambers, and grid cell counts within samples.

SOURCE	SS	df	MS	F_s	
Chamber	1667.8	4	416.95	0.289	ns
Run	7199.9	5	1439.98	8.420	***
Meas.	6840.8	40	171.02		
Total	15708.5	49			

$*F_{.05(4,5)} = 5.19$	$**F_{.01(4,5)} = 11.4$	$***F_{.001(4,5)} = 31.1$
$*F_{.05(5,40)} = 2.45$	$**F_{.01(5,40)} = 3.51$	$***F_{.001(5,40)} = 5.13$

Variance Components - 0% due to Chambers
- 60% due to Runs within Chambers
- 40% due to Measurements within Runs

Results

The analysis in Table 4a,b indicates no significant difference exists between counting chambers difference exists between runs within a chamber at a 99.9% confidence level.

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Variance component calculations indicate there is more variance due to runs (sample taken from one bottle) than to measurements (cell counts) within a run.

Either the technique of withdrawing and preparing a run (sample) needed improving or more runs per chamber needed counting. To check on the latter possibility a second analysis of variance was conducted with more degrees of freedom in both Runs and Measurements. The results are given in Table 5.

Table 5. Nested Analysis of Variance studying the variance in cell counting by defining the effects due to counting chambers, samples within chambers, grid cells within samples. The degrees of freedom were 4, 20, and 225 respectively compared to 4, 5, and 40 from Table 4b.

SOURCE	SS	df	MS	F_s	
Chamber	1092.42	4	273.11	1.55	ns
Run	3516.48	20	175.82	4.34	***
Meas.	9112.70	225	40.50		
Total	13721.60	249			

$*F_{.05(4,20)} = 2.87$	$**F_{.01(4,20)} = 4.43$	$***F_{.001(4,20)} = 7.10$
$*F_{.05(20,\ )} = 1.57$	$**F_{.01(20,\ )} = 1.88$	$***F_{.001(20,\ )} = 2.27$

The analysis in Table 5 indicates no significant difference exists between chambers and a difference exists between runs within a chamber at a 99.9% confidence level. This suggests the technique rather than the degrees of freedom was responsible for the unwanted variance due to withdrawing and

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preparing a run sample for counting. A closer look at the technique was called for.

The method used to prepare a chamber for counting was to (1) swirl the flask about fifty times to insure an even cell suspension, (2) withdraw a sample with a long stem capillary dropper, (3) place a few drops of cell suspension in the chamber, (4) place a cover slip over the chamber, and (5) let the cells settle for a few minutes so they are all on the bottom of the chamber.

When the cells were allowed time to settle, evaporation occured in the chamber. Therefore, a slight excess of cell suspension was placed in the chamber so that the cover slips floated. Upon cell settling and subsequent evaporation the cover slips would come in contact with the chamber walls about the time the cells had completely settled. This method was suspected (after the results in Table 5) to cause error since "floaters" produce unequal chamber volumes.

A modification in the method involved (1) placing a few drops of cell suspension on the chamber, (2) withdrawing excess cell suspension from the port so the cover slip was snug on the chamber wall surface, (3) allowing cells to settle, and (4) injecting distilled water into the port upon evaporation of the cell suspension, but not overfilling the chamber. Table 6 is an Analysis of Variance that used the modification.

Table 6 indicates no significant differences exist between chambers or runs within chambers. The modified method of preparing chambers eliminated the variance due to runs

within chambers. This puts all the variance in counting, which is desirable since one can control the error by simply counting more grids per run. (However, when the cells per grid is very low, such as less than ten cells per grid, one has very little control over variance. The cells are distributed in a Poisson distribution according to Appendix A.) With a significant portion of the variance due to poor technique, as was the case for Table 4b and 5, one has very little control over error.

Table 6. Nested Analysis of Variance studying the variance in cell counting by defining the effects due to counting chambers, samples within chambers, and cells within samples. The degrees of freedom were 4, 20, and 100 respectively.

SOURCE	SS	df	MS	F_s
Chamber	251.41	4	62.85	1.52 ns
Run	898.08	20	44.90	0.104 ns
Meas.	4556.80	100	433.68	
Total	5486.29	124		

$^{*}F_{.05(4,20)} = 2.85$	$^{**}F_{.01(4,20)} = 4.43$	$^{***}F_{.001(4,20)} = 7.10$
$^{*}F_{.05(20,120)} = 1.66$	$^{**}F_{.01(20,120)} = 2.03$	$^{***}F_{.001(20,120)} = 2.53$

Variance Components - 0% due to Chamber
- 0% due to Runs within Chambers
- 100% due to Measurements with Runs

Conclusion

By using a Nested Analysis of Variance an unwanted source of error in cell counting was located, identified, and eliminated by modification of procedures.

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Experiment 2

Correlations between cell counting, in vivo chlorophyll fluorescence, and optical density measured on Selenastrum cell suspensions

Purpose

Correlations were made between cell counts, in vivo chlorophyll fluorescence, and optical density of cell suspensions to determine if a indirect method of monitoring daily Selenastrum growth could be used.

Procedure

A Selenastrum stock culture was diluted nine times from an initial concentration of 3.64×10^6 cells per ml down to 2.20×10^5 cells per ml. Cell counts, optical density, and fluorescence were measured on each of the ten different cell concentrations.

Cell counting consisted of counting two samples per dilution and twenty micrometer grids per sample. Expected counts were calculated and compared with the observed counts (Tables 7 and 8).

Chlorophyll fluorescence was measured using an in vivo method with a Turner®, Model 111 fluorometer. Lorenzen (1966) used the method on the same fluorometer, specially equipped with a red sensitive photomultiplier tube and blue lamp light source, to measure the chlorophyll within phytoplankton

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cells from lake samples. The modification to the fluorometer increases the sensitivity by a factor of ten according to Lorenzen and also Turner (1973). Many have used the method recently in the field due to its sensitivity and simplicity. For this study attempts were made to use the in vivo method without the red sensitive photomultiplier or blue lamp adaption. Since Lorenzen was working with very dilute samples (natural samples) relative to bottle cultures, it was hoped the ten fold loss in sensitivity would not be realized. The cell suspensions were placed in sample holders, matched filters (red and blue) were placed in the fluorometer, fluorescence was measured, and readings were recorded. To obtain the fluorescence units, the readings are multiplied times thirty since the fluorometer was operated at the maximum sensitivity setting of 30x.

Optical density was measured directly on the cell suspensions with a Baush & Lomb, Spectronic 20, at a wavelength of 680 nm and a sample cell width of 1 cm. Table 7 lists the fluorescence and optical density readings.

Results

The correlation coefficients listed in Table 8 were calculated from the data in Table 7. A statistical table (Bailey, 1959) gives the probability of observing a correlation coefficient greater than 0.872 for eight degrees of freedom as 0.001 or 0.1%. The smallest of the five calculated here was 0.988, showing the high degree of correlation for

Table 7. D
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Dilution ratios	F c
stock	
4:1	
3:1	
2:1	
1:1	
1:2	
1:3	
1:4	
1:8	
1:10	

Table 8.

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Observed
[a]Observed
[a]Observed
Expected
Expected

[a]See Figu

Table 7. Data from cell counts, optical density, and *in vivo* chlorophyll fluorescence measured on ten dilutions of a *Selenastrum* cell suspension.

Dilution ratios	Percent of stock	Expected cells/ml	Observed cells/ml	Fluorescence readings	Optical density
stock	100	3.64×10^6	3.64×10^6	100.00	0.139
4:1	80	2.91×10^6	2.79×10^6	84.93	0.102
3:1	75	2.73×10^6	2.63×10^6	78.25	0.096
2:1	67	2.40×10^6	2.19×10^6	78.88	0.089
1:1	50	1.82×10^6	1.57×10^6	55.13	0.067
1:2	33	1.20×10^6	1.28×10^6	40.75	0.044
1:3	25	9.10×10^5	8.40×10^5	27.85	0.020
1:4	20	7.30×10^5	7.50×10^5	19.88	0.009
1:8	12½	4.40×10^5	2.60×10^5	5.50	0.009
1:10	6¼	2.20×10^5	2.40×10^5	2.60	0.011

Table 8. Correlations between expected cell counts, observed cell count, *in vivo* chlorophyll fluorescence, and optical density measured on ten dilutions of a *Selenastrum* cell suspension.

Variables Compared	Correlation Coefficient
Observed - Expected Cell Counts	0.995
[a]Observed Cell Counts - Fluorescence	0.991
[a]Observed Cell Counts - Optical Density	0.988
Expected Cell Counts - Fluorescence	0.991
Expected Cell Counts - Optical Density	0.992

[a]See Figures 2 and 3.

FLUORESCENCE reading
110
100
90
80
70
60
50
40
30
20
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0

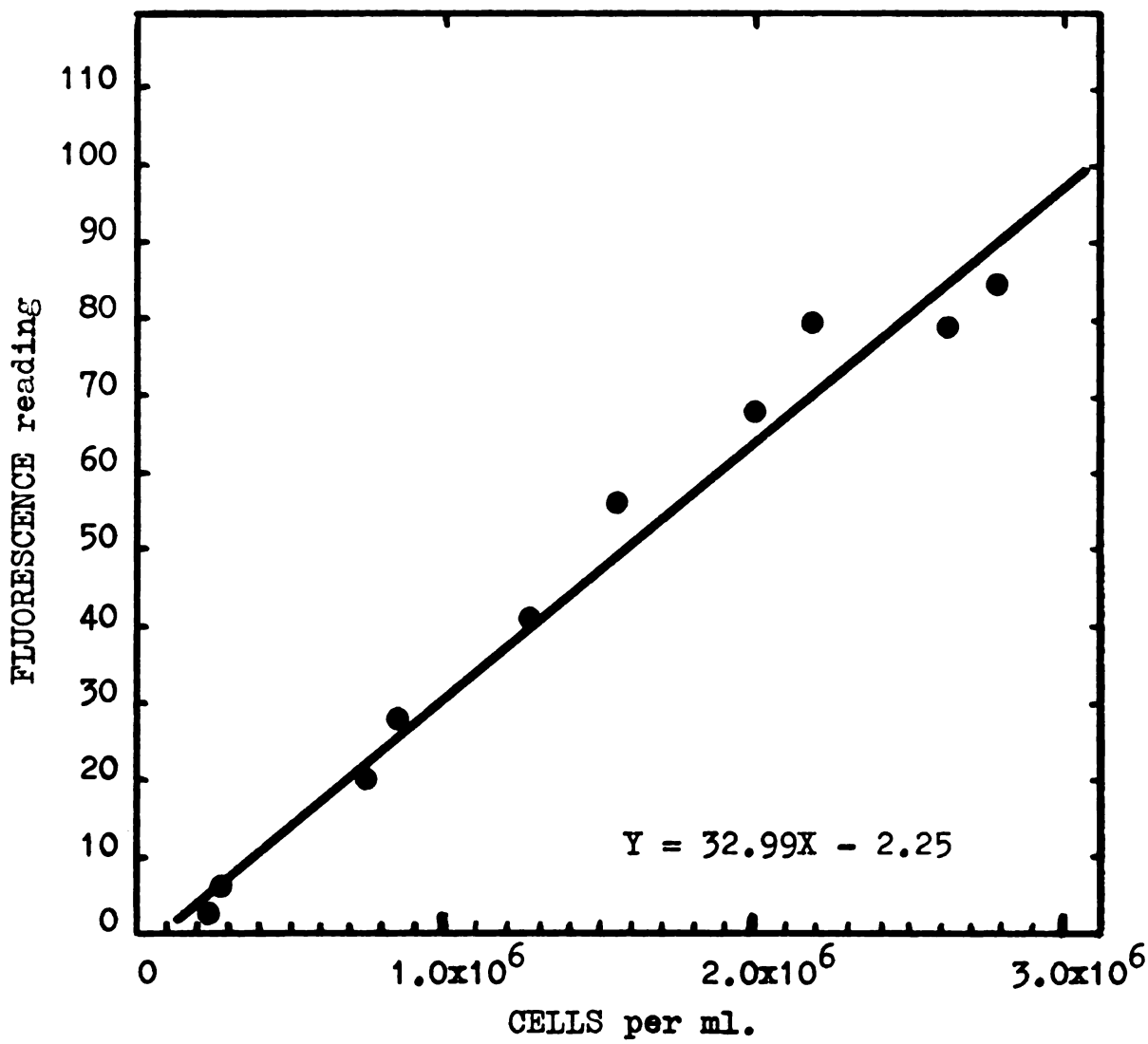

Figure 2. The straight-line relationship between observed cell counts and *in vivo* chlorophyll fluorescence with regression equation.

OPTICAL DENSITY readings

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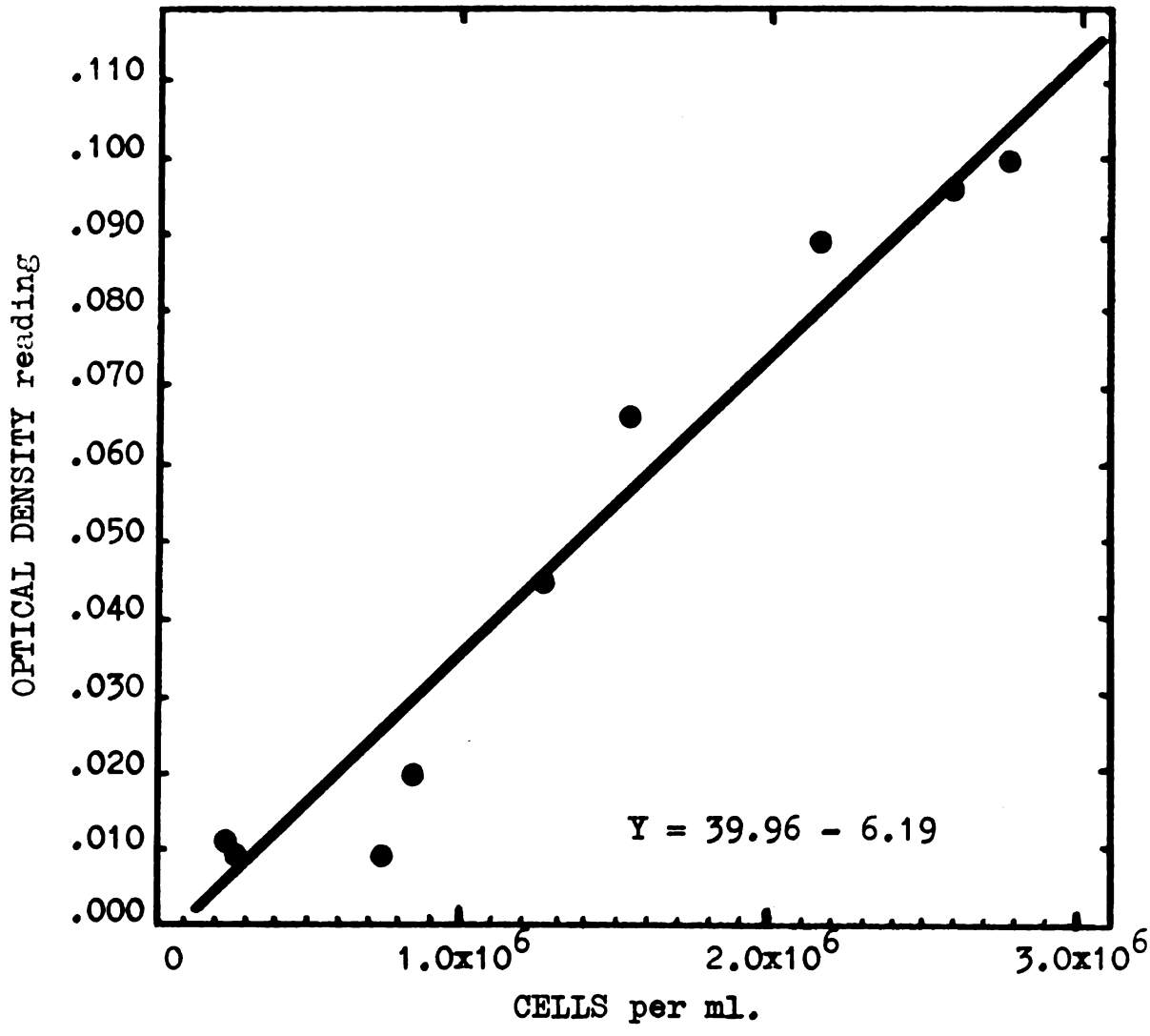

Figure 3. The straight-line relationship between observed cell counts and optical density with regression equation.

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Figures

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Figures 2 and 3 depict the relationships between observed cell counts against optical density and observed cell counts against fluorescence. It is apparent from the figures that the lower detection limit for both instruments is approximately 10^5 cells per ml with the methods tested here.

Conclusions

It was hoped fluorescence or optical density could be calibrated to cell counts thus allowing the growth parameters of daily Specific Growth Rates and Maximum Standing Crops to be indirectly determined. The AAP found most algal bioassays using Selenastrum have the μ_{max} occurring between counts of 10^4 and 10^5 cells per ml and the Maximum Standing Crops occurring above counts of 10^5 cells per ml. Both methods here could be used to determine standing crops. Many experimenters have used with success, Maximum Standing Crops measured by optical density as the only growth parameter for algal bioassays. However, a purpose of this study was to become familiar with algal growth rates. Therefore, fluorescence and optical density were not used in subsequent experiments since they were not sensitive enough for the results desired.

To increase the sensitivity of optical density others have used optimum cell widths (Myers, 1962; Yentsch and Menzel, 1963; and Yentsch, 1957). To increase the sensitivity of fluorescence a red sensitive photomultiplier must be installed on Turner fluorometers. Another method involves concentrating the cells before making measurements.

Effects

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Purpose

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Experiment 3
Effects of four levels of orthophosphate on Selenastrum growth for familiarization with bioassay procedures

Purpose

Primarily, this experiment was for familiarization with Selenastrum culturing, handling, growth measuring, and data collecting. Problems were noted and used to design subsequent experiments. Secondarily, the objective was to determine the sensitivity of the bioassay procedure to low levels of orthophosphate.

Design

Orthophosphate was bioassayed at four levels of 0.00, 0.01, 0.02, and 0.03 mg P/l. Each treatment level was run in four replications. Cell counts were used as the growth indicator.

Procedure

The control treatment was the AAP nutrient medium minus potassium phosphate. Potassium chloride was added to give potassium to the medium at the concentration shown in Table 1. The other three treatments each had potassium phosphate added in amounts which gave the desired phosphorus concentrations.

A six-day-old stock of Selenastrum was used as inoculum. The cells were washed and resuspended in a weak bicarbonate

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solution (15 mg $NaHCO_3$/l) to minimize nutrient carry-over. The prepared inoculum was counted and it was determined 0.55 ml of inoculum should give an initial cell concentration of about $1x10^4$ cells per ml. Inoculum delivery was done with a calibrated 1-ml pipet into fifty ml of one-day-old media treatments. The flasks were randomly inoculated and allotted to the assay platform squares using sixteen of the forty squares which were closest to 400 ft-c.

Cell counts were determined on day 0,1,2,3,5,6, and 10. This consisted of taking two samples per flask and counting twenty micrometer grids per sample. From the cell counts the desired growth parameters were determined.

Results

Figures 4a,b,c,d depict the growth curves for each treatment replication. The cell counts and Coefficient of Variation for counting (C_v) are given in Appendix B tables B1, B2, B3, and B4. The C_v values decrease as the cell counts per grid increase. This may be due to the counting technique rather than the state of the cultures. When the cells per grid are less than about ten, it is apparent the C_v values are generally above 40%. When the cells per grid are greater than about thirty, the C_v values are 15% (the desired level) or lower. Appendix A discussed the Poisson nature of distribution for cells in a counting chamber. For cell counts above ten, the standard deviation calculates to be nearly equal for Poisson and normal distri-

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butions. However, when the means are below ten or so, the standard deviations differ more. For this study, the C_v was calculated from standard deviations calculated from the normal distribution formula. This may have resulted in misleading C_v values for cell counts in the less than ten per grid range.

The cell count tables show the maximums for each replication. In many instances they are not the absolute maximums since they are the last count for which an increase in cell numbers was above five percent per day. This is the procedure suggested in the AAP.

Table 9 gives daily Specific Growth Rates for each treatment replication. The maximums are underlined and occurred between days 1 and 3 for all replications.

Table 10 gives the μ_{max} for each treatment replication. All three phosphorus treatments responded over the control at 95% confidence levels, however, there were no detectable differences between the levels of phosphorus. The μ_{max} values increase as the levels of phosphorus increase, suggesting a correlation. Experimental error may not have allowed the differences in treatment levels to be detected. The C_v values are all above 15% (15% to 38%) which is not an acceptable level of precision according to Bliss (1952) for bioassays.

Table 11 gives the Maximum Standing Crops in cell numbers. Differences were detected between phosphorus treatments at 90% and 95% levels except for one comparison. Phosphorus

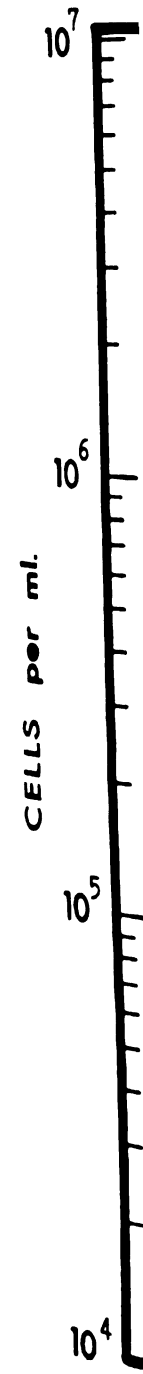


Figure 4. Growth curves for Selenastrum from a ten day bioassay of the effects of four levels of phosphorus in the AAP medium.

a. Page 42 - The 0.00 mg P/l treatment run in four replications.

b. Page 43 - The 0.01 mg P/l treatment run in four replications.

c. Page 44 - The 0.02 mg P/l treatment run in four replications.

d. Page 45 - The 0.03 mg P/l treatment run in four replications.

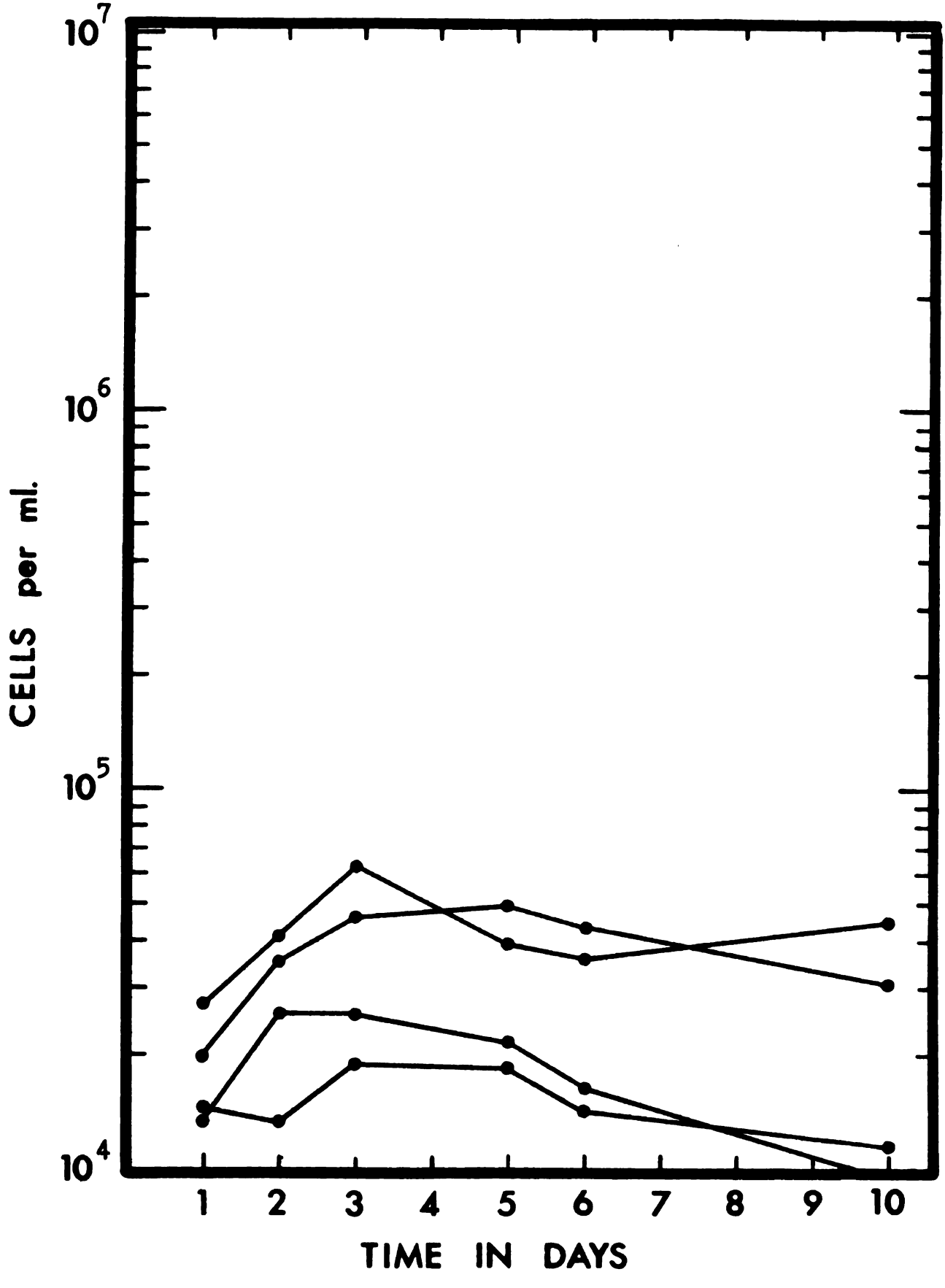
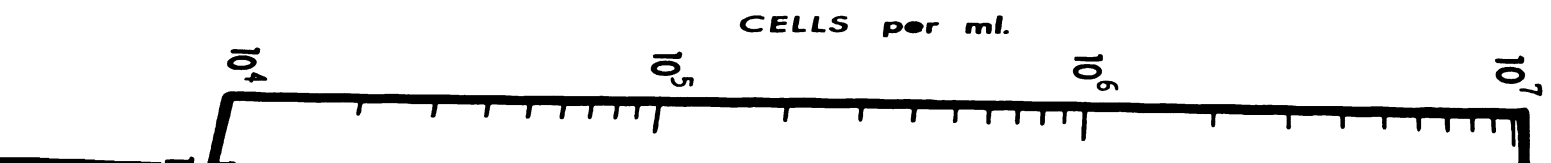


Figure 4a


CELLS per ml.
10^4
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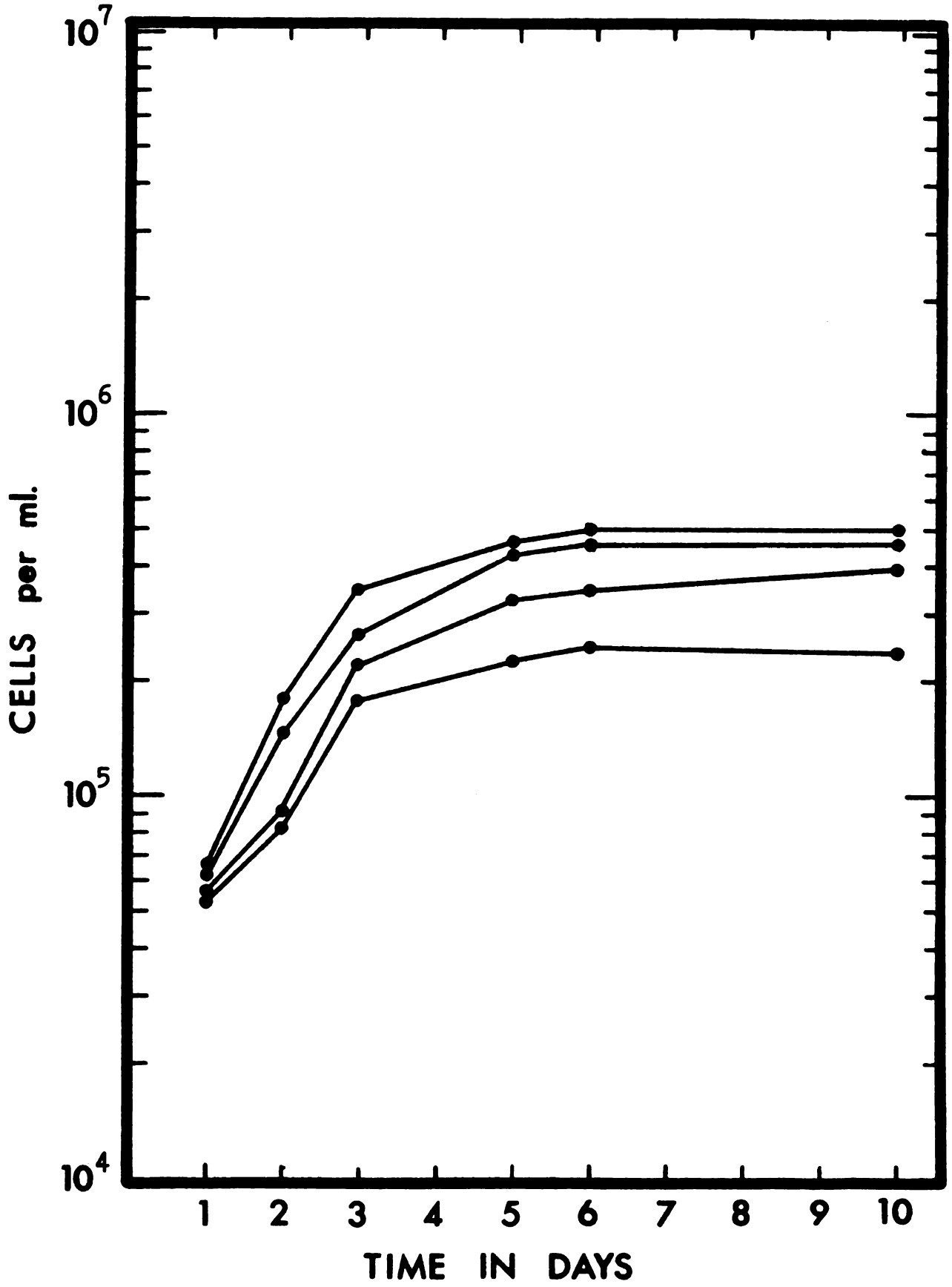

Figure 4b

10^7
10^6
10^5
10^4
CELLS per ml.

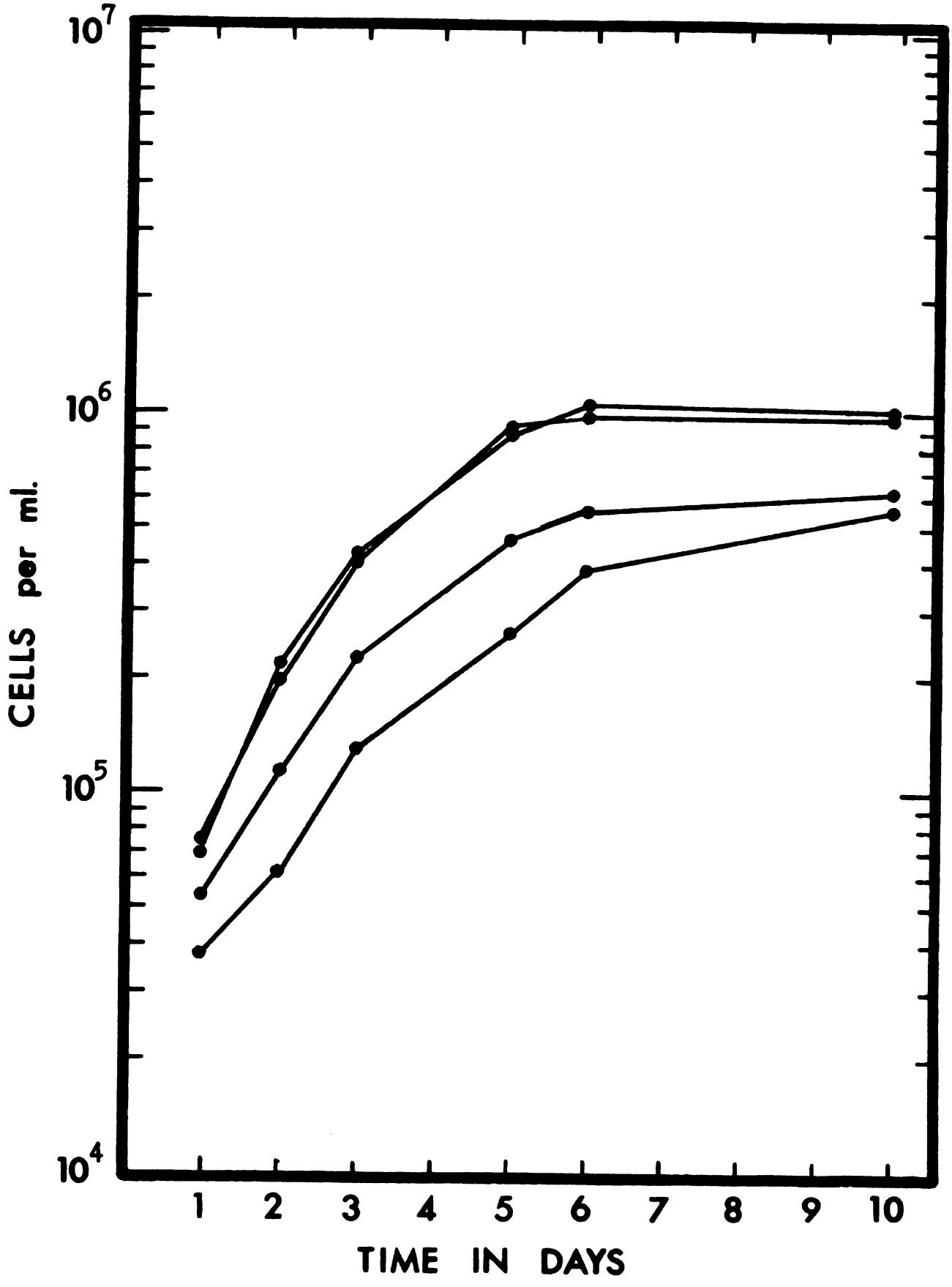

Figure 4c

10^7
10^6
CELLS per ml.
10^5
10^4

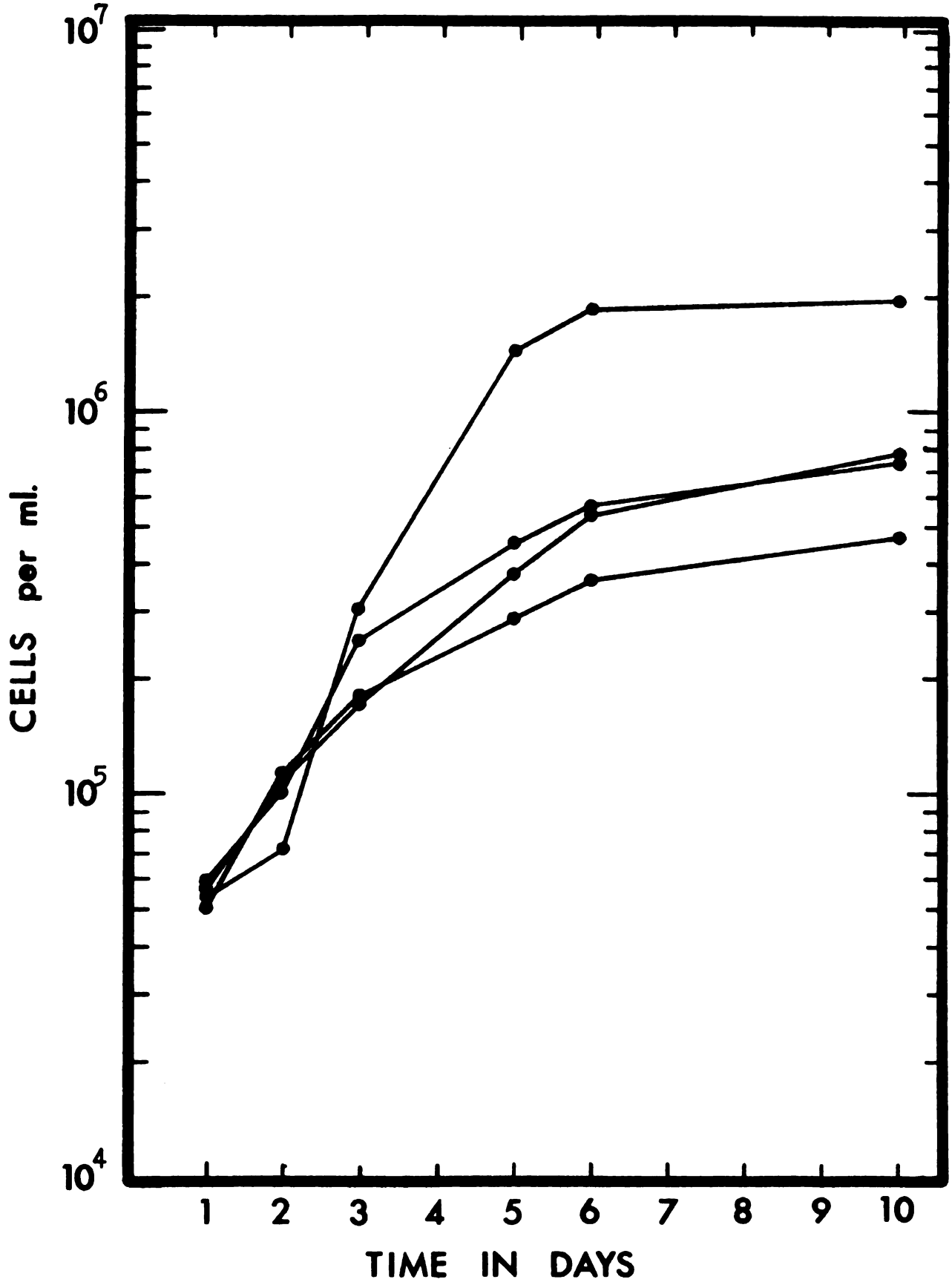

Figure 4d

Table 9.

Time (Days)
0
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3
5
6
10

Time (Days)
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1
2
3
5
6
10

a
b

Table 9. Effects of four levels of phosphorus (0.00, 0.01, 0.02, and 0.03 mg P/l) on *Selenastrum* daily Specific Growth Rates for a ten day static bioassay.

Time (Days)	SPECIFIC GROWTH RATES (μ/day)							
	Control (no P)				Control + 0.01 mg P/l			
	1	2	3	4	1	2	3	4
0								
1	0.25	[b]0.00	0.32	0.00	0.50	0.63	0.70	0.23
2	[a]<u>0.65</u>	0.00	0.41	<u>0.56</u>	0.53	0.37	<u>1.06</u>	<u>0.81</u>
3	0.00	<u>0.34</u>	<u>0.43</u>	0.23	<u>0.88</u>	<u>0.77</u>	0.65	0.58
5	0.00	0.00	0.00	0.03	0.20	0.12	0.12	0.26
6	0.00	0.00	0.00	0.00	0.05	0.08	0.11	0.05
10	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00
Time (Days)	Control + 0.02 mg P/l				Control + 0.03 mg P/l			
	1	2	3	4	1	2	3	4
0								
1	0.23	0.92	0.59	0.84	0.62	0.64	0.54	0.68
2	0.49	<u>0.98</u>	<u>0.75</u>	<u>1.14</u>	0.27	<u>0.64</u>	<u>0.75</u>	0.57
3	<u>0.74</u>	0.71	0.69	0.67	<u>1.44</u>	0.53	0.48	<u>0.88</u>
5	0.35	0.40	0.35	0.37	0.78	0.36	0.25	0.30
6	0.39	0.09	0.17	0.17	0.25	0.37	0.22	0.21
10	0.09	0.00	0.03	0.00	0.01	0.07	0.07	0.07

[a]Specific Growth Rates of 0.00 have been recorded for values of 0.00 or less.
[b]Underlined values are the maximums for each replication.

Table 10. E
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Rep. Number	
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Mean ± 1SD	
C_v	
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Table 11.

Rep. Number	
1	
2	
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4	
Mean ± 1SD	
C_v	
t test (one-tailed	

[a]One-tail

Table 10. Effects of four levels of phosphorus (0.00, 0.01, 0.02, and 0.03 mg P/l) on Selenastrum Maximum Specific Growth Rates with C_v and t testing.

Rep. Number	MAXIMUM SPECIFIC GROWTH RATE (μ_{max})			
	Control (no P)	Control + 0.01 mg P/l	Control + 0.02 mg P/l	Control + 0.03 mg P/l
1	0.65	0.88	0.74	1.44
2	0.34	0.77	0.98	0.64
3	0.43	1.06	0.75	0.75
4	0.56	0.81	1.14	0.88
Mean ± 1SD	0.50 ± 0.14	0.88 ± 0.13	0.90 ± 0.19	0.93 ± 0.36
C_v	27%	15%	21%	38%
a_t test (one-tailed)	1<2 at 95% level 1<3 at 95% level 1<4 at 95% level		2 vs 3 is ns 2 vs 4 is ns 3 vs 4 is ns	

[a]One-tailed test since growth stimulation was expected.

Table 11. Effects of four levels of phosphorus (0.00, 0.01, 0.02, and 0.03 mg P/l) on Selenastrum Maximum Standing Crops in cells/ml with C_v and t testing.

Rep. Number	MAXIMUM STANDING CROPS in cells/ml			
	Control (no P)	Control + 0.01 mg P/l	Control + 0.02 mg P/l	Control + 0.03 mg P/l
1	4.9×10^4	2.4×10^5	5.6×10^5	2.0×10^6
2	6.3×10^4	4.4×10^5	9.8×10^5	7.2×10^5
3	1.9×10^4	3.9×10^5	6.2×10^5	4.7×10^5
4	2.6×10^4	5.0×10^5	1.0×10^6	7.5×10^5
Mean ± 1SD	3.9×10^4 $\pm2.0\times10^4$	3.9×10^5 $\pm1.1\times10^5$	7.9×10^5 $\pm2.3\times10^5$	9.8×10^5 $\pm6.9\times10^5$
C_v	52%	28%	29%	69%
t test (one-tailed)	1<2 at 99% level 1<3 at 99% level 1<4 at 95% level		2<3 at 95% level 2<4 at 90% level 3 vs 4 is ns	

[a]One-tailed test since growth stimulation was expected.

treatments responded over the control at 99% levels. The C_v values are again all above 15% (28% to 69%) indicating poor precision for this growth parameter.

Conclusion

Miller and Maloney (1971) reported Selenastrum responded to phosphorus levels as low as 0.01 mg P/l using the AAP procedures. This experiment supports their finding.

Maloney and Bartsch (1969), as well as others, reported algal bioassay was as sensitive in many cases as chemical determinations. Sensitivity, not to be confused with the detection limit, is the ability to detect a difference between two close levels of the same substance. The closer the levels are to being equal with differences detectable, the more sensitive is the measuring device (Skoog and West, 1971). This experiment showed the bioassay has the potential to be very sensitive. However, before I can place as much confidence in a bioassay (for phosphorus) as in a chemical determination, I will have to further reduce experimental error. This was done in the following experiments by identifying and minimizing error sources.

Experiment 4
Effects of light intensity on Selenastrum growth

Purpose

This experiment attempts to determine the effects of HIGH vs LOW light intensity on Selenastrum growth rates and standing drops under bioassay test conditions.

Design

The treatments were HIGH intensity of 475 ft-c (5111 lux) and LOW intensity of 260 ft-c (2798 lux). The AAP gives 400 ft-c (4304 lux) as the optimum intensity for Selenastrum growth. Each treatment was run in five replications. Cell counts were used to assess growth.

Procedure

The inoculum was prepared in the manner discussed previously from a six-day-old stock culture. The inoculum was counted and it was determined 0.13 ml of inoculum delivered to fifty ml of medium should give an initial cell concentration of 10^3 cells per ml.

Ten 125-ml Erlenmyer flasks were given fifty ml portions of seven-day-old AAP medium that had been stored in the dark (as suggested by the AAP) to prevent any photochemical reactions to the medium. Each flask was randomly inoculated from a calibrated 1-ml pipet with 0.13 ml of inoculum.

Cell counts were made immediately after inoculation and on days 1,2,3,4, and 6. Cell enumeration involved counting two samples per flask and ten micrometer grids per sample.

Results

Appendix table B5 lists the cell counts for each treatment replication. It was apparent the method used to deliver the inoculum was in error from the counts recorded immediately after inoculation. Initial cell concentrations ranged from 6.4×10^3 to 7.0×10^4 cells per ml. Figures 5a and 5b depict this initial error and how it subsequently resulted in poor growth curve precision between treatment replications.

The figures also depict "abnormal" growth curves. Only replication 4 (see also Appendix table B5) of the HIGH intensity treatment gave a "normal" growth curve by going through a logarithmic phase, indicative of nutrients present initially in unlimiting amounts (Pringsheim, 1946). The other cultures do not go through a logarithmic growth phase, suggesting some factor or nutrient was initially limiting algal growth.

To test if some nutrient was limiting growth, two drops of the seven AAP stock solutions were added separately to seven aliquots of replication 2 of the HIGH intensity treatment on day 6. Counts were then made on days 7,8,9, and 10 for the seven aliquots. Table 12 lists the cell counts. A significant growth response at a 99.9% confidence level was obtained from the TRACES treatment. This is depicted in Figure 5b. The other six no-effect treatments have not been graphed separately since they gave no significant responses,

Figure 5. Growth curves for Selenastrum from a six day bioassay of the effects of two levels of light intensity on growth. Differences were judged not detectable due to methodological errors.

a. Page 52 - HIGH intensity treatment of 475 ft-c (5111 lux) run in five replications, showing poor precision for initial cell concentrations and no cultures going through a logarithmic growth phase except for one replication. The response to the TRACES stock added on day 6 is shown. The average response of the other stock nutrients is also shown. This suggests TRACES are limiting algal growth.

b. Page 53 - LOW intensity treatment of 260 ft-c (2798 lux) run in five replications, showing poor precision for initial cell concentration and no cultures going through a logarithmic growth phase.

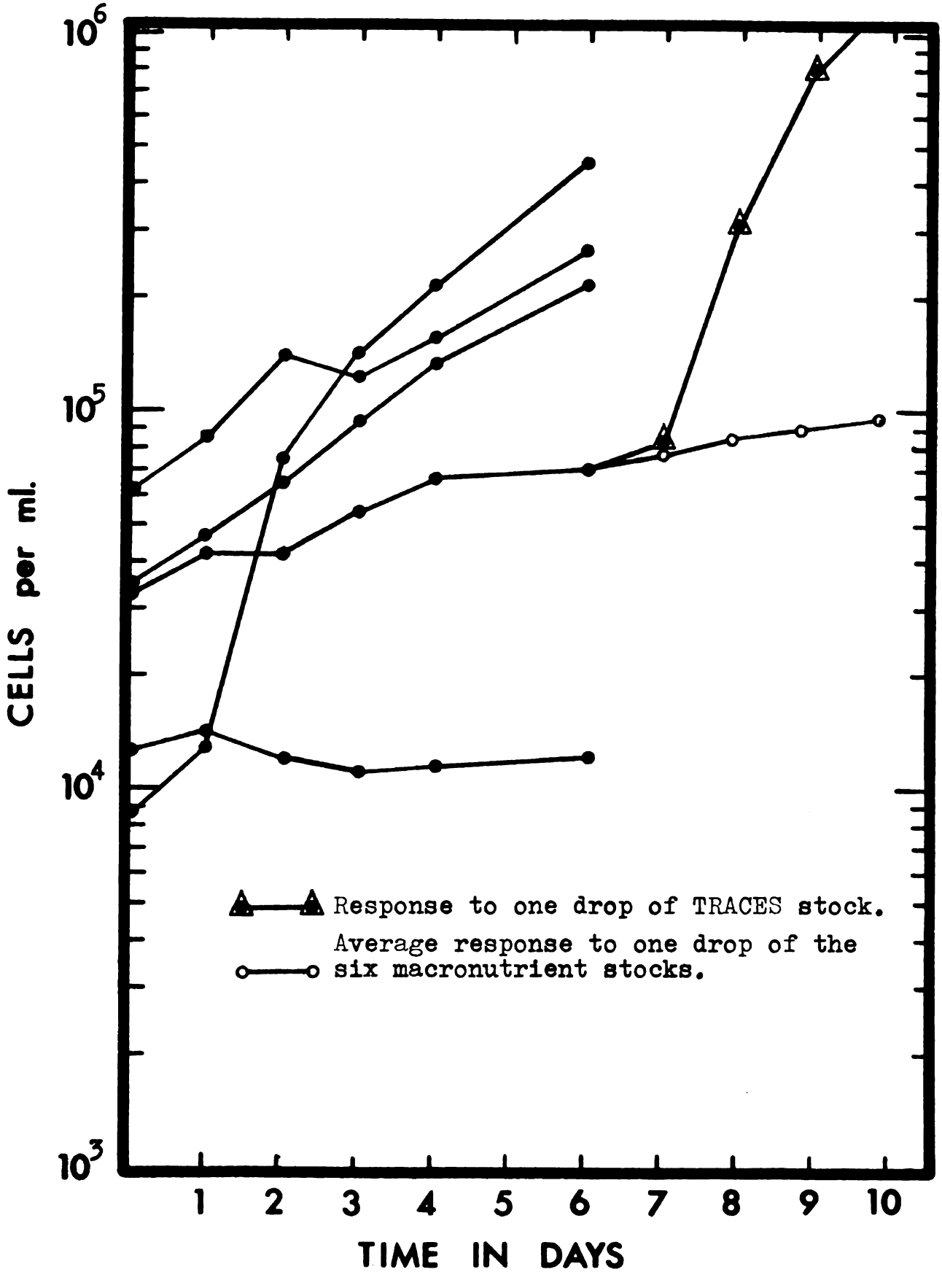
10^6
10^5
10^4
10^3
CELLS per ml.
1
2
3
4
5
6
7
8
9
10
TIME IN DAYS
Response to one drop of TRACES stock.
Average response to one drop of the six macronutrient stocks.

Figure 5a

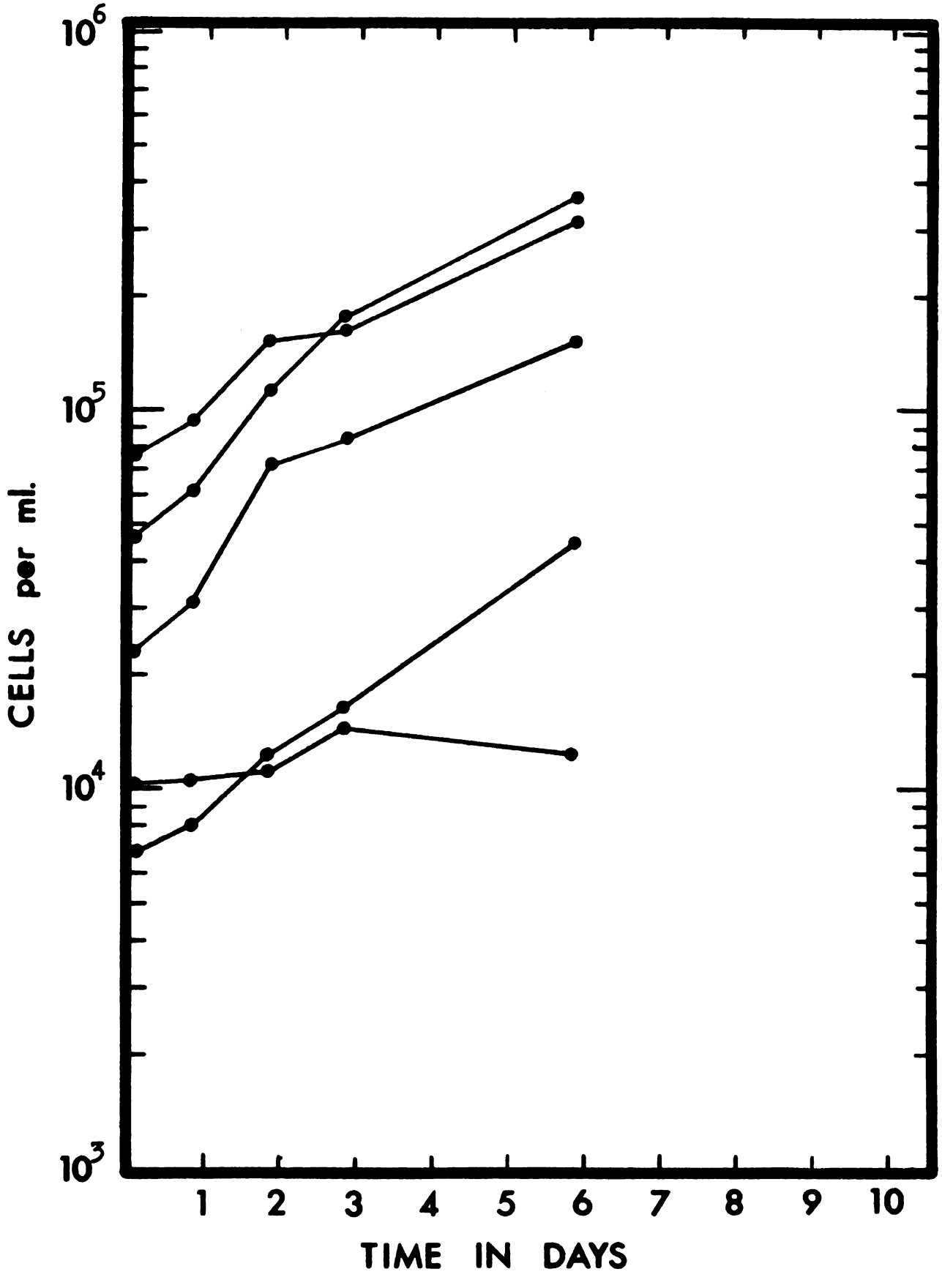

Figure 5b

Table 12. Cell counts of seven aliquot portions of a seven-day-old culture. Each portion was treated with one of the seven nutrient stock solutions used to prepare AAP medium to determine if a stock nutrient was limiting growth.

Day	Treatment solution nutrients (counts in cells/ml)						
	$NaNO_3$	K_2HPO_4	$MgCl_2$	$MgSO_4$	$CaCl_2$	$NaHCO_3$	[a]TRACES
6	$6.9x10^4$	$6.9x10^4$	$6.9x10^4$	$6.9x10^4$	$6.9x10^4$	$6.9x10^4$	$6.9x10^4$
7	$6.9x10^4$	$7.1x10^4$	$6.8x10^4$	$7.0x10^4$	$7.2x10^4$	$7.1x10^4$	$8.4x10^4$
8	$7.0x10^4$	$7.2x10^4$	$6.8x10^4$	$7.1x10^4$	$7.6x10^4$	$7.1x10^4$	$3.2x10^5$
9	$7.1x10^4$	$7.4x10^4$	$6.7x10^4$	$7.3x10^4$	$8.2x10^4$	$7.4x10^4$	$8.2x10^5$
10	$7.0x10^4$	$7.6x10^4$	$6.9x10^4$	$7.5x10^4$	$8.7x10^4$	$7.9x10^4$	$1.2x10^6$

[a]TRACES is a stock solution containing a mixture of all the trace elements used to prepare AAP medium. TRACES was the only treatment giving a significant response according to *t* testing.

Table 13. Cell counts of seven aliquot portions of a ten-day-old culture. Each portion was treated with a trace element solution to determine which element in the stock of TRACES was limiting growth.

Day	[a]Treatment solutions of trace elements (cells/ml)						
	H_3BO_3	$MnCl_2$	$ZnCl_2$	$CoCl_2$	$CuCl_2$	$FeCl_3$	Na_2MoO_4
10	$1.7x10^4$	$1.7x10^4$	$1.7x10^4$	$1.7x10^4$	$1.7x10^4$	$1.7x10^4$	$1.7x10^4$
12	$1.9x10^4$	$1.6x10^4$	$1.9x10^4$	$1.8x10^4$	$1.9x10^4$	$1.7x10^4$	$1.7x10^4$
15	$2.1x10^4$	$1.7x10^4$	$2.4x10^4$	$1.6x10^4$	$1.6x10^4$	$2.1x10^4$	$2.0x10^4$

[a]None of the trace treatments gave a significant response according to *t* testing.

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however they have been averaged and are presented in Figure 5b for comparison purposes.

An attempt was made to determine which trace element was limiting by preparing a solution for each of the seven trace elements at concentrations found in the AAP stock of TRACES mixture. EDTA was added to each solution at the concentration found in the AAP stock of TRACES. Two drops of each trace element solution were added to seven aliquots of replication 5 of the HIGH intensity treatment on day 10. Counts were made and are listed in Table 13. The counts are not shown graphically since none of the seven trace element treatments gave significant responses.

Conclusions

An attempt to determine the effects of two levels of light intensity on *Selenastrum* growth was unsuccessful due to methodological errors.

The procedure used to inoculate the culture medium gave high variance to initial cell concentrations. The expected concentration was 10^3 cells per ml and the observed ranged from 6.4×10^3 to 7.0×10^4 cells per ml.

The medium was found to be initially limiting to algal growth due to lack of TRACES. This may have been omitted upon preparation of medium, may have been added from an improperly prepared stock, or may have become unavailable after addition. An attempt to determine if one specific trace element was limiting growth showed no-effect for all trace treatments tested.

Experiment 5
Effects of three levels of initial cell concentration on Selenastrum growth during a bioassay

Purpose

Experiment 4 indicated that varying initial cell concentrations possibly resulted in excessive growth curve variance between treatment replications. This experiment will investigate specifically the effects of three levels of initial cell concentration on Selenastrum growth rates and standing crops.

Design

The treatments were three levels of initial cell concentration each run in three replications. Cell counts consisted of counting one sample per flask and ten micrometer grids per sample.

Procedure

The inoculum was prepared from five-day-old stock in the previously mentioned manner. A new delivery method was tested in hopes of obtaining better precision than the pipet method gave. The delivery was done with a long stem capillary dropper. The inoculum treatments were four drops, fifteen drops, and fifty drops. The initial cell concentrations were counted to be 1.68×10^3, 6.00×10^3, and 2.10×10^4 cells per ml. This gave a ratio of $1:3\frac{1}{2}:12\frac{1}{2}$ which was approximately

equal to the treatment ratio of 4:15:50. Therefore, it was apparent the drops were uniform in volume and that the dropper method was an improvement over the pipet method for delivering inoculum.

Results

Cell counts are listed in Appendix table B6 for the replication averages of each treatment. These counts were used to depict the growth curves in Figure 6. It shows the growth curves are shifted to the left on the graphs as the initial cell concentrations were increased. This caused the μ_{max} to occur earlier in the assay. It is not desirable to have the μ_{max} occur between days 0 and 1 since there is high counting error on day 0 as discussed previously in this study. Therefore, Figure 6 suggests the initial cell concentration for bioassays should be below 10^4 and close to 10^3 cells per ml if the test alga is Selenastrum.

Table 14 shows the effect of increasing the initial cell concentration was to cause μ_{max} values to increase. If experiments are to be compared, it is apparent the inoculation size must be carefully controlled when μ_{max} is the growth parameter. Table 15 shows the three levels of initial cell concentration had very little effect on Maximum Standing Crops.

Conclusions

The initial cell concentration in an algal bioassay can effect growth rates. This may be one reason why the eight-laboratory evaluation of the Provisional Algal Assay Procedure

Figure 6. Growth curves for Selenastrum from an eleven day bioassay of the effects of three levels of initial cell concentration (1.68×10^3, 6.00×10^3, and 2.10×10^4 cells per ml) on growth.

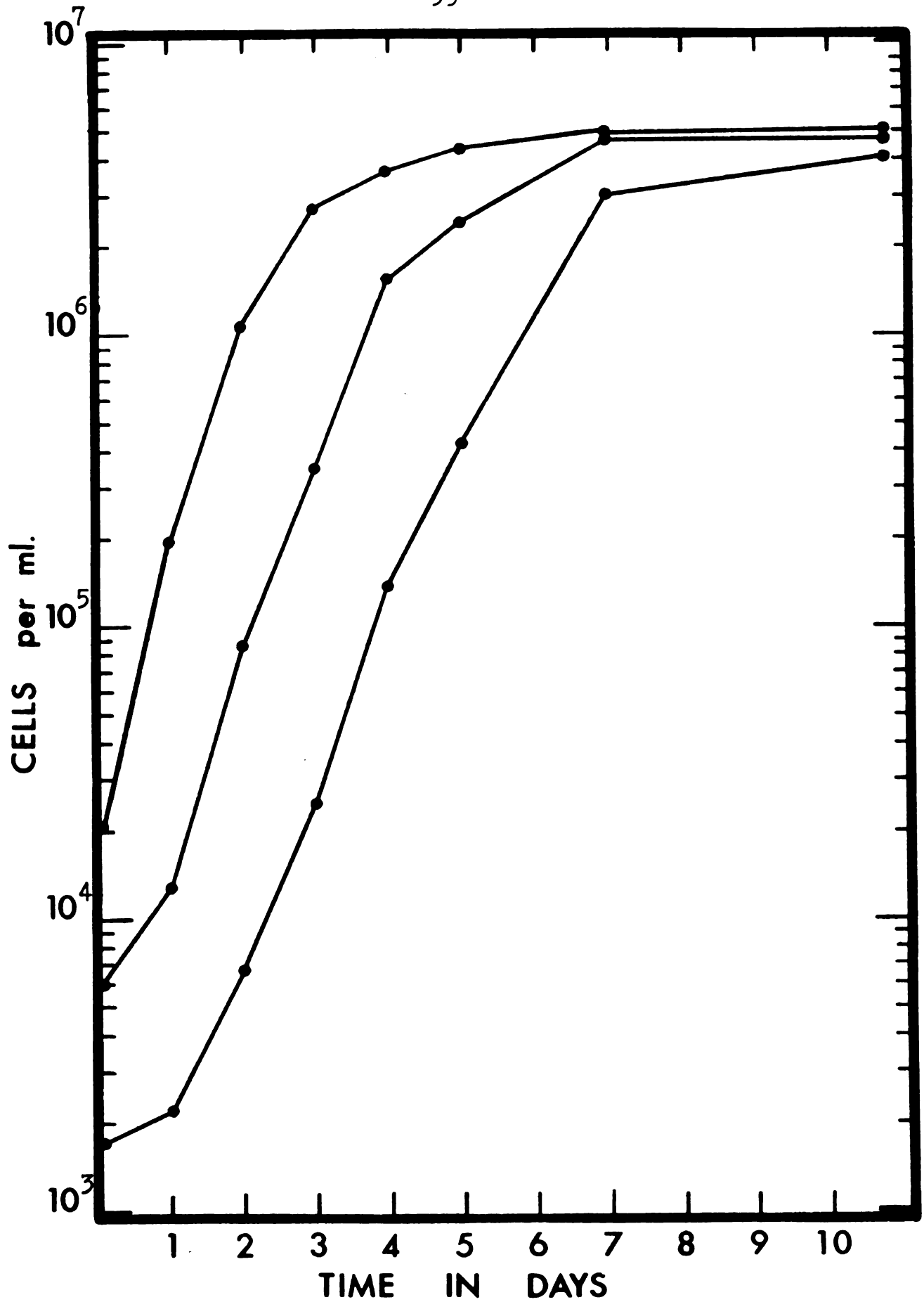


Figure 6

Table 14. Effects of three levels of initial cell concentration (1.68×10^3, 6.00×10^3, and 2.10×10^4 cells per ml) on Selenastrum daily Specific Growth Rates for an eleven day bioassay with treatment replications averaged and t testing on the μ_{max} values. are numbered as 1,2,3 respectively.

Time (Days)	SPECIFIC GROWTH RATE (μ/day)		
	Treatment 1	Treatment 2	Treatment 3
0	0.24	0.77	[a]2.25
1	1.13	[a]1.92	1.66
2	1.31	1.41	1.00
3	[a]1.70	1.46	0.30
4	1.15	0.49	0.15
5	0.64	0.00	0.00
7	0.00	0.00	0.00
11			

[a]Maximums compared by t testing:

Treatment 1 < Treatment 2 at the 95% level.
Treatment 2 < Treatment 3 at the 95% level.
Treatment 1 < Treatment 3 at the 99% level.

Table 15. Effects of three levels of initial cell concentration (1.68×10^3, 6.00×10^3, and 2.10×10^4 cells per ml) on Selenastrum Maximum Standing crops in cells per ml with t testing. The treatments are numbered 1,2,3 respectively.

Treatment 1 yielded	$4.2 \times 10^6 \pm 7.4 \times 10^5$ cells/ml
Treatment 2 yielded	$4.8 \times 10^6 \pm 3.4 \times 10^5$ cells/ml
Treatment 3 yielded	$5.0 \times 10^6 \pm 4.2 \times 10^5$ cells/ml

t testing showed no significant differences.

(Bueltman-Chairman, 1969) found high interlaboratory variance and low intralaboratory variance as noted in the Inter-laboratory Precision Test (Maloney-Deputy Chief, 1971). The PAAP and the AAP give no standardized procedures for inoculating cultures.

It is necessary to use the best available method to deliver a precise inoculum and to keep the initial cell concentration near 10^3 and not above 10^4 cells per ml for Selenastrum bioassays according to the results of this experiment.

Experiment 6
Effects of light intensity on Selenastrum growth
(repeat of Exp. 4)

Purpose

Experiment 6 bioassayed the effects of HIGH vs LOW light intensity on Selenastrum growth rates and standing crops utilizing information gained from Experiments 4 and 5. The variable results for Experiment 4 were attributed to AAP medium lacking stock mixture of trace elements and to poorly controlled initial cell concentrations for the bioassay. Experiment 6 was an attempt to eliminate these sources of variance.

Design

The treatments of HIGH intensity at 475 ft-c (5111 lux) and LOW intensity at 260 ft-c (2798 lux) were run in duplicate replications with cell counts made on days 0,1,2,3,4,5,6,7, and 11, consisting of one sample per flask and twenty micrometer grids per sample. On day 4 the LOW intensity treatments were divided with one half put in a clean culture flask and placed under HIGH intensity while the other half was replaced under LOW intensity.

Procedure

Fresh AAP medium was prepared and randomly inoculated

with five-day-old stock inoculum cultured and prepared in the usual manner. The inoculum was delivered with a long stem capillary dropper and gave an initial cell concentration of $2.9 \times 10^3 \pm 1.4 \times 10^3$ cells per ml upon enumeration immediately after inoculation.

By day 4 the LOW intensity treatment was noticably growing less than the HIGH intensity treatment. For an internal control, half of the LOW intensity treatment was placed under HIGH intensity illumination on day 4 to test if growth could recover and reach the level of the treatment originally placed under HIGH intensity.

Results

The cell counts are listed in Appendix table B7 from which the growth curves, μ_{max}, and Maximum Standing Crops were determined.

Figure 7 depicts the growth curves. It is evident the internal control fully recovered and underwent a logarithmic growth phase. Table 16 lists the daily Specific Growth Rates. The maximums occurred between days 1 and 3. Table 17 gives the μ_{max} values with HIGH intensity greater than LOW intensity at the 80% level. Table 18 gives the Maximum Standing Crops with HIGH intensity greater than LOW intensity at the 99% level.

Conclusions

Light intensity had a marked effect on Maximum Standing Crops and less so on μ_{max} values. A reduction in light inten-

Figure 7. Growth curves for Selenastrum from an eleven day bioassay showing the effects of HIGH and LOW light intensity (474 ft-c and 260 ft-c). Also shown is the response when on day 4 a portion of the LOW intensity treatments was placed under HIGH intensity.

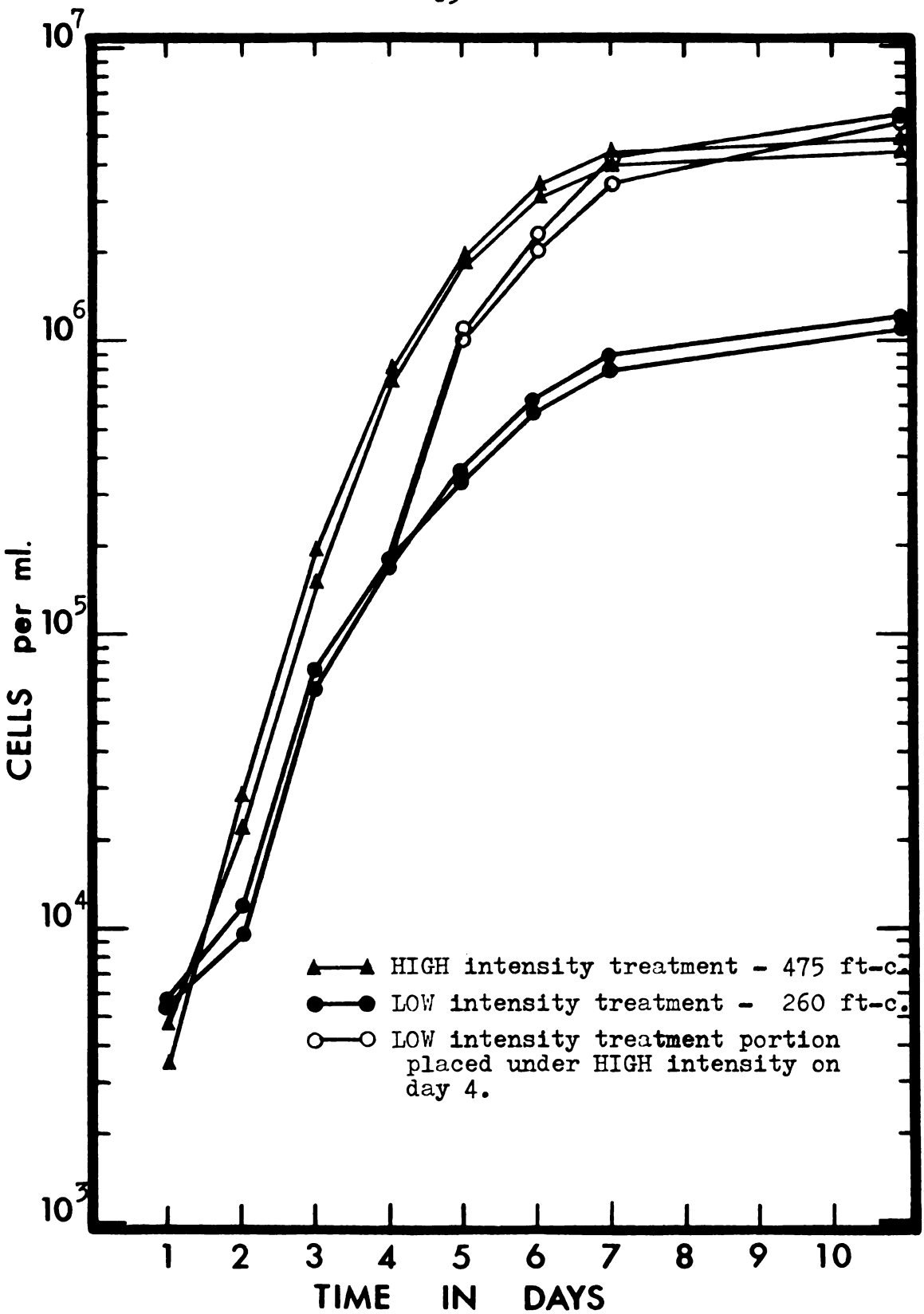

10^7
10^6
10^5
10^4
10^3
CELLS per ml.
1 2 3 4 5 6 7 8 9 10
TIME IN DAYS
HIGH intensity treatment - 475 ft-c.
LOW intensity treatment - 260 ft-c.
LOW intensity treatment portion placed under HIGH intensity on day 4.

Figure 7

Table 16. Effects of two levels of light intensity (475 ft-c and 260 ft-c) on Selenastrum daily Specific Growth Rates for an eleven day static bioassay.

Time (Days)	SPECIFIC GROWTH RATES (μ/day)			
	LOW intensity		HIGH intensity	
	1	2	1	2
0				
1	0.51	0.65	0.69	0.15
2	0.69	0.55	1.53	2.11
3	[a]1.92	1.93	1.94	1.91
4	0.84	0.99	1.56	1.39
5	0.60	0.67	0.97	0.94
6	0.55	0.54	0.50	0.60
7	0.34	0.34	0.06	0.22
11	0.07	0.09	0.00	0.00

[a]Maximums are underlined.

Table 17. Effects of two levels of light intensity (475 ft-c and 260 ft-c) on Selenastrum Maximum Specific Growth Rates with C_v and $\underline{t}$ testing.

Rep. Number	MAXIMUM SPECIFIC GROWTH RATE (μ_{max})	
	LOW intensity	HIGH intensity
1	1.92	1.94
2	1.93	2.11
Mean $\pm$ 1SD	2.025 $\pm$ 0.120	1.929 $\pm$ 0.007
C_v	5.9%	0.4%
$\underline{t}$ test	HIGH intensity is greater than LOW intensity at a 90% level	

Table 18. Effects of two levels of light intensity (475 ft-c and 260 ft-c) on Selenastrum Maximum Standing Crops with C_v and $\underline{t}$ testing.

Rep. Number	MAXIMUM STANDING CROPS in cells/ml	
	LOW intensity	HIGH intensity
1	1.1×10^6	4.4×10^6
2	1.2×10^6	4.7×10^6
Mean $\pm$ 1SD	1.15×10^6 $\pm 0.07 \times 10^6$	4.55×10^6 $\pm 0.21 \times 10^6$
C_v	6%	5%
$\underline{t}$ test	HIGH intensity is greater than LOW intensity at a 99% level	

sity from 475 ft-c to 260 ft-c (about 50%) caused a 75% reduction in the maximum number of Selenastrum cells that could be cultured in a eleven day static bioassay.

A LOW intensity treatment placed under HIGH intensity on day 4 recovered by undergoing another logarithmic growth phase, suggesting light intensity was limiting growth. The limiting effect was reversible since the cultures fully recovered when placed under higher intensity.

Whether light should be considered a limiting factor or a tolerance factor is a matter of definition. This has been brought out on page 8 of this study. Liebig (1840) and Odum (1971) defined the "Law of the Minimum" to include only nutrients. Taylor (1934) and Ruttner (1963) define the "Law of the Minimum" to include light. If one sees the "Law of the Minimum" as the lower end of Shelford's "Law of Tolerance", then light could be included in both "Laws" since it has been shown excessive light can effect algal growth (Murray et al., 1971) The mechanism is believed to be photooxidation of plant pigments (Odum, 1958; Knutson, no date; and Forsythe, 1972).

Because light effects algal growth, it should be carefully controlled in bioassays. When one can not completely control light, as in this study, experimental designs which block out light effects should be used (e.g., Randomized Block Design).

Experiment 7
Effects of carbon availability on Selenastrum growth during a static bioassay

Purpose

The objective of this experiment was to compare the AAP's suggested methods of insuring carbon availability to static algal bioassays. These methods are continuous shaking, venting air, venting CO_2-enriched air, bubbling air, bubbling CO_2-enriched air, and using optimum surface to volume ratios for the medium. All but the continuous shaking methods were investigated.

Design

Six treatments were bioassayed in duplicate replications for eleven days and are shown in Table 19. Specific Growth Rates were determined from daily cell counts through day 7. From the cell counts and dry weights determined on day 11, the "Cell Health" parameter of weight per million cells was calculated.

Procedure

Fresh AAP medium was randomly inoculated with six-day-old stock inoculum cultured and prepared in the usual manner. The inoculum was delivered by a long stem capillary dropper and gave an initial cell concentration of 2.9×10^3 cells per ml upon enumeration immediately after inoculation.

Table 19. Treatments of six levels of carbon dioxide vented above or bubbled into Selenastrum cultures to assess the AAP's suggested methods of insuring carbon availability in static bioassays.

Treatment number	Treatment gas and method of delivery to the cultures	Percent CO_2 by volume in treatment gas	Flow rate of treatment gas in cc/min
I	foam-plug stoppered control	no gas delivered	none
II	air vented	[a]0.003%	25
III	air bubbled	0.003%	5
IV	5% CO_2-enriched air vented	5.003%	25
V	5% CO_2-enriched air bubbled slow	5.003%	5
VI	5% CO_2-enriched air bubbled fast	5.003%	50

[a]from Handbook of Chemistry and Physics, Chemical Rubber Co.

The pH was measured before the treatments began. All treatments were begun on the day of inoculation and delivered through polyethylene tubing equipped at the end with capillary droppers bent to fit the culture flasks. Flow rates for the treatments were controlled with small aquarium valves. Flow rates were determined by bubbling the treatment into a graduated cylinder filled with water and inverted in a large beaker partially filled with water. The bubbling was done at about one inch below the surface of the water in the beaker and in the cultures so that error due to pressure differences would be minimal. Gas flow displacing the water in the graduated cylinder was adjusted until the desired flow in milliliters (cc) per minute was achieved.

Air treatments were delivered using a small aquarium pump. CO_2-enriched air treatments were delivered using a 5% CO_2-enriched air cylinder equipped with a pressure reducing valve and gauge. Flasks were stoppered with foam plugs.

Bubbling was done near the bottom of the cultures and venting was done about two inches above the cultures. An error was noted on day 1 for the flow rate of replication 2 of the air venting treatment (number II in Table 19). The rate was about 50 cc/min and should have been 5 cc/min as replication 1 was. Instead of correcting the flow rate, it was allowed to remain at 50 cc/min.

Cell counts and pH were determined on days 0,1,2,3,4,5, 6,7, and 11 and are listed in Appendix table B8. Figures 8a, b,c,d,e, and f depict the growth curves and Figure 9 summarizes the pH data.

On day 11 dry weights were determined except for three flasks which had undergone excessive media evaporation due to the venting treatments. Aluminum weighing pans were tared by drying overnight at 105°C in a hot air oven and then allowed to come to equilibrium overnight in a desiccator having fresh desiccant. The pans were then weighed on a six-place balance and replaced in the desiccator until samples were ready for drying. The cell suspensions were centrifuged and resuspended in distilled water. This was to eliminate error due to varying amounts of suspended and dissolved solids in the medium. From each suspension, two 20 ml samples were pipeted into two tared pans. The samples were then dried

Figure 8. Growth curves for Selenastrum from an eleven day bioassay showing the effects of six levels of carbon dioxide used to insure carbon availability during algal culturing.

a. Page 73 - No treatment gas delivered to the culture.

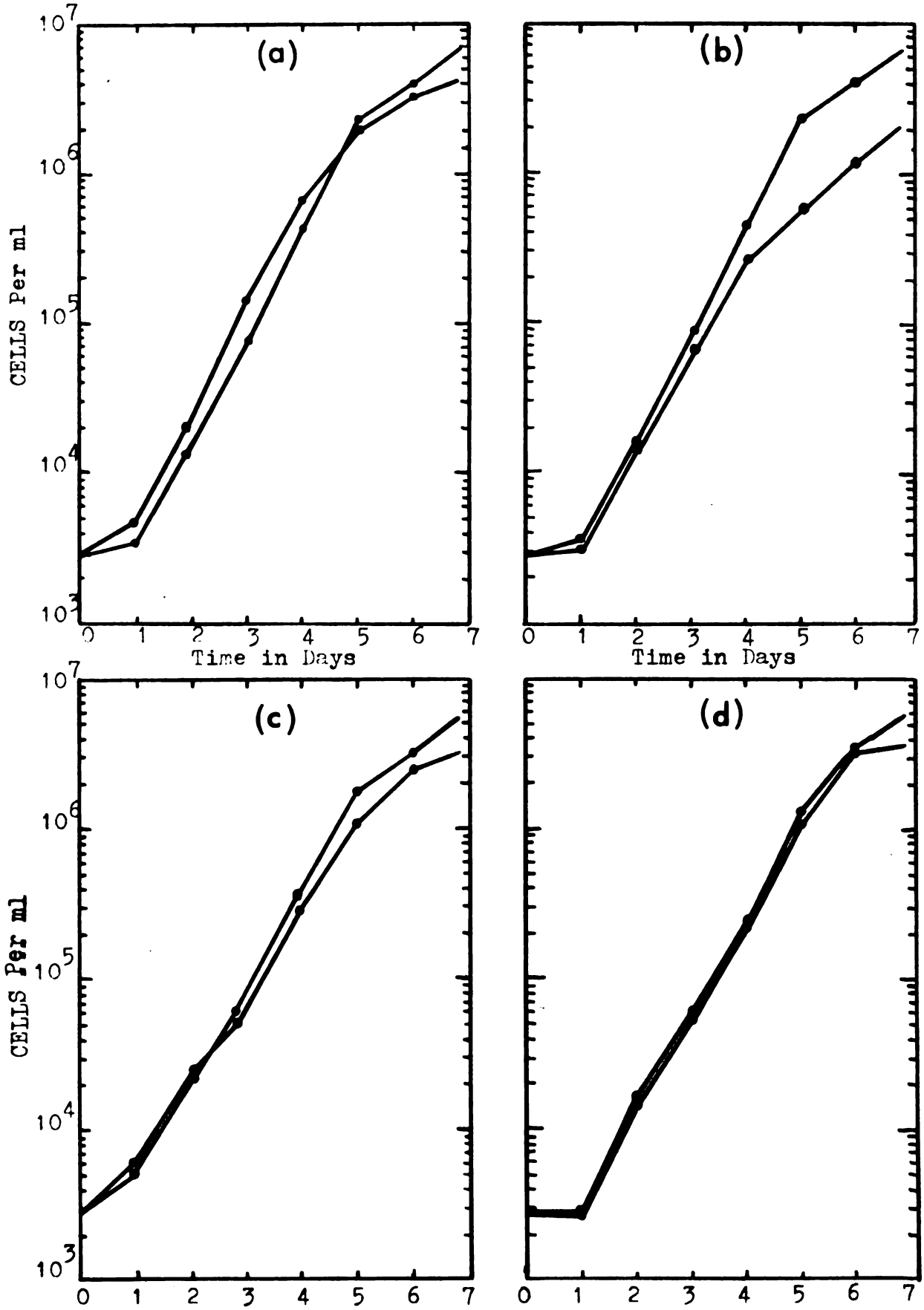
b. Page 73 - Air vented above the culture at 25 cc/min.

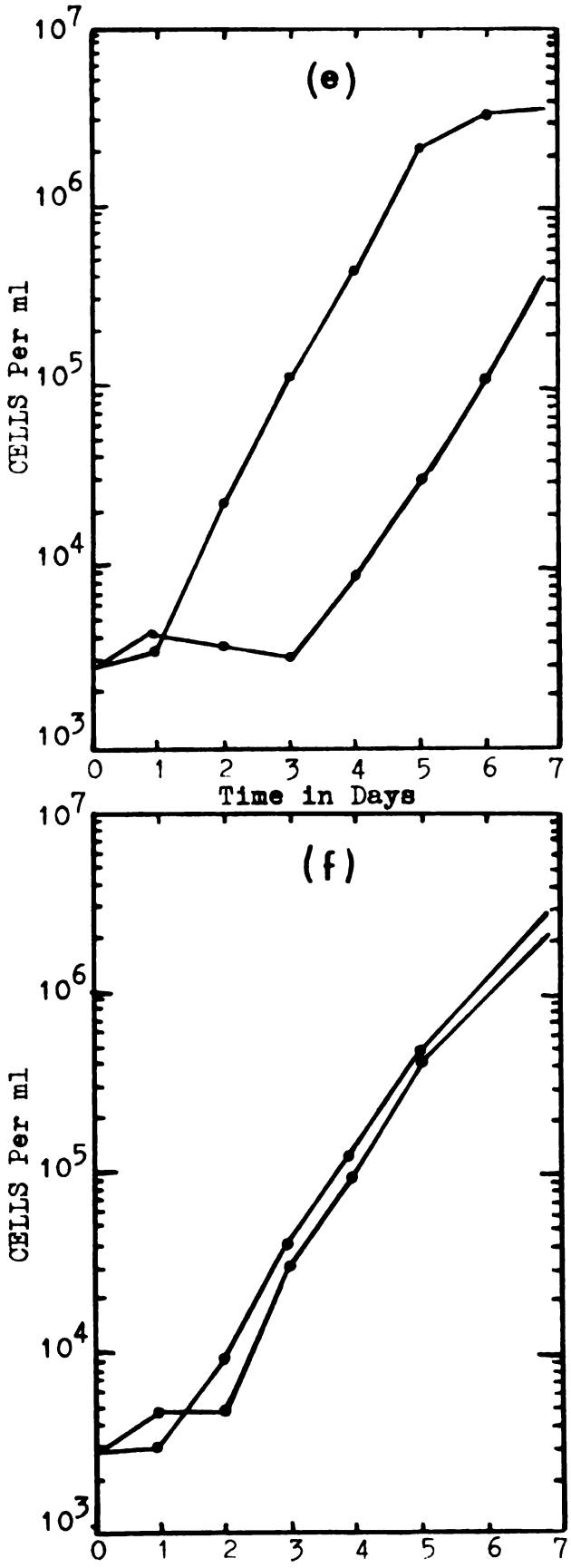
c. Page 73 - Air bubbled into the culture at 5 cc/min, with one replications flow rate in error and found to be 50 cc/min.

d. Page 73 - CO_2-enriched air vented above the culture at 25 cc/min.

e. Page 74 - CO_2-enriched air bubbled into the culture at 5 cc/min.

f. Page 74 - CO_2-enriched air bubbled into the culture at 50 cc/min.


(a)
(b)
(c)
(d)
CELLS Per ml
Time in Days
10^7
10^6
10^5
10^4
10^3
0 1 2 3 4 5 6 7


(e)
CELLS Per ml
10^7
10^6
10^5
10^4
10^3
0 1 2 3 4 5 6 7
Time in Days
(f)
CELLS Per ml
10^7
10^6
10^5
10^4
10^3
0 1 2 3 4 5 6 7

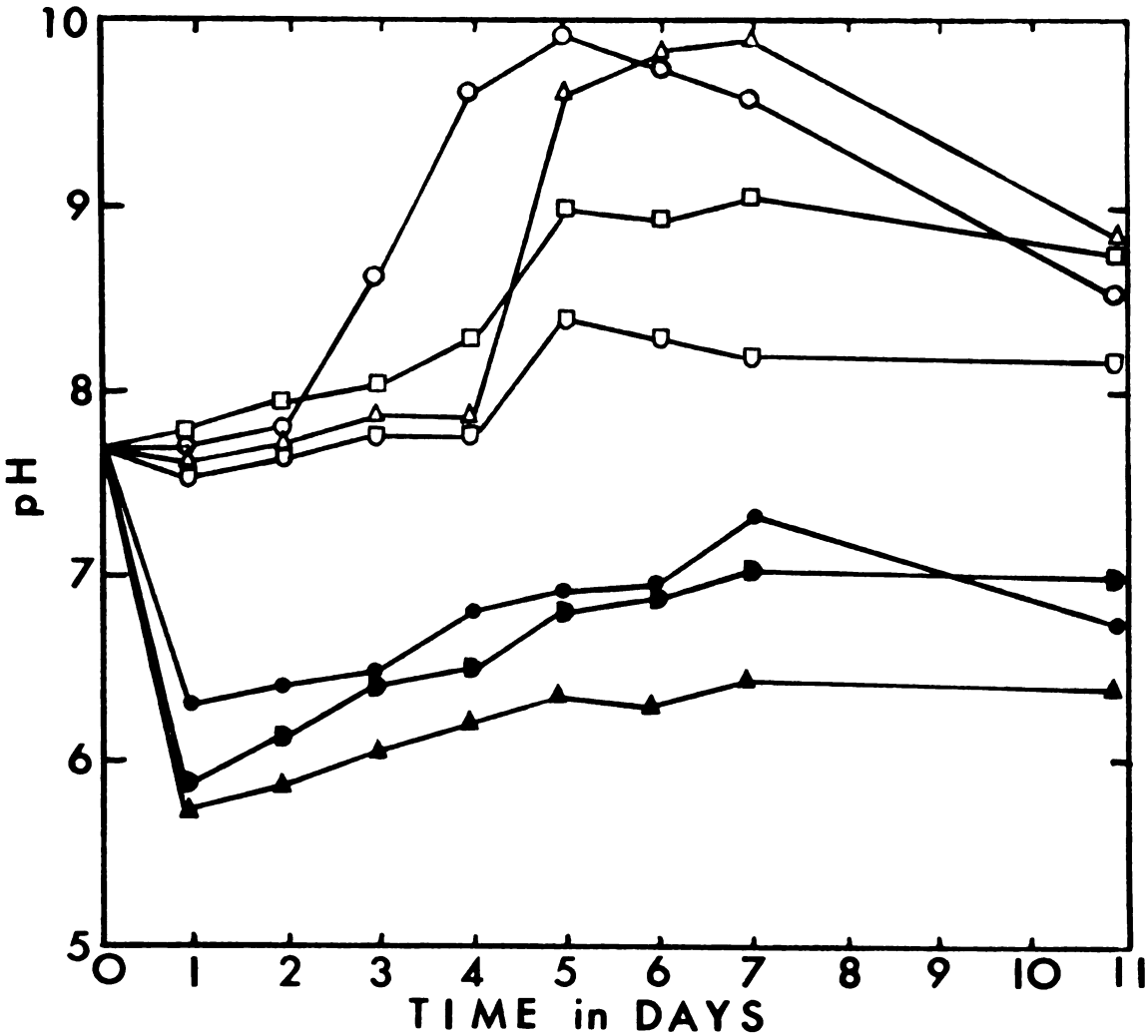


Figure 9. Daily pH measurements from an eleven day bioassay assessing the effects of suggested methods of insuring carbon availability to algal cultures.

overnight and allowed to come to equilibrium in the desiccator. One must use exacting technique to insure high weighing precision (Fritz and Schenk, 1971). A three-place balance was not precise enough for samples of 20 ml since it gave only one or two significant digits for weights. The six-place balance gave four or five significant digits for weight.

Results

The data indicate the AAP's suggested methods of controlling carbon availability yield different degrees of variation in growth parameters both between and within control methods.

Figures 8a,b,c,d,e, and f show how the growth curves are similar in shape for all treatments. For Figure 8e, treatment V, it is apparent growth was initially inhibited. Therefore, treatment gas was turned off temporarily to allow growth to begin since pH was suspected as inhibiting growth. Although the growth curves only depict seven days growth, Appendix table B8 indicates by day 11 the late starting flask had recovered fully compared to its duplicate. Figure 9 suggests the lower tolerance limit for Selenastrum is pH 6 or less. It also depicts the control and the air bubbling treatments (numbers I and II in Table 19) were not effective in controlling pH, with maximums almost reaching pH 10. The air vented treatment somewhat controlled the pH, but would not be acceptable since it caused excessive media evaporation. Slowing the flow rate of air would cause less evaporation but yield higher pH increases.

All the CO_2-air treatments gave low pH readings. Although

low pH was inhibitory in only one instance, it may be undesirable to hold the pH below 7. More experiments would have to be conducted to determine the effects of low pH on Selenastrum.

The air bubbling treatment at 50 cc/min (the flow rate noted earlier as in error) gave the best pH control, holding pH below 8.5 as suggested in the AAP.

Table 20 lists the daily Specific Growth Rates for all treatment replications. The C_v between each of the seven growth rates for a replication were averaged and listed at the bottom of the table. The control yielded the lowest C_v of 15%.

Table 21 lists the μ_{max} for each treatment. The C_v suggests all the methods of insuring carbon yield an acceptable level of precision for μ_{max} since all values were below 15% except for one at 20%. The $\underline{t}$ testing indicates the μ_{max} differ at confidence levels between 60% and 80%, with the control yielding the largest μ_{max}.

Table 22 lists the Maximum Standing Crops in cell numbers for each treatment not undergoing excessive evaporation. All treatments underwent some media evaporation to varying degrees except the control. C_v data indicate the control gave the lowest value for standing crops at 4.4%. The other treatments gave C_v higher than the generally accepted cutoff value for bioassay of 15%. The $\underline{t}$ testing indicates standing crops differ at confidence levels of 70%. A trend appears to be that cell numbers increased as available carbon (free CO_2) decreases. This correlation was tested in Table 26.

Table 20. Effects of six levels of carbon dioxide vented above or bubbled into the Selenastrum cultures on daily Specific Growth Rates. Shown are replication average C_v values for the static bioassay.

Rep Number	Time Days	I Control	II Air vented	III Air bubbled	IV CO_2 vented	V CO_2 bubbled	VI CO_2 bubbled
1	0	0.61	0.15	0.77	0.00	0.16	0.51
	1	1.53	1.57	0.87	1.54	1.90	0.00
	2	1.92	1.57	1.41	1.23	1.50	1.86
	3	1.56	1.70	1.53	1.51	1.41	1.08
	4	0.97	1.77	1.28	1.82	1.68	1.50
	5	0.50	0.54	0.90	0.90	0.31	0.88
	6	0.06	0.44	0.29	0.05	0.09	0.18
	7						
2	0	0.15	0.00	0.29	0.00	0.51	0.00
	1	2.11	1.67	0.28	1.47	0.00	1.15
	2	1.91	1.31	1.62	1.53	0.00	1.50
	3	1.39	1.30	1.53	1.26	1.15	1.08
	4	0.94	1.03	1.68	1.67	1.18	1.26
	5	0.60	0.72	0.58	1.17	1.43	0.96
	6	0.22	0.59	0.57	0.57	1.21	0.79
	7						
Average C_v between the two replication's seven Specific Growth Rates		15%	19%	26%	17%	66%	28%

Table 21. Effects of six levels of carbon dioxide on *Selenastrum* Maximum Specific Growth Rates with C_v and *t* testing.

Rep	Control I	Air vented II	Air bubbled III	CO_2 vented IV	CO_2 bubbled V	CO_2 bubbled VI
1	1.94	1.77	1.53	1.82	1.90	1.86
2	2.11	1.67	1.68	1.67	1.43	1.50
Mean ±1SD	2.025 ±.120	1.720 ±.071	1.605 ±.106	1.745 ±.106	1.670 ±.332	1.680 ±.255
C_v	5.9%	4.1%	6.6%	6.1%	20%	15%
t testing		T-I > T-II 80% level T-I > T-III 80% level			T-I > T-IV 60% level T-I > T-V 60% level T-I > T-VI 60% level	

Table 22. Effects of six levels of carbon dioxide on *Selenastrum* Maximum Standing Crops as cell numbers with C_v and *t* testing.

Rep	Control I	Air vented II	Air bubbled III	CO_2 vented IV	CO_2 bubbled V	CO_2 bubbled VI
1	4.38×10^6	[a] no count	4.77×10^6	4.44×10^6	3.28×10^6	2.51×10^6
2	4.67×10^6	[a] no count	8.17×10^6	[a] no count	4.01×10^6	3.67×10^6
Mean ±1SD	4.52×10^6 $\pm .20 \times 10^6$	—— ——	6.47×10^6 $\pm 2.40 \times 10^6$	4.44×10^6 ——	3.64×10^6 $\pm .52 \times 10^6$	3.09×10^6 $\pm .82 \times 10^6$
C_v	4.4%	—	37%	—	14%	27%
t testing		T-I vs T-III ns T-I > T-V 70% level T-I > T-VI 70% level				

[a] No cell count made because of excessive media evaporation due to gas venting.

Table 23. Effects of six levels of carbon dioxide on Selenastrum Maximum Standing Crops as dry weight in micrograms/ml with C_v and $\underline{t}$ testing.

Rep	Control I	Air vented II	Air bubbled III	CO_2 vented IV	CO_2 bubbled V	CO_2 bubbled VI
1	193	[a]not weighed	221	369	283	225
2	206	[a]not weighed	420	[a]not weighed	441	326
Mean	200	—	320	369	362	276
±1 SD	± 9	—	±141	—	±112	± 71
C_v	4.5%	—	44%	—	31%	26%
$\underline{t}$ testing			T-I vs. T-III	ns		
			T-I < T-V	70% level		
			T-I < T-VI	60% level		

[a]Not weighed because of excessive media evaporation due to gas venting.

Note: Weighing consisted of two 20 ml sample per rep on a six-place balance with the C_v between the two samples as a 9% average showing the weighing technique was precise.

Table 24. Effects of six levels of carbon dioxide on Selenastrum "Cell Health" as dry weight per million cells in micrograms/10^6 cells with C_v and $\underline{t}$ testing.

Rep	Control I	Air vented II	Air bubbled III	CO_2 vented IV	CO_2 bubbled V	CO_2 bubbled VI
1	44.1	[a]—	46.3	83.1	86.3	89.6
2	44.1	[a]—	51.4	[a]—	109.9	88.8
Mean	44.1	—	48.9	83.1	98.1	89.2
±1 SD	±0.0	—	±3.6	—	±16.8	±0.6
C_v	0%	—	7.4%	—	17%	0.7%
$\underline{t}$ testing		T-I < T-III	60% level	T-III < T-V	80% level	
		T-I < T-V	80% level	T-III < T-VI	95% level	
		T-I < T-VI	99% level	T-V vs. T-VI	ns	

[a]Not calculable because of excessive media evaporation.

Note: Parameters calculated by taking a value from Table 23 and dividing it by the respective value from Table 22 then multiplying by one million.

Table 23 lists the Maximum Standing Crops in dry weights for each treatment not undergoing media evaporation. The control again gave the lowest C_v at 4.6%. The other treatments all gave C_v higher than 15%. The *t* testing showed the bubbled CO_2-enriched air treatments yielded the highest dry weights and the control yielded the lowest dry weights. The trend appears to be that while dry weight increases with increasing carbon availability, cell numbers correspondingly decrease. This was visually noted when counting cells. The control's cells were small and irregularly shaped while as the carbon availability increased, the cells were more turgid appearing and much larger. No dimension measurements were made. King (1972) also noted this in cultures of Selenastrum and proposed algae respond to low carbon availability by decreasing their size and increasing their numbers to increase surface area to volume ratios so that carbon dioxide can be more readily taken up by the cells.

Table 24 lists the average dry weights per million cells which is an indication of "Cell Health". Again, the control gave the lowest C_v. The C_v for the other treatments is lower in this table than all others suggesting the "Cell Health" parameter is a good measure for these assays.

Table 26 lists the correlation and confidence levels for comparisons between pH maximums for each treatment and the various growth parameters. The table indicates a very high correlation between pH maximums and the "Cell Health" parameter.

Table 26. Correlation and confidence levels for comparisons between pH maximums reached during the bioassay (this reflects carbon availability) and the growth parameters determined.

Variables Compared	Correlation Coefficient	[a]Confidence level
pH maximum vs Maximum Specific Growth Rates	0.277	ns
pH maximum vs Maximum Standing Crops in cell numbers	0.424	80%
pH maximum vs Maximum Standing Crops in dry weights	0.441	80%
pH maximum vs "Cell Health" in dry weight per million cells	0.857	99%

[a]Taken from tables of Bailey (1959),"confidence levels for correlation coefficients".

Conclusions

Table 25 summarizes all the C_v data. It is evident the control yielded lower variation than the other methods tested for insuring carbon availability. The parameter for "Cell Health" yielded the lowest variance of the four growth parameters measured. This suggests the "Cell Health" parameter is good to use when bioassaying carbon availability. It may be good because it combines two parameters and cancels out variances in cell suspension volumes. In this case, the variance caused by excessive media evaporation was thought to be the major source of variance. Murray et al. (1971) the treatment gases through acidified water to humidify the

Table 25. Summary of the Coefficients of Variation as percents for Experiment 7.

Type of Coefficient of Variation (C_v)	Control I	Air vented II	Air bubbled III	CO_2 vented IV	CO_2 bubbled V	CO_2 bubbled VI
Average of the seven C_v between daily Specific Growth Rates for two treatment reps.	15%	19%	26%	17%	66%	28%
C_v between Maximum Specific Growth Rates for two treatment reps.	5.9%	4.1%	6.6%	6.1%	20%	15%
C_v between Maximum Standing Crops as cells per ml for two treatment reps.	4.5%	[a]—	37%	[a]—	14%	27%
C_v between Maximum Standing Crops as dry weight per ml for two treatment reps.	4.6%	[a]—	44%	[a]—	31%	26%
C_v between "Cell Health" as dry weight per million cells for two treatment reps.	0.0%	[a]—	7.4%	[a]—	17%	0.6%

[a]Not determined because of excessive media evaporation due to gas venting.

gas and minimize the effects of evaporation. This procedure would have helped in this experiment apparently. However, it is evident the "Cell Health" parameter corrects for media evaporation since the volume term is cancelled in calculation.

Because of the loss in precision when attempting to control carbon availability, one may have to concede to no controls other than optimum surface to volume ratios for culture medium. Certainly for studies that look at carbon availability or carbon-nutrient interactions, one should attempt to control carbon.

It is apparent Selenastrum is tolerant of fairly low pH levels (near pH 6). Selenastrum responds to low levels of carbon by increasing cellular surface area to volume ratios (King, 1972). This experiment supports King's finding since I found cell size decreased and cell numbers increased when available carbon (free CO_2) decreased.

Finally, I suggest this carbon control problem may be another reason (see page 57 of this study) for the high interlaboratory variances and the low intralaboratory variances noted in the Inter-Laboratory Precision Test (Maloney-Deputy Chief, 1971). It is likely that before the AAP becomes the "standard" needed, a precise method of controlling carbon availability will have to be developed.

Experiment 8

Effects of culture medium freshness on Selenastrum growth

Purpose

Fresh AAP medium was compared to media one and two weeks old to determine if aged media affected nutrient concentrations. Bioassay was used rather than chemical assessment of the media because bioassay assesses the biological availability of nutrients.

Design

The three treatments were (1) fresh AAP medium, (2) seven-day-old AAP medium, and (3) fourteen-day-old AAP medium all prepared from the same nutrient stock solutions. Each treatment was run in duplicate.

Procedure

The treatments were randomly inoculated with four-day-old inoculum cultured and prepared in the usual manner. The initial cell concentration was about 2×10^3 cells per ml and all six flasks were placed under light intensity of 420 ft-c. On day 10 cell counts and dry weights were determined using procedures described previously.

Results

Table 27 lists averages for cell counts and dry weights, coefficients of variation, and percent reduction in cell counts

and dry weights caused by aged media. The fresh medium yielded significantly higher cell counts and dry weights at 99% confidence levels according to $\underline{t}$ testing.

Table 27. Comparison of fresh AAP medium to aged AAP medium by (a) cell counts and (b) dry weights after ten days growth. Also shown are C_v and percent reductions in cell counts and dry weights due to medium freshness.

(a)

Treatments	Cell counts cells/ml $\pm$ 1SD	C_v	Percent reduction
Fresh medium	$4.5 \times 10^6 \pm .2 \times 10^6$	4.5%	—
Seven-day-old medium	$7.6 \times 10^5 \pm .1 \times 10^5$	1.3%	83%
Fourteen-day-old medium	$6.4 \times 10^5 \pm .1 \times 10^5$	1.6%	87%

(b)

Treatments	Dry weights μg/ml $\pm$ 1SD	C_v	Percent reduction
Fresh medium	199.5 $\pm$ 9.2	4.6%	—
Seven-day-old medium	92.1 $\pm$ 3.0	3.3%	64%
Fourteen-day-old medium	84.6 $\pm$ 3.7	4.4%	68%

A simple test was conducted to determine which nutrients were lost in aged media. Seven aliquot portions from the fourteen-day-old treatment were placed in seven small vials on day 10. Each vial was treated with a few drops of one of the nutrient stock solutions used to prepare AAP medium. After five days only the vial that received the TRACES stock treatment (micronutrient mixture) responded significantly. Table 28 shows the results of this testing. Reductions of up to 87% and 68% for cell counts and dry weights respectively

were observed. It is not known if the nutrients were lost (e.g., adsorbed onto the glass of the flask in which the media treatments were aged) or if the nutrients became biologically unavailable (e.g., chemical precipitation). The media were aged in one-liter flask and poured into the culture flasks on the day of inoculation. By this procedure, the nutrients may have been adsorbed onto the one-liter flask and subsequently not transferred when the medium was poured into culture flasks. Another bioassay would have to be conducted to determine if medium aged in the culture flasks affected nutrient concentrations.

Table 28. Cell counts of Selenastrum cultured in aged medium (fourteen-day-old). On day 10 the culture was divided into seven portions and each treated with one of the AAP nutrient stocks. Cell counts were made after five days of culturing.

Day	Treatment solution nutrients (counts in cells/ml)						
	$NaNO_3$	K_2HPO_4	$MgCl_2$	$MgSO_4$	$CaCl_2$	$NaHCO_3$	[a]TRACES
10	6.4×10^5	6.4×10^5	6.4×10^5	6.4×10^5	6.4×10^5	6.4×10^5	6.4×10^5
15	7.1×10^5	6.8×10^5	7.5×10^5	7.9×10^5	6.8×10^5	6.9×10^5	$\underline{2.4 \times 10^6}$

[a]TRACES is a stock solution containing a mixture of all trace elements (micronutrients) used to prepare AAP medium. TRACES was the only treatment giving a significant growth response according to t testing.

Conclusions

This experiment demonstrates the importance of using freshly prepared AAP medium for bioassays. Many of the problems encountered in earlier experiments in this study may have resulted from using aged AAP medium.

The Algal Assay Procedure: Bottle Test (Bartsch-Director, 1971) does not specify that only fresh medium be used for bioassays. It states,

> "It is recommended that uninoculated sterile reference medium be stored in the dark to avoid any (unknown) photochemical changes."

Murray et al. (1971) observed the order in which the nutrients were added in preparing the AAP medium effected Selenastrum growth. I suggest these problems need to be studied and if proven, the AAP should be modified to eliminate discrepancies resulting from such unmodified procedures.

APPLICATIONS OF ALGAL BIOASSAY

The following six experiments are applications of the bioassay to limnological questions since it was felt the techniques had been mastered sufficiently.

Three Michigan lakes were assayed for (1) possible inhibitory substances reported to prevent algal growth in oligotrophic lakes and (2) the nutrient limiting Selenastrum production in a lake sample.

Lake samples were collected in July of 1973 and bioassayed a day after sampling.

Experiment 9
Bioassay of Torch Lake for inhibitory substances

Purpose

An oligotrophic lake was assayed for unknown substances reported to inhibit algal growth. The lake assayed was Torch Lake, Michigan, near Traverse City along the western side of the state, a few miles inland from Lake Michigan.

McDonald et al. (1970) reported an oligotrophic soft water lake, Lake George, in the Adirondack Mountains of New York, contained inhibitory substances that limited Selenastrum productions in static bioassays. McDonald used the PAAP medium which differs slightly in nutrient concentrations when compared to AAP medium. He put PAAP nutrient stock solutions in two, one-liter volumetric flasks, diluted one flask with distilled water (control), and diluted the other flask with Lake George water which had been membrane filtered to remove indigenous algae. The two treatments were inoculated with Selenastrum and growth was followed. It was found that the nutrients diluted with Lake George water gave significantly lower μ_{max} compared to the control (e.g., 0.98 and 1.32 respectively). He also reported a 29% reduction in dry weight for the treatment where the diluent was Lake George water. He repeated the experiment with Gorham's medium and noted similar results. It was concluded Selenastrum could not use

the increased nutrients added when the lake water was used to dilute the AAP nutrients because some unknown inhibitory substance was apparently in the lake water.

This experiment is designed to be similar to McDonald's study.

Design

The treatments were (1) AAP nutrients diluted with distilled water (control) and (2) AAP nutrients diluted with 0.45μ membrane filtered Torch Lake water. A third treatment was attempted using paper filtered lake sample as a diluent, but gave inconclusive results since the filtering passed through many protozoans. These were much larger than Selenastrum cells so it was likely spores or eggs passed through the filters. One species was very numerous (up to 10^3 per ml) and appeared full of Selenastrum cells. When these protozoans (identified as Collodictyon sp.) were placed in a formalin solution, they burst open and released from ten to twenty algal cells. The membrane filtering passed some bacteria through, however, axenic samples are not strived for using the AAP bioassays.

Each treatment was run in five replications. Cell counts were used to follow growth. A blocking technique was used to block out the effects of slight differences known to exist in light intensity over the assay platform.

Procedure

Torch Lake water, known to be oligotrophic, was collected

and transported under ice in acid rinsed dark bottles to the laboratory where it was immediately membrane filtered. The filtrate was used to dilute a batch of AAP nutrients. The control was a batch of AAP nutrients diluted with distilled water.

The treatments were inoculated shortly after preparation with four-day-old stock Selenastrum prepared and delivered in the usual manner. Cell counts were made on days 0,1,2,3,5,7, and 10.

Results

Daily cell counts and pH are listed in Appendix table B9. The maximum pH reached was about 10.1 for the control and 9.8 for the lake water treatment. Figures 10a,b depict the growth curves and show the precision between treatment replications was high.

Table 29 gives the daily Specific Growth Rates for each treatment replication. The underlined maximums always occurred between days 1 and 3. Table 30 gives the μ_{max} values. The C_v between the treatments replications was low and acceptable for bioassays at 10% and 13%. The t test showed no significant difference between the μ_{max} values.

Table 31 gives the Maximum Standing Crops in cell numbers for each treatment replication. The C_v was 3.2% for the control and 17% for the lake water treatment. The t test showed the control yielded significantly higher standing crops at a 95% confidence level. This finding agrees with McDonald's finding for Lake George.

Figure 10. Growth curves for Selenastrum from a ten day bioassay of the effects of membrane filtered Torch Lake water used to dilute the AAP stock nutrients.

a. Page 94 - The treatment was AAP nutrients diluted with distilled water (control).

b. Page 95 - The treatment was AAP nutrients diluted with membrane filtered Torch Lake water.

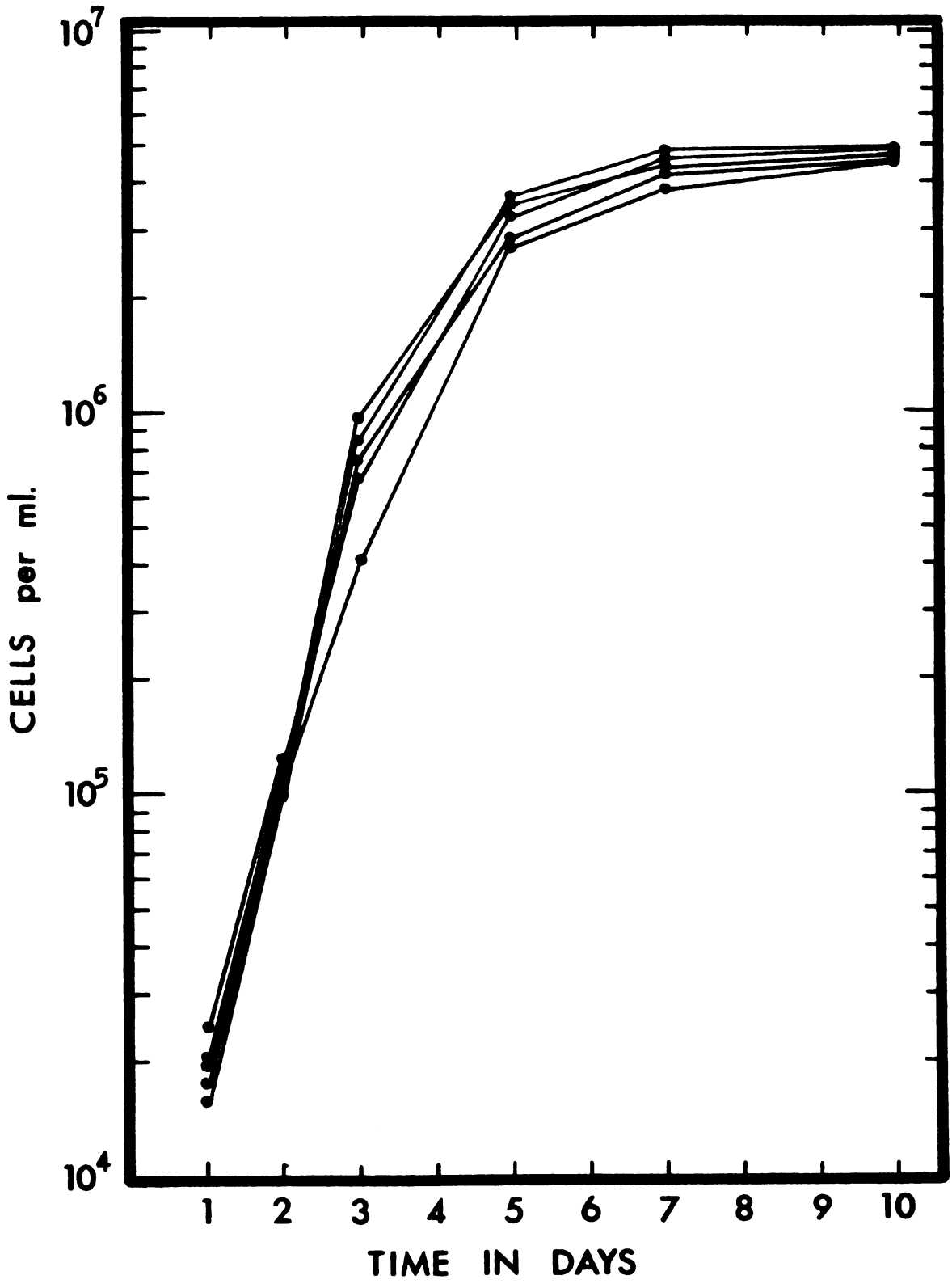


Figure 10 a

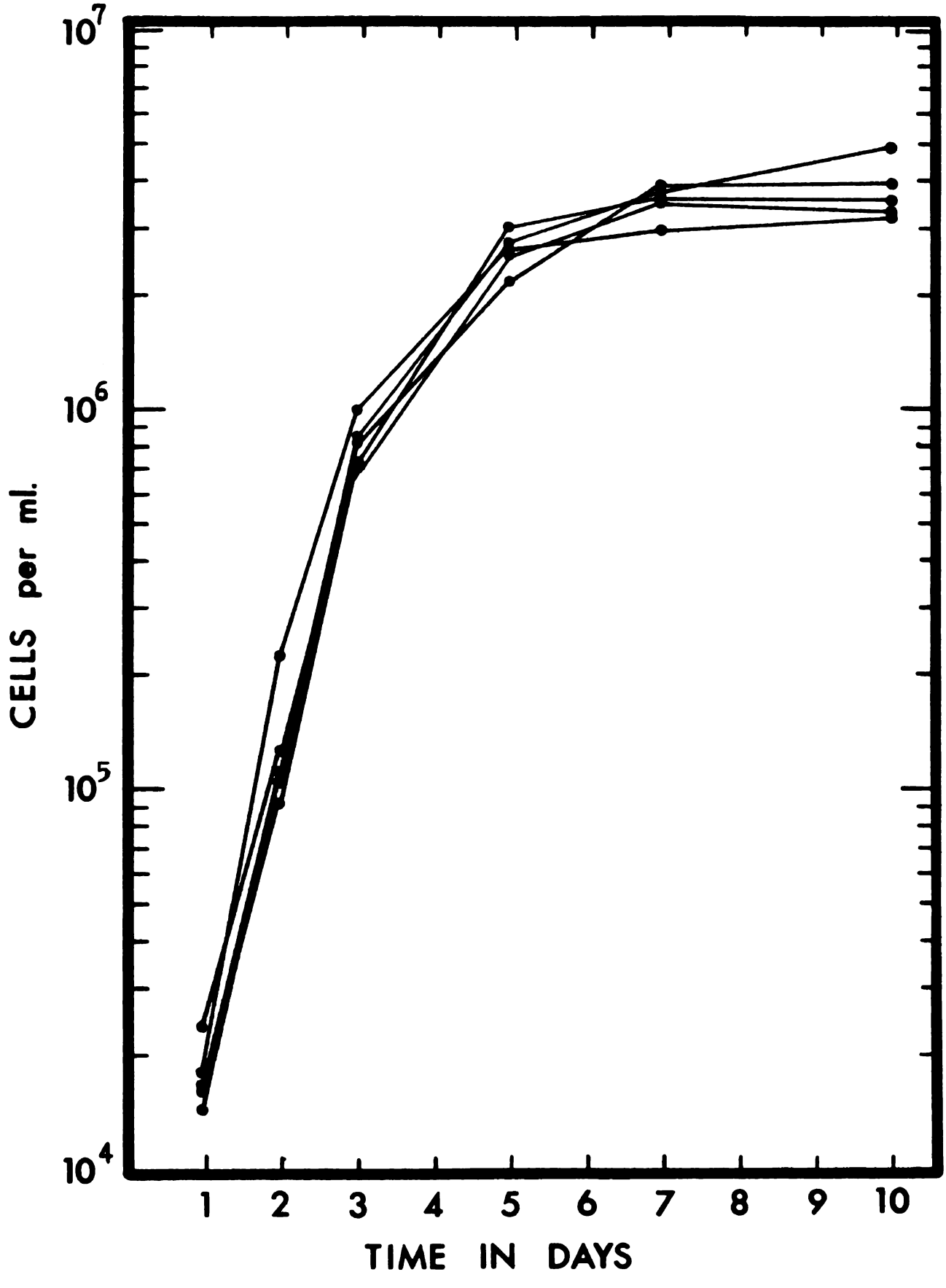


Figure 10b

Table 29. Effects of membrane filtered Torch Lake water as a diluent for AAP nutrients compared to the AAP nutrients diluted with distilled water on Selenastrum daily Specific Growth Rates for a ten day static bioassay.

Time (Days)	SPECIFIC GROWTH RATES (μ/day)									
	Diluent is distilled water					Diluent is Torch Lake water				
	1	2	3	4	5	1	2	3	4	5
0										
1	1.01	0.80	1.24	0.90	1.05	0.86	0.94	0.82	1.21	0.73
2	1.66	1.98	1.45	1.94	1.68	1.86	2.52	1.74	1.62	1.96
3	[a]2.09	1.91	2.22	1.72	1.34	1.87	1.47	2.22	1.91	1.90
5	0.73	0.66	0.65	0.79	0.94	0.77	0.61	0.46	0.65	0.67
7	0.29	0.39	0.15	0.17	0.18	0.06	0.03	0.30	0.08	0.15
10	[b]0.00	0.03	0.04	0.05	0.04	0.03	0.00	0.01	0.00	0.09

[a]Underlined growth rates are the maximums for each replication.

[b]Growth rates recorded as 0.00 may be less than zero.

Table 30. Effects of membrane filtered Torch Lake water as diluent for the AAP nutrients compared to the AAP nutrients diluted with distilled water on *Selenastrum* Maximum Specific Growth Rates. Also listed are C_v and $\underline{t}$ testing data.

Rep Number	MAXIMUM SPECIFIC GROWTH RATES (μ_{max})	
	Diluent is distilled water	Diluent is Torch Lake water
1	2.09	1.87
2	1.98	2.52
3	2.22	2.22
4	1.94	1.91
5	1.68	1.96
Mean $\pm$ 1SD	1.98 $\pm$ 0.20	2.09 $\pm$ 0.27
C_v	10%	13%
$\underline{t}$ test	The treatment means do not differ significantly above the 50% level	

Table 31. Effects of membrane filtered Torch Lake water as a diluent for the AAP nutrients compared to the AAP nutrients diluted with distilled water on *Selenastrum* Maximum Standing Crops in cells numbers. Also listed are C_v and $\underline{t}$ testing data.

Rep Number	MAXIMUM STANDING CROPS in Cell/ml	
	Diluent is distilled water	Diluent is Torch Lake water
1	4.8×10^6	3.1×10^6
2	4.5×10^6	3.5×10^6
3	4.6×10^6	3.8×10^6
4	4.6×10^6	3.5×10^6
5	4.4×10^6	4.8×10^6
Mean $\pm$ 1SD	$4.6 \times 10^6 \pm 0.2 \times 10^6$	$3.7 \times 10^6 \pm 0.6 \times 10^6$
C_v	3.2%	17%
$\underline{t}$ test	The treatment means differ at a 95% level	

Conclusions

McDonald found when the PAAP nutrients were diluted with an oligotrophic lake water and compared to a control of nutrients diluted with distilled water, that the latter gave significantly higher μ_{max} and Maximum Standing Crops.

I found when Torch Lake water was used as a diluent for the AAP nutrients and compared to a control, that the latter gave no detectable differences in μ_{max} and significantly higher Maximum Standing Crops measured in cell numbers.

McDonald suggested inhibitory substances caused this. I suggest from this experiment alone, there is insufficient evidence to conclude the decreases in cell production were caused by inhibitory substances. There are other plausible causes for this study's and McDonald's results. For instance, the added carbonate alkalinity from the lake water treatment may have effected algal growth. Experiment 7 indicated Selenastrum responds to decreased carbon availability by increasing cell numbers and decreasing cell size. King (1972) noted this response and suggested it was to increase the surface to volume ratios of the cells. It was noted during counting in this experiment that the control's cells were small and deformed while the lake water treatment's cells were larger and more turgid (similar to the finding in Experiment 7). This could have accounted for the observed and determined results in this experiment.

McDonald noted a 29% reduction in standing crops. Here a 19% reduction was noted (Table 31). Although these results

were statistically significant, Figures 10a and 10b show the reduction in cell numbers is hardly noticeable from growth curves. The visual observation of varying cell sizes for the treatments is not reflected in the data collected. When dry weights were determined, as in Experiment 11, this observation is verified.

Experiment 10
Bioassay of Deep Lake for inhibitory substances

Purpose

This experiment was similar to Experiment 9 except the lake bioassayed was Deep Lake, located in Barry County, Michigan, within the Yankee Springs State Recreation Area. The lake is free of cottages, spring fed, and oligotrophic. A secchi disk reading of twelve meters was observed in July at the time of sampling. The lake is stocked with Lake and Brown Trout yearly. It gave a good sample to test McDonald's hypothesis that oligotrophic lakes contain unknown inhibitory substances effecting algal production (McDonald et al., 1970).

Design

The set-up was the same as for Experiment 9 except that four treatment replications were used instead of five.

Procedure

The methods are the same as for Experiment 9. Some chemical determinations were made on the lake sample. Phenolphthalein alkalinity was 8 mg/l and total alkalinity was 145 mg/l as calcium carbonate. Total hardness was 166 mg/l as calcium carbonate. Orthophosphate was less than the lower detection limit of 0.01 mg P/l using a molybdate method on the filtered samples. Cell counts were made on Days 0,1,2,3,

4, 6, 10, and 18 (Experiment 9 was assayed for only ten days).

Results

Cell counts, pH, and C_v for each treatment replication are listed in Appendix B10. Figures 11a and 11b depict the growth curves. Table 32 gives the daily Specific Growth Rates with the maximums all occurring between days 1 and 2.

Table 33 gives the μ_{max} values. The C_v between the treatment replications was 6% and 4% which indicates excellent precision. The mean μ_{max} for the Deep Lake water diluent treatment was greater than the distilled water diluent treatment at a 95% confidence level. This is contrary to the findings of McDonald as explained in Experiment 9.

Table 34 gives the Maximum Standing Crops in cell numbers. The C_v between the treatment replications was 9% and 6% which again indicates excellent precision. The mean Maximum Standing Crop for the Deep Lake water diluent treatment was less than the distilled water diluent treatment at a 94% confidence level. This agrees with McDonald's finding.

Bacterial growth was noted when counting the lake treatment, especially after day 6. No attempts were made to quantify or identify these microbes.

Conclusions

AAP nutrients diluted with membrane filtered Deep Lake water compared to AAP nutrients diluted with distilled water gave higher μ_{max} values (1.94 and 1.70 respectively) and lower Maximum Standing Crops (3.8×10^6 and 4.5×10^6 cells per ml respectively).

Figure 11. Growth curves for Selenastrum from an eighteen day bioassay of the effects of membrane filtered Deep Lake water used to dilute the AAP stock nutrients.

a. Page 103- The treatment was AAP nutrients diluted with distilled water (control).

b. Page 104- The treatment was AAP nutrients diluted with membrane filtered Deep Lake water.

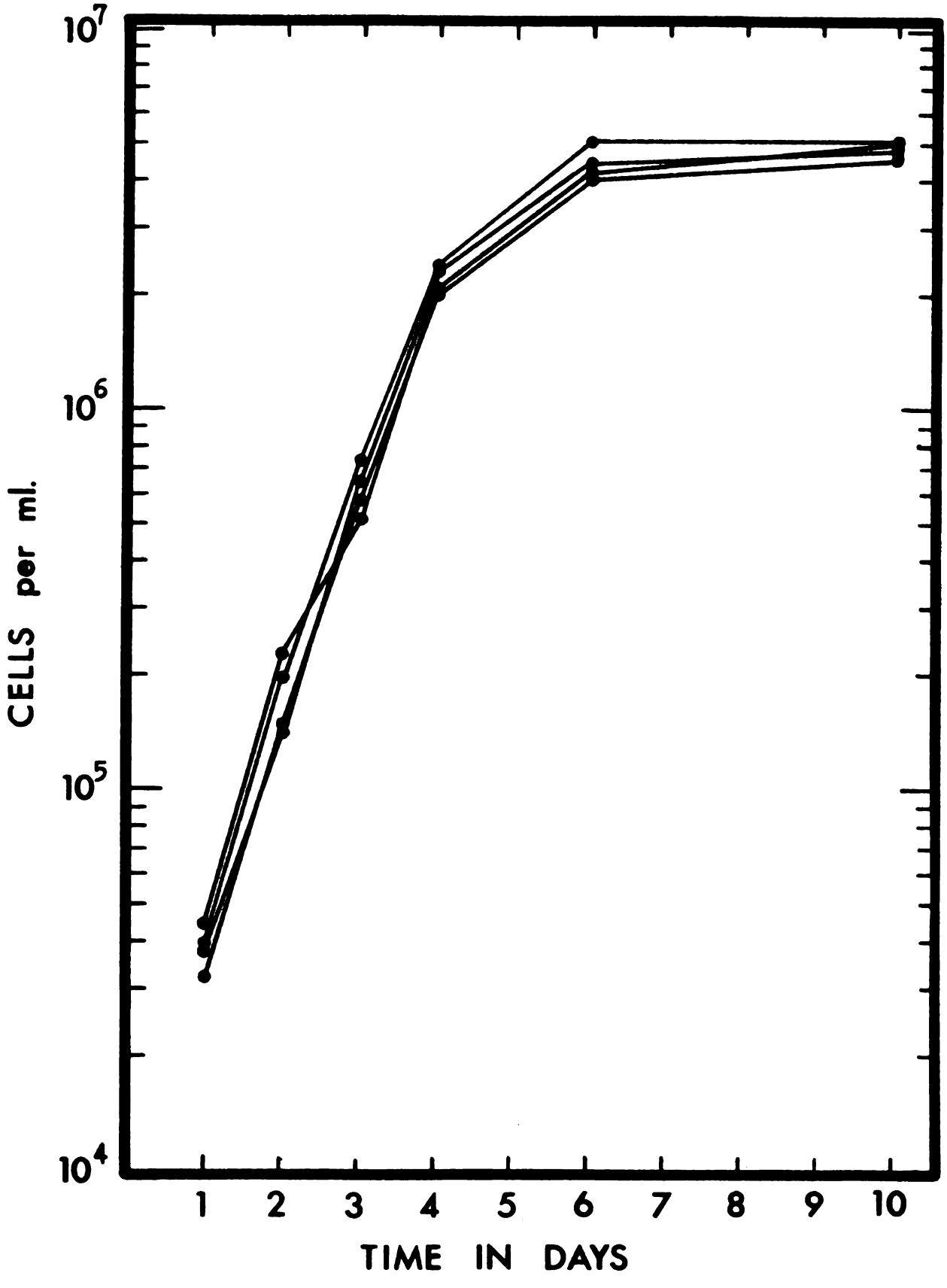


Figure 11a

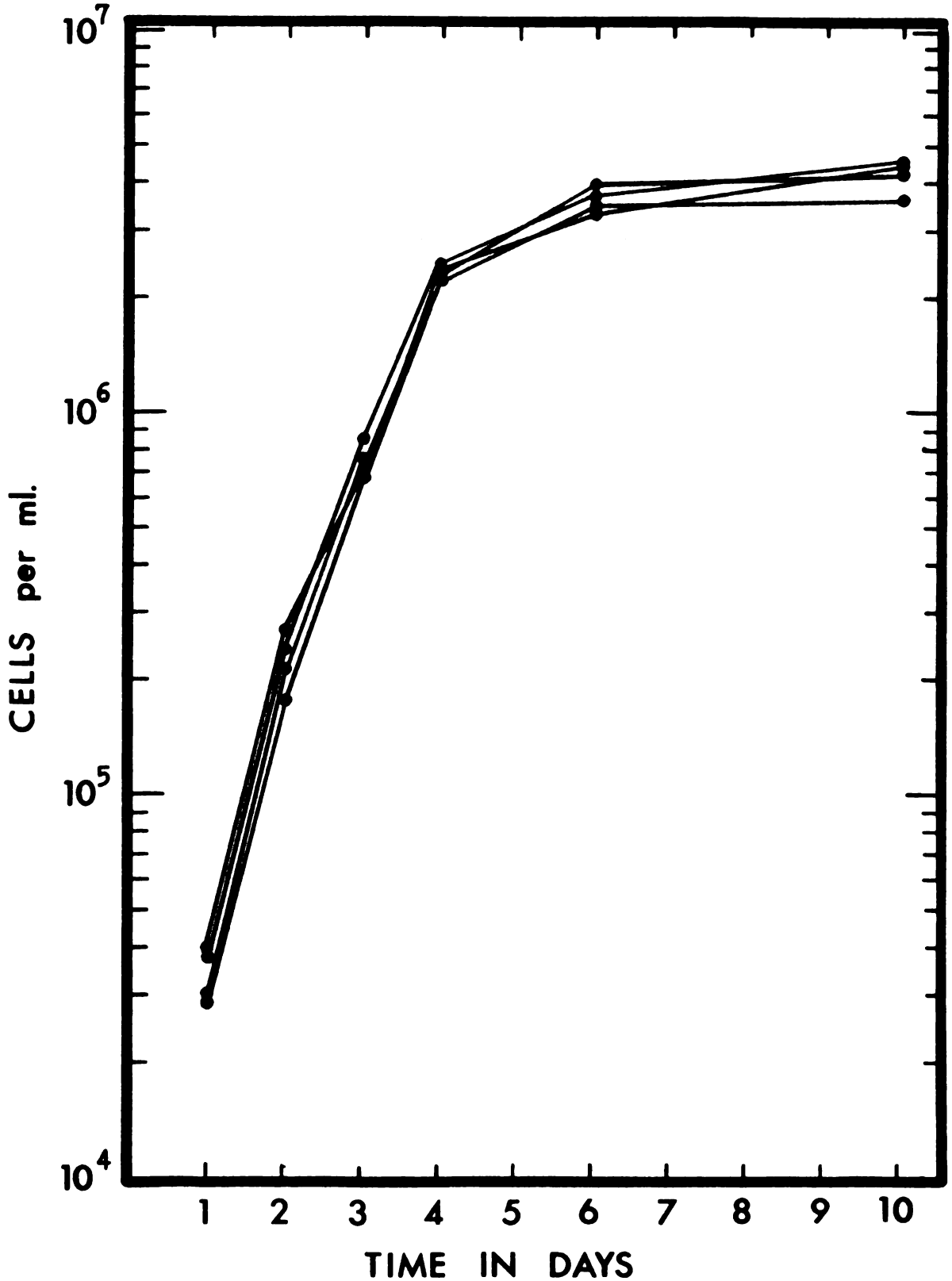


Figure 11b

Table 32. Effects of membrane filtered Deep Lake water as a diluent for the AAP nutrients compared to the AAP nutrients diluted with distilled water on Selenastrum daily Specific Growth Rates for an eighteen day static bioassay.

Day	SPECIFIC GROWTH RATES (μ/day)							
	Diluent is distilled water				Diluent is Deep Lake water			
	1	2	3	4	1	2	3	4
0								
1	1.22	1.36	1.34	1.48	0.88	1.10	1.31	1.39
2	[a]1.68	1.62	1.84	1.65	2.01	1.99	1.96	1.91
3	1.17	1.31	0.99	0.82	1.58	1.24	1.26	0.96
4	1.23	1.17	1.28	1.35	1.20	1.06	1.04	[b]----
6	0.35	0.31	[c]0.00	0.40	0.22	[c]0.00	0.24	[b]----
10	0.03	0.01	0.00	0.00	0.02	0.00	0.04	0.07
18	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00

[a]Underlined growth rates are the maximums for each replication.

[b]Not determined because of "outlier" in Appendix table B10.

[c]Growth rates recorded as 0.00 may be less than zero.

Table 33. Effects of membrane filtered Deep Lake water as a diluent for the AAP nutrients compared to the AAP nutrients diluted with distilled water on Selenastrum Maximum Specific Growth Rates with C_v and t testing.

Rep Number	MAXIMUM SPECIFIC GROWTH RATE (μ_{max})	
	Diluent is distilled water	Diluent is Deep Lake water
1	1.68	2.01
2	1.62	1.99
3	1.84	1.86
4	1.65	1.91
Mean ± 1SD	1.70 ± 0.10	1.94 ± 0.07
C_v	6%	4%
t test	Distilled water diluent mean is smaller than Deep Lake water diluent mean at a 95% level.	

Table 34. Effects of membrane filtered Deep Lake water as a diluent for the AAP nutrients compared to the AAP nutrients diluted with distilled water on Selenastrum Maximum Standing Crops in cell numbers, with C_v and t testing.

Rep Number	MAXIMUM STANDING CROPS in Cells/ml	
	Diluent is distilled water	Diluent is Deep Lake water
1	4.1×10^6	3.9×10^6
2	4.5×10^6	3.5×10^6
3	5.1×10^6	3.7×10^6
4	4.4×10^6	4.0×10^6
Mean ± 1SD	$4.5 \times 10^6 \pm 0.4 \times 10^6$	$3.8 \times 10^6 \pm 0.2 \times 10^6$
C_v	9%	6%
t test	Distilled water diluent greater than lake water diluent at 95% level	

These results were highly significant statistically. However, the biological significance is not apparent. A speculation was given in Experiment 9 on the cause of these results. Another plausible cause might be the effects of the bacteria in the lake sample. The only organic compound in the AAP nutrients is the chelator, EDTA. This chelator is believed to make certain nutrients biologically available. If the chelator was destroyed during the assay, the effects might be nutrients becoming unavailable. Tiedje (personal communication) is presently investigating EDTA decomposition by microbes. He reports that EDTA was not a carbon source for any of the microbes tested. In many instances EDTA acts as an antibacterial agent. In a few instances it was metabolized, but only at extremely slow rates. He doubted metabolism of EDTA in algal bioassay would be significant for short duration tests.

Experiment 11
Bioassay of Lake Lansing for inhibitory substances

Purpose

This experiment, like Experiments 9 and 10, assesses the effects on Selenastrum of a lake water used to dilute the AAP stock nutrients. The lake bioassayed was Lake Lansing, near Lansing, Michigan. Lake Lansing is relatively eutrophic when compared to Torch Lake and Deep Lake.

Design

Dry weights, as well as cell counts, were used to assess growth. A mistake in inoculation resulted in two rather than four replications for the distilled water diluent treatment. The lake sample diluent treatment was run in four replications.

Procedure

Phenolphthalein alkalinity was 13 mg/l and total alkalinity was 110 mg/l as calcium carbonate. Total hardness was 136 mg/l as calcium carbonate. Orthophosphate measured with a molybdate method was 0.03 mg P/l. Total phosphate measured with a persulphate digestion method was 0.08 mg P/l.

The two treatments were randomly inoculated with six-day-old stock inoculum prepared in the usual manner. Two flasks were apparently not inoculated, since no growth was noted. Counts were made on days 0,1,2,3,5,7, and 14. Dry weights

were determined on day 14.

Results

Appendix table B11 lists the cell counts from which the growth curves in Figures 12a and 12b were determined. Table 35 gives the daily Specific Growth Rates which shows the maximums all occurred between days 1 and 2. Table 36 gives the μ_{max} values and Table 37 gives the Maximum Standing Crops. The results are the same as for the Torch Lake and Deep Lake experiments. The AAP nutrients diluted with membrane filtered Lake Lansing water gave higher μ_{max} values and lower Maximum Standing Crops when compared to the AAP nutrients diluted with distilled water.

Table 38 gives the data used to calculate the growth parameter of Maximum Standing Crops in cellular dry weights. The lake water diluent treatment yielded about two and a half times as much dry weight as the distilled water diluent treatment. The difference is obviously highly significant and was not t tested.

Table 39 was calculated from the cell counts on day 14 listed in Appendix table B11 and the dry weights of Table 38. The parameter of "Cell Health" showed the lake water diluent treatment yielded more than three and a half times as much dry weight per million cells as the other control treatment.

The maximum pH reached was 9.8 for the distilled water diluent and 8.7 for the lake water diluent. This suggests both treatments were likely under carbon stress with the

Figure 12. Growth curves for Selenastrum from a fourteen day bioassay of the effects of membrane filtered Lake Lansing water used to dilute the AAP stock nutrients.

a. Page 111 - The treatment was AAP nutrients diluted with distilled water (control). Because of an inoculation error, only two replications were run.

b. Page 112 - The treatment was AAP nutrients diluted with membrane filtered Lake Lansing water.

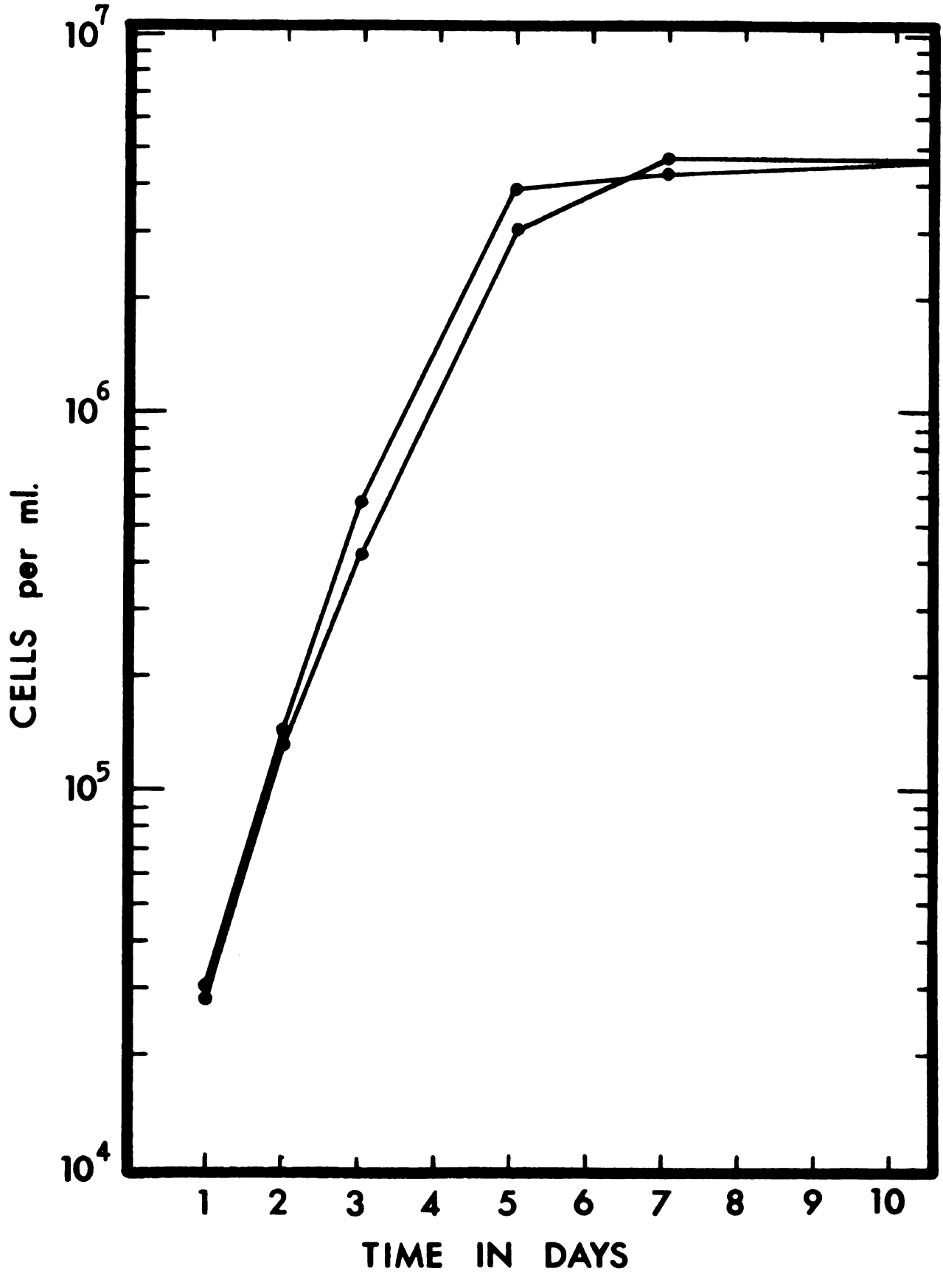


Figure 12a

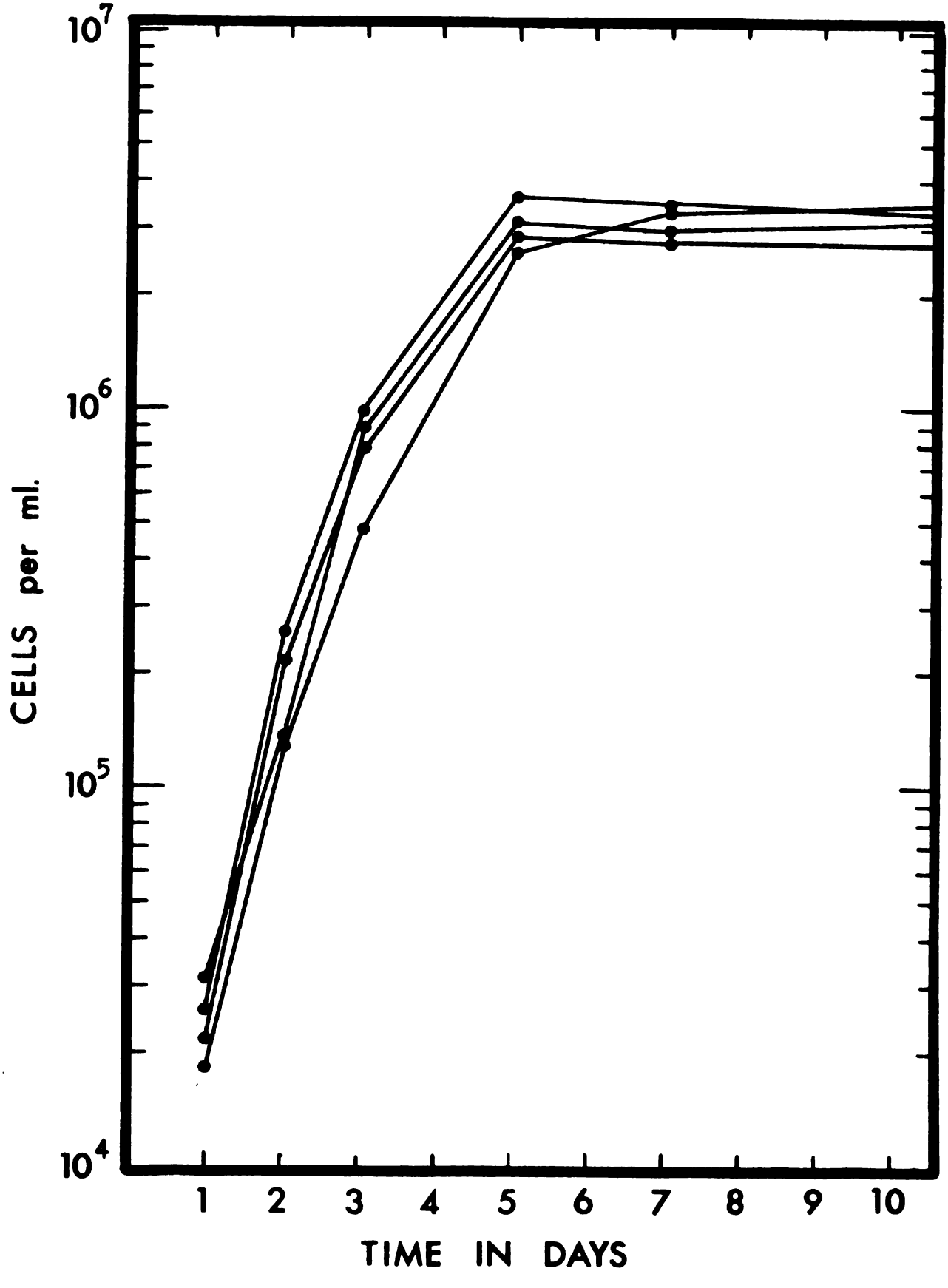


Figure 12b

Table 35. Effects of membrane filtered Lake Lansing water as a diluent for the AAP nutrients compared to the AAP nutrients diluted with distilled water on Selenastrum daily Specific Growth Rates for a fourteen day static bioassay.

Day	SPECIFIC GROWTH RATES (μ/day)							
	Diluent is distilled water		Diluent is Lake Lansing water					
0	1.12	1.16	[a]____	[a]____	0.59	1.13	0.79	1.00
1	[b]1.54	1.48			1.98	2.00	2.26	2.30
2	1.07	1.40			1.33	1.36	1.32	1.33
3	1.01	0.97			0.83	0.61	0.63	0.66
5	0.21	0.05			0.12	0.00	0.00	0.00
7	0.00	0.02			0.00	0.00	0.00	0.00
14								

[a]No replications because of inoculation error.

[b]Underlined values are the replication maximums.

Table 36. Effects of membrane filtered Lake Lansing water as a diluent for the AAP nutrients compared to the AAP nutrients diluted with distilled water on Selenastrum Maximum Specific Growth Rates, with C_v and t testing.

Rep Number	MAXIMUM SPECIFIC GROWTH RATE (μ_{max})	
	Diluent is distilled water	Diluent is Lake Lansing water
1	1.54	1.98
2	1.48	2.00
3	----	2.26
4	----	2.30
Mean $\pm$ 1SD	1.51 $\pm$ 0.04	2.14 $\pm$ 0.17
C_v	3%	8%
t test	Lake Lansing treatment mean is greater than the control mean at a 99% level.	

Table 37. Effects of membrane filtered Lake Lansing water as a diluent for the AAP nutrients compared to the AAP nutrients diluted with distilled water on Selenastrum Maximum Standing Crops in cell numbers, with C_v and t testing.

Rep Number	MAXIMUM STANDING CROPS in Cells/ml	
	Diluent is distilled water	Diluent is Lake Lansing water
1	4.8×10^6	3.3×10^6
2	4.4×10^6	3.1×10^6
3	----	2.8×10^6
4	----	3.7×10^6
Mean $\pm$ 1SD	$4.6 \times 10^6 \pm 2.8 \times 10^5$	$3.2 \times 10^6 \pm 3.8 \times 10^5$
C_v	6%	12%
t test	Lake Lansing treatment mean is less than the control mean at a 95% level.	

Table 38. Dry weight data for the treatments (1) AAP nutrients diluted with distilled water and (2) AAP nutrients diluted with membrane filtered Lake Lansing water determined on day 14. C_v given for precision of samples within replications and replications within treatments.

Treat	Rep	Sample	Sample wt (grams/ml from a 20.0 ml portion)	Replication wt (micrograms/ml ± 1SD with percent C_v)	Treatment wt (micrograms/ml ± 1SD with percent C_v)
1	1	1	0.0002260		
		2	0.0002246	225 ± 1 (.4)	
	2	1	0.0002146		
		2	0.0002078	211 ± 5 (2)	218 ± 10 (5)
2	1	1	0.0004864		
		2	0.0006140	550 ± 90 (16)	
	2	1	0.0004982		
		2	0.0005356	517 ± 26 (5)	
	3	1	0.0005786		
		2	0.0005540	566 ± 17 (3)	
	4	1	0.0004094		
		2	0.0004926	451 ± 59 (13)	521 ± 51 (10)

Table 39. "Cell Health" parameter of dry weight per million cells for treatments (1) AAP nutrients diluted with distilled water and (2) AAP nutrients diluted with membrane filtered Lake Lansing water. C_v given for precision of replications within treatments.

Treat	Rep	DRY WEIGHT per MILLION CELLS	
		Replications	Treatments
		micrograms/10^6 cells	micrograms/10^6 cells
1	1	46.9	
	2	41.4	44.1 ± 3.9 (9)
2	1	148.6	
	2	161.6	
	3	202.1	
	4	132.6	161.3 ± 29.7 (18)

Table 40. Summary of the determined growth parameters of μ_{max}, Maximum Standing Crops in cell numbers, Maximum Standing Crops in dry weights, and "Cell Health" in dry weight per million cells for Experiments 9, 10, and 11. The treatments were (1) AAP nutrients diluted with distilled water and (2) AAP nutrients diluted with membrane filtered lake water sample. The lakes bioassayed were Torch Lake, Deep Lake, and Lake Lansing.

Parameter determined	Treatment Number	Lake Bioassayed		
		Torch	Deep	Lansing
Maximum Specific Growth Rates	1	[a]1.98	1.70	1.51
	2	[a]2.09	1.94	2.14
Maximum Standing Crops in cells/ml	1	4.6×10^6	4.5×10^6	4.6×10^6
	2	3.7×10^6	3.8×10^6	3.2×10^6
Maximum Standing Crops in dry weights (micrograms/ml)	1	---	---	218
	2	---	---	521
"Cell Health" as dry weight per million cells	1	---	---	44.1
	2			161.3

[a]Not significantly different. All other parameters are different at a 95% confidence levels.

distilled water treatment more so.

It was noted while counting, the cells of the lake water treatment were larger and more turgid appearing than the other treatment's cells which were "scrawny" and small although in significantly larger numbers. This observation is similar to the two previous experiments. Bacteria were more numerous in the lake water diluent treatment.

Conclusions

Table 40 summarizes the finding for Experiments 9, 10, and 11, where the lakes bioassayed were Torch Lake, Deep Lake, and Lake Lansing respectively.

For all three lakes the membrane filtered lake water treatments gave higher μ_{max} values, higher Maximum Standing Crops measured as dry weight, and lower Maximum Standing Crops measured as cell numbers. The parameter of "Cell Health" indicated the distilled water diluent treatments gave unhealthy cultures compared to the other treatment.

The results of the three experiments are similar to the findings for Experiment 7, where it was suggested the algal response to carbon availability stress was an increasing cellular surface area to volume ratio accomplished by decreasing cell size and increasing cell numbers. Here it is suggested carbon stress may have caused the noted responses. Algae may respond similarly to other nutrient stresses. In terms of carbon stress, the lake water diluent treatment added available carbon directly (carbonate alkalinity) and indirectly

(organic carbon) as carbon dioxide respired from the microbe heterotrophs.

The growth curves do not depict the treatment responses at a glance. The high degree of experimental precision allowed slight differences to be detected. It was concluded success had been achieved in mastering the techniques used in static algal bioassay.

Experiment 12
Bioassay of Torch Lake for limiting nutrient

Purpose

The limiting nutrient in a membrane filtered Torch Lake sample was determined by static algal bioassay.

Design

The treatments to a membrane filtered sample of Torch Lake water were (1) lake sample control, (2) lake sample plus 0.40 mg N/l, and (3) lake sample plus 0.05 mg P/l. Phosphorus and nitrogen were the only nutrients assayed for because they had been shown to be the nutrients most often limiting algal production in freshwater lakes (e.g., Maloney et al., 1972). Each treatment was run in three replications. Cell counts were used to follow culture growth.

Procedure

Torch Lake water was filtered through a 0.45-μ membrane filter to remove indigenous algae as soon as possible after collection (about eight hours). The treatments were prepared by placing an amount of sodium nitrate to give 0.40 mg N/l in a one-liter volumetric flask, by placing an amount of potassium phosphate to give 0.05 mg P/l in a one-liter volumetric flask, and by then bringing both flasks up to volume with the filtered lake sample. The control was the filtered lake sample with

no nutrients added. Any significant increases in growth were not attributed to the cations in the treatment salts since I am not aware of any reported lakes limited in algal production by sodium or potassium. (This is not to say that sodium and potassium are unimportant in lakes. Provasoli (1969) and Wetzel (1972) have stated these two elements are important components leading to the change in flora induced by eutrophication. Blue-green algae have an absolute need for both elements, but other freshwater algal groups apparently do not according to Provasoli.)

Treatment replications were randomly inoculated with six-day-old Selenastrum stock inoculum prepared in the usual manner. The flasks were randomly allocated to blocks of squares on the assay platform so the effects of light intensity would be blocked out. Cell counts and pH were determined on days 0,1, 2,3,4,5, and 7 and are listed in Appendix table B12.

Results

The pH ranged from 8.2 to 8.6 for all treatments suggesting the cultures were likely not under carbon stress. The C_v values for cell counting were high (averaged about 50% in Appendix table B12) for the control and nitrogen treatments because of variance encountered when cells per micrometer grid are sparse. The phosphorus treatment responded and gave cell counts up to 150 per grid while the other two treatments gave counts of from 2 to 7 per grid.

Figures 13a, 13b, and 13c depict the growth curves. It

Figure 13. Growth curves for Selenastrum from a seven day bioassay to determine the nutrient limiting algal production in a Torch Lake membrane filtered water sample.

a. Page 122 - The treatment was membrane filtered Torch Lake water with no nutrients added (control).

b. Page 123 - The treatment was control plus 0.40 mg N/l.

c. Page 124 - The treatment was control plus 0.05 mg P/l.

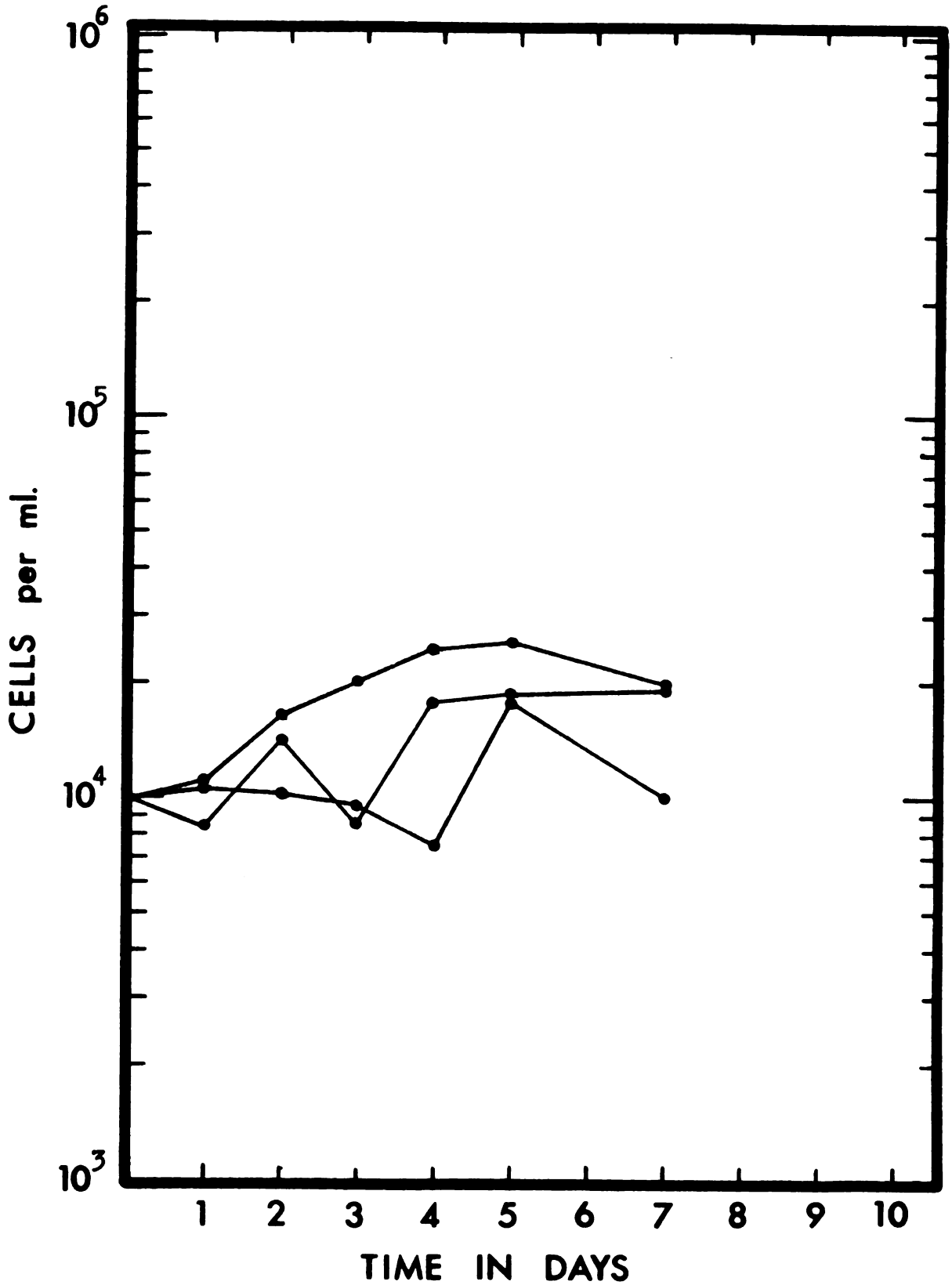


Figure 13a

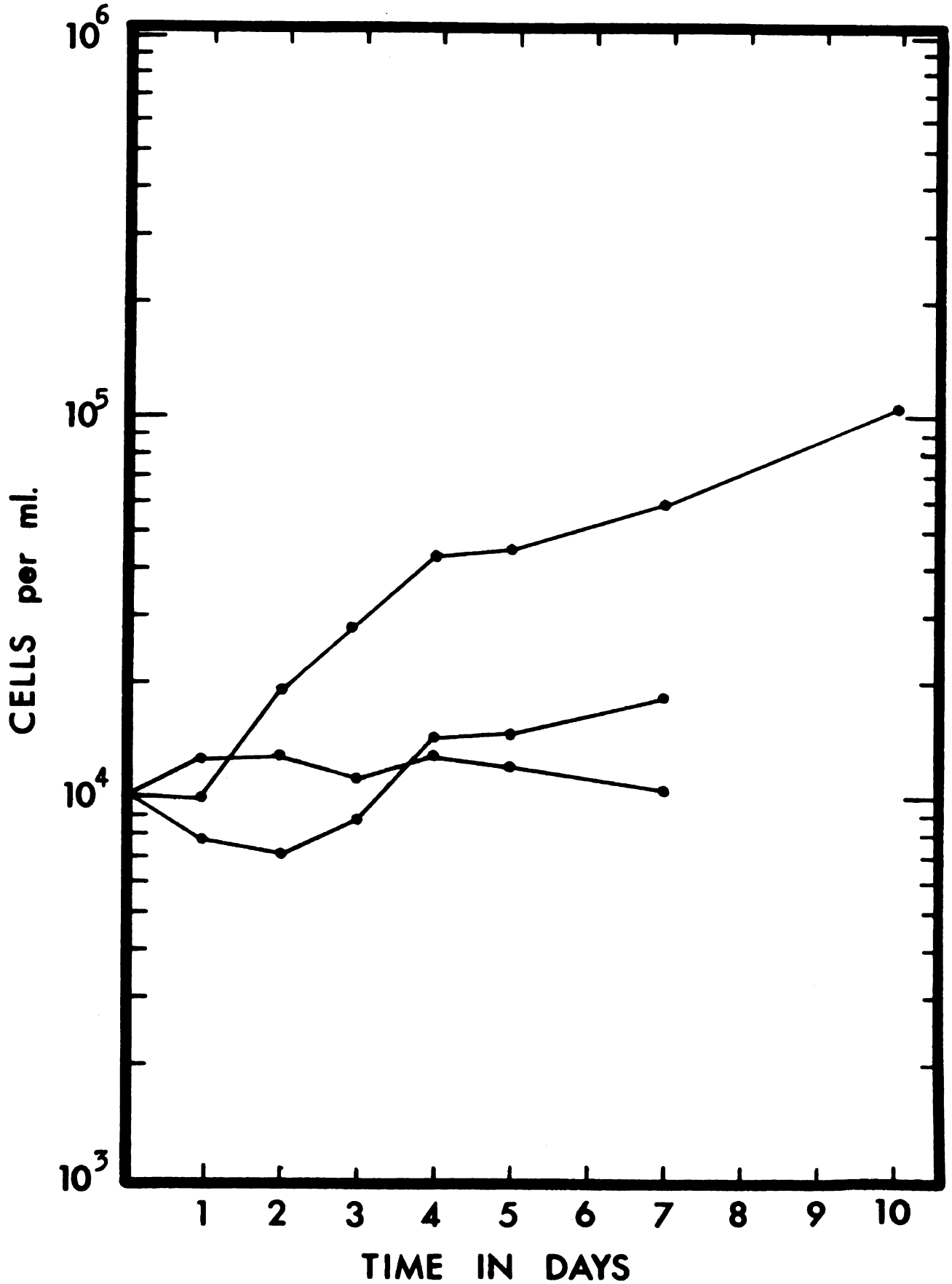


Figure 13b

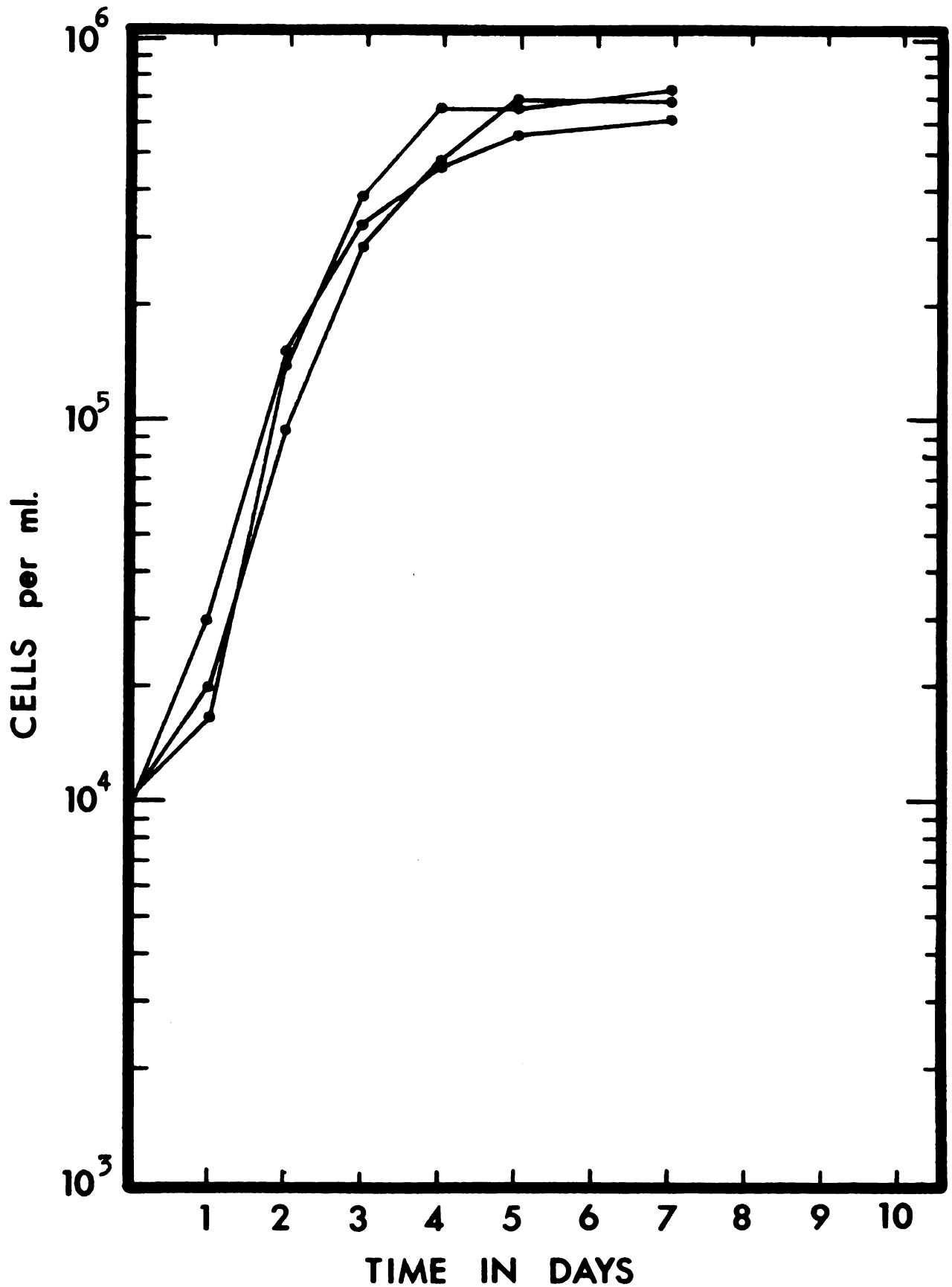


Figure 13c

is evident the phosphorus treatment responded. The third replication of the nitrogen treatment gave a slight response for unknown reasons. The curves for the control and nitrogen treatments are discrepant and may reflect the problems mentioned for counting cells at low concentrations.

Table 41 gives the daily Specific Growth Rates for each treatment replication. The maximums occurred randomly for the control and nitrogen treatments. The maximums for the phosphorus treatment all occurred between days 1 and 2. Table 42 gives the μ_{max} values. The $\underline{t}$ testing between μ_{max} means showed the phosphorus treatment gave higher values than either of the other treatments at a 95% confidence level.

Table 43 gives the Maximum Standing Crops in cell numbers. The $\underline{t}$ testing between means showed the phosphorus treatment yielded higher standing crops at a 99% confidence level. For growth parameters the C_v was high for the control and nitrogen treatments which again may reflect counting problems for low algal concentrations.

Very little bacterial growth was noted throughout the assay when counting the control and the nitrogen treatments. However, the phosphorus treatment had increasing numbers of bacteria as the assay progressed. They were most numerous after about day 4. Whether the bacterial respond directly to the nutrients or indirectly to the algal assimulated organic materials is not known.

Table 41. Effects of two nutrient treatments of 0.40 mg N/l and 0.05 mg P/l to membrane filtered Torch Lake water on Selenastrum daily Specific Growth Rates for a seven day static bioassay.

Day	SPECIFIC GROWTH RATES (μ/day)								
	Control			Control plus 0.40 mg N/l			Control plus 0.05 mg P/l		
	1	2	3	1	2	3	1	2	3
0									
1	0.08	0.10	0.00	[a]0.22	0.00	0.00	0.68	1.09	0.50
2	[b]0.00	0.41	0.53	0.03	0.00	0.66	1.57	1.61	2.08
3	0.00	0.20	0.00	0.00	0.21	0.00	1.09	0.79	1.06
4	0.00	0.22	0.74	0.20	0.52	0.82	0.53	0.39	0.56
5	0.83	0.04	0.04	0.00	0.03	0.05	0.33	0.15	0.00
7	0.02	0.00	0.05	0.00	0.10	0.14	0.01	0.05	0.05

[a]Underlined growth rates are the maximums for that replication.

[b]Growth rates recorded as 0.00 may be less than zero.

Table 42. Effects of two nutrient treatments of 0.40 mg N/l and 0.05 mg P/l to membrane filtered Torch Lake water on Selenastrum Maximum Specific Growth Rates, with C_v and t testing.

Rep Number	MAXIMUM SPECIFIC GROWTH RATE (μ_{max})		
	Control	Control + N	Control + P
1	0.83	0.22	1.57
2	0.41	0.52	1.61
3	0.74	0.82	2.08
Mean ± 1SD	0.66 ± 0.22	0.52 ± 0.30	1.75 ± 0.28
C_v	33%	58%	16%
t testing	Control vs. Control + N is ns. Control < Control + P at a 95% level. Control + N < Control + P at a 95% level.		

Table 43. Effects of two nutrient treatments of 0.40 mg N/l and 0.05 mg P/l to membrane filtered Torch Lake water on Selenastrum Maximum Standing Crops in cell numbers, with C_v and t testing.

Rep Number	MAXIMUM STANDING CROPS as Cells/ml		
	Control	Control + N	Control + P
1	1.8×10^4	1.3×10^4	6.8×10^5
2	2.6×10^4	1.8×10^4	6.2×10^5
3	2.0×10^4	5.8×10^4	7.2×10^5
Mean ± 1SD	2.1×10^4 $\pm 4.2 \times 10^3$	3.0×10^4 $\pm 2.5 \times 10^4$	6.7×10^5 $\pm 5.0 \times 10^4$
C_v	20%	83%	7%
t testing	Control vs. Control + N is ns. Control < Control + P at a 99% level. Control + N < Control + P at a 99% level.		

Conclusions

A membrane filtered Torch Lake sample treated with 0.05 mg P/l phosphorus gave higher μ_{max} values and Maximum Standing Crops than a sample treated with 0.40 mg N/l and a sample not treated with a nutrient (control). The μ_{max} values for the control, nitrogen, and phosphorus treatments were 0.66, 0.52, and 1.75 respectively. The Maximum Standing Crops were respectively 2.1×10^4, 3.0×10^4, and 6.8×10^5 cells per ml. This definite stimulation for the phosphorus treatment indicates the Torch Lake sample limited Selenastrum production under the bioassay test conditions.

Kerr et al. (1972) found additions of inorganic nitrogen and phosphorus directly stimulated heterotrophic growth. She might suggest the responses noted here for Selenastrum were indirect.

Regardless of whether the noted response for Selenastrum was direct or indirect, the results indicate that the addition of phosphorus to Torch Lake from some external source would increase algal production.

Experiment 13
Bioassay of Deep Lake for limiting nutrient

Purpose

The limiting nutrient in a membrane filtered Deep Lake sample was determined by static algal bioassay.

Design

The design was the same as for Experiment 12.

Procedure

The procedure was the same as for Experiment 12, except cell counts were made through day 10 instead of day 7.

Results

Cell counts were determined on days 0,1,2,3,4,6, and 10 and are listed with C_v in Appendix table B13. Figures 14a, 14b, and 14c depict the growth curves.

Table 45 gives the μ_{max} for each treatment replication and the means for values taken from Table 44 of daily Specific Growth Rates. The phosphorus treatment gave a higher mean μ_{max} than the other two treatments at 90% and 87% confidence levels.

Table 46 gives the Maximum Standing Crops in cell numbers. The phosphorus treatment gave a higher mean than the other two treatments at 95% confidence levels. Both growth parameters showed the lowest C_v for the phosphorus treatment.

Figure 14. Growth curves for Selenastrum from a ten day bioassay to determine the nutrient limiting algal production in a membrane filtered Deep Lake sample.

a. Page 131 - The treatment was membrane filtered Deep Lake water with no nutrients added (control).

b. Page 132 - The treatment was control plus 0.40 mg N/l.

c. Page 133 - The treatment was control plus 0.05 mg P/l.

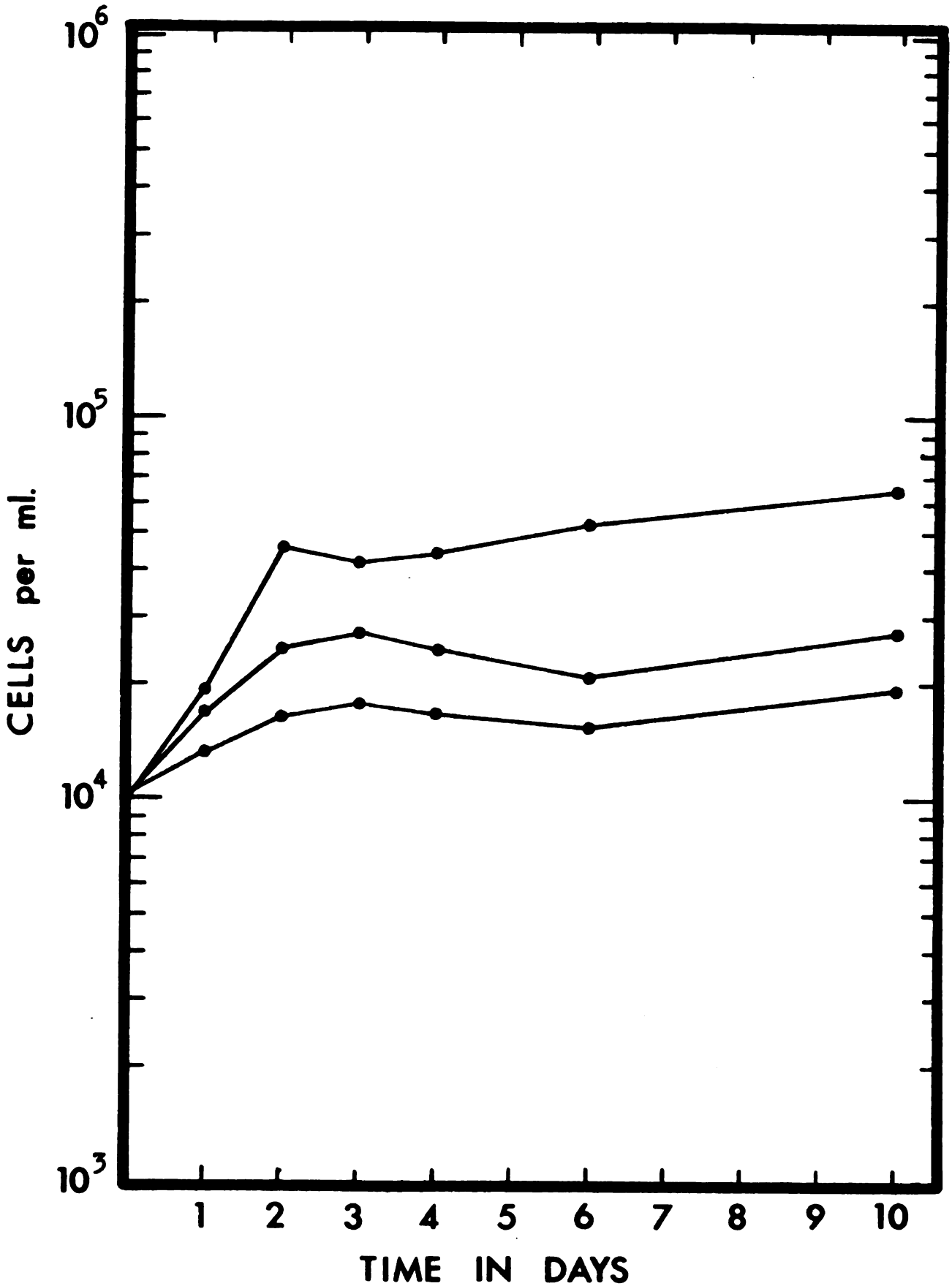


Figure 14a

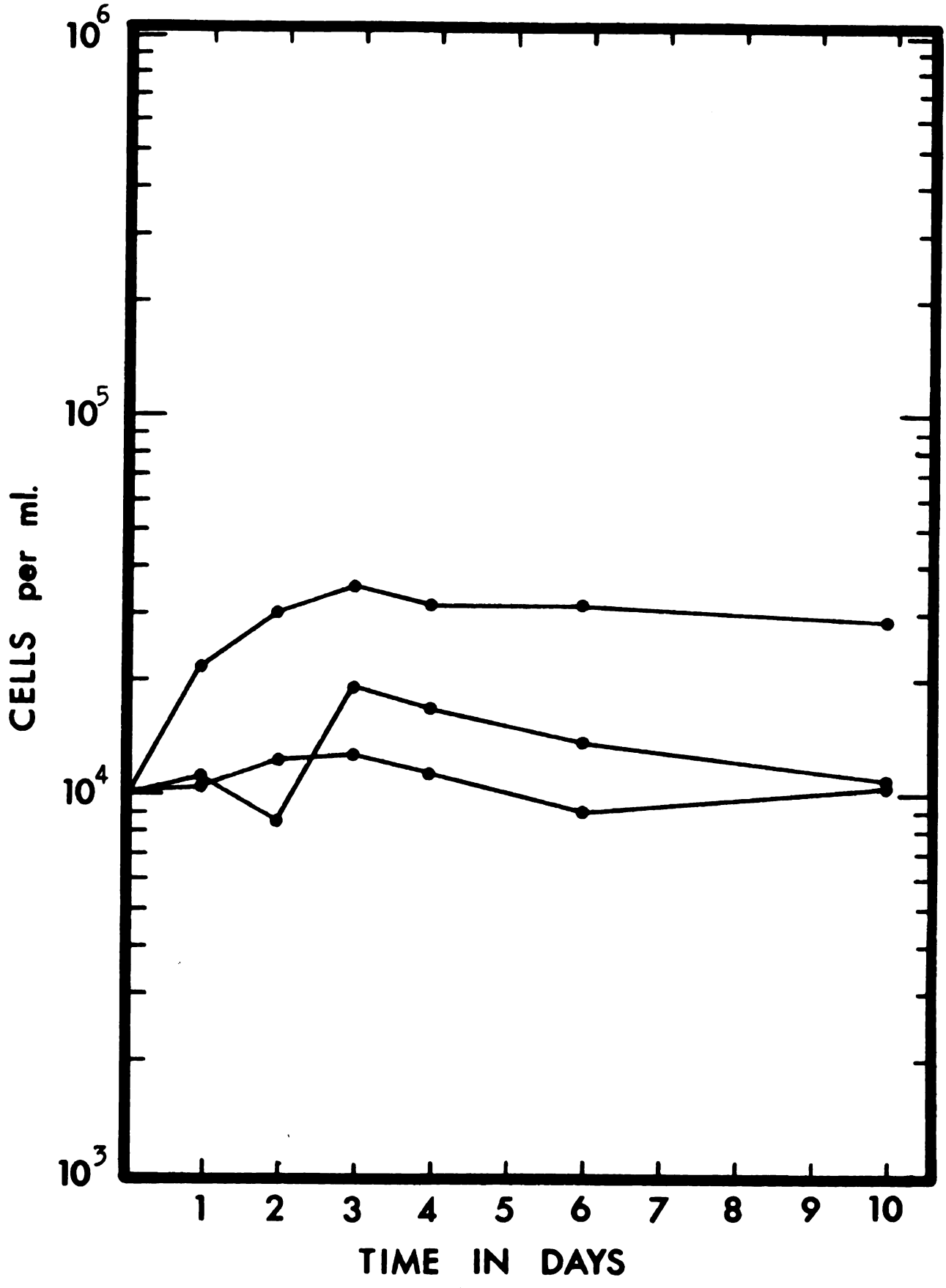


Figure 14b

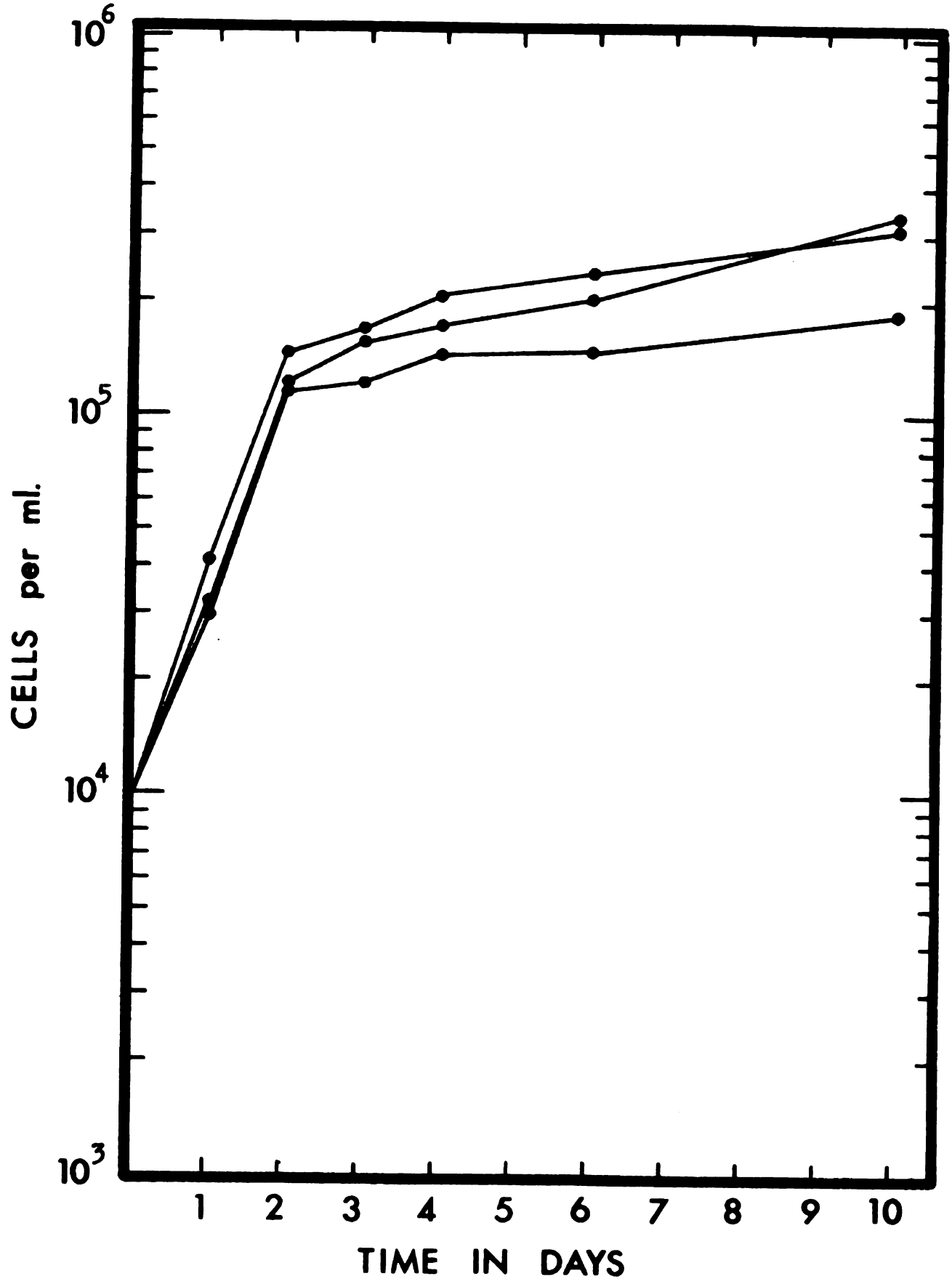


Figure 14c

Table 44. Effects of two nutrient treatments of 0.40 mg N/l and 0.05 mg P/l to membrane filtered Deep Lake water on Selenastrum daily Specific Growth Rates for a ten day static bioassay.

Day	SPECIFIC GROWTH RATES (μ/day)								
	Control			Control plus 0.40 mg N/l			Control plus 0.05 mg P/l		
	1	2	3	1	2	3	1	2	3
0									
1	0.64	[a]0.26	0.53	0.79	0.10	0.10	1.41	1.06	1.16
2	[a]0.88	0.21	0.39	0.34	[b]0.00	0.00	0.23	1.42	1.32
3	0.00	0.11	0.08	0.15	0.00	0.80	0.19	0.09	0.22
4	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.15	0.12
6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16
10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[a]Underlined growth rates are the replication maximums.

[b]Growth rates of 0.00 may be less than zero.

Table 45. Effects of two nutrient treatments of 0.40 mg N/l and 0.05 mg P/l to membrane filtered Deep Lake water on Selenastrum Maximum Specific Growth Rates, with C_v and t testing.

Rep Number	MAXIMUM SPECIFIC GROWTH RATES (μ_{max})		
	Control	Control + N	Control + P
1	0.88	0.79	1.41
2	0.26	0.10	1.06
3	0.53	0.80	1.16
Mean ± 1SD	0.56 ± 0.31	0.56 ± 0.40	1.21 ± 0.18
C_v	55%	72%	15%
t testing	Control vs. Control + N is ns. Control < Control + P at a 90% level. Control + N < Control + P at a 87% level.		

Table 46. Effects of two nutrient treatments of 0.40 mg N/l and 0.05 mg P/l to membrane filtered Deep Lake water on Selenastrum Maximum Standing Crops in cell numbers, with C_v and t testing.

Rep Number	MAXIMUM STANDING CROPS as Cells/ml		
	Control	Control + N	Control + P
1	6.4×10^4	3.6×10^4	3.1×10^5
2	1.9×10^4	1.3×10^4	1.9×10^5
3	2.7×10^4	1.9×10^4	3.4×10^5
Mean ± 1SD	$3.7 \times 10^4 \pm 2.4 \times 10^4$	$2.3 \times 10^4 \pm 1.2 \times 10^4$	$2.8 \times 10^5 \pm 7.9 \times 10^4$
C_v	65%	52%	28%
t testing	Control vs. Control + N is ns. Control < Control + P at a 95% level. Control + N < Control + P at a 95% level.		

Conclusions

The results indicate the nutrient limiting *Selenastrum* production in a membrane filtered sample of Deep Lake water was phosphorus when tested by a static bioassay. The μ_{max} values for the control, nitrogen, and phosphorus treatments were 0.56, 0.56, and 1.21 respectively. The Maximum Standing Crops were respectively 3.7×10^4, 2.3×10^4, and 2.8×10^5 cells per ml.

Experiment 14
Bioassay of Lake Lansing for limiting nutrient

Purpose

The limiting nutrient in a membrane filtered Lake Lansing sample was determined by static algal bioassay.

Design

The design was the same as for Experiment 12.

Procedure

The procedures were the same as for Experiment 12. A fourth treatment was added to this experiment. The treatment is control plus 0.40 mg N/l and 0.05 mg P/l.

Results

Cell counts were determined on days 0,1,2,3,4,5,7, and 10 and are listed with C_v in Appendix table B14. Figures 15a, 15b, 15c, and 15d depict the growth curves. The phosphorus and the phosphorus plus nitrogen treatments showed increases in production. The phosphorus plus nitrogen treatment depicts a decrease in cell numbers after day 7 while the phosphorus treatment shows no such decrease.

Table 47 lists the daily Specific Growth Rates. Table 48 gives the μ_{max} for each replication and the means. The t testing showed the phosphorus treatments had greater μ_{max} values than the control and the nitrogen treatments at 99%

Figure 15. Growth curves for Selenastrum from a ten day bioassay to determine the nutrient limiting algal production in a membrane filtered sample of Lake Lansing water.

a. Page 139 - The treatment was membrane filtered Lake Lansing with no nutrients added (control).

b. Page 140 - The treatment was control plus 0.40 mg N/l.

c. Page 141 - The treatment was control plus 0.05 mg P/l.

d. Page 142 - The treatment was control plus 0.40 mg N/l and 0.05 mg P/l.

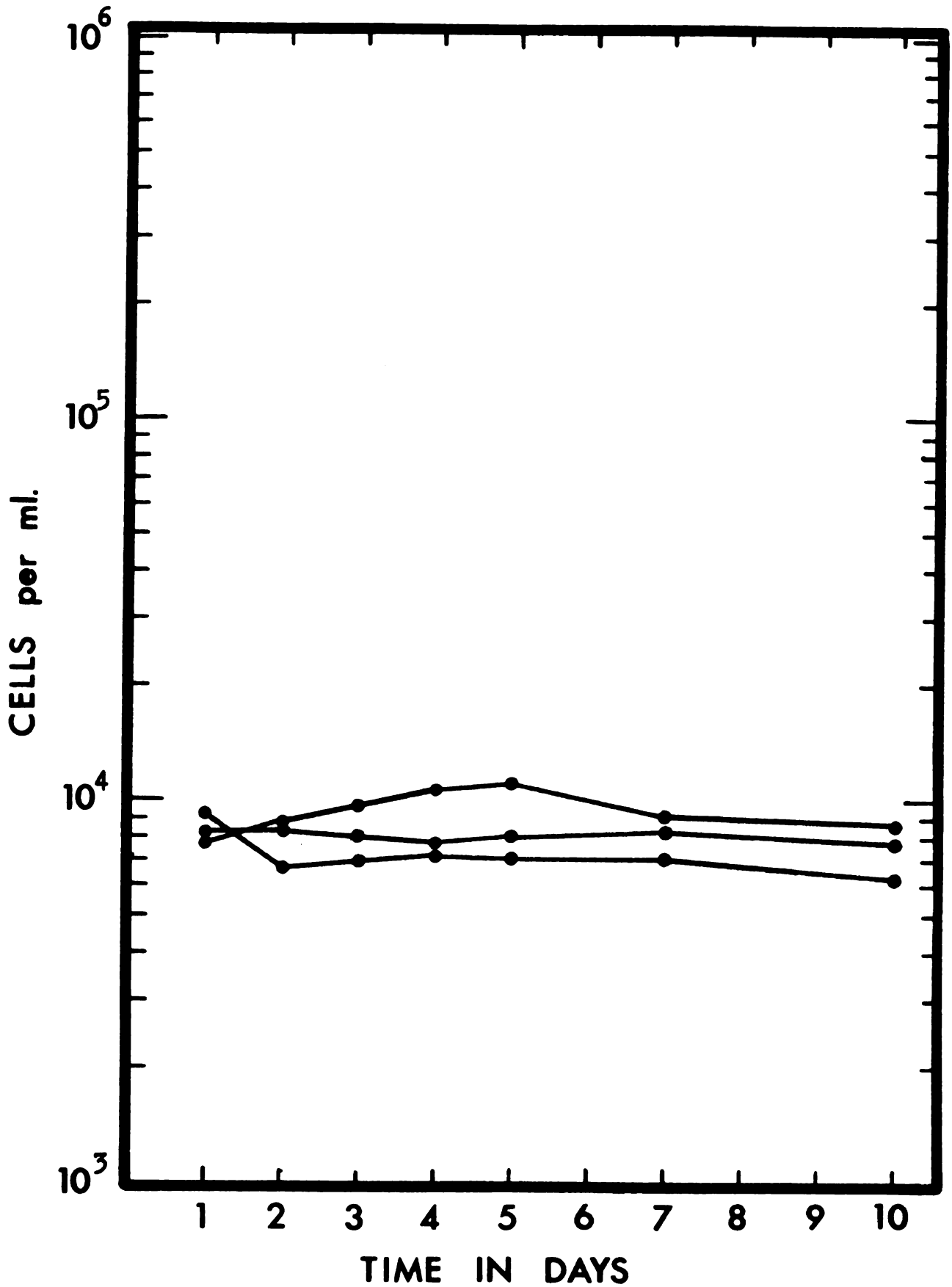


Figure 15a

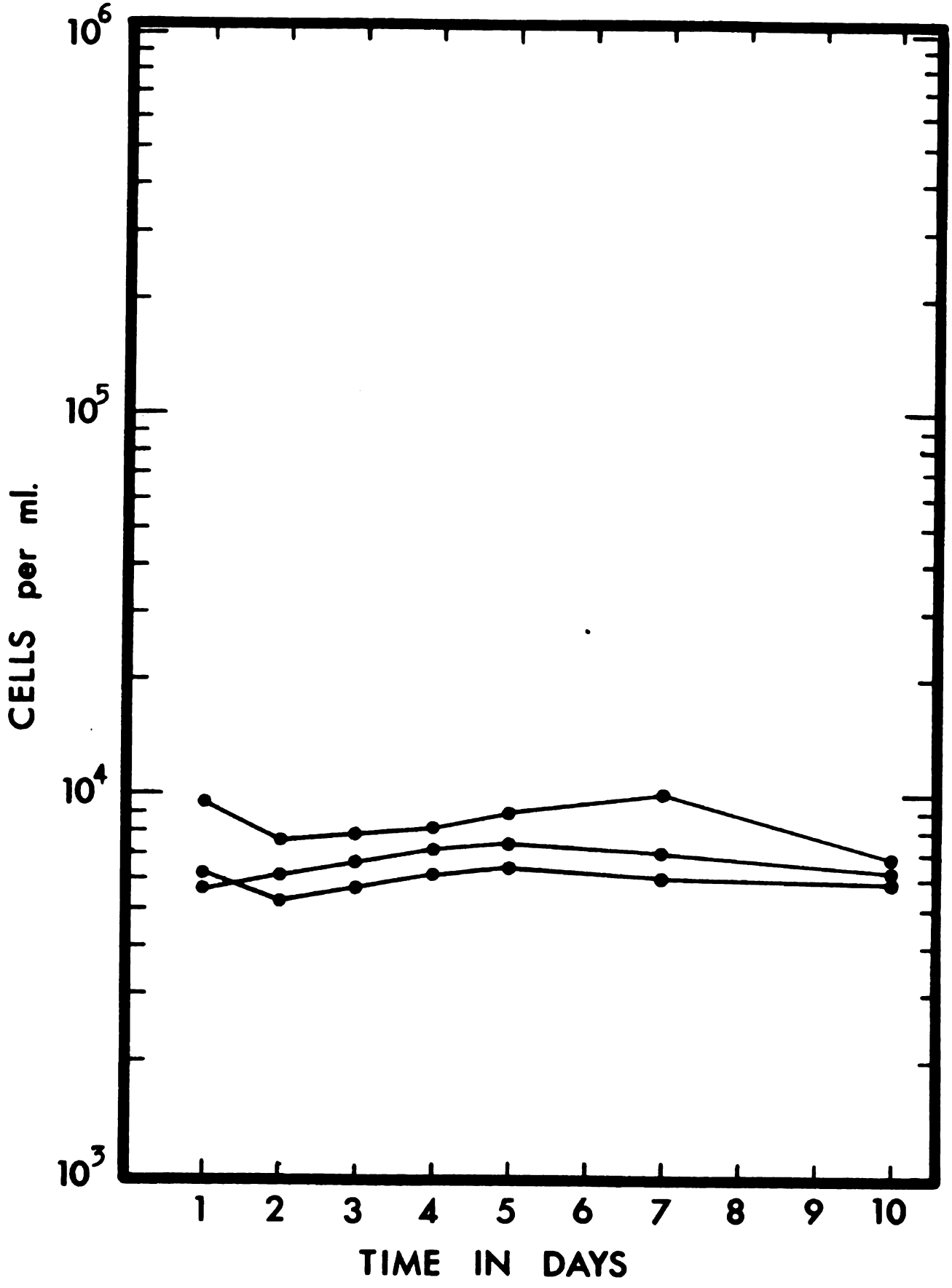


Figure 15b

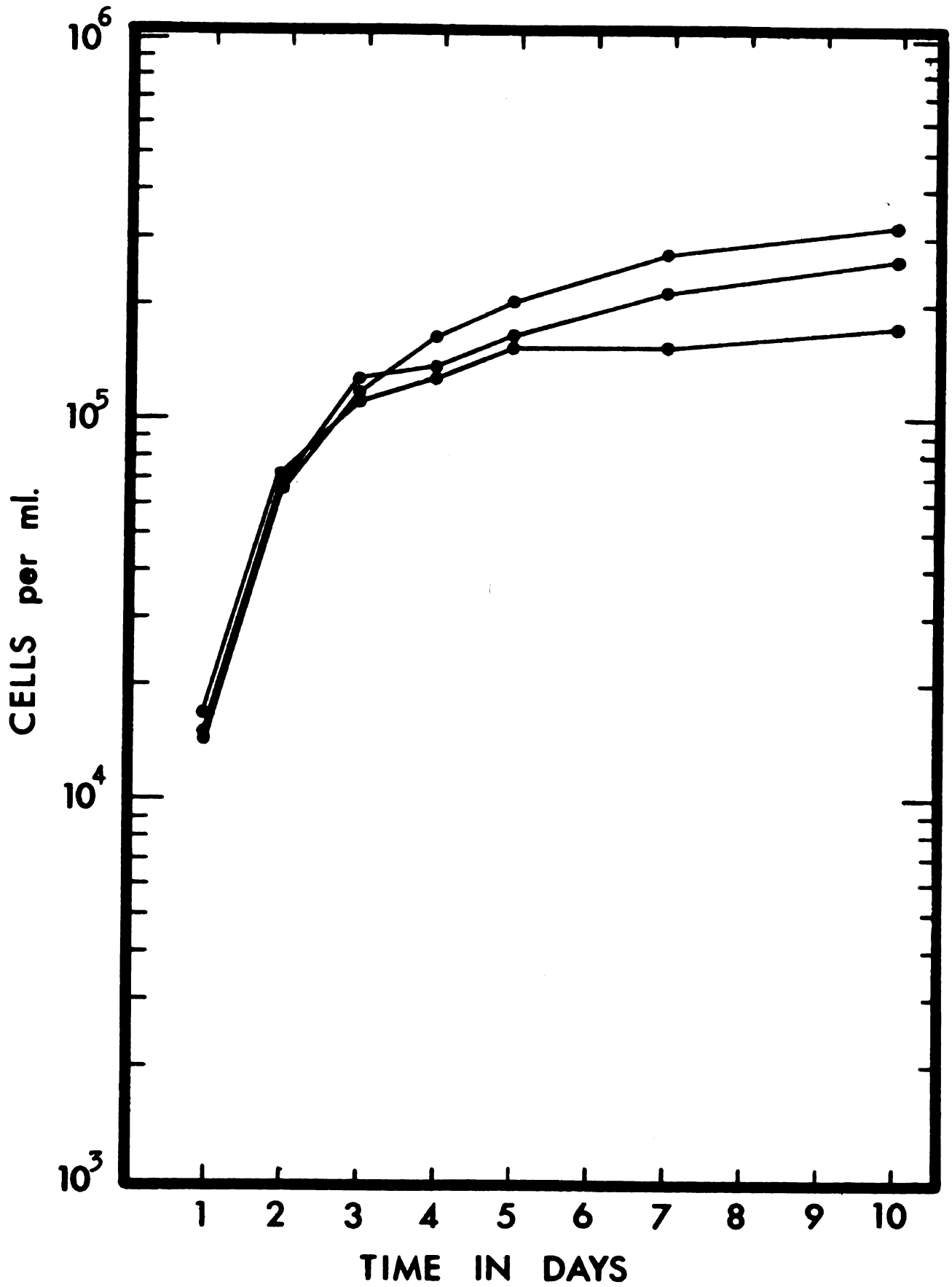


Figure 15c

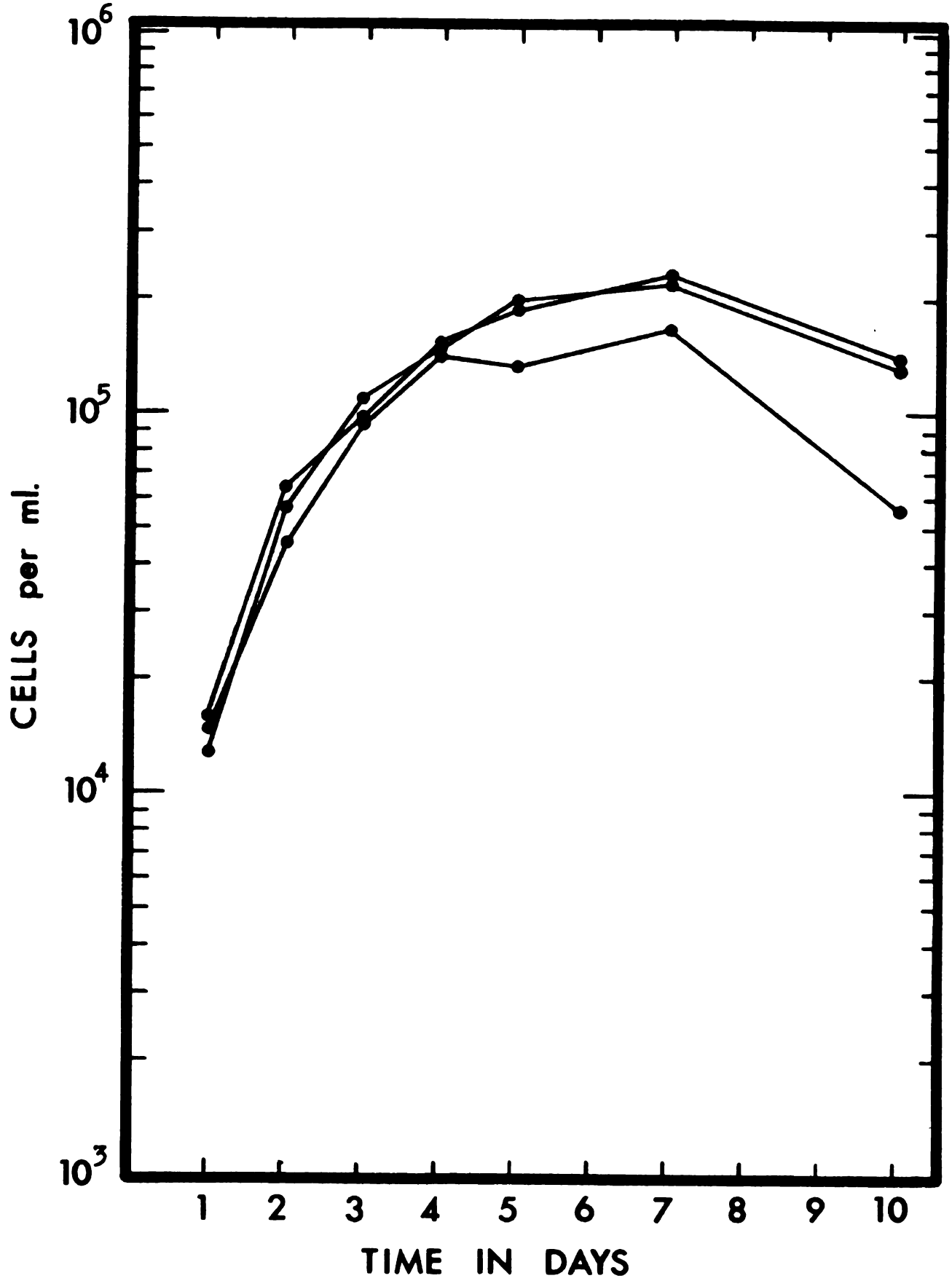


Figure 15d

Table 47. Effects of three nutrient treatments of 0.40 mg N/l, 0.05 mg P/l, and 0.40 mg N/l plus 0.05 mg P/l to membrane filtered Lake Lansing water on Selenastrum daily Specific Growth Rates for a ten day bioassay.

Day	SPECIFIC GROWTH RATES (μ/day)					
	Control			Control plus 0.40 mg N/l		
0						
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.11	0.00	0.05	0.07	0.00	0.00
3	0.10	0.00	0.00	0.08	0.08	0.00
4	0.14	0.04	0.00	0.08	0.09	0.00
5	0.00	0.00	0.00	0.03	0.03	0.20
7	0.00	0.00	0.02	0.00	0.00	0.00
10	0.00	0.00	0.02	0.00	0.03	0.00
Day	Control plus 0.05 mg P/l			Control plus 0.40 mg N/l 0.05 mg P/l		
0						
1	0.41	0.41	0.53	0.26	0.47	0.41
2	1.50	1.58	1.42	1.48	1.36	1.12
3	0.58	0.41	0.62	0.66	0.48	0.72
4	0.35	0.17	0.17	0.31	0.41	0.40
5	0.22	0.21	0.20	0.29	0.24	0.00
7	0.15	0.00	0.12	0.04	0.09	0.13
10	0.10	0.11	0.06	0.00	0.00	0.00

Table 48. Effects of three nutrient treatments of 0.40 mg N/l, 0.05 mg P/l, and 0.40 mg N/l plus 0.05 mg P/l to membrane filtered Lake Lansing on Selenastrum Maximum Specific Growth Rates, with C_v and t testing.

Rep Number	MAXIMUM SPECIFIC GROWTH RATE (μ_{max})			
	Control	Control + N	Control + P	Control + N+P
1	0.14	0.08	1.50	1.48
2	0.04	0.09	1.58	1.36
3	0.05	0.20	1.42	1.12
Mean ±1SD	0.08 ± 0.05	0.12 ± 0.07	1.50 ± 0.08	1.32 ± 0.18
C_v	62%	58%	5%	14%
t testing	Control vs. Control + N is ns. Control < Control + P at a 99% level. Control < Control + N+P at a 99% level. Control + N < Control + P at a 99% level. Control + N < Control + N+P at a 99% level. Control + P > Control + N+P at a 70% level.			

Table 49. Effects of three nutrient treatments of 0.40 mg N/l, 0.05 mg P/l, and 0.40 mg N/l plus 0.05 mg P/l to membrane filtered Lake Lansing water on Selenastrum Maximum Standing Crops as cell numbers, with C_v and t testing.

Rep Number	MAXIMUM STANDING CROPS in Cells/ml			
	Control	Control + N	Control + P	Control + N+P
1	1.1×10^4	7.4×10^3	3.3×10^5	2.2×10^5
2	7.2×10^3	6.4×10^3	1.8×10^5	2.3×10^5
3	8.6×10^3	1.0×10^4	2.7×10^5	1.7×10^5
Mean ±1SD	8.9×10^3 ±3.6×10^3	7.9×10^3 ±1.8×10^3	2.6×10^5 ±7.5×10^4	2.1×10^5 ±3.2×10^4
C_v	40%	23%	29%	15%
t testing	Control vs. Control + N is ns. Control < Control + P at a 95% level. Control < Control + N+P at a 99% level. Control + N < Control + P at a 95% level. Control + N < Control + N+P at a 99% level. Control + P > Control + N+P at a 60% level.			

confidence levels.

Table 49 gives the Maximum Standing Crops in cell numbers and shows the phosphorus treatments yielded greater numbers than the control or the nitrogen treatments at 99% and 95% confidence levels.

Conclusions

The results indicate the nutrient limiting Selenastrum production in a membrane filtered sample of Lake Lansing water was phosphorus when tested by a static bioassay. The μ_{max} values for the control, nitrogen, phosphorus, and phosphorus plus nitrogen treatments were 0.08, 0.12, 1.50, and 1.32 respectively. The Maximum Standing Crops were respectively 8.9×10^3, 7.9×10^3, 2.6×10^5, and 2.1×10^5 cells per ml.

The results suggest the nutrient limiting the decomposition of organic matter (nonliving Selenastrum cells) was nitrogen according to the growth curves depicted in Figure 15d. It is well known nitrogen is a key nutrient substance for microbial growth and hence for organic matter breakdown. Algal cells always contain some nitrogen, but its availability and amount vary greatly. According to Alexander (1961), if the nitrogen of the substrate is high and the element is readily utilized, the microbe satifies its needs from this source, and additional quantities are unnecessary. If the substrate is poor in the element, decomposition is slow, and carbon mineralization will be stimulated by supplemental nitrogen. The AAP medium was intended to culture cells that would be nutrient starved so nutrient carry over would be minimal during inoculation. It

appears the nonliving Selenastrum cells did not contain sufficient quantities of nitrogen for their breakdown to occur by heterotrophic activity. Lake Lansing evidently did not have the supplemental nitrogen needed for breakdown. Therefore, the phosphorus plus nitrogen treatment furnished the necessary nutrient to allow decomposition.

SUMMARY

Following are sources of error encountered for bioassays while conducting this study. These errors affected bioassay precision noticeably:

1. The first tried method of cell counting resulted in unsuitable variance according to Nested Analyses of Variance caused by poor technique in preparing chambers for counting. A modified technique placed all the counting variance in the number of grids counted per sample, which allows control over precision.

2. The first attempt at culturing Selenastrum in a bioassay of four low levels of phosphorus showed much need for improvement since Coefficients of Variation were above 15% (the generally acceptable level for bioassays) for the growth parameters determined.

3. The initial cell concentration in a bioassay culture effected growth. As the initial cell concentration increased, the μ_{max} increased and also occurred earlier in the bioassay. Maximum Standing Crops were not appreciably effected.

4. Light intensity effected both growth rates and standing crops. A 50% reduction in light intensity resulted in a 75% reduction in the maximum number of cells that could be cultured and a significant (at a 90% confidence level)

lowering of μ_{max}. Cultures grown under an intensity of 260 ft-c and later placed under 475 ft-c (after undergoing a logarithmic growth phase) underwent a second logarithmic growth phase, suggesting light can limit growth in bioassays.

5. Culture medium freshness affected the concentrations of biologically available nutrients. Seven-day-old medium resulted in a 68% reduction in the maximum number of cells that could be cultured when compared to fresh medium. One of the trace elements in the micronutrient stock mixture was assessed as becoming unavailable as culture medium aged. A specific trace could not be identified by bioassaying.

6. Suggested methods of insuring carbon availability (given in the AAP) were tested. Six levels of carbon dioxide were introduced to cultures of Selenastrum and resulted in higher growth parameter discrepancies than no carbon dioxide introductions other than what occurred naturally through the foam plugs stoppering the culture flasks. However, cells under carbon stress were small and irregularly shaped compared to the large, turgid cells not under stress. Cultures under carbon stress yielded lower μ_{max} values, lower Maximum Standing Crops measured in dry weights, poorer "Cell Health" measured in dry weights per million cells, and higher Maximum Standing Crops when measured in cell numbers. The responses were attributed to a cellular surface area to volume ratio phenomenon since cell sizes decreased and cell numbers increased when cultured under carbon stress.

High bioassay precision was achieved after these sources of error were realized and controlled. Following are summaries of six applications of the bioassay:

1. Three Michigan lakes, Torch Lake, Deep Lake, and Lake Lansing (oligotrophic, oligotrophic, and eutrophic respectively) were used as a diluent for the nutrient stocks in culture medium preparation and compared to nutrient stocks diluted in the usual manner with distilled water. The regular medium yielded lower Maximum Specific Growth Rates, lower Maximum Standing Crops measured in dry weights, poorer "Cell Health" measured in dry weight per million cells, and higher Maximum Standing Crops measured in cell numbers. The response was plausibly due to carbon stress and the cellular surface area to volume ratio phenomenon since the lake water treatments added carbonate alkalinity.

2. The same three lakes were bioassayed to determine the nutrient limiting primary production. Phosphorus, in all cases, stimulated Selenastrum growth when supplemented to membrane filtered samples of the lake waters and bioassayed. Nitrogen did not stimulate Selenastrum growth. Phosphorus and nitrogen added together to Lake Lansing stimulated growth to the level that phosphorus alone did.

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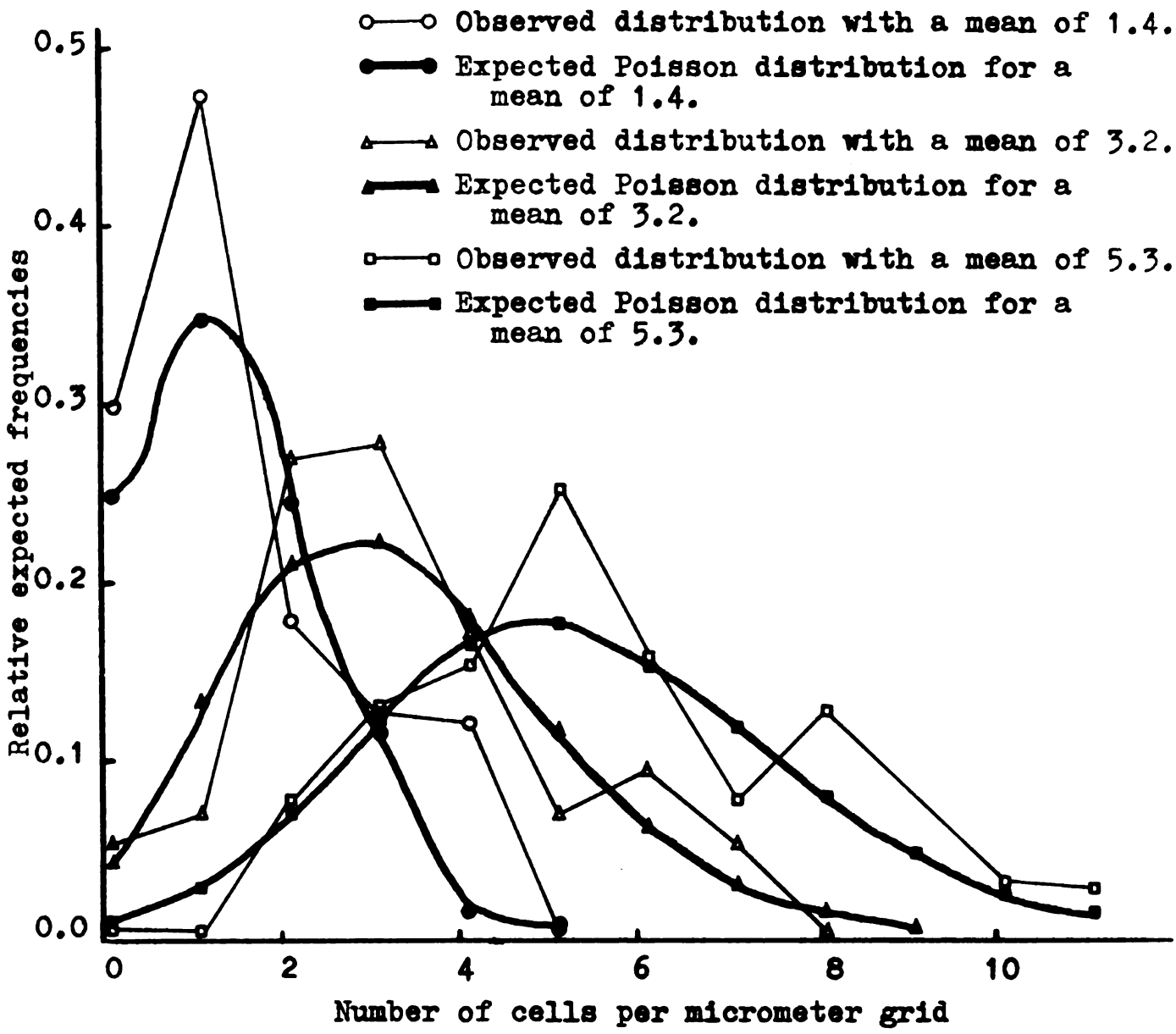
APPENDIX A

APPENDIX A

It is well known that cells enumerated with the aid of counting chambers are distributed in a Poisson fashion. (Sokal and Rohlf, 1969). Bailey (1959) emphasised that although there is an exceedingly small chance of any particular cell finding its way to a given small square of the Whipple micrometer, the total number of cells is so large that quite a lot of the squares are likely to be occupied by one or more cells.

The Poisson distribution is very similar to Gaussian or normal distribution when the means are above 10 or so. However, since Poisson distribution is a function of only the observed mean, it becomes noticeably skewed as the means approach zero.

Knowing the observed mean, one can simply look up the relative expected frequencies from statistical tables (Sokal and Rohlf, 1969). In figure A1, the observed frequencies closely fit the expected frequencies. Chi Square fitting showed for the three cases depicted in figure A1 there is less than a 0.5% chance that the observed values occurred by chance alone. Therefore, the distribution is Poisson.



A1. Figure of the relationship between observed distributions for cell counts (from fifty grid counts per sample and three samples with mean counts of 1.4, 3.2, and 5.3 cells per grid) and expected Poisson distributions for the given means.

APPENDIX B

B1. Cell counts as cells/grid and cells/ml for the treatment control of AAP nutrients minus phosphorus medium of Experiment 3 with maximums marked as * and with C_v.

Rep	Day	Mean cells/grid ± 1SD	Mean cells/ml ± 1SD	C_v %
	0	inoculum	[a]about 10^4	
	1	4.2 ± 1.6	$2.0\times10^4 \pm 7.7\times10^3$	38
	2	7.2 ± 3.4	$3.5\times10^4 \pm 1.6\times10^4$	47
1	3	9.5 ± 3.7	$4.6\times10^4 \pm 1.8\times10^4$	38
	5	10.2 ± 1.4	[b]*$4.9\times10^4 \pm 6.6\times10^3$	13
	6	8.9 ± 3.8	$4.3\times10^4 \pm 1.8\times10^4$	43
	10	6.4 ± 3.0	$3.1\times10^4 \pm 1.4\times10^4$	47
	1	5.7 ± 2.0	$2.7\times10^4 \pm 9.5\times10^3$	33
	2	8.5 ± 3.2	$4.1\times10^4 \pm 1.5\times10^4$	37
2	3	13.2 ± 3.9	*$6.3\times10^4 \pm 1.9\times10^4$	30
	5	8.2 ± 2.7	$3.9\times10^4 \pm 1.3\times10^4$	33
	6	7.4 ± 3.3	$3.6\times10^4 \pm 1.6\times10^4$	44
	10	9.2 ± 3.5	$4.4\times10^4 \pm 1.7\times10^4$	38
	1	3.0 ± 1.7	$1.5\times10^4 \pm 8.2\times10^3$	56
	2	2.8 ± 1.8	$1.3\times10^4 \pm 8.7\times10^3$	65
3	3	4.0 ± 2.2	*$1.9\times10^4 \pm 1.0\times10^4$	53
	5	3.8 ± 2.5	$1.8\times10^4 \pm 1.2\times10^4$	67
	6	3.0 ± 1.2	$1.4\times10^4 \pm 5.9\times10^3$	42
	10	2.4 ± 1.5	$1.2\times10^4 \pm 7.2\times10^3$	60
	1	2.8 ± 1.8	$1.3\times10^4 \pm 8.7\times10^3$	65
	2	5.4 ± 2.2	*$2.6\times10^4 \pm 1.0\times10^4$	38
4	3	5.3 ± 2.0	$2.5\times10^4 \pm 9.8\times10^3$	39
	5	4.4 ± 1.3	$2.1\times10^4 \pm 6.3\times10^3$	30
	6	3.4 ± 1.4	$1.6\times10^4 \pm 6.8\times10^3$	42
	10	1.8 ± 1.4	$8.6\times10^3 \pm 6.9\times10^3$	79

[a]About 10^4 cells/ml for all treatment replication.

[b]According to the AAP, the maximums are the last cell count at which an increase of 5% per day took place. This maximum is often not the absolute maximum.

B2. Cell counts as cells/grid and cells/ml for the control plus 0.01 mg P/l treatment of Experiment 3 with maximums marked as * and with C_v.

Rep	Day	Mean cells/grid ± 1SD	Mean cells/ml ± 1SD	C_v %
	0	inoculum	[a]about 10^4	
	1	11.8 ± 3.9	$5.6\times10^4 \pm 1.9\times10^4$	34
	2	17.2 ± 3.3	$8.3\times10^4 \pm 1.6\times10^4$	19
1	3	37.4 ± 7.4	$1.8\times10^5 \pm 3.6\times10^4$	20
	5	47.0 ± 7.1	$2.2\times10^5 \pm 3.4\times10^4$	15
	6	50.9 ± 5.9	$*2.4\times10^5 \pm 2.8\times10^4$	12
	10	48.5 ± 4.9	$2.3\times10^5 \pm 2.4\times10^4$	10
	1	13.8 ± 4.0	$6.6\times10^4 \pm 1.9\times10^4$	29
	2	30.6 ± 5.4	$1.5\times10^5 \pm 2.6\times10^4$	17
2	3	54.4 ± 7.0	$2.6\times10^5 \pm 3.3\times10^4$	13
	5	91.6 ±11.0	$*4.4\times10^5 \pm 5.0\times10^4$	11
	6	95.7 ± 9.3	$4.6\times10^5 \pm 4.4\times10^4$	10
	10	93.5 ± 7.6	$4.5\times10^5 \pm 3.6\times10^4$	8
	1	11.2 ± 2.4	$5.4\times10^4 \pm 1.2\times10^4$	22
	2	19.1 ± 4.1	$9.2\times10^4 \pm 2.0\times10^4$	22
3	3	46.0 ± 7.9	$2.2\times10^5 \pm 4.4\times10^4$	17
	5	68.7 ± 9.1	$3.3\times10^5 \pm 4.4\times10^4$	13
	6	72.0 ±10.7	$3.4\times10^5 \pm 5.0\times10^4$	15
	10	81.1 ± 7.6	$*3.9\times10^5 \pm 3.6\times10^4$	9
	1	13.1 ± 3.8	$6.3\times10^4 \pm 1.8\times10^4$	29
	2	37.7 ± 4.9	$1.8\times10^5 \pm 2.4\times10^4$	13
4	3	72.6 ±16.8	$3.5\times10^5 \pm 8.0\times10^4$	23
	5	92.3 ±11.9	$4.4\times10^5 \pm 5.7\times10^4$	13
	6	104.1 ± 9.0	$*5.0\times10^5 \pm 4.3\times10^4$	9
	10	102.1 ± 8.2	$4.9\times10 \pm 3.9\times10^4$	8

[a]About 10^4 cells/ml for all treatment replications.

B3. Cell counts as cells/grid and cells/ml for the control plus 0.02 mg P/l treatment of Experiment 3 with maximums marked as * and with C_v.

Rep	Day	Mean cells/grid ± 1SD	Mean cells/ml ± 1SD	C_v %
	0	inoculum	[a]about 10^4	
	1	7.9 ± 2.8	$3.8\times10^4 \pm 1.3\times10^4$	35
	2	12.9 ± 2.8	$6.2\times10^4 \pm 1.3\times10^4$	47
1	3	27.2 ± 7.1	$1.3\times10^5 \pm 3.4\times10^4$	26
	5	54.6 ± 5.5	$2.6\times10^5 \pm 2.6\times10^4$	10
	6	80.3 ±12.4	$3.8\times10^5 \pm 6.0\times10^4$	15
	10	116.6 ± 9.1	$*5.6\times10^5 \pm 4.4\times10^4$	8
	1	15.6 ± 5.2	$7.5\times10^4 \pm 2.5\times10^4$	33
	2	41.4 ± 6.1	$2.0\times10^5 \pm 2.9\times10^4$	15
2	3	84.4 ± 8.8	$4.0\times10^5 \pm 4.2\times10^4$	10
	5	188.3 ±15.9	$9.0\times10^5 \pm 7.6\times10^4$	8
	6	205.2 ±17.0	$*9.8\times10^5 \pm 8.2\times10^4$	8
	10	206.6 ±18.9	$9.9\times10^5 \pm 9.1\times10^4$	9
	1	11.2 ± 3.3	$5.4\times10^4 \pm 1.6\times10^4$	29
	2	23.8 ± 4.7	$1.1\times10^5 \pm 2.3\times10^4$	20
3	3	47.4 ± 5.6	$2.3\times10^5 \pm 2.7\times10^4$	12
	5	96.1 ± 7.9	$4.6\times10^5 \pm 3.8\times10^4$	8
	6	114.6 ±15.0	$5.5\times10^5 \pm 7.2\times10^4$	13
	10	129.2 ±12.4	$*6.2\times10^5 \pm 5.9\times10^4$	10
	1	14.5 ± 4.4	$7.0\times10^4 \pm 2.1\times10^4$	31
	2	45.2 ± 7.2	$2.2\times10^5 \pm 3.5\times10^4$	16
4	3	88.6 ± 8.8	$4.2\times10^5 \pm 4.2\times10^4$	10
	5	185.6 ±22.5	$8.9\times10^5 \pm 1.1\times10^5$	12
	6	218.2 ±24.2	$*1.0\times10^6 \pm 1.2\times10^5$	11
	10	193.3 ±15.9	$9.3\times10^5 \pm 4.4\times10^4$	8

[a]About 10^4 cells/ml for all treatment replications.

B4. Cell counts as cells/grid and cells/ml for the control plus 0.03 mg P/l treatment of Experiment 3 with maximums marked as * and with C_v.

Rep	Day	Mean cells/grid ± 1SD	Mean cells/ml ± 1SD	C_v %
	0	inoculum	[a]about 10^4	
	1	11.6 ± 4.4	$5.6\times10^4 \pm 2.1\times10^4$	38
	2	15.2 ± 3.3	$7.3\times10^4 \pm 1.6\times10^4$	22
1	3	64.0 ±14.1	$3.1\times10^5 \pm 6.7\times10^4$	22
	5	303.6 ±17.3	$1.5\times10^6 \pm 8.3\times10^4$	6
	6	393.2 ±37.2	$1.9\times10^6 \pm 1.8\times10^5$	9
	10	411.2 ±12.7	$*2.0\times10^6 \pm 6.1\times10^4$	3
	1	11.9 ± 3.9	$5.7\times10^4 \pm 1.9\times10^4$	33
	2	22.6 ± 6.1	$1.1\times10^5 \pm 2.9\times10^4$	27
2	3	38.1 ± 5.0	$1.8\times10^5 \pm 2.4\times10^4$	13
	5	79.1 ± 7.6	$3.8\times10^5 \pm 3.6\times10^4$	9
	6	114.2 ± 9.3	$5.5\times10^5 \pm 4.5\times10^4$	8
	10	149.6 ±13.6	$*7.2\times10^5 \pm 6.5\times10^4$	9
	1	10.7 ± 4.0	$5.1\times10^4 \pm 1.9\times10^4$	38
	2	22.7 ± 4.0	$1.1\times10^5 \pm 1.9\times10^4$	18
3	3	36.8 ± 6.6	$1.8\times10^5 \pm 3.2\times10^4$	18
	5	60.4 ± 4.9	$2.9\times10^5 \pm 2.3\times10^4$	8
	6	75.9 ± 7.0	$3.6\times10^5 \pm 3.4\times10^4$	9
	10	98.8 ±11.9	$*4.7\times10^5 \pm 2.3\times10^4$	12
	1	12.4 ± 3.2	$5.9\times10^4 \pm 1.5\times10^4$	26
	2	21.9 ± 4.0	$1.0\times10^5 \pm 1.9\times10^4$	18
4	3	53.0 ± 8.7	$2.5\times10^5 \pm 4.2\times10^4$	16
	5	97.2 ± 9.5	$4.7\times10^5 \pm 4.6\times10^4$	10
	6	119.3 ±10.5	$5.7\times10^5 \pm 5.0\times10^4$	9
	10	157.1 ±12.1	$7.5\times10^5 \pm 5.8\times10^4$	8

[a]About 10^4 cells/ml for all treatment replications.

B5. Cell counts from Experiment 4 bioassaying two levels of light intensity (260 ft-c and 475 ft-c) with C_v.

Rep	Day	LOW intensity Mean cells/ml ± 1SD	C_v %	HIGH intensity Mean cells/ml ± 1SD	C_v %
1	0	$2.0\times10^4 \pm 1.0\times10^4$	50	$3.5\times10^4 \pm 1.8\times10^4$	51
	1	$3.0\times10^4 \pm 1.2\times10^4$	40	$4.6\times10^4 \pm 2.0\times10^4$	44
	2	$7.0\times10^4 \pm 2.6\times10^4$	37	$7.4\times10^4 \pm 1.9\times10^4$	26
	3	$7.6\times10^4 \pm 2.8\times10^4$	37	$9.2\times10^4 \pm 2.0\times10^4$	22
	4	no count made	--	$1.3\times10^5 \pm 2.4\times10^4$	19
	6	$1.6\times10^5 \pm 3.2\times10^4$	21	$2.1\times10^5 \pm 1.5\times10^4$	7
2	0	$1.0\times10^4 \pm 6.0\times10^3$	60	$3.3\times10^4 \pm 1.6\times10^4$	48
	1	$1.0\times10^4 \pm 5.1\times10^3$	51	$4.3\times10^4 \pm 1.3\times10^4$	34
	2	$1.1\times10^4 \pm 5.0\times10^3$	45	$4.3\times10^4 \pm 1.3\times10^4$	34
	3	$1.4\times10^4 \pm 6.5\times10^3$	40	$5.3\times10^4 \pm 7.5\times10^3$	14
	4	no count made	--	$6.6\times10^4 \pm 1.7\times10^4$	26
	6	$1.2\times10^4 \pm 1.1\times10^4$	46	$6.9\times10^4 \pm 1.6\times10^4$	23
3	0	$4.1\times10^4 \pm 2.0\times10^4$	48	$6.0\times10^4 \pm 2.0\times10^4$	33
	1	$5.9\times10^4 \pm 2.3\times10^4$	39	$8.4\times10^4 \pm 2.1\times10^4$	25
	2	$1.1\times10^5 \pm 2.2\times10^4$	20	$1.4\times10^5 \pm 3.1\times10^4$	22
	3	$1.7\times10^5 \pm 2.9\times10^4$	17	$1.2\times10^5 \pm 1.8\times10^4$	14
	4	$2.1\times10^5 \pm 3.2\times10^4$	15	no count made	--
	6	$3.6\times10^5 \pm 5.0\times10^4$	14	$2.6\times10^5 \pm 4.1\times10^4$	16
4	0	$6.4\times10^3 \pm 6.0\times10^3$	94	$8.8\times10^3 \pm 4.4\times10^3$	50
	1	$7.7\times10^3 \pm 7.0\times10^3$	95	$1.3\times10^4 \pm 5.0\times10^3$	38
	2	$1.2\times10^4 \pm 6.5\times10^3$	54	$6.4\times10^4 \pm 2.1\times10^4$	33
	3	$1.6\times10^4 \pm 7.0\times10^3$	44	$1.4\times10^5 \pm 3.7\times10^4$	26
	4	no count made	--	$2.1\times10^5 \pm 3.0\times10^4$	14
	6	$4.5\times10^4 \pm 2.1\times10^4$	47	$4.5\times10^5 \pm 7.5\times10^4$	16
5	0	$7.0\times10^4 \pm 3.4\times10^4$	49	$1.2\times10^4 \pm 8.0\times10^3$	67
	1	$9.1\times10^4 \pm 3.1\times10^4$	34	$1.4\times10^4 \pm 7.0\times10^3$	54
	2	$1.5\times10^5 \pm 3.5\times10^4$	23	$1.2\times10^4 \pm 5.5\times10^3$	48
	3	$1.6\times10^5 \pm 3.1\times10^4$	19	$1.1\times10^4 \pm 6.0\times10^3$	55
	4	no count made	--	no count made	--
	6	$3.1\times10^5 \pm 5.5\times10^4$	16	$1.2\times10^4 \pm 4.8\times10^3$	30

B6. Cell counts, average of three replication, from Experiment 5 bioassaying three levels of initial algal cell concentration ($1.7x10^3$, $6.0x10^3$, and $2.1x10^4$ cells/ml) with C_v between replications. Treatments are shown as I, II, and III respectively.

Treat	Rep	Rep Means cells/ml ± 1SD	C_v %
I	0	$1.7x10^3 \pm 1.6x10^3$	94
	1	$2.2x10^3 \pm 1.8x10^3$	82
	2	$6.7x10^3 \pm 2.4x10^3$	36
	3	$2.5x10^4 \pm 8.7x10^3$	34
	4	$1.4x10^5 \pm 6.0x10^4$	42
	5	$4.3x10^5 \pm 2.4x10^4$	5
	7	$3.1x10^6 \pm 8.0x10^5$	26
	11	$4.2x10^6 \pm 9.0x10^5$	21
II	0	$6.0x10^3 \pm 2.5x10^3$	42
	1	$1.3x10^4 \pm 5.0x10^3$	38
	2	$8.8x10^4 \pm 2.4x10^4$	36
	3	$3.6x10^5 \pm 3.0x10^5$	83
	4	$1.6x10^6 \pm 8.8x10^5$	54
	5	$2.5x10^6 \pm 1.0x10^6$	40
	7	$4.8x10^6 \pm 1.5x10^6$	31
	11	$4.8x10^6 \pm 1.4x10^6$	29
III	0	$2.1x10^4 \pm 1.1x10^4$	52
	1	$2.0x10^5 \pm 1.0x10^5$	50
	2	$1.1x10^6 \pm 5.0x10^5$	45
	3	$2.8x10^6 \pm 1.2x10^6$	43
	4	$3.8x10^6 \pm 1.7x10^6$	45
	5	$4.5x10^6 \pm 2.0x10^6$	44
	7	$5.0x10^6 \pm 2.1x10^6$	32
	11	$5.2x10^6 \pm 2.5x10^6$	48

B7. Cell counts from Experiment 6 bioassaying (a) two levels of light intensity (260 ft-c and 475 ft-c) and (b) cell counts for a portion of the 260 ft-c treatment placed under 475 ft-c on day 4 and cultured through day 11. Both (a) and (b) with C_v.

(a)

Rep	Day	HIGH intensity Mean cells/ml ± 1SD	C_v %	LOW intensity Mean cells/ml ± 1SD	
	0	$2.9\times10^{3} \pm 1.7\times10^{3}$	59	$2.8\times10^{3} \pm 1.7\times10^{3}$	59
	1	$4.8\times10^{3} \pm 2.2\times10^{3}$	46	$5.8\times10^{3} \pm 2.4\times10^{3}$	42
	2	$2.2\times10^{4} \pm 1.2\times10^{4}$	54	$1.2\times10^{4} \pm 7.0\times10^{3}$	58
	3	$1.5\times10^{5} \pm 2.0\times10^{4}$	13	$7.8\times10^{4} \pm 2.9\times10^{4}$	37
1	4	$7.4\times10^{5} \pm 5.1\times10^{4}$	7	$1.8\times10^{5} \pm 3.4\times10^{4}$	19
	5	$1.9\times10^{6} \pm 1.4\times10^{5}$	7	$3.3\times10^{5} \pm 3.5\times10^{4}$	11
	6	$3.2\times10^{6} \pm 5.0\times10^{5}$	16	$5.7\times10^{5} \pm 5.7\times10^{4}$	10
	7	$4.1\times10^{6} \pm 3.4\times10^{5}$	8	$8.0\times10^{5} \pm 7.0\times10^{4}$	9
	0	$2.9\times10^{3} \pm 1.7\times10^{3}$	59	$2.7\times10^{3} \pm 1.6\times10^{3}$	59
	1	$3.4\times10^{3} \pm 1.8\times10^{3}$	54	$5.5\times10^{3} \pm 2.3\times10^{3}$	42
	2	$2.8\times10^{4} \pm 1.2\times10^{4}$	43	$9.6\times10^{3} \pm 5.5\times10^{3}$	57
	3	$1.9\times10^{5} \pm 3.6\times10^{4}$	19	$6.6\times10^{4} \pm 1.8\times10^{4}$	27
2	4	$7.5\times10^{5} \pm 8.6\times10^{4}$	11	$1.8\times10^{5} \pm 2.6\times10^{4}$	14
	5	$1.9\times10^{6} \pm 9.6\times10^{4}$	5	$3.5\times10^{5} \pm 3.6\times10^{4}$	10
	6	$3.5\times10^{6} \pm 3.4\times10^{5}$	10	$6.0\times10^{5} \pm 6.0\times10^{4}$	10
	7	$4.4\times10^{6} \pm 5.6\times10^{5}$	12	$8.4\times10^{5} \pm 8.2\times10^{4}$	9
	11	$*4.7\times10^{6} \pm 2.2\times10^{5}$	5	$*1.2\times10^{6} \pm 1.0\times10^{5}$	8
	[a]11	$*4.4\times10^{6} \pm 6.7\times10^{5}$	15	$*1.1\times10^{6} \pm 1.2\times10^{5}$	11

(b)

Rep	Day	LOW intensity treatment portion from Rep 1 & 2 placed under HIGH intensity of day 4. cells/ml ± 1SD		cells/ml ± 1SD	
		Rep 1		Rep 2	
	4	$1.8\times10^{5} \pm 3.4\times10^{4}$	19	$1.8\times10^{5} \pm 2.6\times10^{4}$	14
	5	$1.1\times10^{6} \pm 8.7\times10^{4}$	8	$1.2\times10^{6} \pm 6.6\times10^{4}$	5
	6	$2.3\times10^{6} \pm 2.3\times10^{5}$	10	$2.5\times10^{6} \pm 2.5\times10^{5}$	10
	7	$3.7\times10^{6} \pm 3.7\times10^{5}$	10	$4.3\times10^{6} \pm 1.0\times10^{6}$	23
	11	$5.6\times10^{6} \pm 8.3\times10^{5}$	15	$5.6\times10^{6} \pm 3.7\times10^{5}$	7

[a]Counts are for rep 1. Maximums are marked with *.

B8. Cell counts and pH from Experiment 7 bioassaying six levels of carbon dioxide used to insure carbon availability in bottle test as suggested in AAP.

Rep	Day	No air or CO_2 vented or bubbled cells/ml ± 1SD	pH	Air vented 25 cc/min cells/ml ± 1SD	pH	Air bubbled [b]5 cc/min cells/ml ± 1SD	pH
1	[c]1	$4.8\times10^{3} \pm 2.2\times10^{3}$	7.7	$3.4\times10^{3} \pm 1.8\times10^{3}$	7.7	$6.2\times10^{3} \pm 2.5\times10^{3}$	7.7
	2	$2.2\times10^{4} \pm 1.2\times10^{4}$	7.8	$1.6\times10^{4} \pm 1.0\times10^{4}$	7.9	$1.5\times10^{4} \pm 5.3\times10^{3}$	7.7
	3	$1.5\times10^{5} \pm 2.0\times10^{4}$	8.6	$7.8\times10^{4} \pm 1.9\times10^{4}$	8.0	$6.1\times10^{4} \pm 1.4\times10^{4}$	7.8
	4	$7.4\times10^{5} \pm 5.1\times10^{4}$	9.5	$4.3\times10^{5} \pm 7.7\times10^{4}$	8.2	$2.8\times10^{5} \pm 2.0\times10^{4}$	7.8
	5	$1.9\times10^{6} \pm 1.4\times10^{5}$	9.9	$2.4\times10^{6} \pm 1.8\times10^{5}$	9.2	$1.0\times10^{6} \pm 5.4\times10^{4}$	9.6
	6	$3.2\times10^{6} \pm 5.0\times10^{5}$	9.7	$4.2\times10^{6} \pm 3.4\times10^{5}$	9.4	$2.5\times10^{6} \pm 4.9\times10^{5}$	9.8
	7	$4.1\times10^{6} \pm 3.4\times10^{5}$	9.5	$6.6\times10^{6} \pm 6.8\times10^{5}$	9.5	$3.4\times10^{6} \pm 6.0\times10^{5}$	9.9
	11	$4.4\times10^{6} \pm 6.7\times10^{5}$	8.4	[a]no count made	8.6	$4.8\times10^{6} \pm 7.5\times10^{5}$	8.8
2	1	$3.4\times10^{3} \pm 2.3\times10^{3}$	7.7	$2.9\times10^{3} \pm 2.3\times10^{3}$	7.6	[b]$3.8\times10^{3} \pm 5.0\times10^{3}$	7.7
	2	$2.8\times10^{4} \pm 1.2\times10^{4}$	7.8	$1.5\times10^{4} \pm 7.4\times10^{3}$	7.7	$1.4\times10^{4} \pm 5.7\times10^{3}$	7.7
	3	$1.9\times10^{5} \pm 3.6\times10^{4}$	8.6	$5.7\times10^{4} \pm 1.2\times10^{4}$	7.8	$7.0\times10^{5} \pm 1.5\times10^{4}$	7.8
	4	$7.4\times10^{5} \pm 8.6\times10^{4}$	9.7	$2.1\times10^{5} \pm 1.4\times10^{4}$	8.0	$3.3\times10^{5} \pm 2.2\times10^{4}$	7.8
	5	$1.9\times10^{6} \pm 9.6\times10^{4}$	9.9	$5.8\times10^{5} \pm 2.0\times10^{4}$	8.3	$1.8\times10^{6} \pm 2.4\times10^{5}$	8.4
	6	$3.5\times10^{6} \pm 3.4\times10^{5}$	9.8	$1.2\times10^{6} \pm 2.6\times10^{5}$	8.2	$3.2\times10^{6} \pm 4.0\times10^{5}$	8.3
	7	$4.4\times10^{6} \pm 5.6\times10^{5}$	9.6	$2.2\times10^{6} \pm 5.0\times10^{5}$	8.1	$5.4\times10^{6} \pm 5.0\times10^{5}$	8.2
	11	$4.7\times10^{6} \pm 2.2\times10^{5}$	8.6	[a]no count made	8.1	$8.2\times10^{6} \pm 4.5\times10^{5}$	8.2

[a]No cell count made because of excessive medium evaporation due to venting.
[b]Air bubbled at 50 cc/min for Rep 2 (note pH compared to Rep 2 bubbled at 5 cc/min).
[c]Initial cell concentration on day 0 was 2.9×10^{3} cells/ml for all treatments and pH was 7.7 for all treatments.

B8 (cont'd).

Rep	Day	CO_2 vented 25 cc/min cells/ml ± 1SD	pH	CO_2 bubbled 5 cc/min cells/ml ± 1SD	pH	CO_2 bubbled 50 cc/min cells/ml ± 1SD	pH
	[a]1	$2.9\times10^{3} \pm 1.7\times10^{3}$	5.8	$3.4\times10^{3} \pm 3.2\times10^{3}$	6.3	$4.8\times10^{3} \pm 2.4\times10^{3}$	5.8
	2	$1.3\times10^{4} \pm 8.0\times10^{3}$	6.0	$2.2\times10^{4} \pm 1.2\times10^{4}$	6.4	$4.8\times10^{3} \pm 4.5\times10^{3}$	5.8
	3	$4.6\times10^{4} \pm 1.5\times10^{4}$	6.6	$1.0\times10^{5} \pm 2.6\times10^{4}$	6.5	$3.1\times10^{4} \pm 1.1\times10^{4}$	6.0
1	4	$2.1\times10^{5} \pm 3.9\times10^{4}$	6.6	$4.1\times10^{5} \pm 9.6\times10^{3}$	6.8	$9.3\times10^{4} \pm 3.8\times10^{4}$	6.2
	5	$1.3\times10^{6} \pm 1.6\times10^{5}$	6.7	$2.2\times10^{6} \pm 3.1\times10^{5}$	6.9	$4.2\times10^{5} \pm 6.6\times10^{4}$	6.3
	6	$3.2\times10^{6} \pm 4.8\times10^{5}$	7.0	$3.0\times10^{6} \pm 6.1\times10^{5}$	6.9	$9.9\times10^{5} \pm 4.9\times10^{4}$	6.2
	7	$3.4\times10^{6} \pm 6.2\times10^{5}$	7.2	$3.3\times10^{6} \pm 4.4\times10^{5}$	7.3	$2.0\times10^{6} \pm 2.1\times10^{5}$	6.3
	11	$4.4\times10^{6} \pm 6.5\times10^{5}$	7.1	$3.3\times10^{6} \pm 5.4\times10^{5}$	6.7	$2.5\times10^{6} \pm 4.9\times10^{5}$	6.3
	1	$2.9\times10^{3} \pm 2.5\times10^{3}$	5.8	$4.8\times10^{3} \pm 2.6\times10^{3}$	6.3	$2.9\times10^{3} \pm 2.1\times10^{3}$	5.8
	2	$1.2\times10^{4} \pm 5.0\times10^{3}$	6.1	$3.4\times10^{3} \pm 3.9\times10^{3}$	6.4	$9.1\times10^{3} \pm 6.6\times10^{3}$	5.9
	3	$5.7\times10^{4} \pm 1.9\times10^{4}$	6.2	[b]$2.9\times10^{3} \pm 1.1\times10^{3}$	6.5	$4.1\times10^{4} \pm 7.9\times10^{3}$	6.0
2	4	$2.0\times10^{5} \pm 3.6\times10^{4}$	6.3	$9.1\times10^{3} \pm 5.3\times10^{3}$	7.4	$1.2\times10^{5} \pm 4.3\times10^{4}$	6.1
	5	$1.1\times10^{6} \pm 8.1\times10^{4}$	6.8	$3.0\times10^{4} \pm 8.7\times10^{3}$	7.6	$4.3\times10^{5} \pm 7.7\times10^{4}$	6.3
	6	$3.4\times10^{6} \pm 6.5\times10^{5}$	6.8	$1.2\times10^{5} \pm 1.8\times10^{4}$	7.6	$1.1\times10^{6} \pm 2.5\times10^{5}$	6.3
	7	$6.1\times10^{6} \pm 7.7\times10^{5}$	6.8	[c]$4.2\times10^{5} \pm 2.5\times10^{4}$	8.3	$2.4\times10^{6} \pm 2.2\times10^{5}$	6.5
	11	[d]no count made		$4.0\times10^{6} \pm 6.4\times10^{5}$	6.8	$3.7\times10^{6} \pm 3.8\times10^{6}$	6.4

[a]Initial cell concentration on day 0 was 2.9×10^{3} cells/ml for all treatments and pH was 7.7 for all treatments.
[b]Bubbling turned off because of no algal growth.
[c]Bubbling turned back on because of growth and rising pH.
[d]No cell count made due noted excessive medium evaporation due to venting.

B9. Cell counts and pH from Experiment 9 comparing by bioassay the AAP nutrients diluted with Torch Lake membrane filtered water to AAP nutrients diluted with distilled water. Maximums marked as *.

Rep	Day	Diluent is DISTILLED WATER cells/ml ± 1SD	pH	Diluent is TORCH LAKE WATER cells/ml ± 1SD	pH
1	0	[a]about 6×10^3	7.7	[a]about 6×10^3	7.7
	1	$1.9\times10^4 \pm 8.8\times10^3$	8.0	$1.6\times10^4 \pm 8.8\times10^3$	8.7
	2	$1.0\times10^5 \pm 2.5\times10^4$	8.3	$1.1\times10^5 \pm 2.7\times10^4$	9.1
	3	$8.2\times10^5 \pm 2.1\times10^4$	10.1	$6.9\times10^5 \pm 7.6\times10^4$	9.8
	5	$3.6\times10^6 \pm 1.4\times10^5$	9.5	$2.6\times10^6 \pm 1.6\times10^5$	9.6
	7	$*4.8\times10^6 \pm 7.0\times10^5$	8.6	$2.9\times10^6 \pm 5.5\times10^5$	9.2
	10	$4.7\times10^6 \pm 1.0\times10^6$	8.5	$*3.1\times10^6 \pm 3.3\times10^5$	8.9
2	1	$1.6\times10^4 \pm 8.8\times10^3$	8.0	$1.8\times10^4 \pm 1.2\times10^4$	8.7
	2	$1.1\times10^5 \pm 2.4\times10^4$	8.3	$2.2\times10^5 \pm 1.7\times10^4$	9.2
	3	$7.6\times10^5 \pm 1.1\times10^5$	10.1	$9.7\times10^5 \pm 1.2\times10^5$	9.8
	5	$2.9\times10^6 \pm 3.3\times10^5$	9.5	$2.6\times10^6 \pm 3.7\times10^5$	9.7
	7	$4.2\times10^6 \pm 4.0\times10^5$	8.6	$*3.5\times10^6 \pm 4.3\times10^5$	9.0
	10	$*4.5\times10^6 \pm 5.4\times10^5$	8.5	$3.4\times10^6 \pm 6.4\times10^5$	8.8
3	1	$2.4\times10^4 \pm 7.3\times10^3$	8.0	$1.6\times10^4 \pm 9.4\times10^3$	8.7
	2	$1.0\times10^5 \pm 1.8\times10^4$	8.3	$9.0\times10^4 \pm 1.7\times10^4$	9.1
	3	$9.5\times10^5 \pm 1.3\times10^5$	10.3	$8.2\times10^5 \pm 8.7\times10^4$	9.8
	5	$3.9\times10^6 \pm 1.7\times10^5$	9.4	$2.1\times10^6 \pm 9.0\times10^4$	9.6
	7	$*4.6\times10^6 \pm 3.2\times10^5$	8.3	$*3.8\times10^6 \pm 5.4\times10^5$	9.2
	10	$4.7\times10^6 \pm 6.5\times10^5$	8.4	$3.8\times10^6 \pm 5.6\times10^5$	8.8
4	1	$1.7\times10^4 \pm 8.7\times10^3$	8.0	$2.3\times10^4 \pm 8.5\times10^3$	8.7
	2	$1.2\times10^5 \pm 2.0\times10^4$	8.3	$1.2\times10^5 \pm 1.8\times10^4$	9.2
	3	$6.7\times10^5 \pm 4.9\times10^4$	10.2	$8.0\times10^5 \pm 8.0\times10^4$	9.7
	5	$3.3\times10^6 \pm 9.7\times10^4$	9.8	$2.9\times10^6 \pm 7.1\times10^4$	9.6
	7	$*4.6\times10^6 \pm 2.2\times10^5$	9.0	$*3.5\times10^6 \pm 5.7\times10^5$	8.9
	10	$4.8\times10^6 \pm 3.4\times10^5$	8.5	$3.2\times10^6 \pm 1.7\times10^5$	8.7
4	1	$2.0\times10^4 \pm 9.0\times10^3$	8.0	$1.5\times10^4 \pm 6.7\times10^3$	8.7
	2	$1.1\times10^5 \pm 3.4\times10^4$	8.3	$1.0\times10^5 \pm 2.0\times10^4$	9.3
	3	$4.0\times10^5 \pm 4.4\times10^4$	10.0	$6.9\times10^5 \pm 7.4\times10^4$	9.7
	5	$2.9\times10^6 \pm 4.2\times10^4$	9.2	$2.7\times10^6 \pm 1.3\times10^5$	9.7
	7	$3.8\times10^6 \pm 6.1\times10^5$	8.6	$3.6\times10^6 \pm 9.6\times10^5$	9.0
	10	$*4.4\times10^6 \pm 2.7\times10^5$	8.6	$*4.8\times10^6 \pm 5.0\times10^5$	8.8

[a]About the same for all replications.

B10. Cell counts, C_v, and pH from Experiment 10 comparing by bioassay the AAP nutrients diluted with Deep Lake membrane filtered water to AAP nutrients diluted with distilled water. Maximums marked as *.

Rep	Day	Diluent is DISTILLED WATER cells/ml ± 1SD	C_v %	pH	Diluent is DEEP LAKE WATER cells/ml ± 1SD	C_v %	pH
1	0	[a]about 10^4		7.7	[a]about 10^4		8.2
	1	$3.4\times10^4 \pm 1.1\times10^4$	32	7.5	$2.4\times10^4 \pm 1.4\times10^4$	58	8.5
	2	$1.8\times10^5 \pm 2.9\times10^4$	16	7.7	$1.8\times10^5 \pm 2.1\times10^4$	12	8.7
	3	$5.8\times10^5 \pm 2.6\times10^4$	5	9.1	$6.9\times10^5 \pm 3.6\times10^4$	5	9.3
	4	$2.0\times10^6 \pm 3.0\times10^5$	15	9.2	$2.3\times10^6 \pm 1.6\times10^5$	7	9.4
	6	$*4.1\times10^6 \pm 4.2\times10^5$	10	9.0	$*3.9\times10^6 \pm 5.0\times10^5$	13	9.0
	10	$4.7\times10^6 \pm 4.8\times10^5$	10	8.5	$4.3\times10^6 \pm 1.0\times10^6$	23	8.5
	18	$5.0\times10^6 \pm 5.0\times10^5$	10	8.5	$4.2\times10^6 \pm 4.2\times10^5$	10	8.5
2	1	$3.8\times10^4 \pm 1.1\times10^4$	29	7.5	$3.0\times10^4 \pm 1.3\times10^4$	43	8.5
	2	$2.0\times10^5 \pm 2.5\times10^4$	12	8.0	$2.2\times10^5 \pm 3.0\times10^4$	14	8.8
	3	$7.3\times10^5 \pm 1.1\times10^5$	15	9.1	$7.6\times10^5 \pm 9.0\times10^4$	12	9.2
	4	$2.3\times10^6 \pm 1.8\times10^5$	8	9.2	$2.2\times10^6 \pm 2.2\times10^5$	10	9.4
	6	$*4.5\times10^6 \pm 6.2\times10^5$	14	8.8	$*3.5\times10^6 \pm 2.9\times10^5$	8	8.9
	10	$4.7\times10^6 \pm 3.3\times10^5$	7	8.8	$3.6\times10^6 \pm 3.1\times10^5$	9	8.5
	18	$5.1\times10^6 \pm 9.6\times10^5$	19	8.5	$4.0\times10^6 \pm 4.0\times10^5$	10	8.5
3	1	$3.8\times10^4 \pm 1.2\times10^4$	32	7.4	$3.0\times10^4 \pm 1.3\times10^4$	43	8.5
	2	$2.4\times10^5 \pm 4.1\times10^4$	17	8.6	$2.4\times10^5 \pm 3.2\times10^4$	13	8.9
	3	$6.5\times10^5 \pm 8.3\times10^3$	13	9.3	$8.5\times10^5 \pm 8.2\times10^4$	10	9.4
	4	$2.3\times10^6 \pm 3.4\times10^4$	15	9.3	$2.4\times10^6 \pm 1.6\times10^4$	7	9.5
	6	$*5.1\times10^6 \pm 4.3\times10^5$	8	8.9	$*3.7\times10^6 \pm 3.2\times10^5$	9	9.0
	10	$5.2\times10^6 \pm 6.8\times10^5$	13	8.8	$4.4\times10^6 \pm 6.4\times10^5$	15	9.0
	18	$5.3\times10^6 \pm 5.8\times10^5$	11	8.6	$4.1\times10^6 \pm 7.0\times10^5$	17	8.7
4	1	$4.4\times10^4 \pm 1.2\times10^4$	27	7.4	$4.0\times10^4 \pm 2.0\times10^4$	50	8.2
	2	$2.3\times10^5 \pm 2.6\times10^4$	11	8.9	$2.7\times10^5 \pm 3.1\times10^4$	12	8.5
	3	$5.2\times10^5 \pm 2.9\times10^4$	6	9.4	$7.0\times10^5 \pm 7.0\times10^4$	10	8.9
	4	$2.0\times10^6 \pm 5.5\times10^4$	3	9.3	[b]$7.6\times10^6 \pm 6.0\times10^6$	79	9.3
	6	$*4.4\times10^6 \pm 2.9\times10^5$	7	9.0	$3.3\times10^6 \pm 3.3\times10^5$	10	9.5
	10	$5.1\times10^6 \pm 4.9\times10^5$	10	8.7	$*4.0\times10^6 \pm 7.2\times10^5$	16	9.0
	18	$5.1\times10^6 \pm 5.9\times10^5$	12	8.6	$4.1\times10^6 \pm 4.8\times10^5$	12	8.6

[a]About 10^4 for all replications.
[b]Tested to be an "outlier", (AAP, Appendix 11, 1971).

B11. Cell counts and C_v from Experiment 11 comparing by bioassay the AAP nutrients diluted with Lake Lansing membrane filtered water to AAP nutrients diluted with distilled water. Maximums marked as *. Inoculation error accounted for only two replications of the distilled water treatment.

Rep	Day	Diluent is DISTILLED WATER cells/ml + 1SD	C_v %	Diluent is LAKE LANSING WATER cells/ml + 1SD	C_v %
1	0	[a]About 10^4		[a]About 10^4	
	1	$3.0\times10^4 \pm 1.0\times10^4$	34	$1.8\times10^4 \pm 8.7\times10^3$	47
	2	$1.4\times10^5 \pm 2.3\times10^4$	16	$1.3\times10^5 \pm 8.2\times10^4$	63
	3	$4.1\times10^5 \pm 1.6\times10^4$	4	$4.9\times10^5 \pm 7.3\times10^4$	15
	5	$3.1\times10^6 \pm 3.1\times10^5$	10	$2.6\times10^6 \pm 4.0\times10^5$	15
	7	$*4.8\times10^6 \pm 4.1\times10^5$	9	$*3.3\times10^6 \pm 7.0\times10^5$	21
	14	$4.8\times10^6 \pm 2.8\times10^5$	6	$3.7\times10^6 \pm 6.1\times10^5$	16
2	1	$3.2\times10^4 \pm 1.4\times10^4$	44	$3.1\times10^4 \pm 1.3\times10^4$	43
	2	$1.4\times10^5 \pm 1.4\times10^4$	10	$2.3\times10^5 \pm 2.8\times10^4$	12
	3	$5.7\times10^5 \pm 3.2\times10^4$	6	$9.0\times10^5 \pm 7.7\times10^4$	9
	5	$4.0\times10^6 \pm 3.6\times10^5$	9	$*3.1\times10^6 \pm 2.9\times10^5$	9
	7	$*4.4\times10^6 \pm 4.2\times10^5$	10	$2.9\times10^6 \pm 5.4\times10^5$	19
	14	$5.1\times10^6 \pm 5.0\times10^5$	10	$3.2\times10^6 \pm 6.2\times10^5$	19
3	1			$2.2\times10^4 \pm 1.2\times10^4$	54
	2			$2.1\times10^5 \pm 2.8\times10^4$	13
	3			$7.9\times10^5 \pm 7.3\times10^4$	9
	5			$*2.8\times10^6 \pm 2.8\times10^5$	10
	7			$2.7\times10^6 \pm 4.7\times10^5$	17
	14			$2.8\times10^6 \pm 3.5\times10^5$	12
4	1			$2.6\times10^4 \pm 9.6\times10^3$	37
	2			$2.6\times10^5 \pm 3.0\times10^4$	12
	3			$9.8\times10^5 \pm 6.5\times10^4$	7
	5			$*3.7\times10^6 \pm 7.7\times10^5$	21
	7			$3.4\times10^6 \pm 7.6\times10^5$	22
	14			$3.4\times10^6 \pm 2.6\times10^5$	8

[a]About 10^4 cells/ml initially for all replications.

B12. Cell counts, C_v, and pH from Experiment 12 for the treatments of (1) membrane filtered Torch Lake control, (2) control plus 0.40 mg N/l, and (3) control plus 0.05 mg P/l. Maximum counts marked as *.

Rep	Day	Control cells/ml + 1SD	C_v %	pH	Control plus 0.40 mg N/l cells/ml + 1SD	C_v %	pH	Control plus 0.05 mg P/l cells/ml + 1SD	C_v %	pH
1	0	[a]About 10^4		8.2	[a]About 10^4		8.2	[a]About 10^4		8.2
	1	$1.1\times10^4 \pm 6.2\times10^3$	57	8.2	$1.2\times10^4 \pm 8.8\times10^3$	71	8.2	$2.0\times10^4 \pm 1.2\times10^4$	60	8.3
	2	$1.0\times10^4 \pm 5.1\times10^3$	51	8.3	$1.3\times10^4 \pm 5.2\times10^3$	40	8.3	$9.4\times10^4 \pm 3.3\times10^4$	35	8.6
	3	$9.6\times10^3 \pm 5.4\times10^3$	56	8.3	$1.1\times10^4 \pm 4.7\times10^3$	42	8.3	$2.8\times10^5 \pm 2.6\times10^4$	9	8.5
	4	$7.7\times10^3 \pm 2.4\times10^3$	32	8.3	$*1.3\times10^4 \pm 5.9\times10^3$	44	8.4	$4.8\times10^5 \pm 5.4\times10^4$	11	8.5
	5	$*1.8\times10^4 \pm 8.2\times10^3$	46	8.4	$1.2\times10^4 \pm 4.6\times10^3$	38	8.4	$6.7\times10^5 \pm 7.7\times10^4$	12	8.4
	7	$1.0\times10^4 \pm 6.4\times10^3$	64	8.4	$1.1\times10^4 \pm 5.4\times10^3$	51	8.1	$*6.8\times10^5 \pm 1.3\times10^4$	2	8.4
2	1	$1.1\times10^4 \pm 6.0\times10^3$	55	8.3	$7.7\times10^3 \pm 3.6\times10^3$	47	8.3	$3.0\times10^4 \pm 1.2\times10^4$	40	8.3
	2	$1.6\times10^4 \pm 8.3\times10^3$	52	8.3	$7.0\times10^3 \pm 4.2\times10^3$	60	8.4	$1.5\times10^5 \pm 3.4\times10^4$	23	8.6
	3	$2.0\times10^4 \pm 1.2\times10^4$	60	8.4	$8.6\times10^3 \pm 2.9\times10^3$	34	8.4	$3.3\times10^5 \pm 2.1\times10^4$	6	8.5
	4	$2.5\times10^4 \pm 1.2\times10^4$	48	8.4	$1.4\times10^4 \pm 8.4\times10^3$	60	8.4	$4.8\times10^5 \pm 4.7\times10^4$	10	8.5
	5	$*2.6\times10^4 \pm 7.2\times10^3$	28	8.4	$1.5\times10^4 \pm 7.3\times10^3$	49	8.4	$5.6\times10^5 \pm 3.9\times10^4$	7	8.4
	7	$2.0\times10^4 \pm 7.4\times10^3$	37	8.3	$*1.8\times10^4 \pm 3.0\times10^3$	17	8.4	$*6.2\times10^5 \pm 6.0\times10^4$	10	8.4
3	1	$8.6\times10^3 \pm 3.7\times10^3$	43	8.2	$9.8\times10^3 \pm 5.9\times10^3$	60	8.3	$1.6\times10^4 \pm 6.9\times10^3$	43	8.4
	2	$1.5\times10^4 \pm 6.5\times10^3$	43	8.3	$1.9\times10^4 \pm 9.0\times10^3$	47	8.4	$1.3\times10^5 \pm 2.5\times10^4$	19	8.6
	3	$8.6\times10^3 \pm 4.4\times10^3$	51	8.4	$1.9\times10^4 \pm 1.1\times10^4$	58	8.4	$3.8\times10^5 \pm 1.7\times10^4$	5	8.6
	4	$1.8\times10^4 \pm 6.8\times10^3$	38	8.4	$4.3\times10^4 \pm 1.3\times10^4$	30	8.5	$6.7\times10^5 \pm 6.0\times10^4$	9	8.5
	5	$1.9\times10^4 \pm 9.6\times10^3$	50	8.3	$4.5\times10^4 \pm 1.5\times10^4$	33	8.5	$6.5\times10^5 \pm 3.6\times10^4$	6	8.4
	7	$*2.0\times10^4 \pm 1.5\times10^4$	75	8.3	$*5.8\times10^4 \pm 1.4\times10^4$	24	8.6	$*7.2\times10^5 \pm 5.0\times10^4$	7	8.4

[a]About 10^4 cells/ml initially for all replications.

B13. Cell counts and C_v from Experiment 13 for the treatments of (1) membrane filtered Deep Lake control, (2) control+0.40 mg N/l, and (3) control plus 0.05 mg P/l. Maximums marked as *.

Rep	Day	Control cells/ml + 1SD	C_v %	Control plus 0.40 mg N/l cells/ml + 1SD	C_v %	Control plus 0.05 mg P/l cells/ml + 1SD	C_v %
1	0	[a]About 10^4		[a]About 10^4		[a]About 10^4	
	1	$1.9\times10^4 \pm 6.8\times10^3$	36	$2.2\times10^4 \pm 9.9\times10^3$	45	$4.1\times10^4 \pm 1.3\times10^4$	32
	2	$4.6\times10^4 \pm 1.3\times10^4$	28	$3.1\times10^4 \pm 1.2\times10^4$	39	$1.4\times10^5 \pm 2.2\times10^4$	16
	3	$4.2\times10^4 \pm 1.4\times10^4$	33	$*3.6\times10^4 \pm 1.2\times10^4$	33	$1.7\times10^5 \pm 2.1\times10^4$	12
	4	$4.4\times10^4 \pm 1.5\times10^4$	34	$3.2\times10^4 \pm 1.0\times10^4$	31	$2.0\times10^5 \pm 3.0\times10^4$	15
	6	$5.2\times10^4 \pm 2.1\times10^4$	40	$3.2\times10^4 \pm 1.4\times10^4$	44	$2.3\times10^5 \pm 2.8\times10^4$	12
	10	$*6.4\times10^4 \pm 4.2\times10^3$	7	$2.9\times10^4 \pm 7.6\times10^3$	26	$*3.1\times10^5 \pm 1.3\times10^4$	4
2	1	$1.3\times10^4 \pm 8.9\times10^3$	68	$1.1\times10^4 \pm 9.1\times10^3$	82	$2.9\times10^4 \pm 7.6\times10^3$	26
	2	$1.6\times10^4 \pm 6.7\times10^3$	24	$1.2\times10^4 \pm 5.0\times10^3$	42	$1.2\times10^5 \pm 2.3\times10^4$	21
	3	$1.8\times10^4 \pm 9.1\times10^3$	50	$*1.3\times10^4 \pm 6.8\times10^3$	52	$1.2\times10^5 \pm 2.3\times10^4$	19
	4	$1.7\times10^4 \pm 7.5\times10^3$	44	$1.1\times10^4 \pm 3.5\times10^3$	32	$1.4\times10^5 \pm 2.0\times10^4$	14
	6	$1.5\times10^4 \pm 8.1\times10^3$	54	$9.1\times10^3 \pm 6.2\times10^3$	68	$1.5\times10^5 \pm 2.4\times10^4$	16
	10	$*1.9\times10^4 \pm 3.4\times10^3$	18	$1.1\times10^4 \pm 6.1\times10^3$	55	$*1.9\times10^5 \pm 3.7\times10^4$	19
3	1	$1.7\times10^4 \pm 8.9\times10^3$	52	$1.1\times10^4 \pm 5.5\times10^3$	50	$3.2\times10^4 \pm 1.0\times10^4$	32
	2	$2.5\times10^4 \pm 7.7\times10^3$	31	$8.6\times10^3 \pm 5.1\times10^3$	59	$1.2\times10^5 \pm 2.0\times10^4$	17
	3	$*2.7\times10^4 \pm 1.2\times10^4$	44	$*1.9\times10^4 \pm 1.3\times10^4$	68	$1.5\times10^5 \pm 3.6\times10^4$	24
	4	$2.5\times10^4 \pm 1.0\times10^4$	40	$1.7\times10^4 \pm 1.0\times10^4$	59	$1.7\times10^5 \pm 3.8\times10^4$	22
	6	$2.1\times10^4 \pm 1.1\times10^4$	52	$1.4\times10^4 \pm 8.3\times10^3$	59	$2.0\times10^5 \pm 1.3\times10^4$	7
	10	$2.7\times10^4 \pm 5.5\times10^3$	20	$1.1\times10^4 \pm 9.0\times10^3$	82	$*3.4\times10^5 \pm 2.3\times10^4$	7

[a]About 10^4 cells/ml initially for all replications.

B14. Cell counts and C_v from Experiment 14 for the treatments (1) membrane filtered Lake Lansing control, (2) control plus 0.40 mg N/l, (3) control plus 0.05 mg P/l, (4) control plus 0.40 mg N/l and 0.05 mg P/l. Maximums marked as *.

Rep	Day	Control cells/ml ± 1SD	%	Control plus 0.40 mg N/l cells/ml ± 1SD	C_v %	Control plus 0.05 mg P/l cells/ml ± 1SD	C_v %	Control plus 0.40 mg N/l 0.05 mg P/l cells/ml ± 1SD	C_v %
1	0	[a]About 8.0×10^3		About 8.0×10^3		About 8.0×10^3		About 8.0×10^3	
	1	$7.7\times10^3 \pm 4.6\times10^3$	60	$5.8\times10^3 \pm 3.0\times10^3$	52	$1.5\times10^4 \pm 7.1\times10^3$	47	$1.3\times10^4 \pm 6.0\times10^3$	46
	2	$8.6\times10^3 \pm 7.4\times10^3$	86	$6.2\times10^3 \pm 3.1\times10^3$	50	$6.7\times10^4 \pm 2.5\times10^4$	37	$5.7\times10^4 \pm 1.0\times10^4$	18
	3	$9.6\times10^3 \pm 7.2\times10^3$	75	$6.7\times10^3 \pm 3.2\times10^3$	48	$1.2\times10^5 \pm 3.0\times10^4$	40	$1.1\times10^5 \pm 1.9\times10^4$	17
	4	$*1.1\times10^4 \pm 3.7\times10^3$	34	$7.2\times10^3 \pm 3.0\times10^3$	42	$1.7\times10^5 \pm 3.5\times10^4$	21	$1.5\times10^5 \pm 2.2\times10^4$	15
	5	$1.1\times10^4 \pm 5.7\times10^3$	52	$*7.4\times10^3 \pm 3.6\times10^3$	49	$2.1\times10^5 \pm 3.2\times10^4$	15	$2.0\times10^5 \pm 2.2\times10^4$	11
	7	$9.0\times10^3 \pm 6.1\times10^3$	68	$7.0\times10^3 \pm 2.8\times10^3$	40	$2.7\times10^5 \pm 3.6\times10^4$	13	$*2.2\times10^5 \pm 2.2\times10^4$	10
	10	$8.6\times10^3 \pm 7.4\times10^3$	86	$5.8\times10^3 \pm 2.9\times10^3$	50	$*3.3\times10^5 \pm 3.9\times10^4$	12	$1.3\times10^5 \pm 2.6\times10^4$	20
2	1	$9.1\times10^3 \pm 6.9\times10^3$	76	$6.2\times10^3 \pm 4.0\times10^3$	65	$1.5\times10^4 \pm 7.1\times10^3$	47	$1.6\times10^4 \pm 5.3\times10^3$	33
	2	$8.2\times10^3 \pm 5.1\times10^3$	62	$5.3\times10^3 \pm 2.9\times10^3$	55	$7.3\times10^4 \pm 2.2\times10^4$	30	$6.2\times10^4 \pm 3.0\times10^3$	48
	3	$6.9\times10^3 \pm 4.1\times10^3$	59	$5.7\times10^3 \pm 3.2\times10^3$	56	$1.1\times10^5 \pm 2.5\times10^4$	23	$1.0\times10^5 \pm 2.3\times10^3$	23
	4	$*7.2\times10^3 \pm 5.6\times10^3$	78	$6.2\times10^3 \pm 3.1\times10^3$	50	$1.3\times10^5 \pm 2.9\times10^4$	25	$1.5\times10^5 \pm 1.5\times10^4$	10
	5	$7.0\times10^3 \pm 4.4\times10^3$	63	$*6.4\times10^3 \pm 3.2\times10^3$	50	$1.6\times10^5 \pm 2.6\times10^4$	16	$1.9\times10^5 \pm 1.8\times10^4$	9
	7	$7.0\times10^3 \pm 3.9\times10^3$	56	$6.0\times10^3 \pm 2.1\times10^3$	35	$1.6\times10^5 \pm 2.4\times10^4$	15	$*2.3\times10^5 \pm 2.2\times10^4$	10
	10	$6.2\times10^3 \pm 3.2\times10^3$	52	$6.2\times10^3 \pm 2.6\times10^3$	42	$*1.8\times10^5 \pm 2.3\times10^4$	13	$1.3\times10^5 \pm 2.1\times10^4$	16
3	1	$8.2\times10^3 \pm 4.6\times10^3$	56	$9.6\times10^3 \pm 6.1\times10^3$	64	$1.7\times10^4 \pm 7.3\times10^3$	43	$1.5\times10^4 \pm 6.2\times10^3$	41
	2	$*8.6\times10^3 \pm 5.0\times10^3$	58	$7.7\times10^3 \pm 4.5\times10^3$	58	$7.0\times10^4 \pm 3.1\times10^4$	45	$4.6\times10^4 \pm 1.0\times10^4$	22
	3	$8.0\times10^3 \pm 5.2\times10^3$	65	$9.0\times10^3 \pm 3.5\times10^3$	39	$1.3\times10^5 \pm 2.7\times10^4$	21	$9.4\times10^4 \pm 3.1\times10^4$	33
	4	$7.7\times10^3 \pm 6.9\times10^3$	90	$8.2\times10^3 \pm 4.1\times10^3$	50	$1.4\times10^5 \pm 3.2\times10^4$	23	$1.4\times10^5 \pm 4.0\times10^4$	28
	5	$7.0\times10^3 \pm 5.0\times10^3$	71	$*1.0\times10^4 \pm 4.6\times10^3$	46	$1.7\times10^5 \pm 5.1\times10^4$	30	$1.3\times10^5 \pm 1.5\times10^4$	12
	7	$7.2\times10^3 \pm 5.3\times10^3$	74	$9.0\times10^3 \pm 3.6\times10^3$	40	$2.2\times10^5 \pm 2.2\times10^4$	10	$*1.7\times10^5 \pm 1.7\times10^4$	10
	10	$7.7\times10^3 \pm 6.5\times10^3$	84	$6.7\times10^3 \pm 3.6\times10^3$	54	$*2.7\times10^5 \pm 2.7\times10^4$	10	$5.6\times10^4 \pm 1.0\times10^4$	18

[a]About 8.0×10^3 cells/ml initially for all replications.