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Title: Growth Control of a Marine Diatom by Low and Limiting Levels of Nitrate-Nitrogen

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Herbert C. Curl

Batch and continuous flow growth and uptake experiments were conducted to determine the control low concentrations of nitrate nitrogen exerted over the growth response and selected physiological characteristics of Thalassiosira pseudonana (CN) Hasle and Heimdal, a marine centric diatom. Initial experiments were conducted to determine the suitability of this species to be used as a test alga for the Environmental Protection Agency's program to assess the fertility of estuarine and marine waters. These experiments indicated that T. pseudonana possessed many of the characteristics desireable in a test organism: rapid growth, ease of electronic counting, broad salinity response, good suspension characteristics and good growth in artificial sea water. Other experiments indicated a nitrate-nitrogen yield of 2.3×10^7 cells/µg-at NO_3-N and a phosphate-

phosphorus yield of 1.06 x 10^9 cells/µg-at $PO_4^{\equiv}$ -P. The utility of knowledge of yield coefficients is discussed. Light response studies indicated light saturation at about 2000 µw/cm^2; light inhibition was not evident at the highest intensity (3900 µw/cm^2) used.

Nitrate-nitrogen limited batch and continuous flow experiments indicated that the specific growth rate (measured as changes in cell numbers, total cell volume, or particulate carbon) was not directly related to the nitrate-nitrogen concentration of the medium but rather to the physiological state of the population which is in turn controlled by the availability of nitrogen in the system. The nitrogen specific growth rate (or nitrogen assimilation) may be more closely related to the nitrate-nitrogen concentration in the medium. The physiological state of the population (with respect to nitrogen) can be expressed in terms of a carbon:nitrogen ratio; low carbon:nitrogen ratios (5-8 by atoms) indicate a nitrogen sufficient condition while high carbon:nitrogen ratios are indicative of nitrogen deficient conditions. Values in excess of 20 were observed in both batch and continuous flow systems. An inverse relationship between carbon specific growth rate and carbon:nitrogen ratio is presented.

Nitrate-nitrogen uptake characteristics of T. pseudonana may be controlled by the nitrogen physiological state of the population. In batch systems, V_{max} normalized to population nitrogen is similar to the maximum specific growth rate of the population but is more than twice as high for a nitrogen deficient population. In continuous

flow systems, V_{max} increased as the dilution rate of the system increased. K_s values were low, typical of communities in low nitrogen waters. Though not significant at the 95% confidence level, K_s values for batch grown populations were about twice as high as those for continuously grown populations. The influence which variable uptake characteristics have upon classical continuous flow theory is discussed.

Comparison of ranges of carbon:nitrogen ratios in laboratory systems with those available for field studies indicate a lower range of carbon:nitrogen ratios in natural communities, suggesting that although ambient nitrogen levels are often low in natural systems, mechanisms are available which maintain these communities in a nitrogen sufficient state. Possible mechanisms are discussed.

Growth Control of a Marine Diatom by Low and Limiting Levels of Nitrate-Nitrogen

by

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GROWTH CONTROL OF A MARINE DIATOM BY LOW AND LIMITING LEVELS OF NITRATE-NITROGEN

INTRODUCTION

The process of eutrophication of a body of water has been variously described as a progression in the state of the body of water from one characterized by low critical nutrient levels (that nutrient or those nutrients which limit algal growth), low standing stocks of phytoplankton with resultant high clarity of water, low rates of transfer of critical nutrients through the system, and low production of organic matter with resultant low biological oxygen demand to one characterized by high critical nutrient levels, high standing stocks of phytoplankton (which may be of obnoxious algae), low clarity of water, high rates of transfer of nutrients, high production of organic material and high biological oxygen demand. (Stewart and Rohlich, 1967; Hutchinson, 1969; Rodhe, 1969; Lee, 1970; Likens, 1972.) The above attributes of a eutrophic system are produced by an increase in the critical nutrient flux through and availability in the system. The sediments often act as a trap or reservoir in which organic materials may be stored, decomposed, oxidized to critical nutrients and act as a feedback into the system to enhance nutrient flux and availability.

The sequence of events associated with the eutrophic process may occur naturally over geologic time scales. However, man's impact has been to compress the time scale over which the progression of events occurs by increasing the flux of

critical nutrients into and through the system. Where the natural process may have taken hundreds of thousands of years, man has compressed this rate many orders of magnitude to a time scale of decades or less. The result of this compression of time scales is that, where unaffected by man few bodies of water would exhibit conditions described as eutrophic, a great number of aquatic systems now fall into that category.

With respect to man, the attributes of a eutrophic system are often undesirable--recreational value of the system declines because of such phenomena as proliferation of algae which produce noxious odors (either due to decomposition of the algae or as byproducts of the algae themselves), proliferation of macrophytes, production of trash fish, production of algal mats or scum befouling the waters; aesthetic values decline for many of the same reasons; the water often becomes unhealthy for human consumption requiring greater costs to reclaim it; economic fisheries as well as sport fisheries production declines.

The problem of eutrophication then becomes a social issue, involving man and his environment. A conflict of interest often arises among different segments of society. Public outcry generates political pressure to "solve the problem". In response to the building eutrophication problem as well as the increasing vocal public complaint, the then Federal Water Pollution Control Administration established a National Eutrophication Research Program (NERP) in 1967 at Corvallis, Oregon, with the broad

duties of establishing programs to promote the solution of problems dealing with eutrophication, to develop methods for understanding the eutrophic process, controlling and reversing it.

The primary thrust of NERP was to develop programs to assess the fertility of a body of water and to determine methods by which the fertility might be altered. One of the tactics chosen was to develop various algal bioassay methods which could be used as tools to evaluate the level of fertility. Those chosen were a batch test, a continuous flow test, and an in situ ^{14}C uptake test. All three suggest that the growth potential of a natural body of water can be determined by incubating a sample of the water body for a specified time, observing the biomass production with time and relating this biomass production to the chemical characteristics of the body of water. Various nutrients spikes would demonstrate whether rate of production or biomass yield was stimulated by those spikes and therefore indicate that one or more elements was present in growth limiting concentrations in the original body of water.

A further effort was initiated to establish a standardized assay system using single clones of algae which could be used uniformly throughout the country and internationally if need be. The focus of this effort has been the development of the Bottle Test and at present it has been set forth as the Algal Assay Procedure-Bottle Test (1971). The emphasis has been placed upon analysis of fresh water systems, primarily lakes.

A growing need for the assessment of eutrophication in estuarine and coastal systems resulted in an expansion of the program to include the development of an assay system which would be helpful in this task. Basic to the development of such a system is the selection of a suitable test organism based upon an understanding of its growth characteristics as they may relate to characteristics desirable in test organisms. (Joint Task Force, 1969). One of the test organisms required was a diatom species.

Coincidently, in 1970 the then Federal Water Quality Administration established a Graduate Fellowship Program which provided for the employment of students working toward Master's or Doctoral degrees.

> "This program provides for students at the master's degree or doctoral level, who have substantially completed their graduate course requirements except for the thesis, to be employed for a period of time by the Federal Water Quality Administration (FWQA) at one of their laboratories in the United States. During the employment the student will accomplish the research, analysis or other practical work that will supply the basic material for his graduate degree thesis on a subject area preselected by mutual agreement among the student, the University, and FWQA officials. The thesis topic must concentrate on a subject of current need related to the mission of

the FWQA. In many cases the thesis will serve as the student's final report to the employing office although interim reports may be required from time to time."[1]

My interest in the kinetics of nutrient limitation of phytoplankton growth coincided well with the desire on the part of NERP to obtain background information concerning a diatom species suitable for use as a test organism for the evaluation of the potential fertility of estuarine waters. This thesis then describes the general growth response of Thalassiosira pseudonana Hasle and Heimdal (Cyclotella nana Hustedt) as it may apply as a test organism. Particular emphasis is placed upon its growth response to low and limiting levels of nitrate nitrogen.

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[1]Statement of Agreement between Federal Water Quality Administration and Graduate Associate.

GENERAL GROWTH CHARACTERISTICS OF *THALASSIOSIRA PSEUDONANA* HASLE AND HEIMDAL AS THEY RELATE TO ITS PROPOSED USE AS AN ESTUARINE TEST ORGANISM

Introduction

Thalassiosira pseudonana Hasle and Heimdal was first isolated from the River Weser by Hustedt (1957) and described as *Cyclotella nana*. Subsequently he suggested that its taxonomic position be revised (1959). Guillard and Ryther (1962) isolated several clones of *T. pseudonana* (identified as *Cyclotella nana* by Hustedt from electronmicrographs) from widely varying locales ranging from the nearly fresh water of Forge River and Senix Creek, Moriches Bay, Long Island, N. Y., to the saline waters of the Sargasso Sea. They also suggested that its taxonomic position be clarified. The taxonomic position was finally settled by Hasle and Heimdal (1970) who obtained isolates from the Gulf of Naples and Mamaia, Black Sea, as well as net samples from affluents of the Weser River. Morphological characteristics based upon light and electron microscopic observations are presented in Hasle and Heimdal (1970) as well as in Guillard and Ryther (1962).

T. pseudonana seems to be widely distributed, clones having been isolated from nearly fresh water (River Weser and Swan River, W. Australia), several regions of coastal waters (Japan, East Coast of U.S., and Mediterranean), and the Sargasso Sea.

Growth responses to various regimes of temperature and salinity were determined by Guillard and Ryther (1962) for the several clones of T. pseudonana which they isolated. Those isolated from estuarine locales exhibited broad salinity responses, growing well over a range of 0.5°/$_{oo}$ to 32°/$_{oo}$ while the clones isolated from the Sargasso Sea and continental shelf waters exhibited marked declines in specific growth rates at lower salinities. Maximum division rates were slightly greater than 3 day^{-1} under optimum laboratory conditions. Estuarine clones grew best at 20-25°C at slightly greater than three divisions per day while the clone isolated from continental shelf waters grew best over a temperature range of 10-20°C at slightly greater than two divisions per day and the Sargasso Sea clone between 20-25°C at slightly less than two divisions per day. Responses to various light intensity regimes were not determined. A requirement for vitamin B-12 was demonstrated for all clones, but no other vitamins were required.

T. pseudonana has been used as a test organism by a variety of investigators. The assay work followed two lines--that of determining yield responses in natural waters to assess whether "good" or "bad" water existed and that of determining the utility of T. pseudonana as a vitamin B-12 bioassay organism. Smayda (1970) and Gold (1965) used T. pseudonana (clone 13-1) to determine the growth potential of waters in and near Phosphorescent Bay, Puerto Rico, in order to try to understand the persistent blooms of luminescent dinoflagellates. No details are given for Gold's work; Smayda used T. pseudonana as one of four test diatoms, and while growth of the three other diatoms

(Skeletonema costatum, Bacteriastrum hyalinum, and Thalassiosira rotula) was restricted at various of the stations, T. pseudonana responded well at all stations. T. pseudonana (clone 13-1) has also been used to assay waters in the Sargasso Sea area to assess the effect of various nutrient additions to filtered sea water (Smayda, 1964).

Guillard and Ryther's (1962) initial studies concerning the physiological responses of T. pseudonana indicated a vitamin B-12 requirement and led them to test the ability of this organism to act as a bioassay indicator of B-12 concentration. Using clone 3H they obtained a linear response which related cell numbers to B-12 concentration over a range from 0-2 μμg B-12/ml (Ryther and Guillard 1962). The yield coefficient varied from 5.5-7.9x10^5 cells/μμg of B-12 for clone 3H and 2.9x10^5 cells/μμg of B-12 for clone 13-1. Gold (1964) related the ^{14}C uptake (per 24 hours) by vitamin deficient cells to the B-12 concentration over a similar range. Carlucci and Silbernagel (1969) describe the effect of limiting levels of B-12 on the growth, $^{14}CO_2$ uptake rates and chlorophyll content of T. pseudonana (13-1); a yield coefficient of 2.1-3.1x10^5 cells/μμg of B-12 is reported. Davis and Nicol (1967) using a clone of T. pseudonana isolated from Swan River, Western Australia, determined a yield coefficient of 4x10^5 cells/μμg of B-12 and tested for B-12 levels in estuarine waters as well as in preparations of blood serum and extracts of brain tissue. Menzel and Spaeth (1962) applied the techniques developed by Ryther and Guillard (1962) to test for B-12 in the Sargasso Sea.

Additional studies using T. pseudonana include an analysis of the osmotic and ionic requirements of clone 13-1 (Guillard and Myklestad, 1970). A study of the response to chlorinated hydrocarbons (Menzel, Anderson, and Randke, 1970) indicated inhibitory levels of DDT, dieldrin, endrin at levels of 0.1 to 1.0 parts per billion. The effect of inorganic nitrogen nutrition on macromolecular synthesis (DNA, RNA, etc.) was determined by Hobson and Pariser (1971). Fuhs (1969) and Fuhs et al., (1972) conducted a rather thorough investigation of the response of T. pseudonana (clone 3H and an unidentified clone) to phosphate limitation under different regimes of temperature and light utilizing both batch and continuous flow systems.

The literature survey above indicates that several clones of T. pseudonana may be quite useful as a candidate test organism for studies involving bioassay of estuarine waters. Its characteristics (small size of 4-10 μ in diameter, single cell characteristics, rapid growth, tolerance to temperatures between 20-25°C, broad natural distribution) seem adequate for use as a test organism. Several clones of T. pseudonana were obtained from the National Marine Water Quality Laboratory, Kingston, Rhode Island, and further tested to determine some of the physiological responses of this organism as they might apply to its use as a bioassay organism for estuarine waters. The tests included salinity responses, light responses, nitrate and phosphate yield responses, and growth characteristics in enriched artificial sea water. The capabilities and limitations of algal bioassays are discussed as they relate to the assessment of the growth promoting characteristics of natural waters.

Materials And Methods

Four clones of T. pseudonana were obtained from Dr. P. Hargraves, University of Rhode Island, West Kingston, RI. These clones are CN (which was indicated to be the same as 13-1), 13-1 (isolated from the Sargasso Sea, 33° 11'N, 65° 15'W), BIS (isolated from Hamilton Harbor, Bermuda), and 3H (isolated from Forge River, Moriches Bay, Long Island.) The cultures were maintained in natural sea water enriched with Guillard and Ryther's f/2 (Guillard and Ryther, 1962) medium except that the nitrate concentration was reduced to 100 µg-at/l of final medium. Cultures were maintained at a salinity of 20°/$_{oo}$, a temperature of 22.5°C and cool white fluorescent illumination of ≃1800 µw/cm^2 (≃400 foot-candles). Stock cultures were transferred once a week.

Sea water was obtained off the Oregon coast (>45 miles offshore), aged at least two months in 15 gallon polyethylene containers, filtered through 0.45 µ Millipore^R filter, treated with activated charcoal for one day (1g/l), refiltered, adjusted to desired salinity with double distilled water, autoclaved at 15 psi for fifteen minutes and enriched with nutrients.

Cell numbers were used as indicators of biomass. Both Coulter Counter Model B and Model T were used with an attached mean cell volume computer. Mean cell volume computers were calibrated with latex spherical particles whose mean diameter was 3.49 µ.

Growth constants were calculated using the formula $k = \frac{\ln (N/N_o)}{t-t_o}$ where N and N_o were biomass measures at times t and t_o and k has units of time^{-1}.

Culture vessels used were 500 ml Erhlenmeyer flasks stoppered with polyurethane plugs. Culture volume was 250 ml with initial cell densities of 100 cells/ml.

Light intensities were measured with an ISCO model SR spectroradiometer calibrated with an ISCO model SRC spectro-radiometer calibrator.

Salinity response experiments. Sea water collected 165 miles off the Oregon coast was aged at least two months, filtered through 0.45 μ Millipore^R membrane filters, and treated with activated charcoal (1g/l). Salinities were adjusted to 30°/$_{oo}$, 25°/$_{oo}$, 20°/$_{oo}$, 15°/$_{oo}$, 10°/$_{oo}$, and 5°/$_{oo}$ and the medium was enriched with the above nutrient array. Flasks (triplicate) were inoculated with clone CN such that the initial population density was about 100 cells/ml and were incubated at 22.5°C (±1.0°C) under constant cool white fluorescent illumination of ≃1800 μw/cm^2. Cell counts were obtained every twelve hours, and the growth constant calculated based upon the highest rate attained over a given time period. Two runs were conducted, the second of which differed from the first in that $NaHCO_3$ was added to all replicates of 20°/$_{oo}$ or less to insure that carbon limitation did not restrict growth.

Clones 13-1, 3H, and BIS were maintained on enriched sea water adjusted to 20°/$_{oo}$ for several months prior to determining their growth responses at 20°/$_{oo}$. The experimental design was similar to that above.

In an estuarine situation one is likely to encounter salinities of a broad range. It would be of value if the test organism displayed characteristics which would not require that it be acclimitized to various salinity regimes prior to its use as an inoculum. Ideally it would be maintained at a single salinity midway over a range which might be encountered; 20°/$_{oo}$ has been selected somewhat arbitrarily. In order to determine whether it is necessary to acclimatize a population to a given salinity, the response of a population (clone CN) acclimatized at 5°/$_{oo}$ was compared to that of a population acclimatized at 20°/$_{oo}$. Acclimatization at 5°/$_{oo}$ spanned two weeks (two transfers) while acclimatization at 20°/$_{oo}$ spanned several months (maintenance salinity.) Both were tested at 5°/$_{oo}$; two separate runs of triplicate flasks were conducted. The length of lag phase as well as maximum specific growth rate were determined.

Artificial sea water experiments. The growth response of clone CN in two artificial sea water recipes (Lyman and Fleming, 1940; Burkholder, 1962; Table 1) enriched with modified f/2 after filtering through a 0.45 μ Millipore^{R} filter and autoclaving at 15 psi for 15 minutes was determined. Triplicate flasks were incubated at 22.5°C and 1800 μw/cm^2 cool white fluorescent illumination. The experiment was conducted twice.

TABLE 1: COMPOSITION OF ARTIFICIAL SEA WATER RECIPES TESTED.

1. Burkholder's (1962) ASW to make 20°/$_{oo}$

SALT	WEIGHT
NaCl	13.420g
Na_2SO_4	2.240
$NaHCO_3$	0.109
KCl	0.377
KBr	0.055
H_3BO_3	0.015
$MgCl_2 \cdot 6H_2O$	6.063
$SrCl_2 \cdot 6H_2O$	0.023
$CaCl_2 \cdot 6H_2O$	0.839
H_2O to	1000ml

2. Lyman and Fleming's (1942) ASW to make 20°/$_{oo}$

SALT	WEIGHT
NaCl	14.445g
$MgCl_2 \cdot 6H_2O$	6.544
$Na_2SO_4 \cdot 10H_2O$	5.467
$CaCl_2$	0.678
KCl	0.409
H_3BO_3	0.016
H_2O to	1000ml

Light response experiments. Clone CN was grown in enriched sea water adjusted to 20°/$_{oo}$ under radiant energy fluxes of about 550, 1300, 1950, 2450, and 3900 μw/cm^2 (380-700 nm cool white fluorescent illumination; highest intensity was achieved with the addition of one high intensity fluorescent lamp to the cool white array.) Growth constants were calculated as above, based upon cell counts determined every twelve hours. Each treatment was conducted in duplicate.

Phosphorus yield coefficient. Aliquots of a phosphate starved population (clone CN) were inoculated into artificial sea water media made up to 20°/$_{oo}$ (Lyman and Fleming, 1940, enriched as above except that phosphate was deleted.) The following phosphate concentrations were obtained by adding KH_2PO_4 dried at 50°C for 24 hours: 0, 0.025, 0.050, and 0.10 μg-at $PO_4^{\equiv}$ -P/l. Triplicate flasks were incubated at 22.5°C uner ≃1800 μw/cm^2 cool white illumination until stationary phase was achieved. The yield coefficient was obtained from the slope of a regression of cell numbers against initial phosphate concentration.

Nitrogen yield coefficient. Nitrogen depleted natural sea water adjusted to 20°/$_{oo}$ enriched with f/2 less nitrate was made up to initiial concentrations of 5.0, 10.0, and 15.0 μg-at NO_3^- -N/l with KNO_3 dried at 50°C for 24 hours. Nitrate starved aliquots of a population of clone CN were added to triplicate flasks such that the initial concentration was about 100 cells/ml. The flasks were incubated at 22.5°C under ≃1800 μw/cm^2. The yield determination

was carried out twice, separated by about four months. The yield coefficient was determined from the slope of a regression of cell numbers against initial nitrate concentration.

Results And Discussion

Salinity response. Clone CN exhibited a rather broad salinity response, being nearly constant over the range of 10°/₀₀ to 30°/₀₀ at about 2.75 divisions per day (Fig. 1). A slight decline at 5°/₀₀ to a rate of slightly greater than two divisions per day indicated that the diatom was still capable of growing rapidly at this salinity. Both clones BIS and 13-1 exhibited a poor growth response at 20°/₀₀ (1.65 divisions per day, σ=0.04 for BIS and 1.35 divisions per day, σ=0.11 for 13-1) while the response of 3H at 20°/₀₀ (2.80 divisions per day, σ=0.09) was indistinguishable from that of clone CN. Additionally, BIS and 13-1 tended to adhere to the sides of the flasks during growth response studies as well as during normal maintenance.

Both BIS and 13-1 were isolated from regions where the salinity would be expected to be rather high, and would therefore not exhibit good growth characteristics at lower salinities. Hargraves (personal communication) indicated that clone CN might be synonymous with 13-1. However, this experiment does not support that idea. Clone CN may be identical with 3H based upon their nearly identical growth response at 20°/₀₀. Guillard and Ryther (1962) indicate a rather broad response of 3H, being nearly constant over a range of 0.5°/₀₀ to 32.0°/₀₀ at a rate of slightly greater than three divisions per day over a range of 16°/₀₀ to 32°/₀₀ and slightly less than three divisions per day at 0.5°/₀₀ to 8°/₀₀. A slightly higher light intensity was used in the Guillard and Ryther study than was used

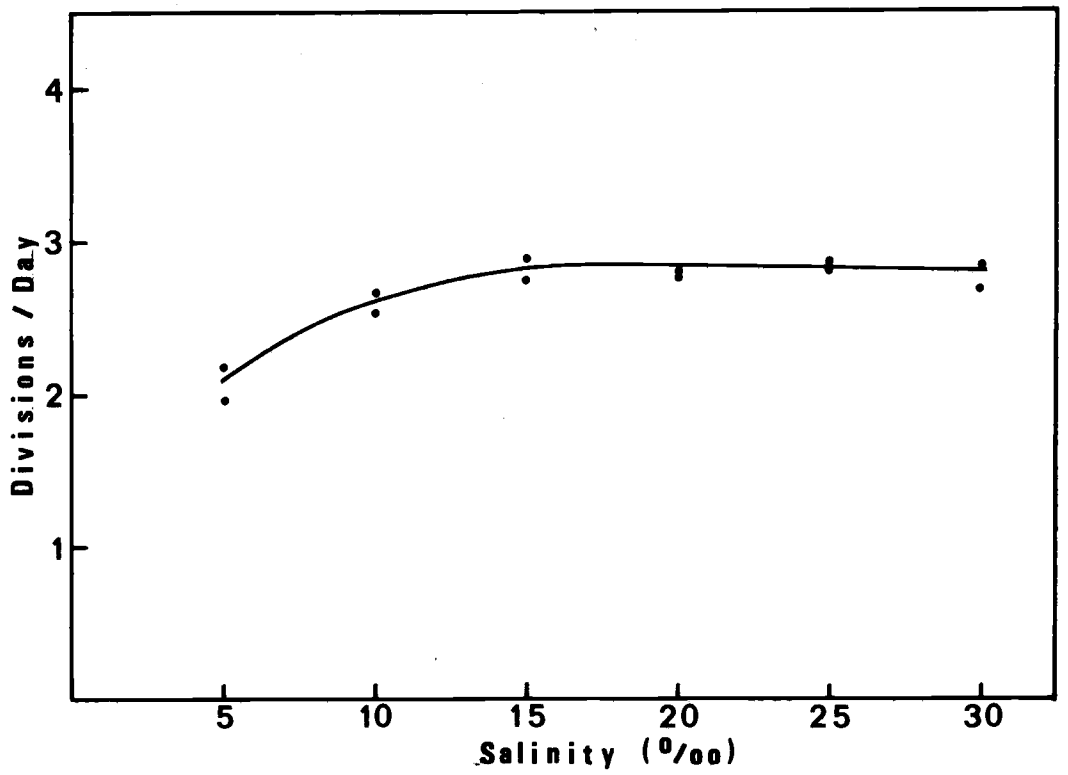


Figure 1. Salinity response of T. pseudonana. Each datum point represents the mean of three replicates whose average standard deviation was 0.06 divisions per day.

in these studies. The response of clone 13-1 dropped off markedly at salinities less than 24°/$_{oo}$ in their study, adding support to the idea that CN and 13-1 are not synonymous.

The growth constants (Table 2) which allow comparison between acclimated and unacclimated populations indicated no significant difference between the two treatments and compare well with the growth constants obtained during the earlier salinity response studies. Also the length of the lag phase observed prior to the establishment of rapid growth was about the same for both acclimated and unacclimated populations, lasting approximately one day. It therefore seems unnecessary to maintain cultures at various salinities. A median culture salinity of about 20°/$_{oo}$ is adequate.

Light response. Although the range of radiant energy fluxes is limited, the lower two intensities definitely restricted the maximum specific growth rate of clone CN (Fig. 2). Maximum rate of cell division appeared to be achieved at about 2000 μw/cm^2 (0.29 ly/min), or at a somewhat lower level. Light inhibition was not evident at the highest intensity, 3900 μw/cm^2 (0.56 ly/min).

Ryther (1956) demonstrated that maximum photosynthesis appears at approximately 0.06 ly/min for several diatom species. Results were reported as an average curve so that species variations cannot be detected. Jitts et al., (1964) and McAllister et al., (1964) showed that maximum growth rates (cell division) and maximum photosynthesis (^{14}C uptake) were achieved at about 0.1 ly/min for Skeletonema costatum and maximum cell division rates (Jitts et al.) for

TABLE 2: COMPARISON BETWEEN ACCLIMATED AND UNACCLIMATED POPULATIONS OF T. PSEUDONANA GROWN ON 5°/$_{oo}$ SALINITY SEA WATER TREATMENTS WERE TRIPLICATED. AVERAGE STANDARD DEVIATION WAS 0.06 DIVISIONS PER DAY.

	Acclimated	Unacclimated
Run 1	2.08 divisions per day	2.24
Run 2	2.19	2.23

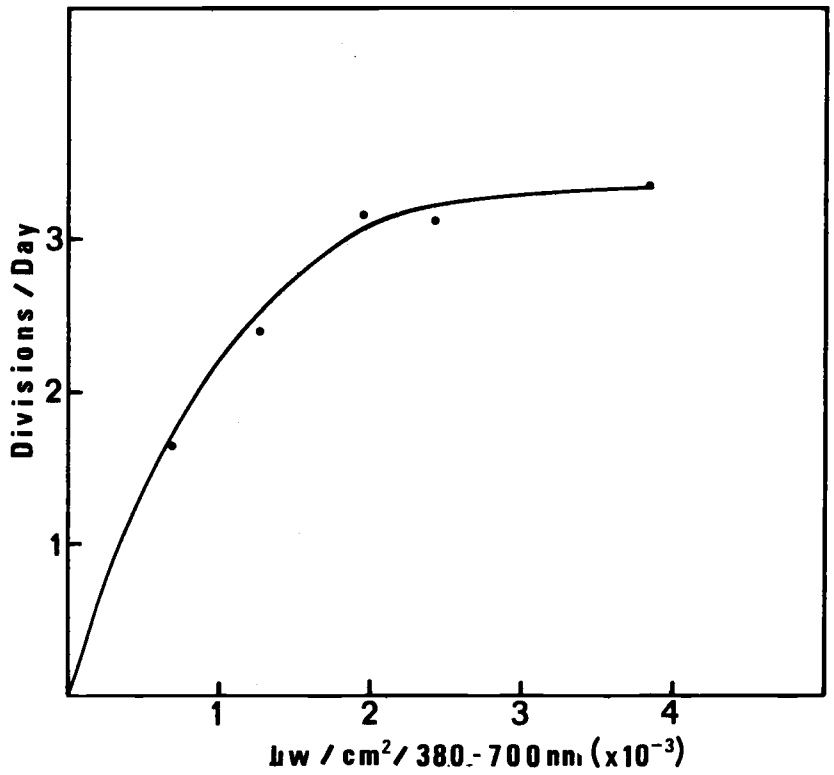


Figure 2. Light response of T. pseudonana. Each datum point represents the mean of two replicates. The maximum difference between replicates was 0.09 divisions per day.

Thalassiosira nordenskioldii was achieved at a value less than 0.05 ly/min at optimum temperatures. Lundy (personal communication) reports that maximum ^{14}C uptake for S. costatum occurred at about 0.03 ly/min.

The maximum specific growth rate observed here is similar to that reported by Guillard and Ryther for clone 3H (3.35 divisions per day); all other clones responded with lower maximum specific growth rates. Their experiments were carried out at 20°C and 4500 Klux. It is difficult to convert units of luminous flux to energy units without proper luminosity factors, however, 4500 Klux can be assumed to be an intensity which does not restrict growth.

Phosphate - phosphorus and nitrate - nitrogen yield coefficients. A yield coefficient of 1.06×10^9 cells/µg-at $PO_4^{\equiv}$-P (0.94×10^{-15} g-at P/cell) was determined from a regression of cell numbers against phosphate-P concentration (Fig. 3). The intercept indicates the number of cells produced by the control - no added phosphate and is indicative of the level of phosphorus contamination, perhaps from the reagent grade chemicals.

Fuhs (1969) demonstrated that a minimum of 0.9×10^{-15} g-at P was required per cell (clone 3H) and determined that this value was unaffected by light over a range of 2000 to 6000 Klux and temperatures over a range of 13.5°C to 24°C. This value compares well with the present yield determination of 0.94×10^{-15} g-at P/cell for clone CN.

Nitrate yield coefficients for the two determinations were 2.22×10^7 and 2.37×10^7 cells/µg-at NO_3-N (Fig. 4). Variability in nitrate yield coefficients is discussed in a later section.

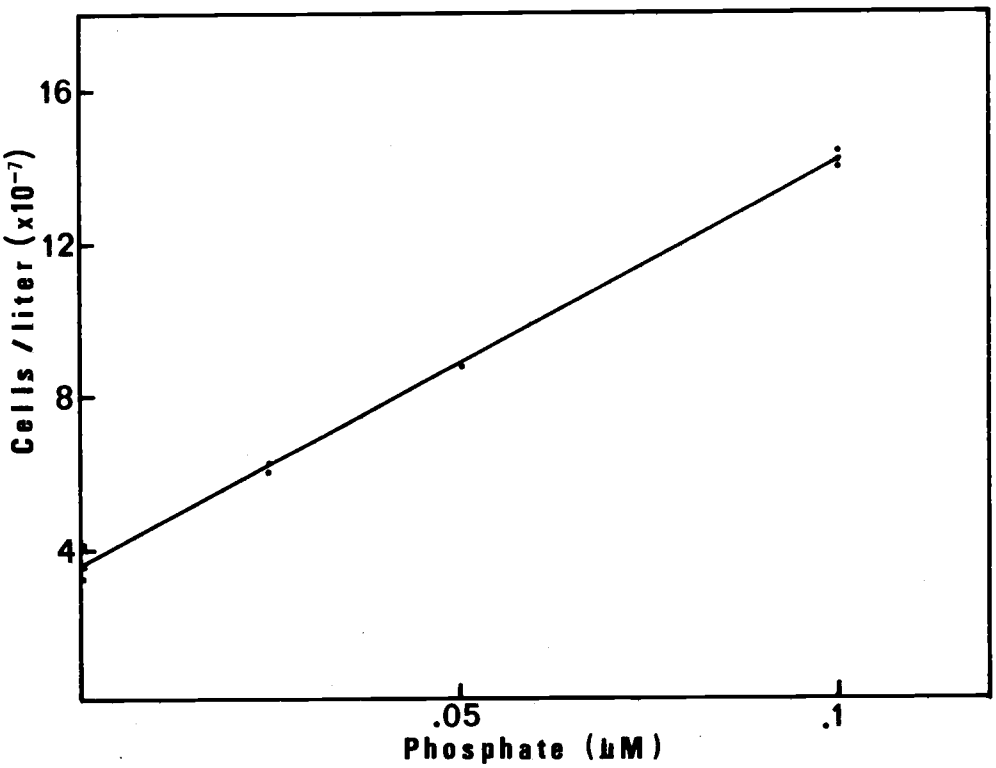
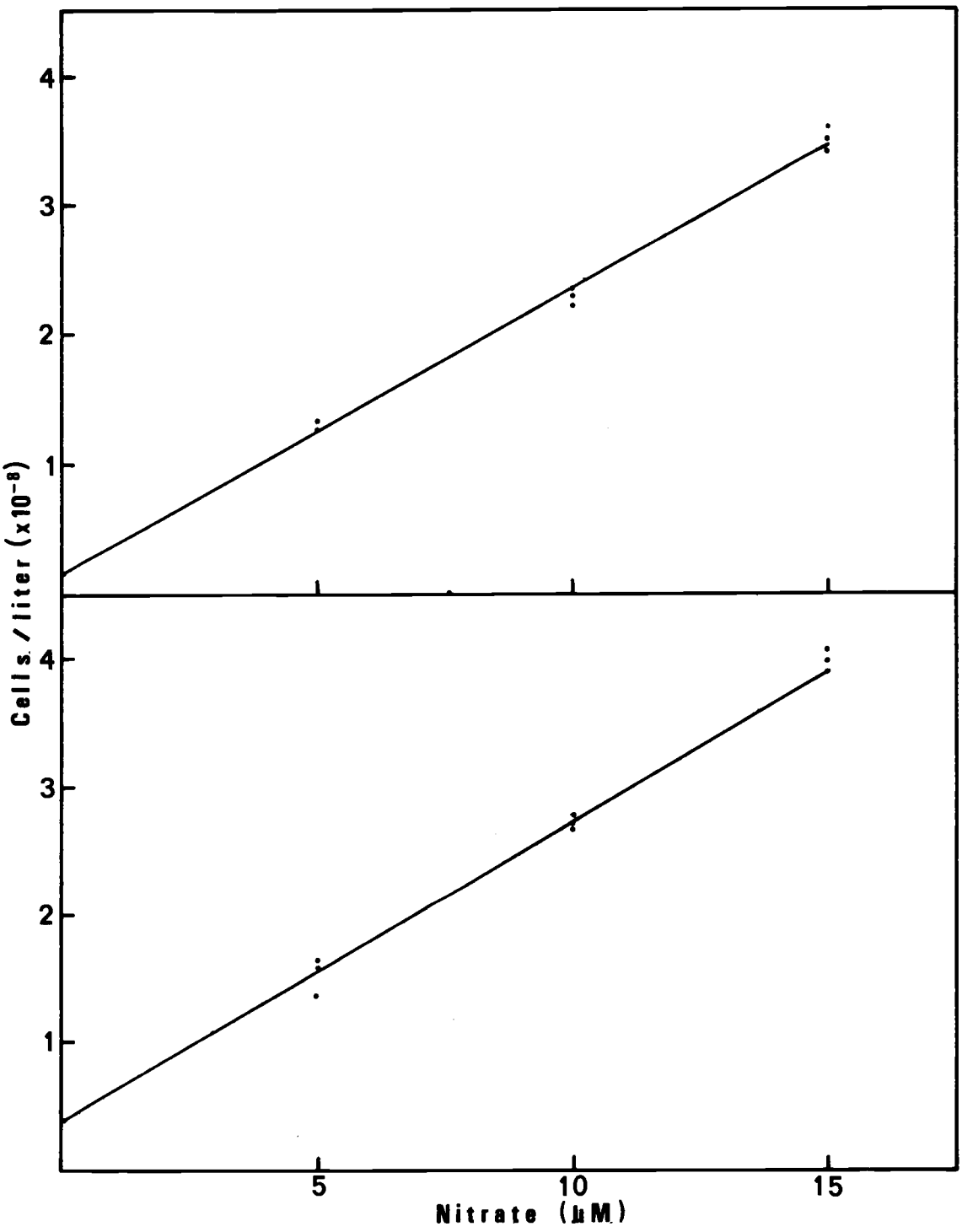


Figure 3. Phosphate-phosphorus yield response of T. pseudonana. Cell numbers = $1.06 \times 10^9 \times m\ PO_4^{\equiv} + 3.54 \times 10^7$; $r \doteq 0.998$.

Figure 4 Nitrate-nitrogen yield response of *T. pseudonana*.

A. Cell numbers = $2.22 \times 10^7 \times \mu m\ NO_3^- + 1.39 \times 10^7$; $r = 0.998$.

B. Cell numbers = $2.37 \times 10^7 \times \mu m\ NO_3^- + 3.42 \times 10^7$; $r = 0.99$.


Cells / liter (x10⁻⁸)
4
3
2
1
4
3
2
1
5
10
15
Nitrate (µM)

A knowledge of yield coefficients of test organisms for various growth requiring elements can be used as a check upon the internal consistency of the test. Chemical analyses of test waters indicate the concentration of elements in question. Growth experiments spiked and unspiked indicate the biomass yield of the test water as well as the element suspected to be in short supply. A calculation based upon yield coefficient and elemental concentration and comparison with unspiked treatment would serve as a check upon the conclusion drawn from information obtained from spiking experiments. Also, knowledge of yield coefficients allows for direct estimation of yield potential of a given water based upon elemental concentrations.

Growth response on artificial sea water. The maximum specific growth rate observed for clone CN grown on enriched Lyman and Fleming's ASW was 2.49 divisions per day (σ =0.19) and on enriched Burkholder's ASW was 2.19 divisions per day (σ =0.15). Both these growth constants are lower than those determined for enriched natural sea water under similar illumination (2.75 divisions per day, σ =0.09) however, they are not so low as to preclude the use of an artificial sea water medium for maintenance of cultures and for preparation of cultures for inocula minimizing nutrient carryover.

The algal bioassay: capabilities and limitations. In the absence of growth inhibiting substances, it is possible to approximate the yield potential of a body of water if a sufficient number of chemical parameters are measured, particularly those whose concentrations are suspected to limit biomass yield. Conversion factors

are necessary which relate measured parameters to the appropriate biomass parameter. Although in most cases a reasonable estimate of expected biomass can be obtained, there is no assurance that all the necessary parameters have been measured. Perhaps a limiting factor had not been measured. Additionally, there would be no indication of whether there were any factors present which either inhibited or completely halted growth. Thus, in order to determine the potential fertility (growth capacity) of a system, a batch algal assay test has been developed (Anon, 1971) for freshwater environments. Others have developed algal bioassays along similar lines for both fresh water and marine systems (Skulberg, 1967; Smayda, 1970).

The results from an ideal algal bioassay might answer the following questions about the natural system to which it is applied: 1) what is the potential for algal biomass production within the system? 2) how fast does the algal biomass proliferate? 3) which species are responsible for the production? and 4) what limits or controls the biomass production, particularly which nutrient or nutrients limit the rate of production and yield, if such nutrient limitation exists? To what extent is an algal bioassay capable of answering these questions?

Total dry algal biomass produced by an aquatic system is composed by weight of primarily carbon, oxygen, nitrogen, sulfur, and phosphorus, and in the case of diatoms, silicon. The remainder of the required elements composes an insignificant amount of the biomass by weight. The major elements occur in a reasonably

constant ratio of one to another independent of algal species composition. The ratios may alter depending upon the nutritional status of the algae, however, different species seem to respond in a similar manner to similar types of nutrient deficiency (Fleming, 1940; Scott, 1943; Spoehr and Milner, 1949; Knauss and Porter, 1953; Redfield, 1958; Strickland, 1960; Lewin, 1962; Redfield et al., 1963).

Therefore, in terms of potential biomass production, a test organism yield would in general be representative of the biomass produced by a mixed population of algae subjected to similar conditions. However, if conditions exist in the natural body of water which limit the biomass production of the test organism yet allow other species to proliferate, results of the test would be inapplicable. Such conditions often exist in lakes where inorganic nitrogen has been depleted, allowing only those algae capable of nitrogen fixation to continue production. Situations may exist where a depletion of silicon preferentially allows non-silicon requiring algae to proliferate. Similarly, the organic composition of natural waters may exert a selective influence over the organisms present--if the test organism where incapable of utilizing the available nutrient sources, while other species were capable of using these same nutrient sources, misleading results would be obtained, as might be observed in situations where presence or absence of vitamins or other organic factors

may play a role in the selection of species (Provasoli, 1963). Additionally, trace substances may be present in the water which selectively inhibit one species or another (Moser, et al., 1972).

Some answers to these criticisms exist--necessary nutrients peculiar to the given test organism may be provided. For example, silicon spikes may be added when using diatoms as test organisms, or specific vitamin requirements may be added for those requiring vitamins. It becomes more difficult if faced with an organic milieu for the identity of the selective factor may be unknown and difficult to determine. Test algae from several different phyla may be used in the hopes that the most representative responses may be covered.

Since the rate of growth of algae in natural systems is controlled by so many factors (such as species present, ambient light, temperature, nutrient levels, grazing and turbulence) not duplicated under the standard laboratory conditions, it should not be expected that algal assay growth rates be comparable in any way to those in natural systems. Nor should it be expected that the algal assay provide information concerning the factors which control the rate of growth of natural assemblages of algae. It can provide information concerning which nutrient (or nutrients) may be in short supply and therefore be expected to exert control over the rate of growth without specifying what that control might be. So often, the rate of growth is controlled by a succession of factors, or even a superposition of factors, such as light

and temperature, or light, temperature and nutrient concentration, that to duplicate such control would add such complexity to a standardized assay system so as to render it overly burdensome.

Spatial and temporal environmental heterogeneity complicate the issue, in that a complete characterization of the aquatic system may require repeated tests, the frequency of which would be dictated by the frequency of change in the environment. Similarly, spatial variation would dictate the location of sampling sites.

Finally, the algal assay system uses selected species as test organisms, and therefore is not designed to determine what causes the selection of one species over another. Indeed, in many cases the test organism may not be one of the species present in the test water.

In summary, the strength of the bioassay is its potential ability to: 1) determine whether factors are present which restrict the specific growth rate of the test algae, and therefore may inhibit the growth of the natural assemblage, 2) identify nutrients which may limit biomass production if nutrient limitation is present, and 3) approximate the natural algal biomass yield expected in the absence of such loss factors as sinking, grazing, and turbulence.

Conclusions

McGauhey, et al., (1968) summarize the characteristics which an algal assay test organism should display to the greatest extent possible. These characteristics include: 1) broad nutrient response, 2) distinctive shape, 3) uniform size, 4) clear cellular division, 5) nonattachment to glass, 6) good suspension properties, 7) non-clumping, 8) ease of laboratory culture, 9) nonauxotrophic nature, and 10) lack of production of autotoxins. Additionally, an estuarine test organism should exhibit a wide salinity tolerance.

The morphological and physiological characteristics of I. pseudonana seem to be well suited for the selection of this organism as a test organism for the bioassay of estuarine waters. Its single cell characteristic allows for rapid electronic counting. Its rapid growth rate and suspension characteristics enable bioassays to be performed over reasonable lengths of time. Its broad salinity response suits it well to test estuarine waters with a minimum of manipulation. There is a considerable amount of information concerning its physiological characteristics, particularly with respect to phosphorus, and with this study, with respect to nitrogen, the two elements for which estuarine waters will probably be initially bioassayed and which probably are the elements likely to be responsible for nutrient limitation when nutrient limitation occurs. I. pseudonana grows well over a broad range of nutrient concentrations, particularly nitrogen and phosphorus whose concentration in f/2 enriched media are as high as to be expected in potentially

eutrophic estuarine waters. Fuhs (1969) and the present study indicate that this diatom grows well at low concentrations of nitrogen and phosphorus. T. pseudonana utilizes a variety of nitrogen sources including NO_3^-, NO_2^-, NH_3^+, urea, and perhaps amino acids (Guillard, 1963). There has been no evidence to suggest that T. pseudonana produces autotoxins.

It is therefore suggested that this diatom species be used as a test species for the bioassay of estuarine waters.

SOME GROWTH AND UPTAKE CHARACTERISTICS OF *THALASSIOSIRA PSEUDONANA* HASLE AND HEIMDAL AT LOW AND LIMITING CONCENTRATIONS OF NITRATE-NITROGEN

Introduction

Nitrogen has been identified as one of the elements whose concentration in marine waters is most commonly associated with limiting both the rate of production and ultimate biomass yield of marine phytoplankton (Ryther and Dunstan, 1970; Thomas, 1970; Glooshenko and Curl, 1970; and others). Thus, in order to understand the nature of phytoplanktonic growth it is necessary in part to investigate the response of phytoplankton to low and limiting levels of environmental nitrogen.

The response of algae to low levels of nitrogen can be considered as either an uptake (or absorption) response or as a growth response. The former refers to the nature of removal of nitrogenous compounds from the environment and the latter to the nature of proliferation of biomass commonly measured as cell carbon, cell numbers, cell nitrogen, or cell chlorophyll *a*.

Ketchum (1939 a, b) first and others many years later (Dugdale, 1967; Eppley and Coatsworth, 1968; Eppley, Rogers, and McCarthy, 1969; MacIsaac and Dugdale, 1969; Carpenter and Guillard, 1971; and McCarthy, 1972) demonstrated that the relationship between the rate of absorption of various nitrogenous compounds (nitrate, nitrite, ammonia, and urea) by phytoplankton and the concentration of these ions in the environment

could be described by an expression similar to the Langmuir adsorption isotherm or the Michaelis-Menten expression for saturation enzyme kinetics. This latter expression relates the specific uptake rate to the nutrient concentration in the following manner; $v = \frac{Vmax\ S}{K_s + S}$, where v is the specific rate of uptake of nutrient, Vmax the maximum specific rate of uptake of the nutrient, S the nutrient concentration, and K_s the concentration of nutrient at which v is 1/2 Vmax.

It has been demonstrated that phytoplankton associated with environments whose concentrations of nitrogen are naturally low (oceanic environment) exhibit low K_s (nitrate) values (on the order of <0.2 µg-at N/l) while phytoplankton associated with environments whose concentrations of nitrogen are generally high (coastal and upwelling regions) exhibit higher K_s (nitrate) values (>1.0 µg-at N/l) (MacIsaac and Dugdale, 1969). It has also been suggested that the succession of species in an environment of declining nitrogen concentration may be controlled by the ability of succeeding species to remove nitrogen at lower and lower concentrations and therefore exhibit decreasingly lower K_s values (Eppley and Coatsworth, 1968).

The relationship between growth response and concentration of nitrogenous nutrient is less clearly understood. Ketchum (1939 b) was unable to detect a change in specific growth rate (measured as changes in cell numbers with time) over the range of nitrate concentrations used to demonstrate the control of nitrate over the rate of absorption. Others (Eppley and Thomas, 1969; Thomas, 1970)

have demonstrated that the specific growth rate (measured by changes in cell number by the former and by changes in fluorescence by the latter) could be adequately related to the concentration of nitrogen in the medium by the hyperbolic expression.

Unable to demonstrate a similar relationship utilizing continuous flow techniques, Caperon (1965, 1967) and Williams (1965, 1967, 1970) postulated that the specific growth rate (measured as changes in cell number with time) is not directly a function of the limiting concentration of nitrogen in the medium but is rather related to an internal store of nitrogen. Others suggest that phytoplankton may exist in various states of nutrient deficiency and that the specific rate of growth may be related to the state nutrient deficiency, or physiological state of the population (Fitzgerald, 1968; Thomas, 1970; and Morris, et al., 1971; Caperon and Meyer, 1972 a, b).

The purpose of this investigation was to examine in further detail the nature of phytoplankton growth response to low and limiting levels of nitrate-nitrogen using both batch and continuous flow techniques. Several hypotheses may be advanced to describe the relationship between specific growth rate and limiting concentrations of nitrogenous nutrients:

1) the specific growth rate is related to the concentration of nitrogenous nutrient in the medium and this relationship can be adequately described by the hyperbolic expression,

2) the specific growth rate is related to the concentration of nitrogenous nutrient in the medium in a manner not best expressed by the hyperbolic expression, or

3) the specific growth rate is not related to the concentration of nitrogenous nutrient in the medium but rather to the physiological state of the population which in turn is a function of nitrogen nutrition.

The results of this investigation are discussed with reference to the control which low concentrations of nitrogen exert over the growth of marine phytoplankton in both laboratory and natural situations.

Materials and Methods

Unialgal cultures of Thalassiosira pseudonana Hasle and Heimdal (clone CN) obtained from Dr. Paul Hargraves, University of Rhode Island, were used as the experimental organism for these studies.

Medium preparation. Nitrogen free water was prepared in the following manner. Water collected off the Oregon coast (>45 miles) was filtered through 0.45 μ Millipore^{R} filters, adjusted to 20°/$_{oo}$ with double distilled water, enriched with phosphate and iron (Guillard and Ryther, 1962) inoculated with a small population of T. pseudonana and incubated under cool white fluorescent light (1800 μw/cm^2) at 22.5°C until maximum density was obtained. The culture was then filtered through 0.45 μ Millipore^{R} filter. The filtrate was treated with activated charcoal (1 g/l) for one day, and filtered again. Two aliquots were removed, one of which was enriched with f/2 (Guillard and Ryther, 1962) less nitrate and the other of which was enriched with f/2, in order to test the efficiency of nitrogen removal. A lack of growth in the aliquot without nitrate added indicated that nitrogen removal was sufficient. The medium was then autoclaved at 15 psi for 15 minutes. Nutrients (Guillard and Ryther's f/2, except nitrate was added to obtain a desired concentration) were filtered sterilized through 0.22 μ Millipore^{R} filter and added asceptically. For experiments utilizing radiocarbon, a $H^{14}CO_3^-$, solution was added in a similar manner to produce an activity in the medium of about 5 μ ci/l.

Measure of biomass. Cell numbers and total cell volume were determined using either a Model B or ZB Coulter Counter with mean cell volume computer attached. For size distribution determinations a Model J plotter was used connected to the Model B Coulter Counter. The mean cell volume computers were calibrated using latex spherical particles whose diameter was 3.49 μ. Particulate carbon was determined by calculating a conversion factor which would relate ^{14}C activity of filtered mmaterial to particulate carbon measurements made with a Hewlett-Packard Carbon-Hydrogen-Nitrogen analyzer. Measurement of populations of low biomass could thus be obtained without filtering large quantities of media.

A Turner Fluorometer was used to measure fluorescence of suspended algal cells as an indicator of chlorophyll concentration. The instrument was not calibrated. The intent of the measurement was to determine relative changes in chlorophyll. The fluorescence of either a suspension of plant cells or an extract of chlorophyll a with a suitable solvent has been used to measure the concentration of chlorophyll a present (Lorenzen, 1966). The concentration of chlorophyll a has often been used as a measure of plant biomass (Hansmann, 1973) hence observations of its changes in relation to changes in other biomass measures and in relation to nutrient concentration were desirable.

Nitrate analyses were performed according to the method of Wood et al., (1967) as updated by Strickland and Parsons (1968).

Batch growth experiments. Batch growth experiments were conducted under three general regimes in order to determine the relationship between the decline in nitrate concentration and the increase in the various biomass parameters. Three experiments were conducted at light saturation (3900 $\mu w/cm^2$ cool white fluorescent light), one at a low growth restricting intensity (550 $\mu w/cm^2$), and two using a light (3900 $\mu w/cm^2$)-dark cycle. The inception of the dark phase depended upon the stage of growth of the population.

Media prepared as above were inoculated with a population of cells grown such that their yield would be nitrogen limited in order to prevent nutrient carryover. Initial populations were about 100 cells/ml and initial nitrate concentrations were about 10 µg-at/l (measured for each experiment). Erhlenmeyer flasks varying in size from one to three liters were used as culture flasks. Experiments were carried out at 22.5°C (±1.0°C). Measurements of biomass and nitrate-nitrogen concentration were obtained at frequent intervals; the interval between samples depended upon the amount of growth activity present.

Uptake experiments. Five uptake experiments were performed using populations harvested from 1) a 10 µg-at NO_3^- -N batch culture as soon as nitrogen was depleted but cell division was still vigorous (uptake experiment #1, UE1), 2) a 5 µg-at NO_3^- -N batch culture which had depleted nitrogen but had not quite reached stationary phase (uptake experiment #2, UE2), 3) a continuous flow system whose dilution rate was 0.079 hr^{-1} (uptake experiment #3, UE3), 4) a continuous

flow system whose dilution rate was 0.04 hr^{-1} (uptake experiment #4, UE4), and 5) the 12 hour overflow from the 0.079 hr^{-1} continuous flow system representing a population of mixed physiological state (uptake experiment #5, UE5). Uptake experiments were carried out as described by Eppley and Coatsworth (1968) with the exception that at low nitrate concentrations (<1.0 µg-at NO_3^--N/l) nonlinearities appeared (Fig. 5). In these cases, the mean concentration between each time of sampling was used as the concentration; the slope of the line between the two sampling times was used as the rate. The rate of uptake vs. concentration curves were transformed according to the method described by Dowd and Riggs (1965) such that the intercept would be V_{max} and the slope $-K_s$.

Continuous flow experiments. Continuous flow experiments were designed in order to determine 1) the relationship between specific growth rate and limiting nutrient concentration, 2) the variation of steady state biomass with variation in flow rate (i.e. with variation in specific growth rate), and 3) the nature of the approach to steady state.

For the experiments designed to observe the approach to steady state, a small initial inoculum was used so that the initial concentration of cells was less than 50/ml. Pumping commenced immediately at the desired flow rate. Samples were periodically withdrawn to observe the increase in population biomass with concomitant decrease in nitrate-nitrogen concentration.

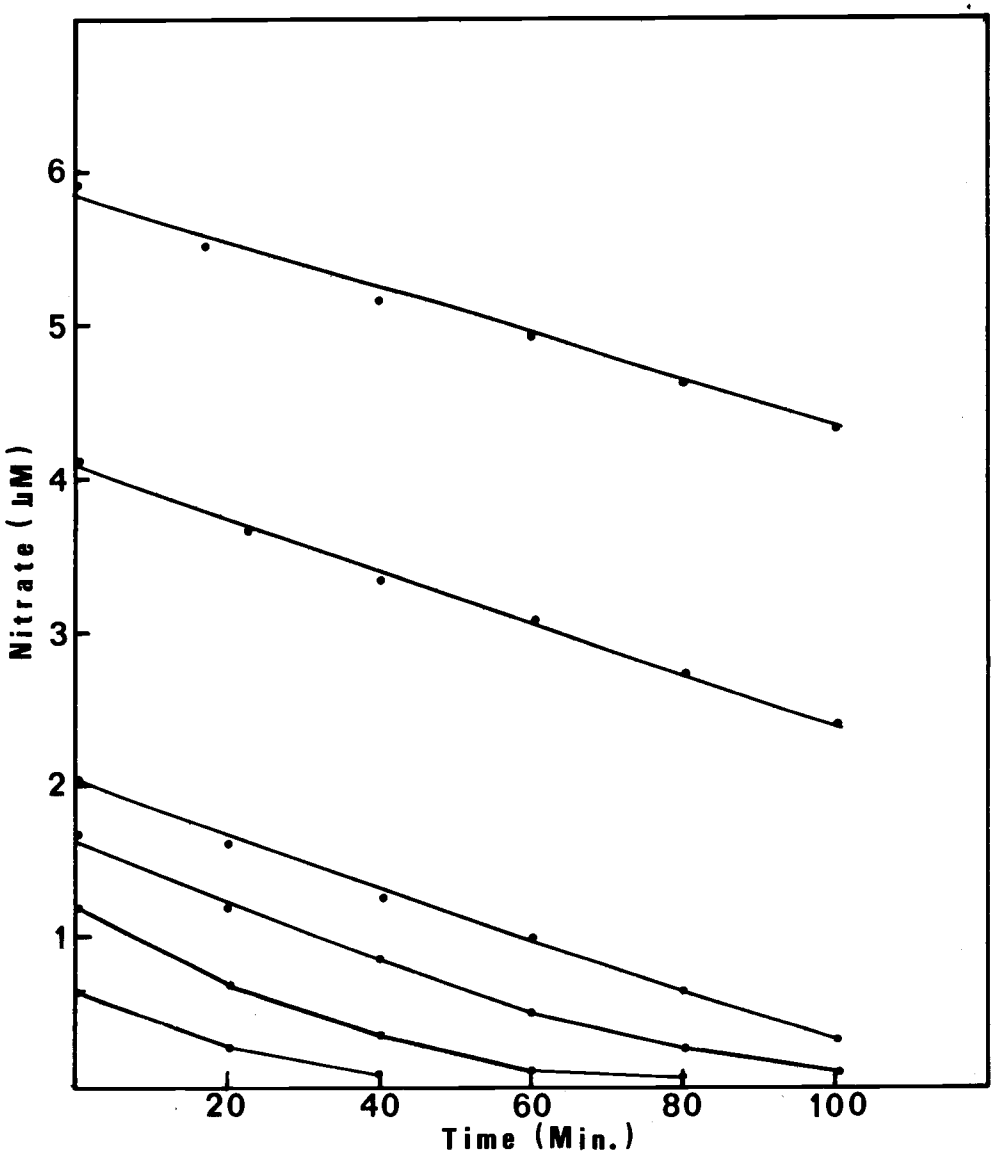
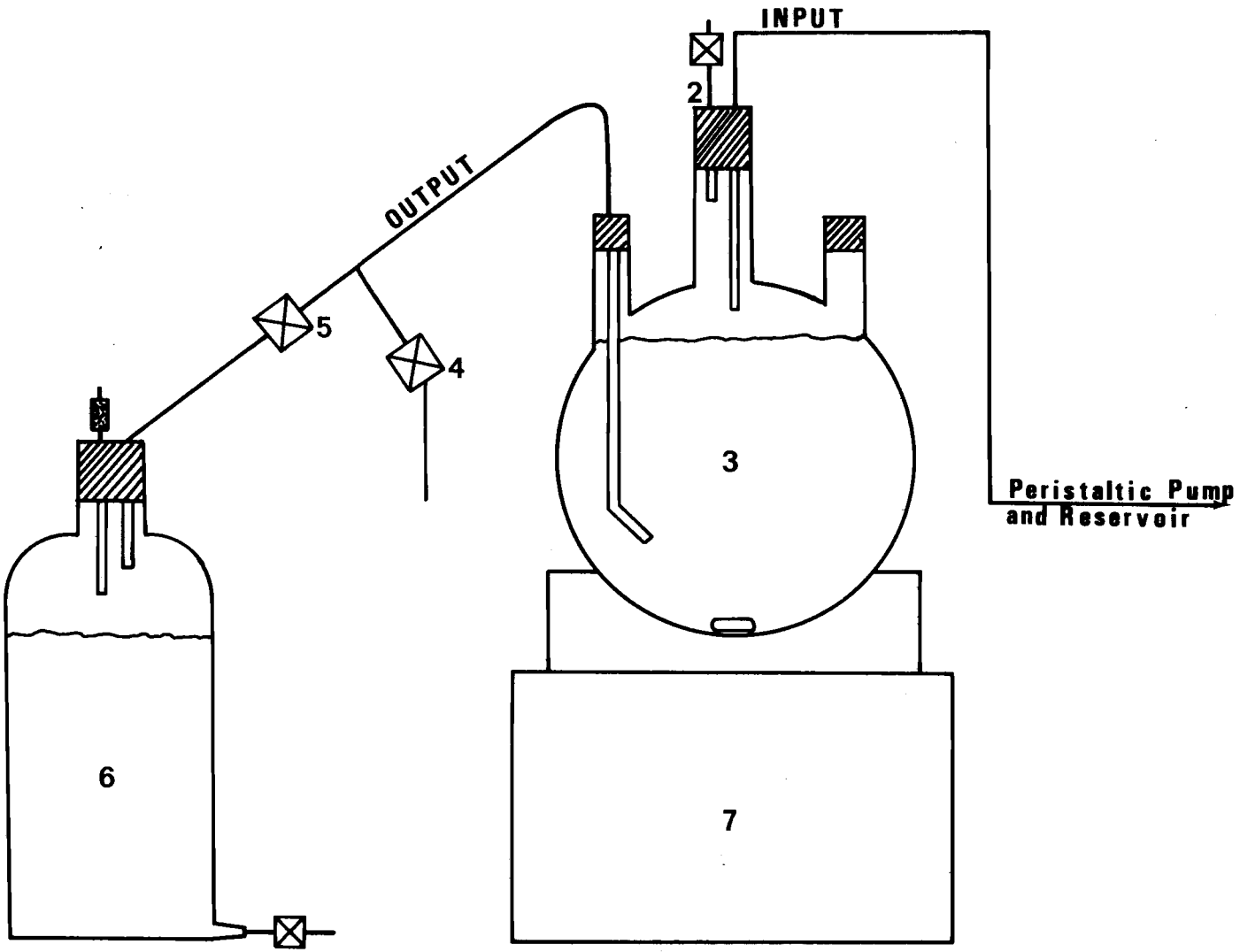


Figure 5. Removal of nitrate by T. pseudonana at differing initial concentrations of nitrate. Population density was 10^8 cells/l.

For the experiments designed to observe the steady state populations, an initial inoculum of less than 50 cells/ml was allowed to grow until a density of approximately 10,000 cells/ml was obtained at which time pumping commenced. Steady state was assumed to be achieved when population biomass remained constant for at least three days, at which time the flow rate was changed or the experiment terminated depending upon the amount of media remaining in the reservoir. Biomass measurement and nitrate concentration were determined at each steady state level. No more than two steady state observations were obtained from a given experimental run.

Continuous flow system. Either 500 or 2000 ml round bottomed flasks were used as growth chambers (Fig. 6); the 500 ml flasks were used for the experiments involving high specific growth rates. The growth chambers were supplied with media from five gallon glass jugs through polyurethane tubing (MP 1485, 1/16" I.D., 1/8" O.D.; Molded Products Co., Easthampton, Mass. 01027). Flow rates were regulated with a Buchler Polystaltic^{R} pump (model #2-6100, Buchler Instruments, Inc., 1327 16th St., Ft. Lee, N.J.). Overflow was collected in aspirator bottles and the volume measured to determine rates of flow. Samples were collected by siphoning the desired volume into a graduated cylinder. The media and cells so removed were replaced by rapidly pumping in fresh media. Sample volumes ranged from 30-50 mls depending upon the analyses to be performed.

Figure 6. Apparatus used for continuous culture of T pseudonana. 1. Inoculation port. 2. Equilibration valve open while siphoning media for biomass and nutrient measurements; closed during normal operation. 3. Growth chamber. 4. Sampling valve open during sampling; closed during normal operation. 5. Outlet valve closed during sampling; open during normal operation. 6. Overflow bottle. 7. Magnetic stirrer and stand.


INPUT
2
OUTPUT
5
4
3
Peristaltic Pump
and Reservoir
6
7

Stability of ^{14}C activity in batch and continuous flow systems. The total activity (media and cells) of ^{14}C in batch experiments changed slightly with time, the loss in activity amounting to about 3-5% per day, a loss relatively insignificant compared with the increases in biomass of rapidly growing populations. The loss of activity occurred because the batch systems were open to the atmosphere and rigorous mixing prior to sampling undoubtedly hastened the loss of activity to the atmosphere.

The activity of ^{14}C in the reservoirs remained relatively constant throughout the experimental period; variation over the experiments was about ±2% with no downward trend in activity. Similarly, total activity (media and cells) of samples withdrawn from continuous flow culture vessel showed daily variation of ±2% with no evident trend.

Liquid scintillation efficiency determinations. Efficiencies with which ^{14}C labelled samples were counted were determined in the following manner. A culture of T. pseudonana was grown on enriched media with H $^{14}CO_3^-$ present. The yield was limited by the addition of only 10 µg-at NO_3^- -N/l as nitrogen source. After stationary phase had been reached, four replicates of 10, 20, 30, 40, and 50 mls each were filtered onto both 25 and 45mm 0.45 µ Millipore^R membrane filters. The filters were dried at 50°C for 24 hours. Two of the four replicates of each volume were dissolved in 100% reagent grade acetone in scintillation vials. The 25mm filters required 1.5 mls for complete dissolution, and the 45mm filters required 2.5 mls.

After dissolution of membrane filters (approximately two hours), Aquasol^{R} was added to each vial to bring the volume to 14 mls. Diatom material did not dissolve completely but appeared as flakes which gradually settled to the bottom of the counting vial. Samples were twice counted for five minutes each in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3375 optimized for ^{14}C counting. The samples were then spiked with 10 µl ^{14}C toluene standard (4.35×10^{6} cpm/ml) and recounted. Efficiencies calculated from the spike were then applied to the diatom counts such that an estimate of the activity of the diatoms present was determined.

The remaining pair of filters of each volume were then counted in a toluene-butyl-PBD (0.5% w/v) cocktail. The efficiencies with which these were counted were calculated based upon the activities of the diatoms determined previously. These samples were then spiked with 10 µl ^{14}C toluene standard, recounted, and efficiencies again determined and applied to the counted material.

Efficiencies with which filtered samples were counted in toluene-butyl-PBD determined by the acetone dissolution method were 96.3% (σ = 1.3%, n = 10) for 25mm filters and 96.1% (σ = 1.2%, n = 8) for 45mm filters. Efficiencies determined by spiking with standard ^{14}C were 93.6 % (σ = 0.7%, n = 10) for 25mm filters and 96.1% (σ = 1.2%, n = 8) for 45mm filters. There was little evidence for self absorption by filtered material as indicated by the linearity of the relationship between amount filtered and activity (Fig. 7).

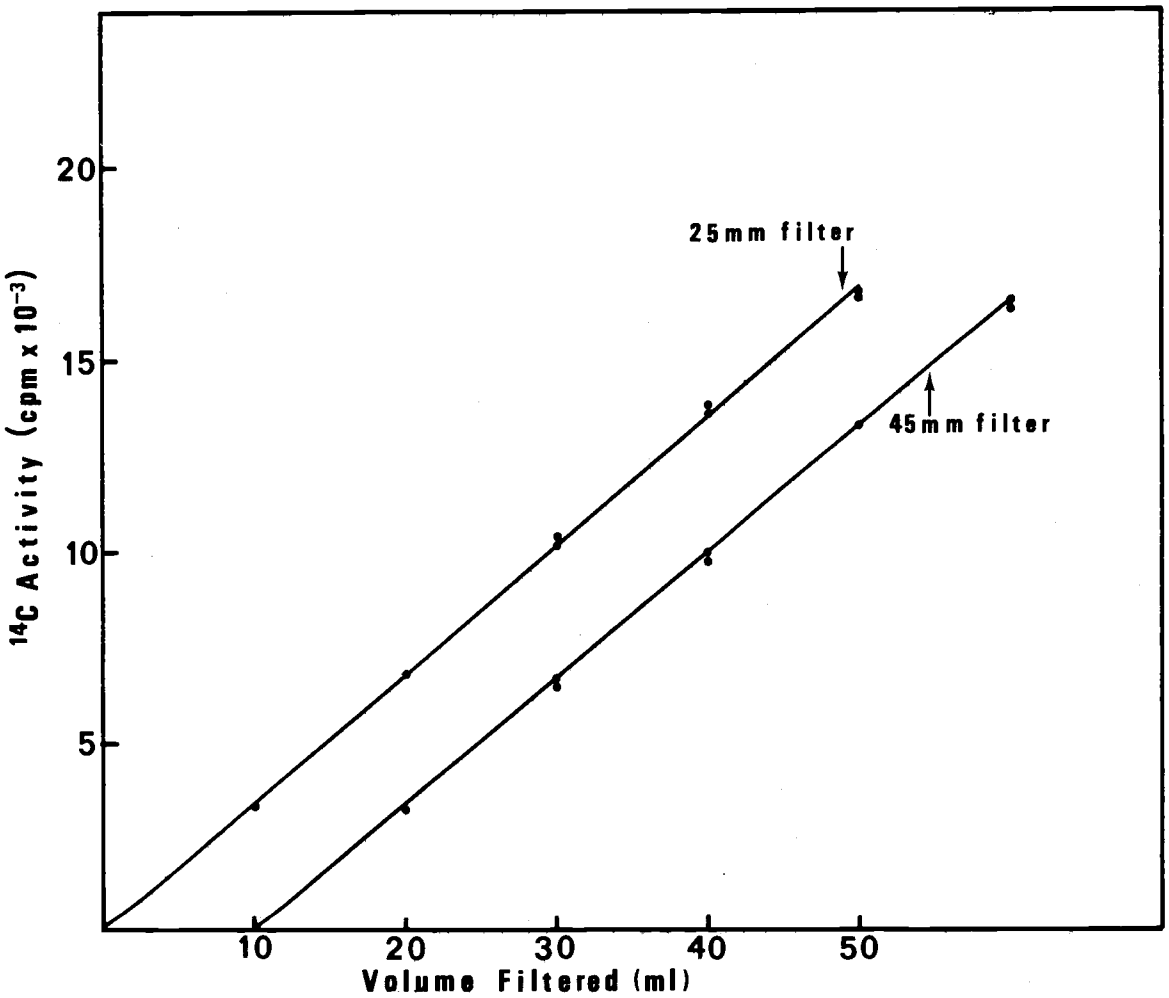


Figure 7. Relationship between ^{14}C activity and volume of culture filtered indicating lack of appreciable self absorption. Axis was shifted to the right for the 45 mm filter experiment.

Since there was little variation in efficiency of counting with increasing thickness of diatom material, an average efficiency of 95.5% was used for unknowns counted in toluene-butyl-PBD.

Results

Batch growth response: saturating light intensity. The growth of T. pseudonana at saturating light intensity followed the typical pattern of algal growth in batch systems. An initial lag phase was followed by an exponential increase in biomass and then a cessation of growth after a critical nutrient had been depleted (Figs. 8, 9, and 10). However, the amount of growth which occurred subsequent to the depletion of the critical nutrient, nitrate, was perhaps somewhat unexpected. Growth measured as increases in cell numbers and total cell volume continued at a rapid rate seemingly initially unaffected by the lack of nitrate in the medium. Stable population densities were achieved at biomass levels approximately three times that at the time of nitrate disappearance (Table 3). The indication is that a healthy rapidly growing population has about three times as much nitrogen associated with it as does one which has reached stationary phase on N-restriction.

The pattern was repeated for changes in particulate carbon where carbon increases continued after nitrate had been depleted. The increase was slightly less than three fold.

Carbon:nitrogen ratios expected to be about 7 by atoms (Redfield 1958, 1965) ranged from about 8 during the exponential phase of growth to about 23 at stationary phase. These ratios were calculated in the following manner: carbon values were determined from a conversion of

Figure 8 Batch response of T. pseudonana. Light saturation experiment 1.

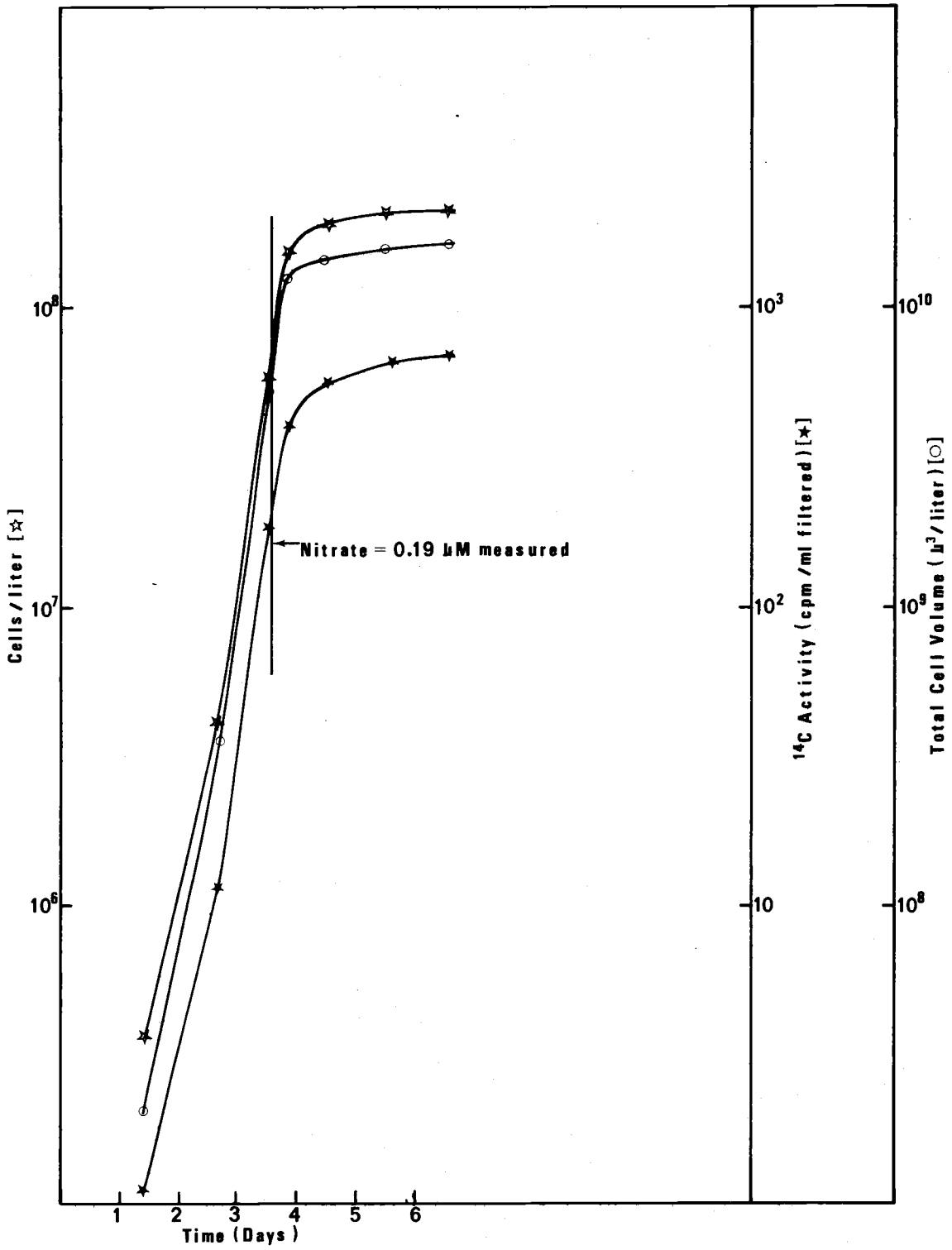

Cells / liter [☆]
10^8
10^7
10^6
Nitrate = 0.19 µM measured
^{14}C Activity (cpm / ml filtered) [★]
10^3
10^2
10
Total Cell Volume (μ^3 / liter) [○]
10^{10}
10^9
10^8
1
2
3
4
5
6
Time (Days)

Figure 9. Batch growth response of T. pseudonana. Light saturation experiment 2.

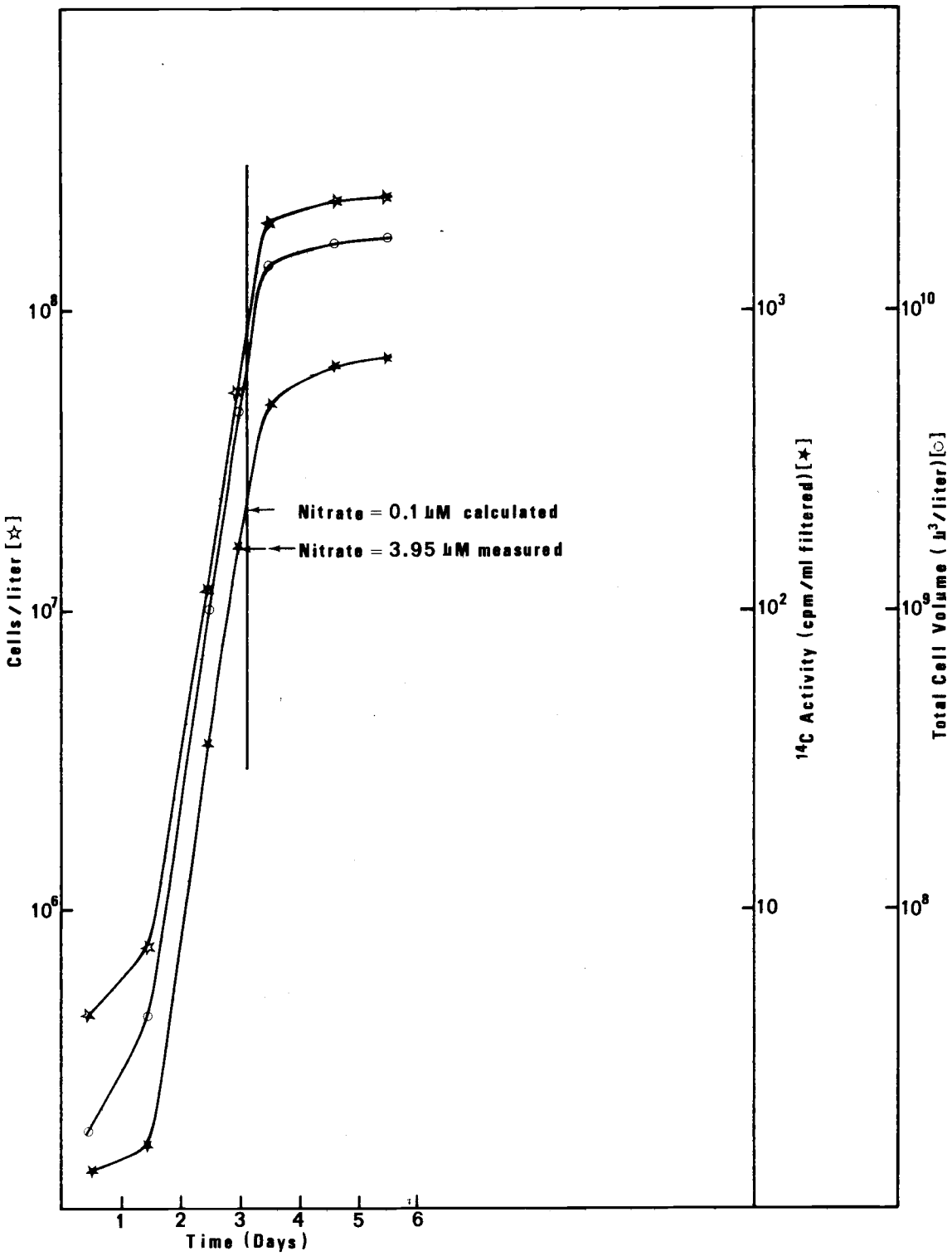

Cells / liter [☆]
10^8
10^7
10^6
Nitrate = 0.1 µM calculated
Nitrate = 3.95 µM measured
10^3
10^2
10
^{14}C Activity (cpm/ml filtered)[★]
10^{10}
10^9
10^8
Total Cell Volume (µ^3/liter)[○]
1
2
3
4
5
6
Time (Days)

Figure 10. Batch growth response of T. pseudonana. Light saturation experiments 1 & 2. Linear presentation demonstrates the considerable growth after nutrient depletion.

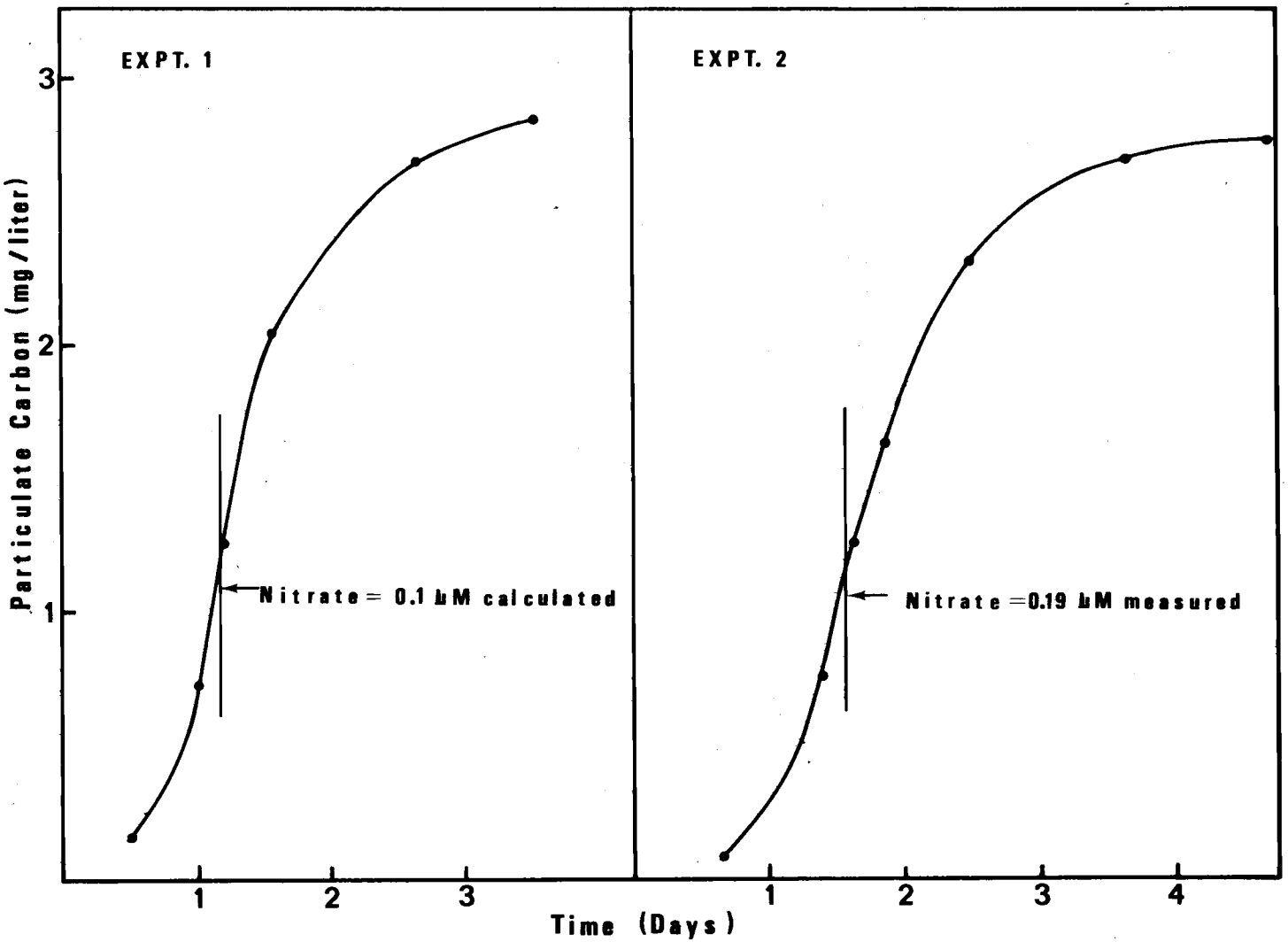

EXPT. 1
EXPT. 2
Particulate Carbon (mg/liter)
3
2
1
Nitrate = 0.1 µM calculated
Nitrate =0.19 µM measured
1
2
3
1
2
3
4
Time (Days)

TABLE 3. SUMMARY OF BATCH GROWTH CHARACTERISTICS OF *T. PSEUDONANA*. LIGHT INTENSITY WAS 3900 $\mu w/cm^2$.

	Experiment 1	Experiment 2
Initial NO_3^- Conc.	9.8 µg-at/l	9.7 µg-at/l
Cell Density at NO_3^- Depletion	7.5×10^4 cells/ml (calculated)	6.3×10^4 cells/ml
Final Cell Density	23.4×10^4 cell/ml 17.2×10^6 μ^3/ml	21.1×10^4 cells/ml 16.2×10^6 μ^3/ml
Growth Constant	0.139 (^{14}C) $hr.^{-1}$ 0.137 (Cell #) 0.139 (Total Cell Volume)	0.144 (^{14}C) 0.137 (Cell #) 0.139 (Total Cell Volume)
C:N Range By Atoms	8.9 - 22.6	8.0 - 22.3
N Measured During Stationary Phase	10.2 µg-at N/l 10.0 9.7 Av = 9.97	10.6 10.5 Av = 10.55

^{14}C activity to particulate carbon; nitrogen values were determined by difference between initial nitrate concentration and measured nitrate concentration at the time of determination. Mass balance comparisons of nitrogen calculated from nitrate removal and that measured with carbon-nitrogen analyser indicated a slightly greater particulate N concentration than that calculated by nitrate removal (Table 3). This would lower slightly carbon:nitrogen ratios calculated above by increasing the nitrogen value. The various parameters associated with these growth experiments are summarized in Table 3.

In another similar experiment, fluorescence changes were monitored (Fig. 11). The increase in fluorescence terminated when nitrate was depleted (measured NO_3^- concentration = 0.15 µg-at/l), however, cell numbers as well as particulate carbon (as ^{14}C) continued to increase, nearly doubling during the next twenty hours.

Batch growth response: low light intensity. The growth of a population grown at a low light intensity (550 µw/cm^2) restricting the growth constant (k = 0.07/hr) was similar to that observed at a high (saturating) light intensity. The initial lag phase was followed by a period of exponential growth during which time nutrients were rapidly consumed. Upon complete removal of critical nutrient (NO_3^-) growth continued, declined, and eventually stabilized (Fig. 12). The nitrogen per cell calculated during exponential growth was similar to the nitrogen requirement of populations grown at saturating intensities (Table 4). The final yield (cell numbers) was slightly greater than two times that at the calculated time of nitrate depletion, whereas, there was an approximately three-fold increase

Figure 11. Batch growth response of T. pseudonana. Light saturation experiment 3.

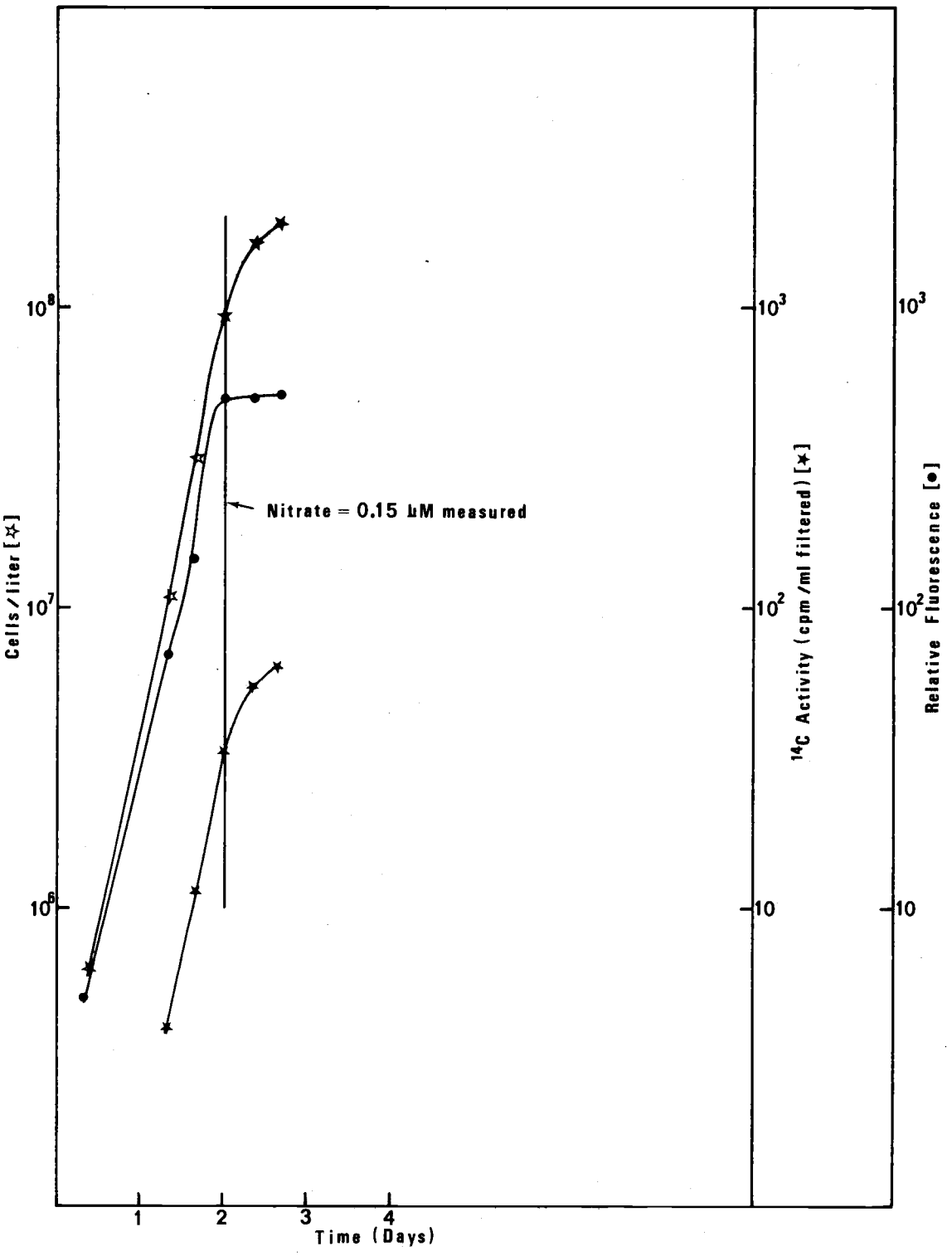

Cells / liter [☆]
10^8
10^7
10^6
Nitrate = 0.15 µM measured
10^3
10^2
10
^{14}C Activity (cpm / ml filtered) [★]
10^3
10^2
10
Relative Fluorescence [●]
1
2
3
4
Time (Days)

Figure 12. Batch growth response of *T. pseudonana* at low light intensity.

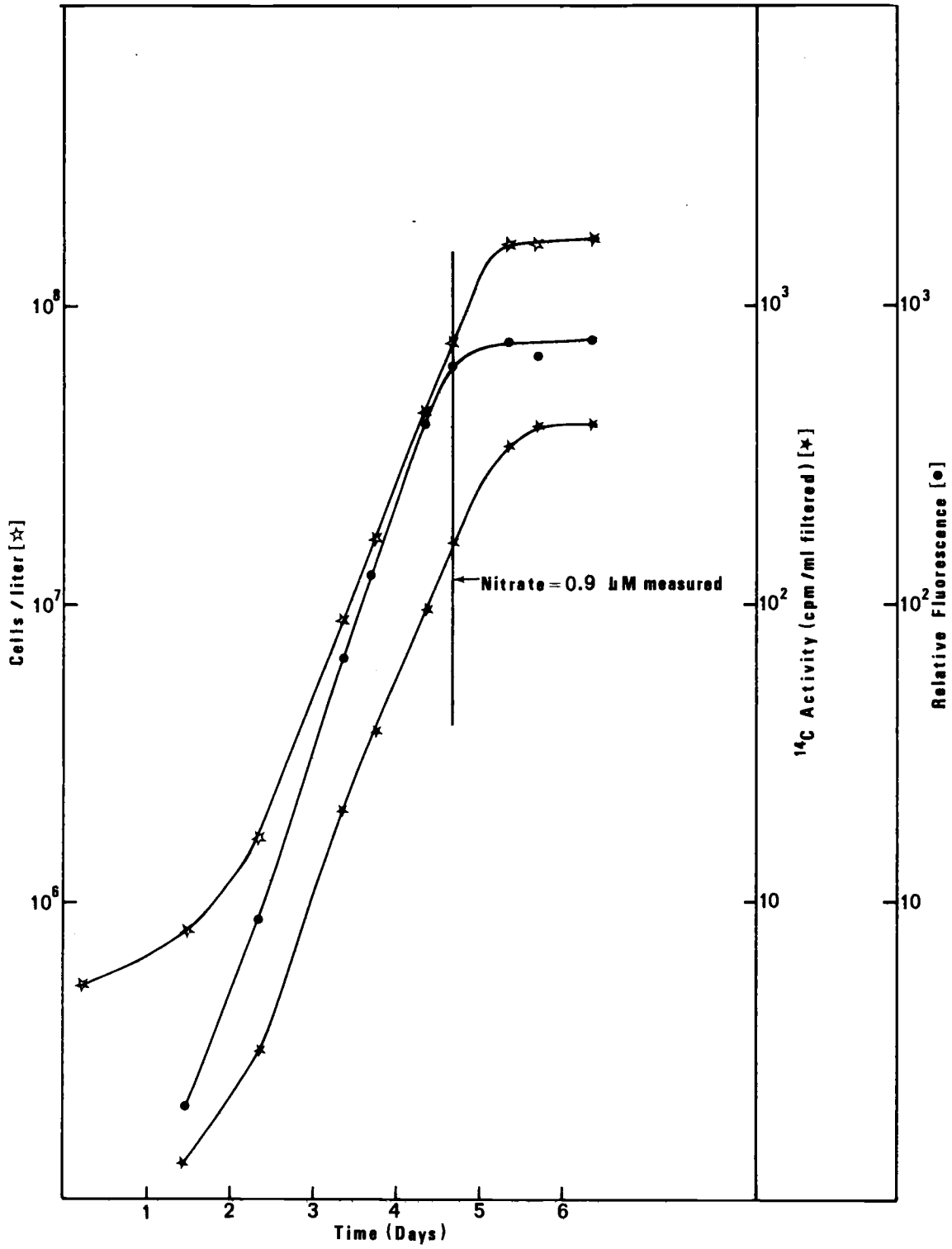

Cells / liter [☆]
10^8
10^7
10^6
Nitrate = 0.9 µM measured
^{14}C Activity (cpm/ml filtered) [★]
10^3
10^2
10
Relative Fluorescence [●]
10^3
10^2
10
1
2
3
4
5
6
Time (Days)

TABLE 4. YIELD OF HEALTHY T. PSEUDONANA GROWN AT A LIGHT INTENSITY OF 550 μw/cm^2.

DATE/TIME	Cells per ml	$[NO_3^-]$ μg-at/l	Δ NO_3^-	Δ Cell #	Cells/μg-at NO_3^- -N
3/13 0800	1670	11.2			
3/14 0800	8970	10.5			
			1.0	7730	7.730×10^6
3/14 1720	16700	9.5			
			3.7	27500	7.432×10^6
3/15 0800	44200	5.8			
			4.9	29400	6.000×10^6
3/15 1600	73600	0.9			
					7.053×10^6

in cell numbers after nitrogen depletion for populations grown at saturating intensities. Particulate carbon measured as ^{14}C activity increased approximately 2.5 fold after nitrogen depletion.

As observed previously, increase in fluorescence ceased at approximately the time of nitrogen depletion. Its slight increase did not correspond to the increases in cell numbers and particulate carbon (Fig. 12).

Batch growth response: light-dark cycles. The response of various parameters to light-dark regimes is summarized in Figs. 13 and 14. At the inception of a dark regime, the increase in labelled particulate carbon ceased. The concentration of labelled particulate carbon began to decline immediately at a rate of about 2% per hour as a result of a combination of respiratory losses and release of organic material. The labelled carbon specific uptake of nitrate continued in the dark at about 40% of the rate in the light. In both experiments a substantial amount of nitrate was consumed in the dark. Over an eight hour period this amounted to an equivalent of about 40% of the nitrogen biomass present at the inception of the dark period. There was no immediately discernable change in the increase in cell numbers upon the inception of the dark regime.

Both the rate of uptake of carbon and nitrate responded immediately to the initiation of the light phase after eight hours of dark (Fig. 14). Cell division did not respond immediately. However, the population had attained a density of 142,000 cells/ml twelve hours later.

Figure 13. Light-dark batch growth response of T. pseudonana. Experiment 1.

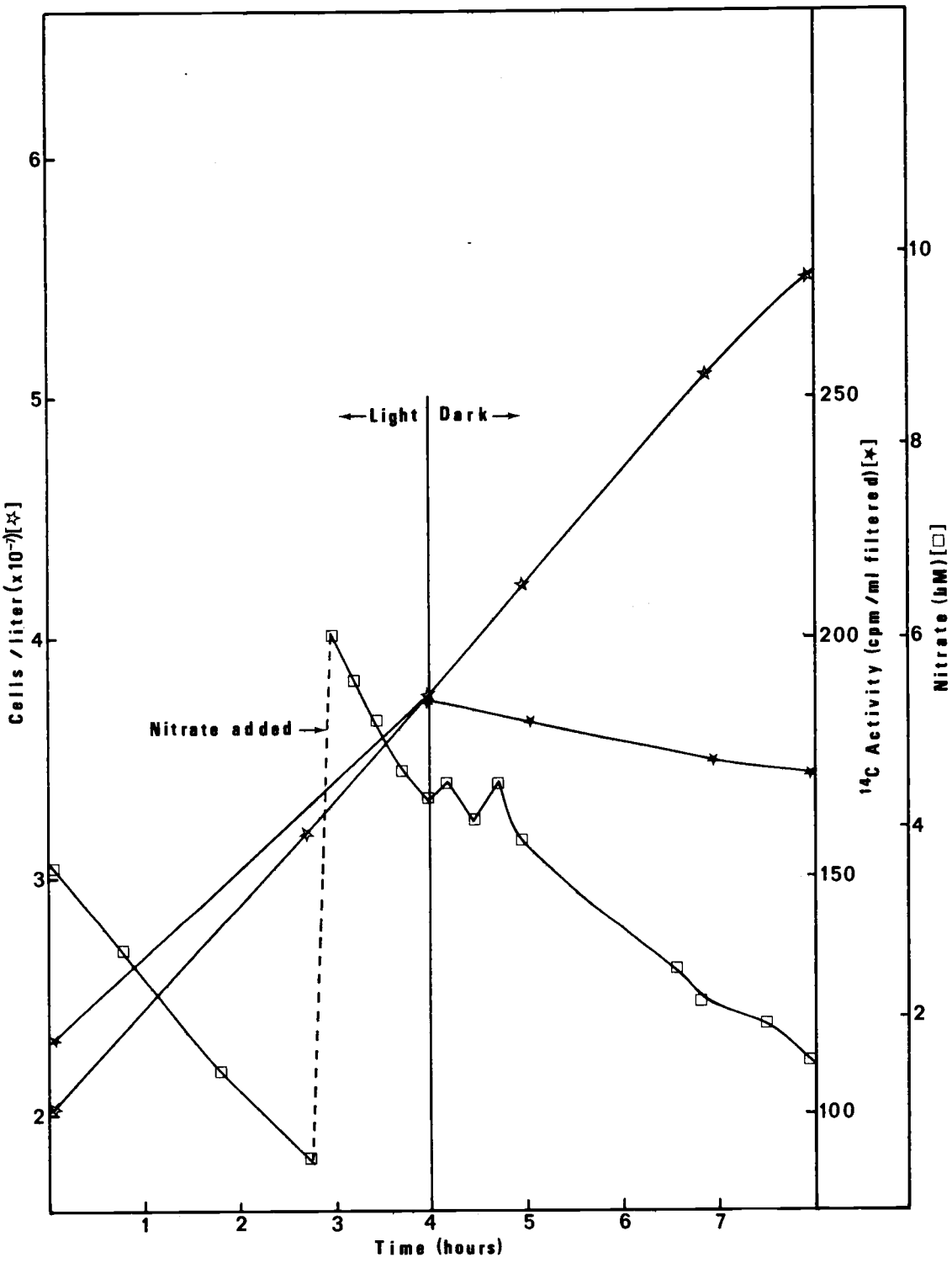
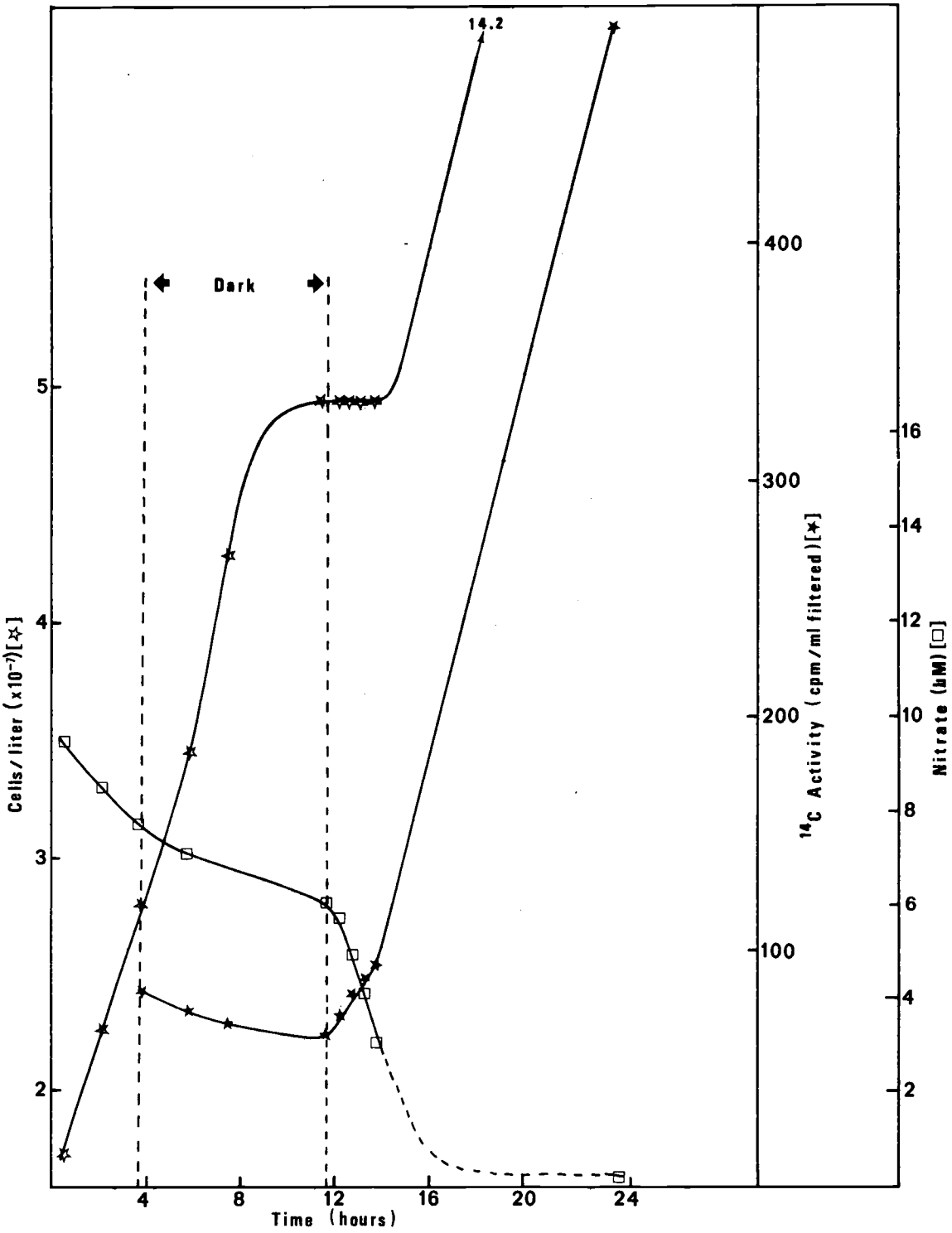

Cells / liter (x10⁻⁷)[☆]
¹⁴C Activity (cpm/ml filtered)[★]
Nitrate (μM)[□]
←Light | Dark→
Nitrate added
Time (hours)
6
5
4
3
2
10
8
6
4
2
250
200
150
100
1
2
3
4
5
6
7

Figure 14. Light-dark batch growth response of *T. pseudonana*. Experiment 2.


14.2
Dark
Cells/liter (x10⁻⁷)[☆]
¹⁴C Activity (cpm/ml filtered)[★]
Nitrate (μM)[□]
Time (hours)
2
3
4
5
100
200
300
400
2
4
6
8
10
12
14
16
4
8
12
16
20
24

Batch growth response: uptake characteristics. In all cases, the uptake curves displayed the hyperbolic characteristic. The rate of uptake was normalized to 10^7 cells (Fig. 15). Values were determined for V_{max} and K_s based upon a mean transformation of the Michaelis-Menten equation such that the y-intercept is V_{max} and the slope $-K_s$ (Fig. 16). The units of V_{max} so determined are μg-at NO_3^- -N/10^7 cells/hr. In order to express this value in terms comparable to a specific growth rate, it was necessary to determine the nitrogen equivalent of 10^7 cells for each situation. These range from 0.90×10^7 to 2.95×10^7 cells/μg-at NO_3^- -N (Table 5) and compare well with yield coefficients determined from other batch and continuous flow experiments. Corrected V_{max} values range from 0.137 hr^{-1} for the population harvested at NO_3^- depletion to 0.334 hr^{-1} for the population harvested just prior to reaching stationary phase. K_s values range from 0.10 to 0.35 μg-at NO_3^- -N/l. Uptake characteristics are summarized in Table 5.

Continuous flow growth response: approach to steady state. For the slow system (dilution rate = 0.02 hr^{-1}) the time course of growth of the approach to steady state was similar to that observed in batch systems approaching stationary phase except that the growth constant was decreased by the dilution rate. Corrected maximum specific growth rates were 0.14 hr^{-1} for changes in cell numbers and particulate ^{14}C activity (Fig. 17). Cell numbers increased three-fold after nitrogen had been depleted while particulate carbon (^{14}C) and total cell volume doubled. Mean cell volume of about 100 μ^3 during rapid growth

Figure 15. Nitrate uptake response of T. pseudonana normalized to 10^7 cells/l. Populations utilized represented a variety of physiological states.

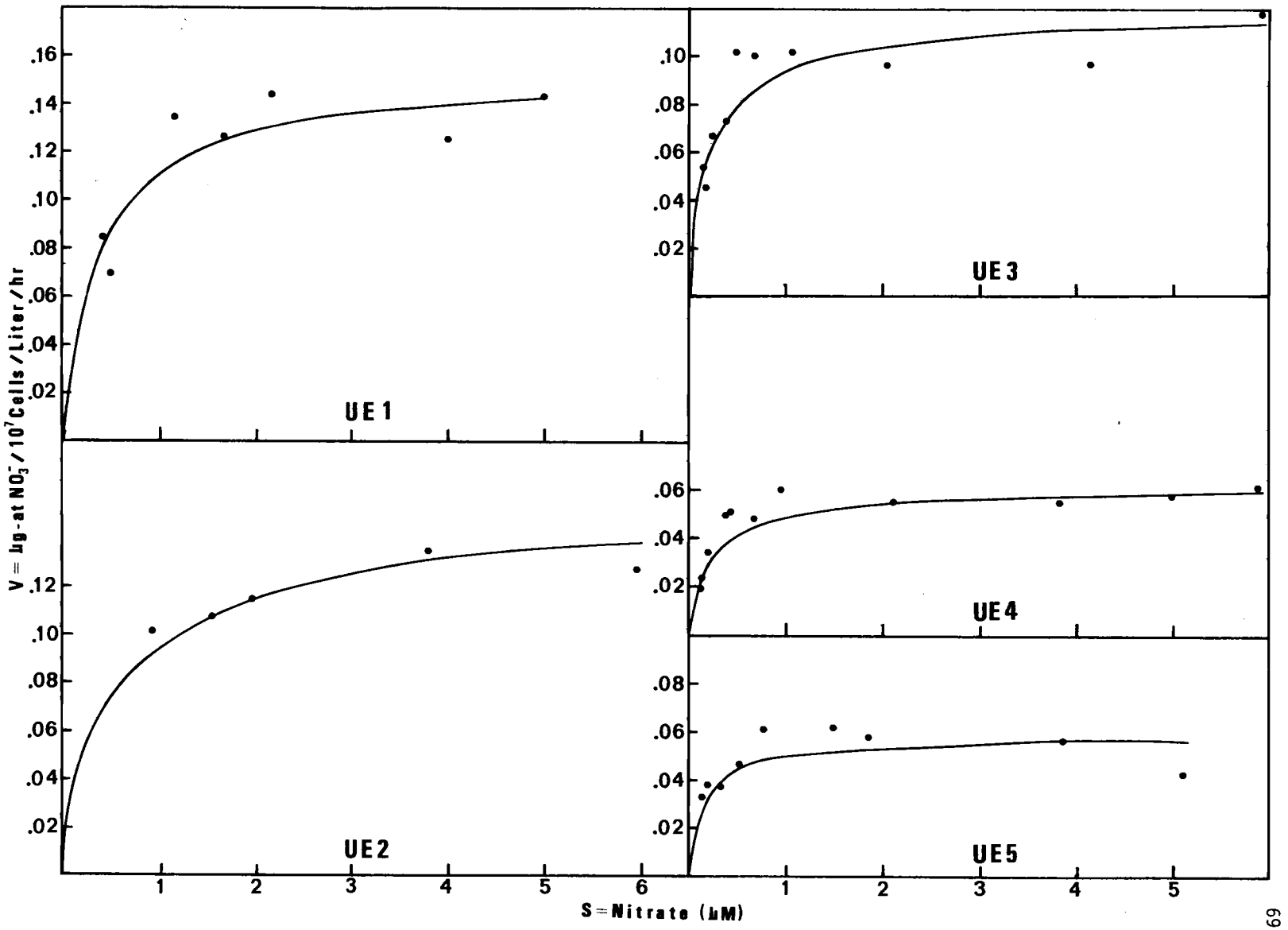

V = µg-at NO3- / 10^7 Cells / Liter / hr
S = Nitrate (µM)
UE 1
UE 2
UE 3
UE 4
UE 5

Figure 16. Nitrate uptake response of T. pseudonana transformed such that the y-intercept is V_{max} and the slope is $-K_s$ (Dowd and Riggs, 1965).

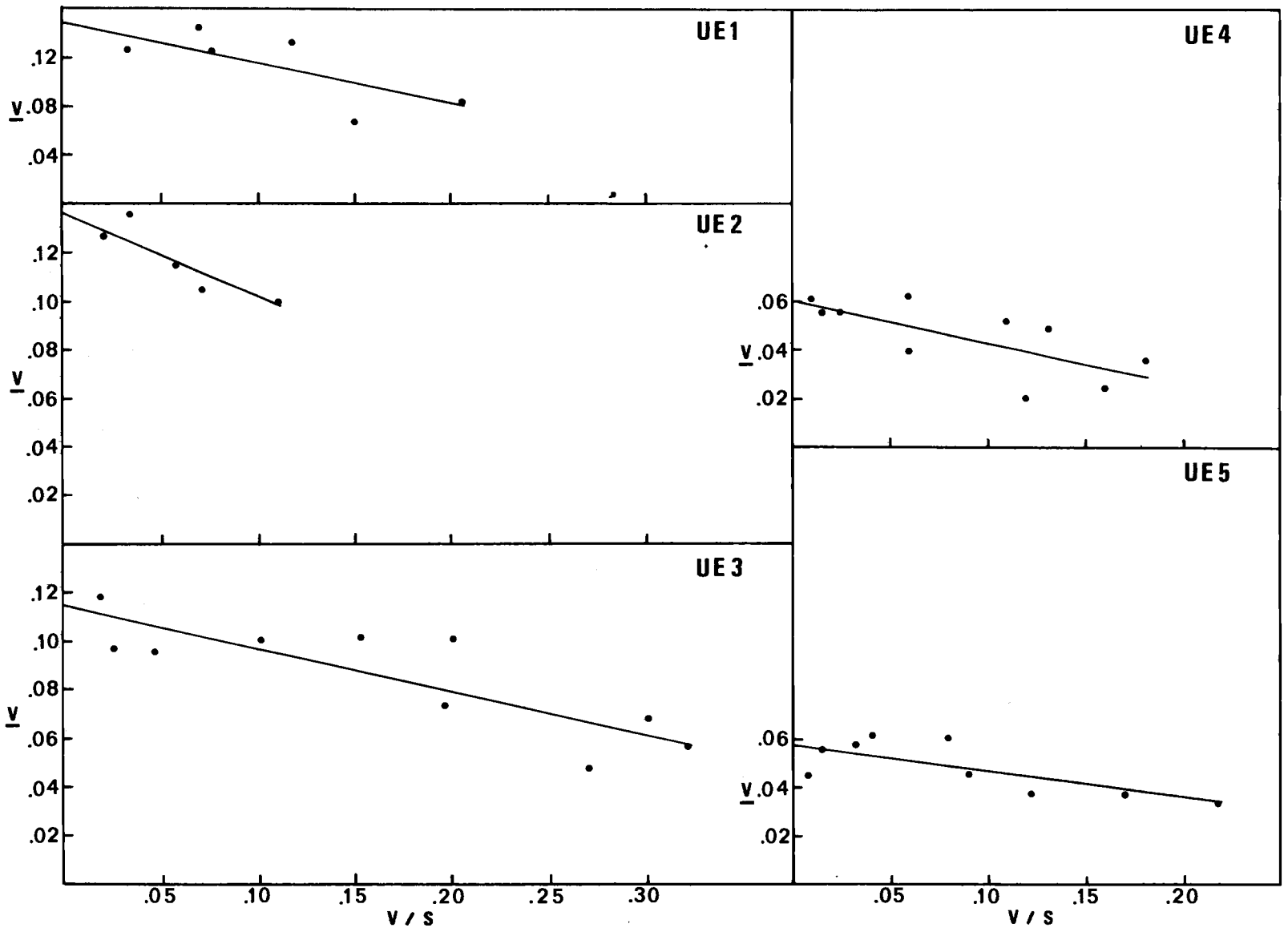
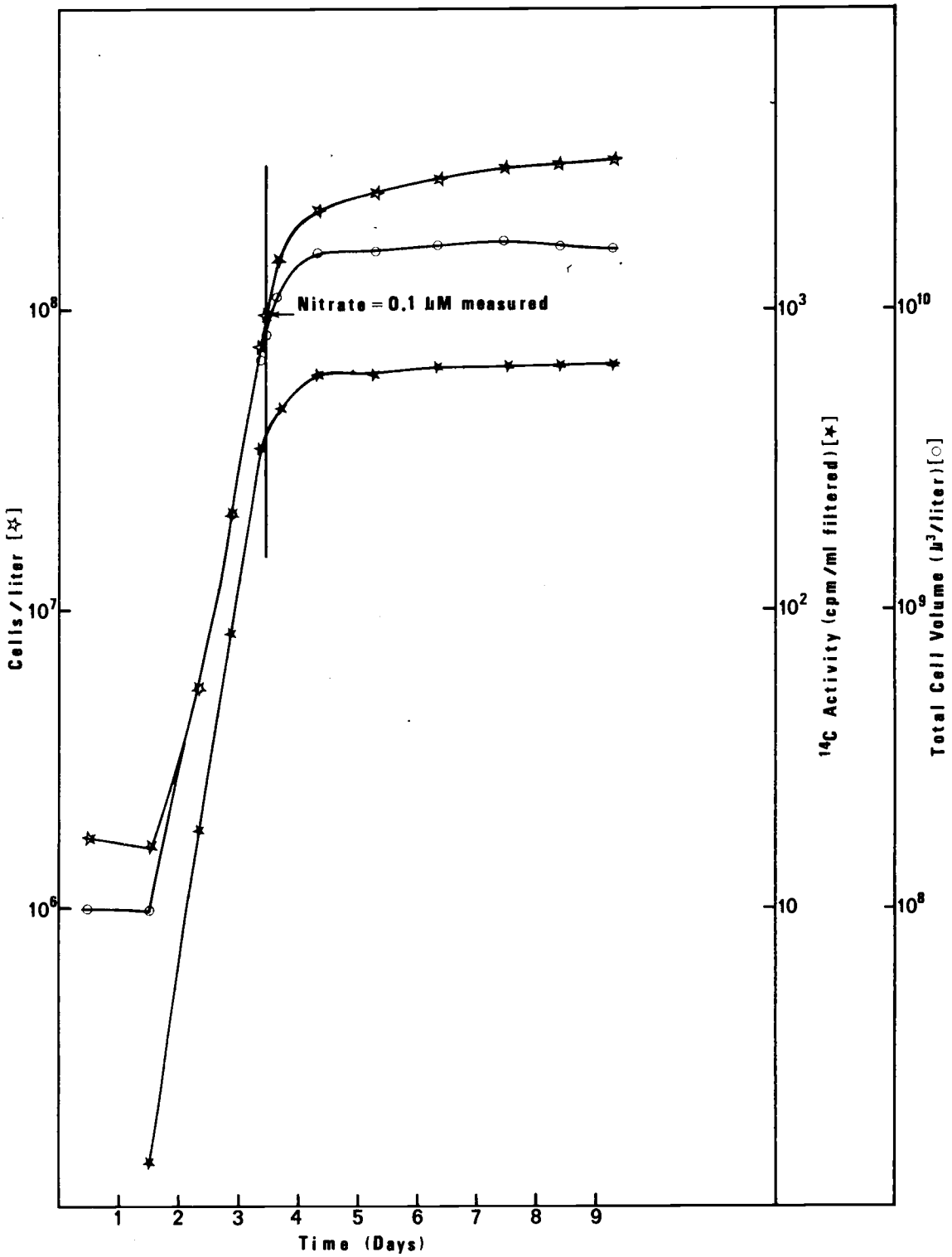

UE1
UE2
UE3
UE4
UE5
V
V / S
.12
.10
.08
.06
.04
.02
.05
.10
.15
.20
.25
.30

TABLE 5: SUMMARY OF NITRATE UPTAKE CHARACTERISTICS OF T. PSEUDONANA.

Experiment	Yield (cells/ µg-at N)	V_{max} (±95% confidence interval)	V_{max} (µg-at N/ 10^7 cells/hr)	K_s (µg-at NO_3^- -N) ±95% confidence interval	MCV μ^3
UE1	0.90×10^7	0.136 ± .036	0.152	0.35 ± .33	92
UE2	2.44×10^7	0.334 ± .042	0.138	0.33 ± .25	117
UE3	2.01×10^7	0.228 ± .030	0.114	0.17 ± .07	58
UE4	2.72×10^7	0.165 ± .030	0.060	0.18 ± .10	50
UE5	2.93×10^7	0.167 ± 0.027	0.057	0.10 ± .07	55

Figure 17. Continuous flow growth response. Approach to steady state. Dilution rate = 0.02 hr^{-1}.


Nitrate = 0.1 µM measured
Cells / liter [☆]
10^8
10^7
10^6
10^3
10^2
10
10^{10}
10^9
10^8
^{14}C Activity (cpm/ml filtered) [★]
Total Cell Volume (μ^3/liter) [○]
1
2
3
4
5
6
7
8
9
Time (Days)

declined to about 50 μ^3 at the end of the experiment. Cell density never really reached a steady state although both particulate ^{14}C and total cell volume were relatively stable over five days.

For the rapid continuous flow system (Fig. 18, dilution rate = 0.12/hr), the corrected specific growth rate was 0.15 hr^{-1}. The density at which nitrate was depleted was not observed, however, at a cell density of 17.8×10^3/ml, the nitrate concentration had declined from 7.4 to 2.3 µg-at/l, indicating a yield of 0.36×10^7 cells/µg-at N, about twice as much nitrogen associated per cell as that determined for a healthy, rapidly growing population in batch systems. Steady state cell concentrations of about 68,000/ml resulted in a yield of about 0.924×10^7 cells/µg-at N, a value similar to that observed for a rapidly growing population under batch conditions. Mean cell volume, initially about 100 μ^3, stabilized at about 57 μ^3. Measured steady state nitrate concentrations were about 0.20 µg-at NO_3^- -N/l (Table 6).

Continuous flow growth response: steady state. Steady state population densities (measured as cell numbers and total cell volume) declined with increasing flow rate in an apparently linear fashion (Fig. 19). Since the nitrate concentration in the reservoir varied from run to run (from 5 to 10 µg-at NO_3^- -N/l) the population densities were normalized to that produced by 1.0 µg-at NO_3^- -N. Residual nitrate concentrations in the growth chambers varied from 0.1 - 0.3 µg-at/l and showed no trend with changes in dilution

Figure 18. Continuous flow growth response. Approach to steady state. Dilution rate = 0.12 hr^{-1}.

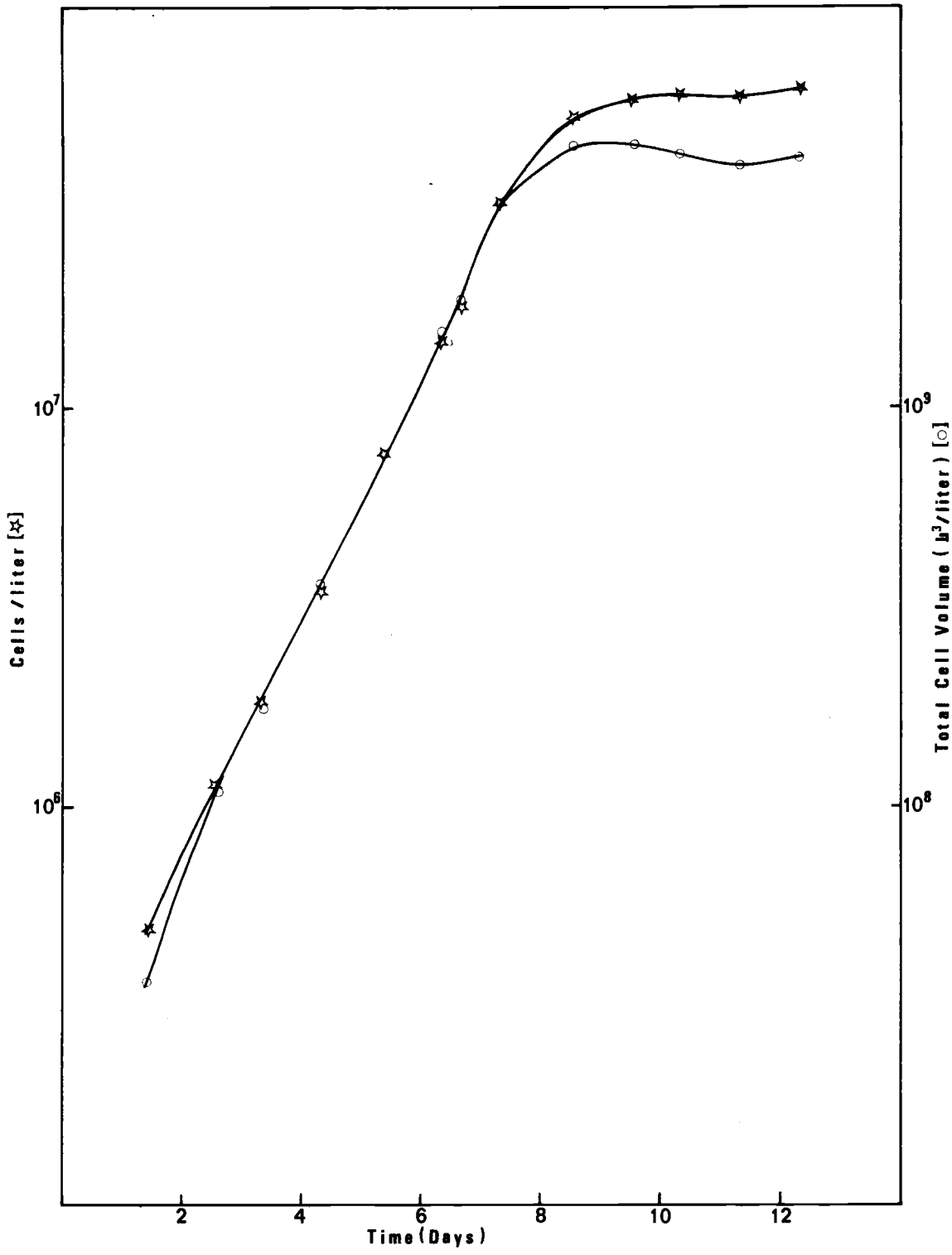
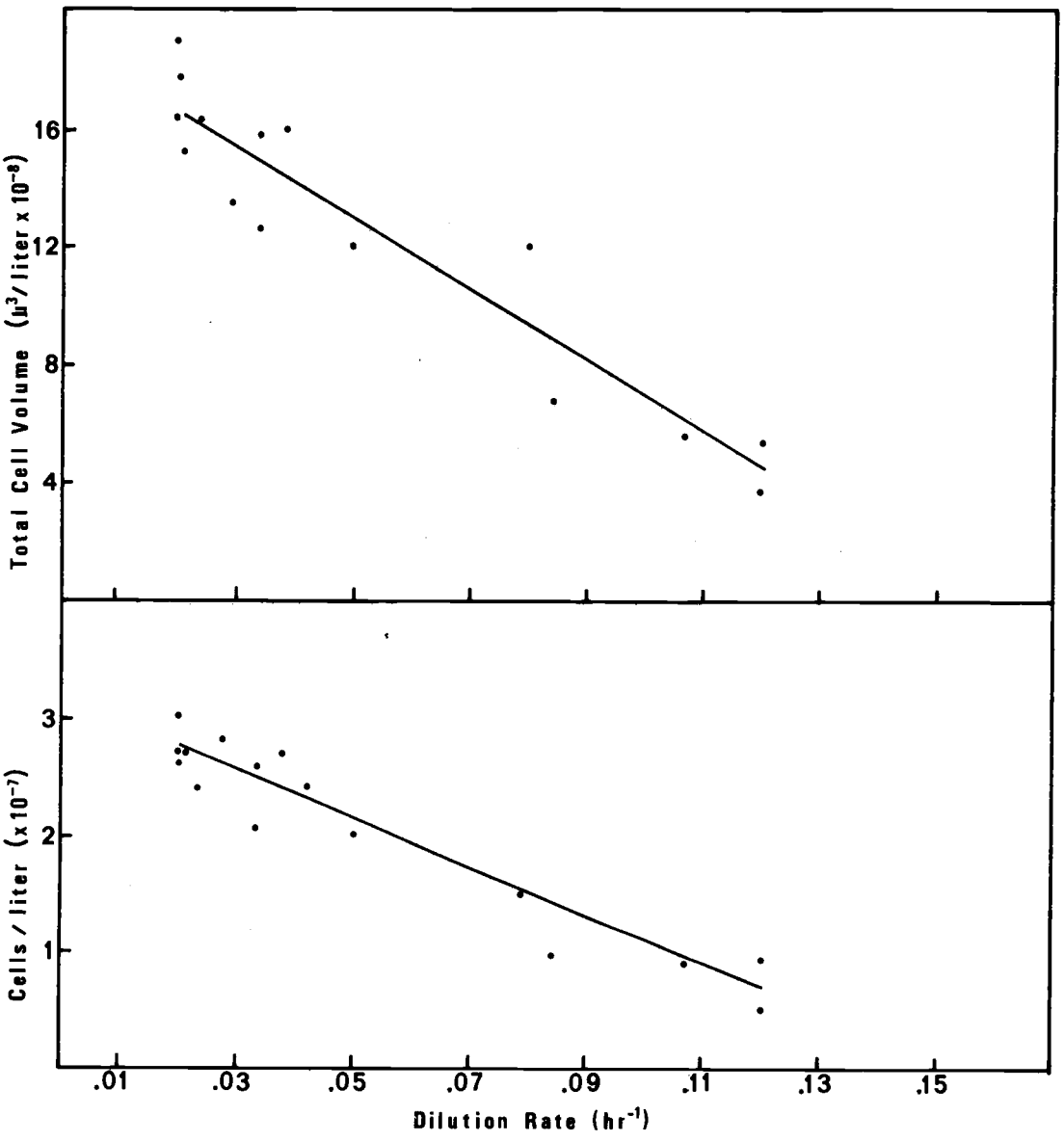

Cells / liter [☆]
Total Cell Volume (μ³/liter) [○]
10⁷
10⁶
10⁹
10⁸
2
4
6
8
10
12
Time (Days)

TABLE 6: TYPICAL NITRATE CONCENTRATIONS AT STEADY STATE FOR HIGH FLOW RATE CONTINUOUS FLOW SYSTEM.

DATE/TIME	Nitrate Concentration µg-at/l
11/0805	0.18
12/1230	0.26
13/1340	0.12
14/0805	0.28
15/0810	0.16
16/0805	0.23
	$\bar{x}$ = 0.20

Figure 19. Relationship between steady state population densities and flow rate. Cells/l ($x10^7$) = -21.0 x flow rate (hr^{-1}) + 3.19; r = -0.95. Total cell volume/l (μ^3 x 10^8) = -122 x flow rate (hr^{-1}) + 19.05; r = -0.95.


Total Cell Volume (μ^3/liter x 10^{-8})
16
12
8
4
Cells / liter (x10^{-7})
3
2
1
.01
.03
.05
.07
.09
.11
.13
.15
Dilution Rate (hr^{-1})

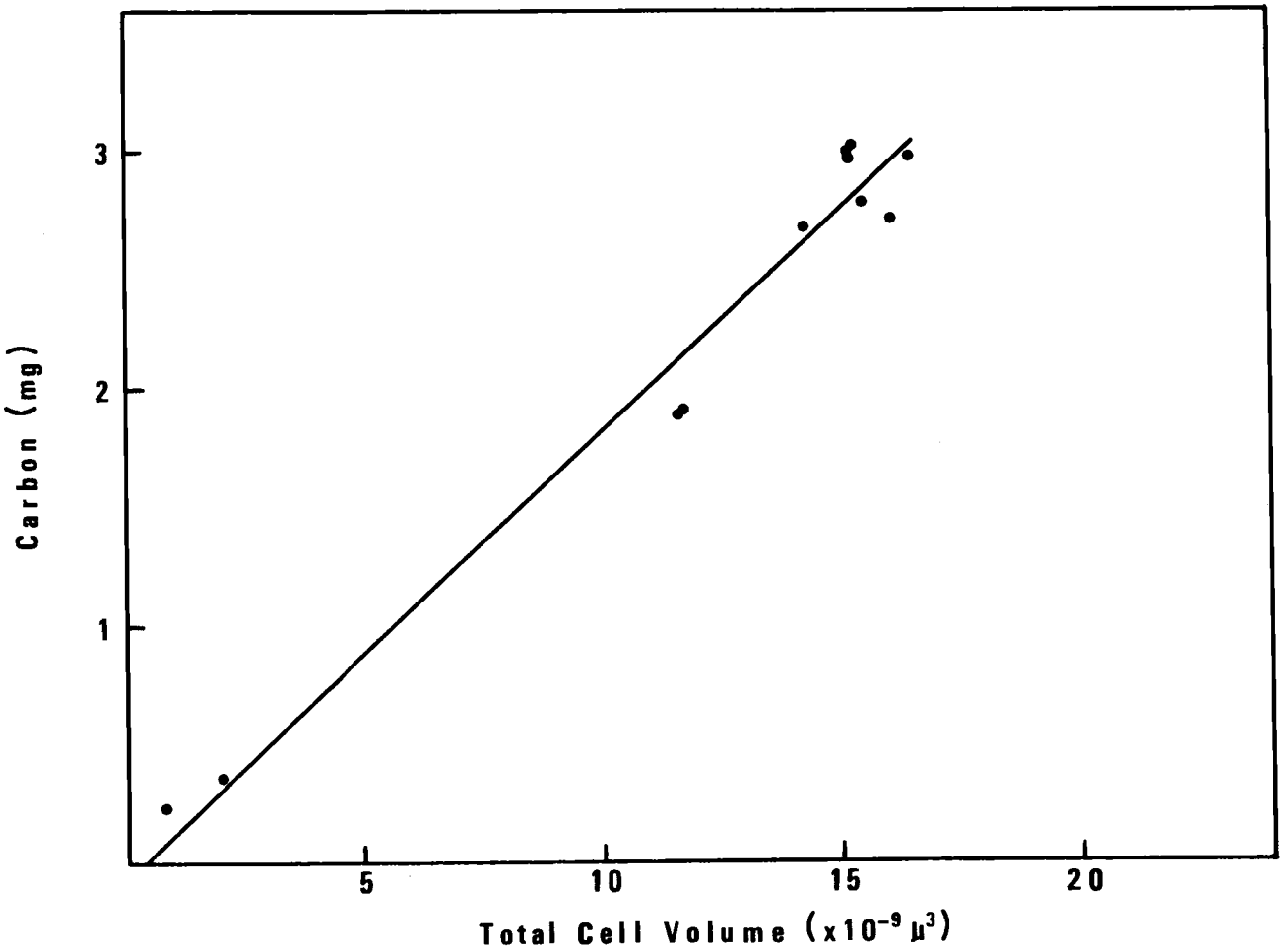
rate. Typical measured values of nitrate concentration for a run at a dilution rate of 86% of the maximum specific growth rate are given in Table 6.

The intercept of cell numbers regressed against dilution rate indicated a yield of 3.2×10^7 cells/µg-at NO_3^- -N at zero dilution rate, or batch conditions. This value is significantly higher than that determined for batch conditions (2.3×10^7 cells/µg-at NO_3^- -N, Fig. 4) or that determined for the two batch growth experiments (2.2×10^7 cells/µg-at NO_3^- -N; Table 3). The total cell volume at zero dilution rate (1.90×10^9 μ^3/µg-at NO_3^- -N) is only slightly higher than the total cell volume for the batch cultures (1.7×10^9 μ^3/µg-at NO_3^- -N Table 3).

Measurement of particulate carbon for a variety of batch and continuous flow runs revealed a conversion of 15.5 µg-at C/10^9 μ^3 (Fig. 20), therefore, the total cell volume steady state values could be expressed in terms of particulate carbon. The range of estimated particulate carbon values is 26 µg-at C/µg-at NO_3^- -N to 5.6 µg-at C/µg-at NO_3^- -N or a carbon:nitrogen range from 5.6:1 to 26:1 (by atoms) over the range of flow rates studied, a slightly broader range than that observed for batch experiments.

Size distribution shift of populations grown under continuous culture. Utilizing the population biomass parameter, cell numbers, for determining population steady state values of continuous flow systems can lead to confusion since there was a definite increase in cell density with time, although particulate carbon (measured as ^{14}C activity) and total cell volume remained relatively constant at

Figure 20. Relationship between particulate carbon and total cell volume. Particulate carbon (mg) = 0.187 x TCV (10^9 μ^3) -0.079; r = 0.986.


Carbon (mg)
3
2
1
5
10
15
20
Total Cell Volume (x10⁻⁹ µ³)

various steady state values. With the increase in cell number, there was a concomitant decrease in mean cell volume such that the total cell volume remained relatively constant (Table 7). That the changes in mean cell volume did indeed represent changes in size distribution was demonstrated by following the size distribution changes for a continuous flow run (Fig. 21). The size distributions were normalized such that the count obtained in each window of the plotter was expressed as a fraction of the maximum number obtained for that run. The initial distribution represented a population in exponential growth phase (Day 1)--the median was about twice that for the first "steady state" distribution (Day 3). The median thence shifted from a relative volume of eight to a relative volume of four over a period of seven days.

Characteristics of these smaller cells were retained in subsequent batch systems. The yield coefficient was significantly lower than that obtained for populations which had not been subjected to continuous flow conditions. A yield of 2.90×10^7 cells/µg-at NO_3^- -N (Fig. 22) was determined for a population of cells harvested from a continuous flow system (0.21 hr^{-1}) by spiking with various concentrations of nitrate and observing the additional biomass yield and is similar to that of 3.2×10^7 cells/µg-at NO_3^- -N obtained at a zero dilution rate of continuous flow systems (Fig. 19). Batch grown populations had a yield of 2.2×10^7 cells/µg-at NO_3^- -N. Mean cell volume for batch grown populations was about 100 μ^3 (Table 3), while that for the population grown in continuous flow system was about 48 μ^3.

TABLE 7: STEADY STATE PARAMETERS INDICATING STABILITY OF BIOMASS MEASURES TOTAL CELL VOLUME AND ^{14}C BUT INCREASE IN CELL NUMBER.

DATE	TIME	^{14}C (cpm/10ml)	Cells/ml	MCV (μ^3)	TCV (μ^3/ml)	Flow rate
6/14/71	1100	702	203×10^3	80	167×10^5	
6/15	1030	734.5	234×10^3	73	168×10^5	0.51 day^{-1} (0.02 hr^{-1})
6/16	1030	737	253×10^3	65	163×10^5	
6/17	1100	730	287×10^3	59	168×10^5	
9/24/71	0930	657	275×10^3	60	165×10^5	
9/25	1130	656	299×10^2	58	171×10^5	0.50 day^{-1} (0.02 hr^{-1})
9/26	1000	650	309×10^3	52	162×10^5	
9/27	0805	674	318×10^3	50	160×10^5	

Figure 21. Size distribution shift for a population of T. pseudonana at steady state. Dilution rate = 0.021 hr^{-1}.

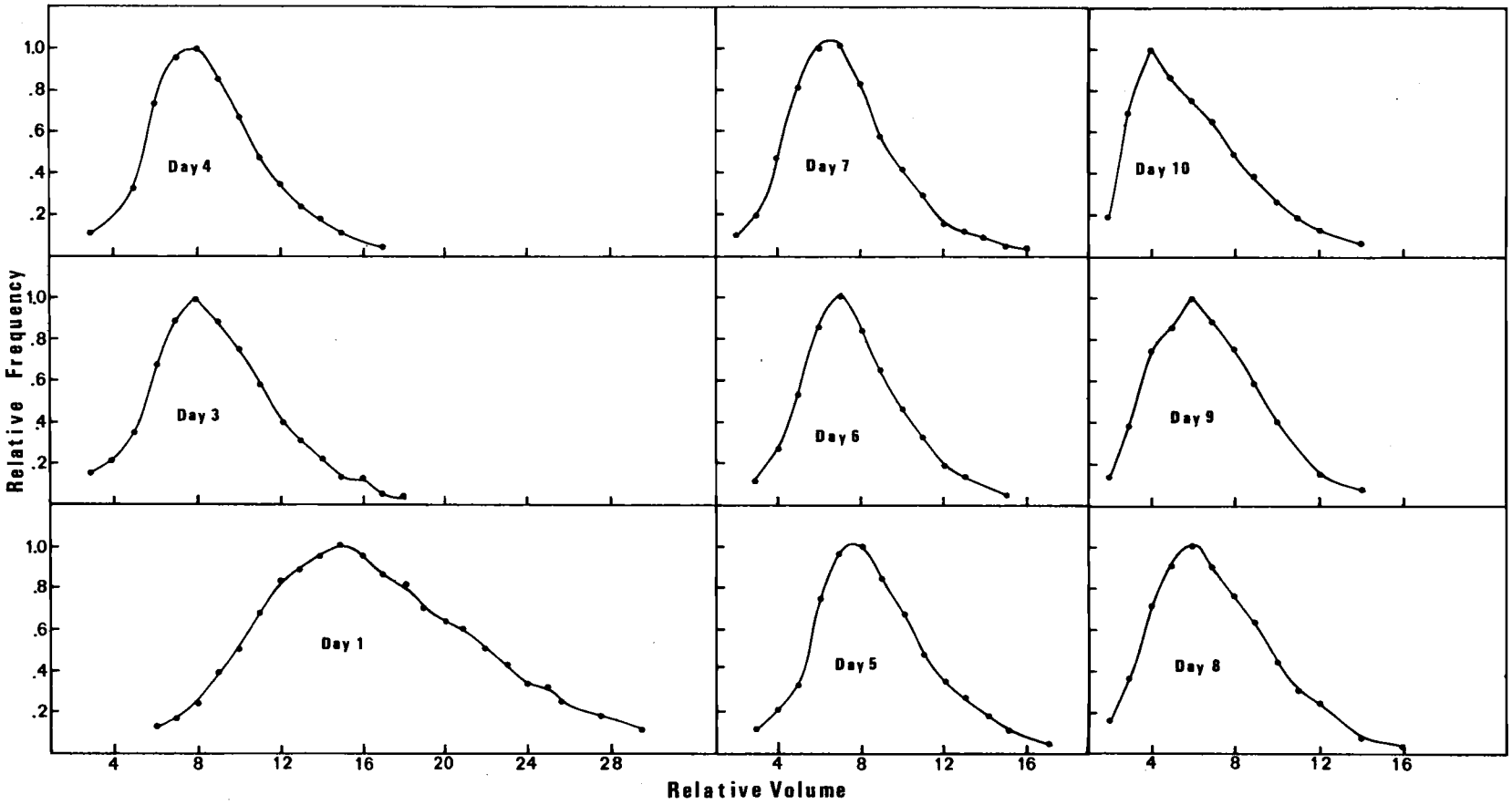
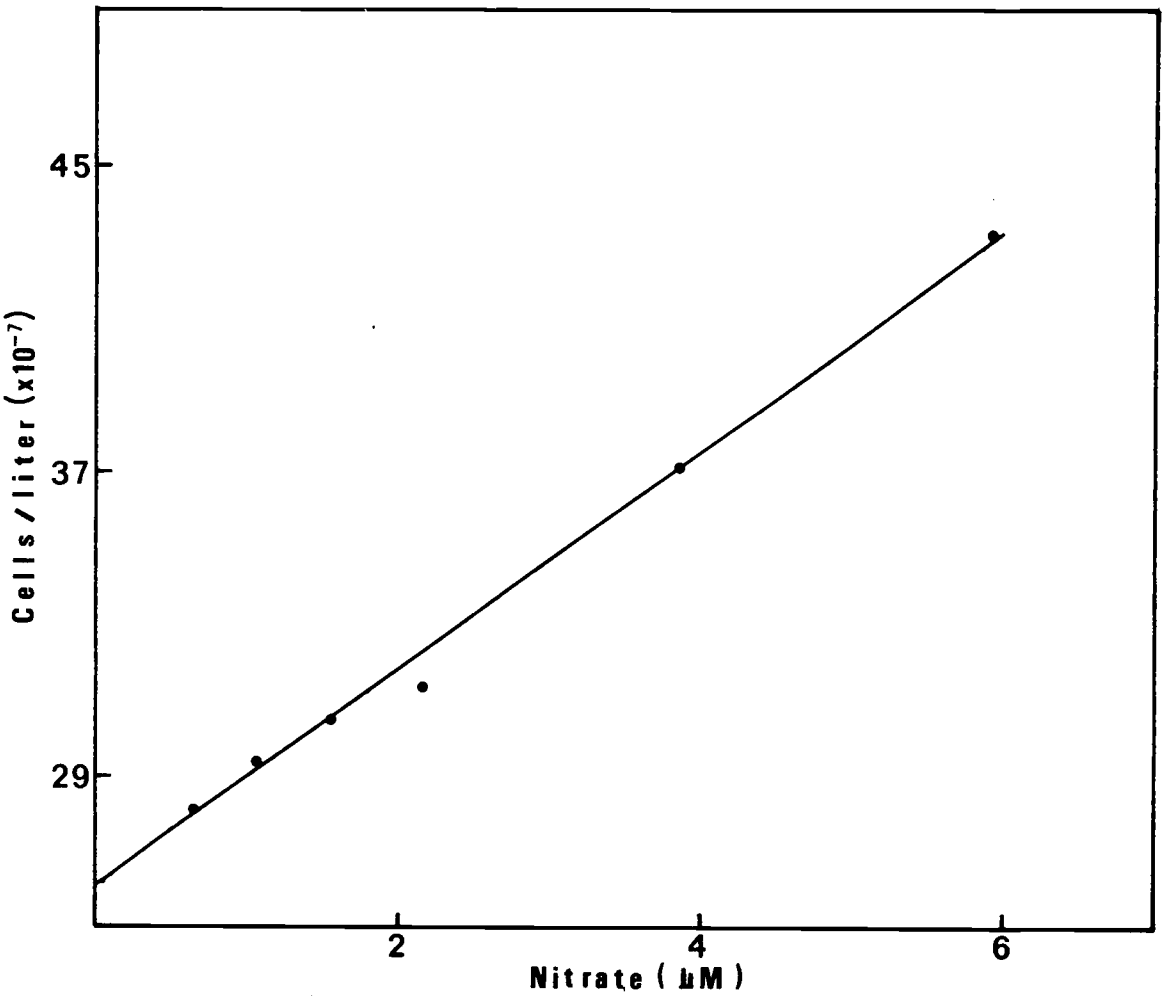

Day 4
Day 7
Day 10
Day 3
Day 6
Day 9
Day 1
Day 5
Day 8
Relative Frequency
Relative Volume
1.0
.8
.6
.4
.2
4
8
12
16
20
24
28

Figure 22. Nitrate-nitrogen yield response of T. pseudonana harvested from a continuous flow system. Cells/l ($x10^7$) = 2.90 x μm NO_3^- + 25.95; r = 0.99.


45
37
29
Cells / liter (x10⁻⁷)
2
4
6
Nitrate (µM)

Discussion

Growth vs. nitrate-nitrogen concentration. There are several nutrient limited conditions under which algae may grow. These fall into four evident categories: 1) a batch system in which the nutrient concentration is initially at such a level so as to be non-limiting, 2) a batch system in which the nutrient concentration is initially at a limiting level, 3) a continuous flow system in which nutrient concentration in the reservoir is non-limiting, and 4) a continuous flow system in which the nutrient concentration in the supply is limiting. The present study offers insight into categories (1) and (3). Cases (2) and (4) were not studied due to the extremely low concentrations of nitrate at which uptake seemed to be restricted for T. pseudonana and resulting difficulties measuring biomass parameters and changes in nutrient concentrations.

That growth (measured as increases in particulate carbon, cell numbers, or total cell volume) continued seemingly uninterrupted for a period of time after the depletion of nitrate-nitrogen and is therefore not directly related to the concentration in the medium is evident from an examination of the growth response of T. pseudonana under both batch and continuous flow systems. The continued increase in particulate carbon, cell numbers, and total cell volume seems to be related to the ability of the population to synthesize organic material using internal stores of nitrogenous compounds whose

levels are at a maximum during exponential growth but whose levels then decrease after depletion from the medium. Eventually the internal levels become depleted and continued synthesis ceases.

In the absence of information concerning the nature of growth controlling mechanisms and their relationships to ambient nutrient concentrations, it has been suggested that populations or communities of phytoplankton may exist in various states of nutrient deficiency and that the deficiency might be expressed by some measure of the physiological state of the population (community) such as a carbon:chlorophyll a ratio, a chlorophyll:carotenoid ratio or an assimilation ratio (Yentsch and Vaccaro, 1958; Manny, 1969; Thomas, 1970, 1972).

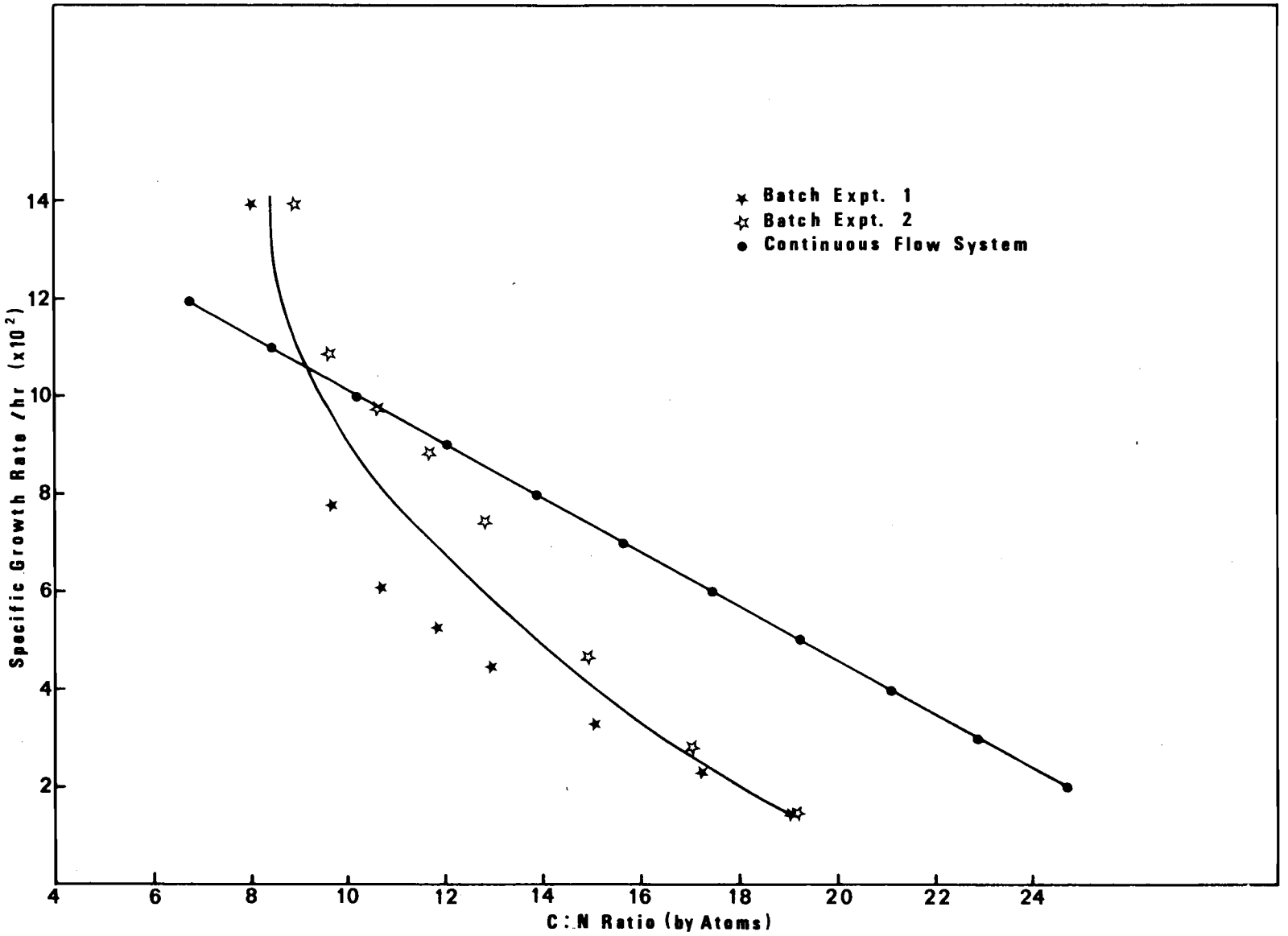
The present study suggests the possibility of quantifying the physiological state of a population (community) in terms of a carbon:nitrogen ratio and relating the specific growth rate to this value. For the continuous flow system, carbon:nitrogen ratios varied over a range from 26:1 to 5.6:1 covering specific growth rates ranging from 14% to 86% of the maximum specific growth rate. For the batch systems, the carbon:nitrogen ratios ranged between 22.5 and about 8.5. In both cases, the minimum carbon:nitrogen ratio is associated with the highest specific growth rate and the maximum carbon:nitrogen ratio is associated with the lowest specific growth rate. The variation in carbon:nitrogen ratios of populations subjected to nitrogen deficiency is well documented (Strickland, 1960; Redfield, 1965; Syrett, 1965).

A relationship between specific growth rate and carbon:nitrogen ratio for batch systems (Fig. 23) was obtained in the following manner. The specific growth rate was determined by obtaining graphically the slope of the growth curve (µg-C vs. time, Fig. 10) at various points and dividing this value by the carbon value at this point, thus obtaining a value of the form $\frac{1}{N}\frac{dN}{dt}$ with units t^{-1}. The carbon:nitrogen ratio was determined by dividing the carbon value by the initial nitrate-nitrogen value, assuming all nitrate was incorporated into particulate nitrogen.

For the continuous flow studies, a relationship between specific growth rate and carbon:nitrogen ratio (Fig. 23) was obtained as follows. The specific growth rate is equivalent to the dilution rate for continuous flow systems operated at steady state. The carbon:nitrogen ratio was obtained by converting the total cell volume at each dilution rate to particulate carbon using the previously determined ratio of 15.5 µg-at C/10^9 μ^3 (Fig. 20) and dividing that by the amount of nitrogen consumed.

The most striking difference between the relationship obtained from batch systems and that obtained from continuous flow systems is that the relationship for batch systems is clearly nonlinear whereas that for the continuous flow system is linear. The difference may be due to the inherent difference between the two culturing systems. For the batch system, population characteristics change rapidly subsequent to nitrogen depletion so that the instantaneous growth rate may be expressing a lag, responding to previous characteristics.

Figure 23. Relationship between specific growth rate and carbon:nitrogen ratio for batch and continuous flow systems.


Batch Expt. 1
Batch Expt. 2
Continuous Flow System
Specific Growth Rate /hr (x10²)
C : N Ratio (by Atoms)
14
12
10
8
6
4
2
4
6
8
10
12
14
16
18
20
22
24

Continuous flow systems, on the other hand, operated at steady state, allow population characteristics to stabilize to those dictated by the requirements to support a particular specific growth rate.

Recently, Caperon and Meyer (1972 a, b) have demonstrated that the rate of growth of several species of marine phytoplankton under continuous flow conditions could be related to a nitrogen:carbon ratio which increased as the growth rate increased and declined to a minimum value at low growth rates. Nitrogen:carbon ratios varied from 0.22 at high growth rates to 0.045 at low growth rates for C. pseudonana. The corresponding ranges in nitrogen:carbon ratios in the present study were 0.174 to 0.037.

The observation that the carbon:nitrogen ratio may vary depending upon the nitrogen source might cast doubt upon the utility of carbon:nitrogen ratios as an index of nitrogen deficiency. Eppley et al. (1971) using natural communities cultured shipboard in 200 liter polyethylene vats, determined that carbon:nitrogen ratios in two experiments were 5.9 and 5.2 using nitrate, 3.9 and 4.3 using ammonium and 5.6 and 7.0 using urea. The extent to which carbon:nitrogen ratios vary depending upon nitrogen source remains to be investigated. The differences reported by Eppley et al. (1971) are not large compared with the wide variations one can encounter.

If the carbon:nitrogen ratio is taken to be indicative of the general physiological state of populations of phytoplankton, one might expect to find wide variations in natural systems,

particularly in regions of low ambient nitrogen. For the situation in which natural communities of phytoplankton grown under environmental conditions excluding advection, grazing, turbulence, etc., such as the big bag experiments of McAllister, et al. (1961) or the deep tank experiments of Strickland, et al. (1969), carbon:nitrogen ratios ranged from 9.5 to 15; values greater than 10 occurred subsequent to nitrogen depletion. Extensive analyses of carbon:nitrogen ratios determined for communities grown under natural conditions in which the crop harvested was primarily of phytoplanktonic origin reveal carbon:nitrogen ratios which average 6.6:1 by atoms (Redfield, 1934; Fleming, 1940). Work of a more recent nature in Auke Bay, Alaska found carbon:nitrogen ratios by atoms consistently to be on the order of 5-7:1 (Zakar, 1972, personal communication) in an environment generally depleted of ambient nitrogen, conditions under which one might expect higher values. Also, Eppley et al., (1971) observed carbon:nitrogen ratios averaged about 5:1 for shipboard grown cultures of natural assemblages of phytoplankton whose nitrogen source was either ammonia, nitrate, or urea. Ramberg (1970) has observed that carbon:nitrogen ratios of filtered material obtained from Oregon coastal waters during periods of upwelling range from about 6 to 15 and higher, and noticed a consistent trend with minimum values closest to shore and higher values off shore. It thus seems that natural phytoplanktonic communities exhibit characteristics which are attributable to a healthy state although the ambient nitrogen concentrations may be near zero.

The carbon:nitrogen ratio of phytoplankton communities can be used as a qualitative measure of the physiological state of phytoplanktonic communities. That the occurrence of low carbon:nitrogen ratios is generally widespread perhaps indicates that except for short periods of time, mechanisms are available by which phytoplanktonic populations are maintained in a healthy state.

Assimilation ratio. That the synthesis of chlorophyll a ceases at the time of nitrogen depletion is compatible with observations demonstrating that non-nitrogenous compounds (carbohydrates and lipids) continue to be produced during periods of nitrogen deficiency (McAllister et al., 1961; Antia et al., 1963; Strickland et al., 1969; Platt and Subba Rao, 1970; Eppley et al., 1971). Curl and Small (1965) and Thomas (1970) suggest that the assimilation ratio varies with the ambient nitrogen regime. Lower values of the assimilation are associated with low ambient nitrogen concentrations. The present observations on chlorophyll a synthesis also suggest that the assimilation ratio may be a function of the physiological state of the population. If the chlorophyll a concentration were constant and carbon accumulation continued at a declining rate subsequent to nutrient depletion (Fig. 11), the assimilation ratio would decline as the population became further and further deficient. It is likely that the assimilation ratio is indicative of the physiological state of the population (Thomas, 1972) and that the physiological state of the population is a function of a variety of parameters which

affect the supply of nutrients to the population so that a measure of ambient nitrogen concentration may not provide information concerning the state of the population.

Nitrate uptake characteristics. The hyperbolic expression which relates rate of uptake of nutrient to nutrient concentration has been derived in a manner analagous to the derivation of the Michaelis-Menten expression for enzyme kinetics (Caperon, 1968). For uptake, the external nutrient is considered to be the substrate. A mechanism transporting nutrient across the cell wall is analagous to the enzyme, and the nutrient within the cell is analagous to the product. The K_s value for uptake incorporates the ratio of the rate constants $\frac{K_3+K_2}{K_1}$ where K_1 is the rate constant for formation of membrane-nutrient complex and K_2 and K_3 are rate constants for dissociation of membrane-nutrient complex, K_2 for dissociation prior to transport, releasing nutrient back into the environment and K_3 for dissociation across the cell wall into the cell interior. K_s values are peculiar to the particular mechanism of tranport just as is K_m peculiar for a particular enzyme, hence, variability within a particular species would not be expected (Caperon and Meyer, 1972b). Further if the uptake mechanism is similar from species to species, comparative K_s values observed should not vary more than that dictated by experimental constraints (Caperon and Meyer, 1972b).

In the present study although the K_s values seem to group themselves into two categories--0.35 and 0.33 µg-at NO_3^- -N/l for the batch grown populations and 0.18, 0.17, and 0.10 µg-at NO_3^- -N/l

for the continuously cultured populations--they are not significantly different from each other at the 95% confidence level (Table 5). Caperon and Meyer, (1972b) were unable to detect any statistical difference among K_s values determined for several species of marine phytoplankton preconditioned at different steady state growth rates in continuous flow systems. That considerable variability exists among species has been documented (Eppley, et al., 1969; MacIsaac and Dugdale, 1969). Carpenter and Guillard (1971) report intraspecific variation in K_s for nitrate for various clones of T. pseudonana. These are 1.87 for 3H, 1.19 for 7-15, and 0.38 for 13-1. They suggest that variation in K_s is due to an adaptation by the clone to the nitrate regime of its environment, 13-1 being indigenous to the low nitrate waters of the Sargasso Sea and 3H being a characteristically estuarine species encountering high ambient nitrogen levels.

It is therefore apparent that K_s values do vary both among and within species. This variation suggests that the mechanism for transport may be different among and even within species. However, it seems that too much emphasis may be placed upon conforming the kinetics of nutrient uptake to that of enzyme kinetics. The relationship generated by the conceptual analogy is mathematically convenient and useful, but the details of the process appear to be more complex than the analogy allows. An interesting experiment could be performed to determine whether K_s variability can be induced within a particular clone. This is to cultivate a species whose K_s value has been

determined to be high in a continuous flow system to subject the species to low ambient nutrient levels. A lowering in K_s value would suggest that the transport mechanism is under physiological control; a constant K_s would indicate that its value is characteristic of that particular clone.

Values for V_{max} (per unit nitrogen) indicate a general tendency to increase as the physiological state of the population declines, exemplified by the extremes in values for the two batch grown populations (Table 5). V_{max} for uptake experiment 1 is nearly identical with the growth constant for increases in particulate carbon and cell numbers. The batch population harvested during stationary phase (UE 2) exhibited the largest V_{max} while those harvested from continuous flow systems exhibited intermediate values. It might be expected, however, that the population harvested from the high flow chemostat (UE 3) would exhibit a lower V_{max} than one harvested from a low flow chemostat (UE 4).

Caperon and Meyer (1972 b) have shown that the V_{max} (per cell) increases as the preconditioning growth rate increases. The change is attributed to change in availability of uptake sites, thought to be associated with V_{max}. This is consistent with the present observations that V_{max} (per cell) is higher for the 0.079 hr^{-1} chemostat than for the 0.04 hr^{-1} chemostat (Table 5). V_{max} (per cell), however, is similar for both batch populations, perhaps suggesting that the availability of uptake sites has not yet been affected by nitrogen deficiency in the population harvested near stationary phase.

Although emphasis has primarily been placed upon the value of K_s as an indicator of the ability with which a species is capable of removing nutrients at low level, variation in V_{max} can change the ability with which a population removes nutrients. The present study indicates that V_{max} may vary up to slightly greater than two times the maximum specific growth rate, and other studies indicate this value may be as high as four times the maximum specific growth rate (Eppley and Thomas, 1969). Therefore, depending upon the nitrogen deficiency of the population (or community), the ability with which a nutrient at a limiting concentration may be removed may vary considerably.

Continuous flow system: theory vs. observations. Continuous flow theory (Kubitschek, 1970; Tempest, 1970) suggests that it is possible to predict residual limiting nutrient concentrations at steady state based upon a knowledge of the uptake response of the population to that nutrient as well as the dilution rate of the system according to the following equation: $S = K_s \frac{\omega}{V_{max} - \omega}$, where S, K_s, and V_{max} have been defined previously and ω is the dilution rate (hr^{-1}). If, for example, the uptake constants are taken as 0.35 µg-at NO_3^--N/l for K_s and 0.14 hr^{-1} for V_{max} as determined above, easily measurable nitrate concentrations of greater than 0.3 µg-at NO_3^--N/l should be obtained at dilution rates of 0.06 hr^{-1} and greater. However, if the value of V_{max} is variable, and dependent upon physiological state of the population as is indicated by this study, then it is possible to increase the ability to remove nitrate by increasing the value of V_{max}. For example, if V_{max} were

taken as 0.23 hr^{-1}, as was determined for a population harvested at a dilution rate of 0.079 hr^{-1} and K_s kept at 0.35 µg-at/l, an expected nitrate concentration of greater than 0.3 µg-at/ would only occur at dilution rates of greater than 0.1 hr^{-1}. A similar argument may be applied to variability of K_s. However, its value was not demonstrably dependent upon physiological state. The observations that V_{max} varies with physiological state and that K_s is generally quite low, and may be variable, suggest that phytoplankton populations have the capability to deplete nitrate concentrations to such levels that variation in nitrate concentration as a function of flow rate may not be detectable using standard analytical methods, as have been pointed out by Caperon and Meyer (1972b). It is thus difficult to generalize about uptake kinetic parameters associated with populations grown under continuous flow conditions.

Continuous flow theory (Kubitschek, 1970; Tempest, 1970) also predicts that the steady state biomass is dependent upon the dilution rate, the concentration of the critical nutrient in the reservoir, and the uptake characteristics of the population. The measure of biomass is critical when interpreting results from a continuous flow system. Hypothetically, consider a population with the uptake characteristics determined for this study, i.e. K_s = 0.35 µg-at NO_3^--N/l and V_{max} = 0.14 hr^{-1} (as determined for the population harvested from the 10 µg-at NO_3^--N/l batch culture). Predicted steady state nitrogen biomass at different dilution rates can be determined from the following equation: $X = \left(S_o - K_s \frac{\omega}{V_{max} - \omega} \right)$, where X is

the nitrogen biomass and S_o the concentration of nitrogen in the reservoir. If the reservoir concentration is 10 µg-at NO_3^-/l, the theoretical steady state biomass vs. dilution rate curve is given in Fig. 24. The nitrogen biomass yield for the present study was constant for all flow rates up to 0.12 hr^{-1} assuming that all nitrate consumed was present as biomass. Similar constant biomass measures would be obtained for the other studies above. The lack of agreement between theory and observation again seems to be attributable to variation in uptake parameters.

The critical issue then becomes how do the other measures of biomass distribute themselves at steady state and how do the other measures of biomass depend upon the specific growth rate (dilution rate). The distribution of nitrogen into cells is related to the dilution rate in a manner which suggests a high requirement for nitrogen at higher dilution rates (i.e. higher specific growth rates) in that the N per cell is greatest at the highest dilution rates. This observation is consistent with observations of batch growth where the N per cell is greatest during exponential growth when presumably the population is growing at its most rapid rate. The distribution of nitrogen per unit carbon biomass is similarly related. The greatest amount of nitrogen associated per unit carbon occurs at the highest dilution rates (highest specific growth rates) for the continuous flow system and at the highest specific growth rates for batch systems.

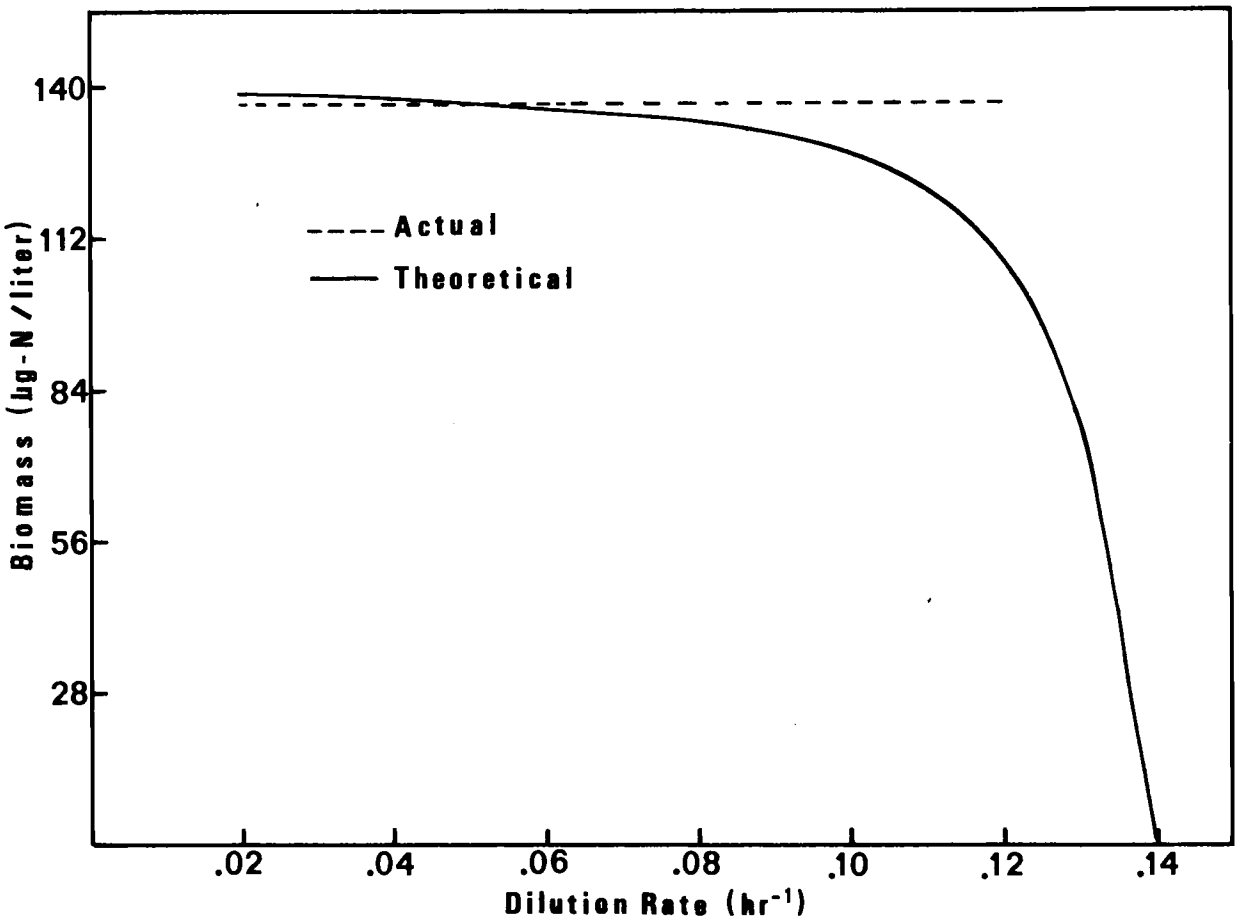


Figure 24. Theoretical and observed nitrogen biomass at steady state as a function of dilution rate.

It is reasonable to suggest that a high level of nitrogen (distributed into its various compartments of pools, precursors, enzymes, DNA, RNA, etc.) is required to support the high specific growth rates, and that this high level of nitrogen reflects itself in N per cell or per unit carbon. However, one might also expect the converse to be true, i.e., a lower nitrogen requirement in situations where the maximum specific growth rate were restricted, for example, by light limitiation. That the N requirement per cell during low light exponential growth phase (where the maximum specific growth rate is reduced to 1/2) is similar to that at high light indicates that the converse is not true.

At present the manner in which nitrogen is distributed into cellular compartments or is associated with carbon cannot be explained mechanistically. It is thus difficult to represent any of the relationships mathematically except by assuming some arbitrary parameters and relationships (Caperon, 1965, 1967, 1968; Williams, 1967, 1970; Grenney, 1972) whose values are not demonstrable experimentally. The model which Grenney proposed assumed a constant protein-cell yield relationship, a relationship which is not supported experimentally. However, this does not necessarily invalidate the conclusions drawn from model simulation runs. If the relationship were valid, one would assume that the increase in particulate carbon subsequent to nitrogen depletion would be associated with further increases in protein. Although not measured in this study, protein increases have been shown to cease after N depletion (Antia et al., 1961; McAllister et al., 1963; Strickland et al., 1969). Continued

increases in particulate carbon are associated with increases in carbohydrates or lipids. Williams (1967, 1970) suggested that carbon uptake ceased at the time of N-deletion, an observation not supported by present studies. Caperon and Meyer (1972 a, b) recently described a model which incorporates the ideas of variable nitrogen:carbon ratios, variable V_{max} related to preconditioned steady state growth rate and the concept of physiological state to explain the steady state nutrient uptake and growth by populations of phytoplankton as they relate to the concentration of nitrogen in the medium.

Diel growth cycle. The present study allows one to speculate about the nature of carbon and nitrogen uptake and assimilation by phytoplankton in natural systems as functions of the diel cycle. This rather "microscopic" view allows one to "think as a phytoplankter" and points out the necessity to bear in mind that the techniques used to measure the responses of phytoplankton can mask the responses because the responses may occur at time intervals which are short relative to the time required to make the observations.

Eppley and Rogers (1970) present what is perhaps the most pertinent discussion on the uptake and assimilation of nitrogen by a marine diatom Ditylum brightwelli. The discussion might be generalized to include communities of phytoplankton. Intracellular inorganic nitrogen is conceived of as being distributed into "pools" which may or may not be common. The reducing enzymes act upon these "pools" as substrates converting from one form into another eventually forming amino acids and thus, perhaps,

assimilating nitrogen. The "pools" are supplied by nitrogen from the external medium, the mechanism of choice being some sort of active transport. Specific rates of uptake are described as being concentration dependent. Assimilated nitrogen then is incorporated into the various structural, genetic, and synthetic portions of the population promoting and being part of continued generations of phytoplankton.

A healthy population growing rapidly is perhaps characterized by "pools" being "full" or at an optimum level for growth requirements, and cellular components would be similarly at an optimum level. The supply of nitrogenous nutrients would be at a sufficient level to support the requirements of the population.

Often, however, the concentrations of nitrogenous nutrients in the marine environment are at a level which restrict the rate at which the nutrients are removed. The following pattern might be observed for natural communities of phytoplankton. During the day-night cycle, the assimilation of carbon is controlled by the ambient light level. Superimposed upon the response to the light regime would be a modification of the physiological state of the community based upon the supply and its demand for nitrogen. During periods of low light, particularly at night, inorganic nitrogen can be taken up (present study; Fitzgerald, 1968; Eppley and Coatsworth, 1969) so that in the early morning the population may be non-deficient or less-deficient with respect to nitrogen. Increasing light levels drive the photoassimilation of carbon,

the relative rate of which may then exceed the relative rate of uptake of inorganic nitrogen present at low concentrations. The population may become N deficient thereby restricting the carbon assimilatory power. Decreasing levels of light may then restrict carbon assimilation such that uptake of inorganic nitrogen may then exceed carbon assimilation, thereby allowing the community to become less nitrogen deficient.

One of the difficulties of substantiating such a sequence for natural systems is that the time required to make observations is long relative to the time required for the events; N^{15} experiments on natural populations are typically carried out over a twelve to twenty-four hour period (Dugdale and Goering, 1967; MacIsaac and Dugdale, 1969) in closed containers. Ambient N levels may be easily depleted in shorter time intervals than is required for the conduct of the experiment. ^{14}C experiments are generally carried out over periods of two hours or longer. Resolution is therefore better in these cases. In a dynamically balanced system, the assimilation of carbon and nitrogen might be expected to be in a relatively constant ratio. However, to try to relate the assimilation of carbon directly to the ambient inorganic nitrogen level may not be possible except in a very general and perhaps averaging sense.

Conclusions

The hypothesis which states that the specific rate of growth of phytoplankton is not related (or coupled) directly to the concentration of nitrogenous nutrient in short supply is supported by this study. A certain amount of clarification is necessary. It appears that the concentration of nitrogen in short supply controls the rate of removal or uptake of that nutrient from the environment, but this control can be modified by the physiological state of the population. The rate of growth of the population may be expressed or determined by measurement of changes of various biomass parameters with time. The relationship between growth and either concentration of limiting nutrient or physiological state can only be determined by specifying the particular biomass parameter selected, for the changes in various parameters seem to be independent of each other, at least over short time intervals.

The net rate of increase in particulate carbon and cell numbers is not immediately responsive to the depletion of nitrogen from the medium, but seems to be controlled by the physiological state of the population with regard to nitrogen. Growth continues but the specific rate of growth declines as the population becomes further nitrogen deficient. Mechanisms exist whereby recovery from deficiency is possible in that the maximum rate of uptake of nitrogen can exceed the maximum rate of growth (carbon or cell numbers) and in that nitrogen can be taken up in the dark. Both processes tend to increase the internal nitrogen content relative to the carbon content.

The rate of assimilation of nitrogen (considered as the incorporation of inorganic nitrogen into organic nitrogen) seems to be more closely coupled to the concentration of nitrogen in the medium than are either the assimilation of carbon or changes in cell numbers. The following argument might be advanced to explain this phenomenon. If the rate of assimilation of nitrogen is related to the size of an internal store of inorganic nitrogen, or is controlled by the cellular concentration of one of the forms of inorganic nitrogen, and the size of this store is small relative to the amount of assimilated nitrogen, then subsequent to the depletion of these nutrients from the environment, the internal pool would be rapidly depleted. There would then be a correspondingly rapid cessation of the increase in assimilated nitrogen. There is little evidence at present concerning the size of nitrogen pools.

Extrapolation of the results of this study to characteristics of natural communities necessitates a rough classification of the environment with respect to growth of phytoplankton and the response of the phytoplankton related to these environments. Three general regimes can be identified: 1) an environment in which batch conditions are approximated, i.e. initial high nutrient levels are depleted by rapidly growing phytoplankton communities; the resupply of nutrients is slow, therefore the biomass produced can no longer be supported by the supply of nutrients; such conditions might be found in temperate regions where spring and fall blooms dominate the annual cycle or in areas characterized by intermittent upwelling; 2) an environment in which quasi-steady state conditions exist where

populations present are supported by the rather low supply of nutrients from a deeper nutrient rich region and by internal cycling of nutrients; such regions are evident in the tropical oceanic areas and temperate regions after the spring bloom runs its course, where there is generally an adequate supply of light, and a well established pycnocline provides a barrier between nutrient rich and nutrient depleted regions; 3) an environment in which the supply of nutrients is in excess of that required by the phytoplanktonic populations such as in regions of consistent upwelling or coastal regions where the supply of nutrients from land sources is sufficient that growth is controlled by grazing, turbulence and average light conditions. This third case is somewhat trivial in that the phytoplankton community should express nutrient sufficient characteristics at all times.

One might expect phytoplankton present in a situation where the supply of nutrients is insufficient to support continued growth (i.e. condition 1) to exhibit nutrient deficient characteristics. That relatively few observations indicate high carbon:nitrogen ratios is natural systems suggests the phenomenon may be transient or mechanisms exist which prevent the occurrence of extreme deficiency. Two such mechanisms which would adjust the community size to that supportable by available nutrient supply are increased grazing pressures toward the end of blooms and increased sinking rates of nitrogen deficient populations (Eppley, 1967). Both mechanisms tend to decrease the size of the phytoplankton community. Grazing, additionally, recycles nutrients, thus increases the rate of supply.

It might also be expected that phytoplankton communities existing in nutrient poor systems (such as represented by the second type of environment) would exhibit nutrient deficient characteristics. An analogy might be drawn between a continuous flow system and a two layered oceanic system. In both, a nutrient reservoir exists. If the slow supply of nutrients through a pycnocline were analagous to the situation of a slow flow rate chemostat, one would expect similarly deficient phytoplankton. Thomas (1970) has observed that the assimilation ratio of phytoplankton in the nutrient depleted areas off Costa Rica was slightly but significantly lower than that of phytoplankton harvested from the nutrient rich Costa Rica Dome. Others indicate carbon:nitrogen ratios which are characteristic of nonnitrogen deficient communities. Extreme deficiency perhaps does not occur for several reasons. The chemostat analogy may not be appropriate, hence, deficiency may not be expected. In an oceanic two layered system the total nitrogen (phytoplankton and nutrients) in the "growth chamber" is much lower than that in the deeper "reservoir"; for a chemostat, the two are approximately equal.

Further, it is to be expected, given sufficient time to attain a quasi-steady state, that ecological control mechanisms would adjust the various communities (phytoplankton, zooplankton, bacteria, etc.,) such that their physiological characteristics would be described as nutrient sufficient. It may indeed be that physiological characteristics

of these populations are different from those of populations of differing nutrient regimes and hence any measure of a characteristic such as nutrient deficiency may only be applicable relative to an environmental type.

That many have observed carbon:nitrogen ratios to be on the average 6:1 indicates for the most part, natural populations are in a nutrient sufficient state and that perhaps nutrient deficient conditions are transient. No reports exist which attempt to characterize the physiological state of phytoplankton with regard to the environment from which they have been harvested with the exception of the one report by Thomas. Furthermore, refined investigations may reveal considerable variation in carbon:nitrogen ratio. The greatest difficulty in adequately determining carbon:nitrogen ratios of natural communities is the unknown abundance of non-plant particulate carbon and nitrogen.

The tacit assumption in the above discussion has been that nitrogen is the nutrient in short supply. Redfield (1965) has demonstrated that inorganic phosphorus and nitrogen in the deep waters of the ocean exist in ratios which are similar to those ratios of the average composition of algae. He argues that the depletion of these nutrients should occur in similar fashion such that they may both become in short supply at the same time. Ryther (1970) suggests that the rapid exchange or cycling of phosphorus within the immediate environment is sufficient to prevent the algae from becoming phosphorus limited. Others (Glooshenko and Curl, 1971;

Menzel and Ryther, 1961) have demonstrated that iron may control the rate of growth of phytoplankton. Thus, although a particular environment may be nitrogen limited to the ultimate yield of phytoplankton, the rate of growth (or carbon fixation) may be controlled by the low concentrations and supply of other required nutrients.

Based on this and other similar studies, it seems that the physiological state of phytoplankton populations should be measured in connection with other observations concerning the growth of natural populations. Although the carbon:nitrogen ratio may provide a qualitative measure of nitrogen deficiency, difficulties in obtaining measurements sufficiently clear of contaminating material such as non-plant carbon and nitrogen as well as variations in carbon:nitrogen ratios due to nitrogen source may preclude its utility except under limited conditions. Further detailed examinations of the changes in assimilation ratio with nutrient deficiency of phytoplankton populations may provide better evidence as to the relative deficiency of natural populations.

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