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Culture and Growth of *Closterium ehrenbergii* (Desmidiaceae)

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AN ABSTRACT OF THE THESIS OF David Bruce Renstrom for the Master of Science in Biology presented July 23, 1979.

Title: Culture and Growth of *Closterium ehrenbergii* (Desmidiaceae)

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

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Methods were devised for the isolation and pure culture in defined mineral medium of *Closterium ehrenbergii* Menegh. (Chlorophyceae). The effect of pH, type of nitrogen source, nitrate concentration, total hardness, and various buffers on growth were investigated. Best growth rates were obtained at pH 8 to 9, but good growth was observed even at pH 9.4. Nitrate, ammonia, or urea nitrogen could be utilized, but at concentrations greater than 10^{-2} M urea was toxic to growth. At higher concentrations of ammonia and

nitrate longer lag phases of growth were obtained but eventual higher cell yields were obtained. Good growth rates were obtained with Tris-(hydroxymethyl)-aminomethane buffer at up to 10^{-2} M; higher concentrations were inhibitory.

Assimilation of nitrate occurred both under light and dark conditions, however the assimilation was slower in the dark. After four days in nitrate-depleted medium, assimilation of nitrate occurred immediately after nitrate was added. This indicated relative stability of the assimilation enzymes. Attempts to determine the K_m for nitrate uptake yielded results that did not fit the classic Michaelis-Menton hyperbola but may be instead described by sigmoidal kinetics. Daily fluctuations in nitrogen per cell occurred due to synchronous cell division. Attempts to produce a sexual response in defined mineral medium with a known homothallic strain failed. A review of cultural and observational studies of desmid physiology is included.

CULTURE AND GROWTH OF
CLOSTERIUM EHRENBORGII (Desmidiaceae)

by

DAVID BRUCE RENSTROM

A thesis submitted in partial fulfillment of the
requirements for the degree of

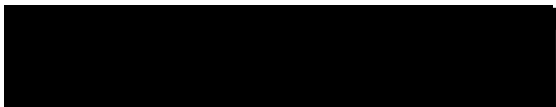
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in
BIOLOGY

Portland State University

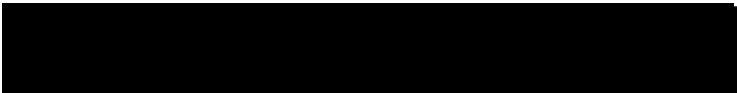
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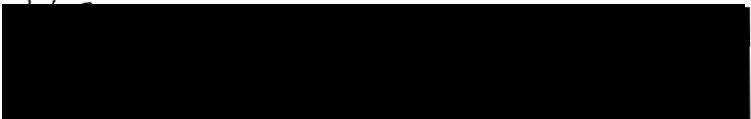
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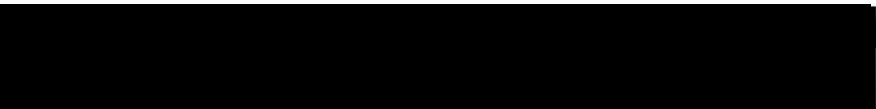

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TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vi
LIST OF FIGURES	vii
INTRODUCTION	1
MATERIALS AND METHODS	11
Growth Measurements	13
pH Experiments	15
Buffer Experiments	15
Hardness Experiment	17
Nitrate Concentration Variation Experiment	17
Nitrate Uptake Experiments	17
Uptake Kinetics	19
RESULTS	20
pH Experiments	20
Buffer Experiments	20
Hardness Experiment	23
Nitrate Concentration Variation Experiment	23
Nitrate Uptake Experiments	25
Nitrate Uptake Experiment #1 with Strain #3	25
Synchrony	28
Uptake Kinetics	30

PAGE

CONCLUSIONS	33
REFERENCES CITED	36
APPENDIX	40

LIST OF TABLES

TABLE		PAGE
I	Preparation of Medium A	12
II	Preparation of Medium A-1	16
III	Cell yield of <i>Closterium ehrenbergii</i> #9 with Tris-(hydroxymethyl)-aminomethane buffer . .	22
IV	Yield of <i>Closterium ehrenbergii</i> #9 with sodium- carbonate buffer	22

LIST OF FIGURES

FIGURE	PAGE
1. The effect of initial pH on yield of <i>Closterium ehrenbergii</i> (#9)	21
2. The effect of medium concentration on growth of <i>Closterium ehrenbergii</i> (#3)	24
3. The effect of nitrate concentration on growth of <i>Closterium ehrenbergii</i> (#9)	26
4. Nitrogen/cell in synchronous cultures of <i>Closterium ehrenbergii</i> (#3)	27
5. Synchronous cell division of <i>Closterium ehrenbergii</i> (#15)	29
6. Nitrate uptake by <i>Closterium ehrenbergii</i> (#15)	31

INTRODUCTION

The Zygnematales (or Conjugales) are an order of three families of green algae distinguished by their unique sexual conjugation. Pores in the cell wall are absent in the filamentous Zygnemataceae, including the common genera *Spirogyra* and *Zygnema*, and the Mesotaeniaceae (or saccoderm desmids) while such pores (1) and a median constriction between the two semi-cells usually are present in the Desmidiaceae (placoderm or "true" desmids). Thorough discussions of the morphology and taxonomy of the saccoderm and placoderm desmids of North America have appeared recently (2, 3, 4).

In the sexual conjugation process, potential gametes align themselves and form a conjugation tube or a loose mucilagenous matrix through which the protoplasts migrate and fuse to form a zygospore. In the zygospore nuclear fusion eventually occurs, but this usually occurs just prior to germination after a period of dormancy (5). The diploid zygospore is generally the only spore formed by the desmids but aplanospores and other haploid spores have been reported (6, 7, 8). The zygospore is the typical resistant stage in the life cycle, and can withstand periods of dessication. In habitats subject to dessication, sexuality would be assumed to be a well-preserved trait. It has been noted that planktonic desmids of large lakes rarely, if ever, form zygospores (9). In these habitats the desmids probably reproduce only by cell division. In contrast, zygospores of sexual strains are commonly collected in mud samples from seasonal waters (10).

Sexual strains of desmids make suitable models for the study of physical and chemical factors which may trigger sexuality. The loss of sexuality in algae during prolonged cultures has been a difficulty experienced by many workers (11). This loss of sexuality during laboratory culture is found with algae in general. It is possible that changes in physiological properties occurring during laboratory culture may be a common problem (11). The possibility that the inadvertent selection of mutant genomes adapted to the culture method used has been suggested as a reason for the loss of sexuality (12).

Factors that have been indicated as affecting the sexual response of desmids and other algae in laboratory cultures are (a) light, (b) temperature, (c) the composition of the medium, and (d) carbon dioxide (10, 13, 14).

Starr (10) developed a method to induce conjugation in which log phase cells are suspended in nutrient depleted medium in a watch glass or spot plate and incubated in a petri dish. He found that atmospheric diffusion of carbon dioxide into the medium might be important: a watch glass with its larger surface area produced a higher percentage of conjugating cells and conjugation seemed to occur more readily near the surface of the medium. Increasing the carbon dioxide concentration of the atmosphere by placing a 5% sodium bicarbonate solution in the petri dish bottom increased the percentage of conjugants. It was also found that removal of carbon dioxide with a 5% sodium hydroxide solution in the petri dish bottom prevented conjugation. Best results were obtained at a light intensity of about 5500 lux with a daily light cycle of at least 16 hours. Reduction of the light intensity,

duration of the light cycle, or decreasing the incubation temperature to below 20°C decreased or halted conjugation. Lippert (13, 14) reported similar results with *Closterium ehrenbergii* and *Closterium moniliferum*. The effects of carbon dioxide and light intensity are generally assumed to be due to enhanced photosynthesis (15). However, the possible reduction of pH in cultures incubated in the presence of increased CO₂ may favor zygospore production independently (14). With *Closterium ehrenbergii*, 5% CO₂ treatment enhanced zygospore production regardless of pH but the effect was greater at pH 6.2 than at pH 8.8 (14). Dubois-Tylski (16) reported that light duration, intensity, and quality were all important in sexual induction with desmids.

The use of fresh, rather than nutrient depleted medium, results in a period of cell division prior to conjugation (10). This "depleted medium factor" is probably the nitrogen source(s) in the soil-water medium used by Starr (10), since nitrogen-free medium has been commonly used to induce conjugation in desmids (12, 13, 14). However, Hoshaw (17) induced sexuality within 5 to 7 days after transfer to fresh medium by increasing the temperature to 26 °C from 22 °C. Starr (10) suggests that the factors which appear to be operating in laboratory experiments may be important in those natural situations where zygospores are generally found, i.e. temporary waters.

Preliminary experiments (14) indicated enhancement of zygospore formation at 0.02 to 2.0 mg/l kinetin but complete inhibition at 5.0 to 10.0 mg/l kinetin. Indole-3-acetic acid (IAA) and naphthalene-acetic acid (NAA) did not seem to have any effect but were possibly inhibitory at 1.0 mg/l IAA or NAA. Difficulties in reproducibility of

of experiments (Lippert, personal communication) may be related to the difficulty in controlling other pertinent factors such as nitrogen in soil-water medium.

Starr (10) and Lippert (13, 14) used Pringsheim's soil-water medium (18) with calcium carbonate added to grow those species preferring an alkaline medium. The medium "contains nitrates, ammonia, vitamins, an array of bacterial metabolites, and trace metals chelated by humic acids" (14, p. 742). The fact that some algae could be grown only on this undefined medium was one of the clues which led to the search for the then unknown factors, such as vitamins, which were required by these algae. Many algae have since been shown to require one or more of the vitamins—thiamine, biotin, or B₁₂ (cyanocobalamine). Only B₁₂ has been shown to be required by any of the desmids (11, 19, 20). Axenic (pure) cultures of *Closterium strigosum* initially required vitamin B₁₂. After extended culture in mineral medium plus soil extract this organism did not require it any longer (20). As previously noted, such changes in synthetic ability may be a common problem with laboratory cultures (11).

The culture of algae in mineral media dates back to the 1890's, when Beijerinck, Molisch, and Miguel succeeded in culturing a number of green algae and diatoms (11). Their success with these autotrophic organisms obscured the fact that up to 70% of algal species have now been shown to require one or more growth factors and consequently would not grow in their strictly mineral media (11). Pure cultures of desmids were probably first obtained in 1926 by Czurda (21), a student of Pringsheim.

The use of axenic cultures may be a theoretical, rather than a practical, necessity for the determination of many physiological characteristics when using mineral media. Several desmid researchers (19, 20) have considered the results of unialgal cultures in mineral medium to be equally valid to those in axenic culture. Extracellular polysaccharide production by algae is usually minimal during exponential growth (22). This is possibly due to the slow rate of autolysis compared to lag and stationary growth phases. Experimental results, using exponentially growing cultures are probably not affected by bacterial interferences. Moss (19) accepts growth measurements obtained with vitamin utilization in non-axenic cultures partly because even with bacteria present the alga required the addition of vitamins.

Culture research with desmids has often paralleled field research which attempts to describe the physical and chemical determinants of desmid distribution in natural waters. Field data frequently led to the hypothesis that desmids were calciphobic and favored by a high ratio of monovalent to divalent cations. However, Moss (23) compared the effect of calcium concentration and the ratio of monovalent to divalent cations on a number of desmid and other algal species that are characteristic of soft and hard waters. He concluded that these factors probably are unimportant in determining the distribution of soft water (oligotrophic) and hard water (eutrophic) species. These terms are used in the original sense in this paper, i.e. based on hardness, rather than in accord with more recent usage equating eutrophy to high productivity and oligotrophy to low productivity (24).

This new usage ties the terms to nutrient availability, which may not be apparent from levels of dissolved nutrients.

Moss (23) also reported different minimum calcium requirements for algae. Some oligotrophic, filamentous desmids had the highest minimum requirements (1 to 3 mg/l Ca). Calcium concentrations of this order are unlikely to be limiting in any natural waters. Growth of desmids endemic to soft waters (less than 90 mg/l hardness as CaCO_3) was not affected by levels of calcium up to 100 mg/l or by the ratio of monovalent to divalent cations. Algae of harder waters (up to 360 mg/l hardness as CaCO_3) were unaffected by low (1 mg/l) or high (100 mg/l) calcium. Tassigny (20) reported that cultures of *Staurastrum sebaldi* Reinch var. *ornatum* Nordst., *Closterium strigosum* Ehr. and *Micrasterias crux-melitensis* (Ehr.) Hassal. had a 20 to 30% increase in generation times at increased calcium ion concentration. *Staurastrum paradoxum* Meyen, common in eutrophic waters, was indifferent to calcium ion concentration. These results are criticized by Moss (23) on the basis that unnaturally high ionic concentrations were used in the media. Gough (25) examined the growth of *Closterium moniliferum* and *Cosmarium granatum* from hard waters and *Triploceras gracile* from an acid bog at high (50 mg/l) and low (3 mg/l) calcium ion concentration. Each calcium concentration was tested at high (8.5) and low (6.0) pH. Low pH and low calcium concentration favored the growth of *Triploceras* but high pH at either calcium concentration favored *Closterium*. He contended that both high pH and high calcium concentration favored the growth of *C. granatum*, but his interpretation was based on relatively small differences in growth rate at the two calcium

concentrations.

The presence of a diverse desmid flora (more than 20 species) is considered indicative of oligotrophy (23) when oligotrophy is used in the original sense of soft waters. Hardness is commonly measured as alkalinity (weak acid salt concentration) and expressed as bicarbonate (HCO_3^-) or as CaCO_3 since these commonly make the greatest contribution. Conductivity may be roughly converted to alkalinity by the relation $125 \mu\text{mho/ml} = 1 \text{ m-equiv. HCO}_3^-$ (23). Moss (23) uses a citation from Rodhe to estimate the concentration of the other major ions which may be expected to occur with a given bicarbonate concentration in natural waters.

Highly productive, often nutrient enriched, soft waters may retain much of their "oligotrophic" algal flora but unproductive hard waters have similar flora to that of productive hard waters (23). The use of algal species as indicators of trophic status is critically reviewed by Brook (26), who apparently defines eutrophy/oligotrophy in terms of productivity. Tassigny (27) presents considerable field data on the use of algae, particularly desmid species, as trophic indicators. Much of the confusion associated with the use of trophic status terminology seems to be due to the failure of some authors to adequately define their use of the terms, or even to attach different meanings in different context.

Moss (28) examined the growth of 25 marker species and found, relative to the carbon dioxide-bicarbonate-pH system, no differences in the minimum pH tolerated by eutrophic versus oligotrophic species. However, eutrophic species usually tolerated a higher maximum pH (>9.3)

than oligotrophic species (<8.6). Experimental evidence is presented to exclude ionic content and trace metal availability as explanations for his results. The simplest and perhaps most reasonable explanation is the availability of free carbon dioxide for photosynthesis. As the pH increased above 7.7, free CO₂ in the medium decreases to a minimum, and growth rates of the oligotrophic organisms decreased correspondingly. The continued growth of some algal species at higher pH is probably due to active absorption of bicarbonate, which is converted to carbon dioxide by the activity of the enzyme carbonic anhydrase. A rigorous proof for the utilization of bicarbonate may be impossible (29) since some free CO₂ is always present in solution at any pH. It should be noted that those desmids with a typically wider distribution, such as certain species of *Staurastrum*, *Cosmarium*, and *Closterium*, had a much wider distribution in waters of varying types than did other desmid genera. The vascular plant *Utricularia*, supported the widest diversity of desmids wherever it occurred. "High generic diversity was correlated with low conductivity, calcium, and alkalinity, pH of 5.1 to 7.0 and the presence of free carbon dioxide" (32).

Division rates in exponentially growing desmid cultures are slow compared to other algae (0.1 to 1.0 divisions/day). Direct comparisons of yield are difficult to obtain and are seldom reported. Using cell organic content as a measure of size, Moss (19) demonstrated little correlation between size and growth rate. The expected (by this author) correlation may only occur at optimal growth rates. Moss also investigated the relationship between growth rate and temperature (19). The broad range of optimal temperatures in his results may be

due to culture conditions in which temperature is not clearly the limiting factor; two other potentially limiting variables, i.e., light and turbulence, were not investigated. More rigorous experiments with light and temperature limited growth of *Chlorella* (34) showed that "temperature has little effect on photosynthesis and growth in light-limited systems" (34, p. 723). With these synchronous cultures of *Chlorella* under temperature-limited conditions, an exponential increase in yield occurred from 20 to 30 °C with a Q_{10} of about 6. It would appear that growth must be optimized before the effect of temperature and perhaps other variables on growth can be accurately investigated.

The effect of cell division synchrony on growth rate determinations may be a special problem with desmids. Division occurs predominantly or exclusively at night with most species of desmids (35, 36), so that a degree of cell division synchrony is always present in growing desmid cultures. Schulle (37) reports synchronous division of *Staurastrum pingue* Teil. at a definite time after the beginning of the light period. His claims for the first synchronous desmid cultures are, however, unjustified (see 35, 36).

Many species of algae have been shown to be able to use a wide variety of nitrogen sources, including nitrate, ammonia, urea, various amino acids and other organic nitrogenous compounds. Moss (19) investigated growth of desmids with nitrate or ammonia as a nitrogen source and obtained similar growth rates with either.

My work with *Closterium ehrenbergii* was primarily intended to develop efficient and reliable techniques for axenically culturing the organism in mineral medium. Axenic cultures contain no organisms

other than the desired species and are synonymous with pure cultures. The use of pure cultures for physiological experiments precludes the possibility of interferences which may arise from the presence of contaminant microorganisms. Mineral media may be defined, i.e. the concentrations of all components are known. This is necessary for most physiological experimentation, especially where growth requirements are being investigated. The lack of carbon sources in mineral medium is an added benefit in that the growth of contaminant bacteria and fungi is inhibited by the lack of carbon sources other than the extracellular products of the research organism.

After good growth of pure *Closterium ehrenbergii* in defined mineral medium was obtained, a series of growth experiments were conducted to investigate the effects of (a) total salt concentration, (b) pH, (c) various buffers at varied pH, (d) nitrogen source, and (e) nitrate concentration. The effects of pH and nitrogen on growth were investigated because they have been indicated to be important to the sexual response of desmids. Further experiments were conducted with the uptake of nitrate as exclusive nitrogen source.

MATERIALS AND METHODS

Closterium ehrenbergii Menegh. (Desmidiaceae, Chlorophyceae) was found in abundance on the surface of submerged decaying alder (*Alnus rubra*) leaves in very shallow spring-fed seepage ponds near Oaks Park, Portland, Oregon in March 1976. A number of collections from discrete masses of nearly unialgal cells were made and 12 unialgal clones were successfully grown from single cells isolated by capillary pipette into soil-water medium with added CaCO_3 (18).

Using the method of Starr (39), I found that one clone was homothallic, 4 were one member of a heterothallic pair (+ strain) and 7 were the other member (- strain). Visually observed differences in growth on soil-water medium may indicate that the various plus (+) or minus (-) strains are not necessarily physiological equivalents but for the purposes of further study, the homothallic strain (#3) and one each of the plus (#9) and the minus (#15) were isolated axenically by manipulation with a glass microneedle on agar medium so as to remove accompanying microorganisms (40). Initially axenic (pure) cultures were established in soil-water medium preconditioned by growth of unialgal *Closterium*. The growth medium was filter sterilized with a 0.22 μ millipore filter (0.45 μ filters selectively passed one strain of bacteria). Later a mineral medium [medium A (Table I)] was devised which supported good growth and axenic cultures were established directly on medium A agar (1% Difco) by micro-manipulation and transferred to liquid medium A or its later modifications. Vigorously

TABLE I
PREPARATION OF MEDIUM A

	Medium Concentration (mg/l)	Molarity
<u>Major ions</u> ^a		
NaNO ₃	62.5	7.25 x 10 ⁻⁴
MgSO ₄ ·7H ₂ O	18.7	5.10 x 10 ⁻⁵
CaCl ₂ ·2H ₂ O	25.0	1.7 x 10 ⁻⁴
(NH ₄) ₂ HPO ₄	20.0	1.5 x 10 ⁻⁴
<u>Bold's minor element solutions (44)</u> ^a		
1. Na ₂ EDTA	50.0	
KOH	31.0	
2. FeSO ₄ ·7H ₂ O	5.0	acidified with 1 ml/l H ₂ SO ₄
3. H ₃ BO ₃	11.4	
4. ZnSO ₄ ·7H ₂ O	8.82	
MnCl ₂ ·4H ₂ O	1.44	
MoO ₃	0.85	
CuSO ₄ ·5H ₂ O	1.57	
Co(NO ₃)·6H ₂ O	0.49	
<u>Vitamin solution</u> ^b		
thiamine -HCl	1.0	
biotin	2.6 x 10 ⁻³	
B ₁₂	96.2 x 10 ⁻³	

^aAR grade chemicals were used for media preparation.

^bA combined vitamin solution was prepared. 12% water hydration was allowed when weighing B₁₂ and 4% for biotin (42). This vitamin solution was acidified to pH 5 with concentrated HCl and autoclaved separately from the medium. One ml/l was added after cooling to give the required medium concentration.

growing cells are used much more successfully to establish pure cultures.

Axenic condition was tested for in cultures used for inoculation and at the conclusion of experiments. Cultures were plated on nutrient agar (Difco) plus 3 g/l yeast extract and on medium A agar. Plates of each medium were incubated at 25 °C and at room temperature for up to 2 weeks. Occasionally, platings were made on a variety of other bacterial media. Microscopic examination of cultures with the oil immersion objective of a compound microscope failed to indicate the presence of bacteria if they were not also indicated by the plating.

Axenic cultures were routinely grown in a culture room at 20 °C \pm 1 °C on a 16/8 hour light/dark cycle. Light (approximately 3300 lux at the surface of experimental culture vessels) was provided by cool white fluorescent fixtures. For maintenance of cultures, the cultures were exposed to 3300 lux until good growth was evident and then were stored at much lower light levels (approximately 550 lux).

All glassware used as culture chambers or in the preparation of media was cleaned by soaking in a solution of concentrated nitric acid and sulfuric acid (40/60) for at least 24 hours. Each piece was then rinsed in 25 volumes of glass distilled water. Glass distilled water was used for all media and reagent preparation.

GROWTH MEASUREMENTS

Change in cell number was used as a measure of growth; measurements of optical density (turbidity) did not give the necessary precision in these dilute cultures and dry weight measurements

required too large a culture volume. Cell number was determined by a minimum of 3 counts of 1 ml aliquots from the culture vessel. Since sample size was small (10-500 cells/ml), the entire aliquot was counted. Cells were dispersed within the growth chamber by swirling the flask or tube until cells no longer adhered to the walls of the vessel. It was interesting to note that cells adsorbed to the glassware more during early stages of growth and ceased to adsorb as cells approached stationary phase. The percent standard deviation of replicate counts seldom exceeded 10%, and the methods for dispersion within the growth chamber and sampling error were deemed acceptable.

In graphical presentations of results, mean cell numbers \pm one standard deviation are presented. Numerically, this gives very similar results to precision at the 95% confidence level.

The following expression describes growth rate (41):

$$k = \frac{\log_2(N_1/N_0)}{t_1 - t_0}$$

where N_0 = initial cell number at t_0 , N_1 = cell number at t_1 , t_1 and t_0 = days, and k = specific growth rate constant in divisions/day.

A degree of synchronous cell division is always apparent in growing cultures of *C. ehrenbergii*, since cell division occurs almost exclusively during the last four hours of the dark period. The degree of synchronous cell division is considered to be the percentage of cells dividing during a given dark period. Enhanced synchronous cell division is that which occurs above the normal (or average) percentage of cell division during exponential growth.

pH EXPERIMENTS

The effect of pH on growth of strain #9 was investigated in unbuffered medium A. The initial pH was adjusted with HCl or NaOH at increments of one pH unit over the range of pH 4 to 10. The pH of the unadjusted medium was 9.0. No attempt was made to control the pH during the course of the experiment. Yields of strain #9 were determined by cell counts at stationary phase. Three replicate cultures in 18 X 150 mm test tubes with 10 ml of medium were used at each pH. Culture conditions were as previously stated.

BUFFER EXPERIMENTS

In order to determine if buffer and buffer concentration affect the final yield, the cell number of strain #9 was determined when cells reached stationary phase after growth in medium A-1 with Tris-(hydroxymethyl)-aminomethane buffer (Tris) at 10^{-1} , 10^{-2} , and 10^{-3} molar. Each concentration was tested at pH 7.8, 8.3, and 8.8. A similar experiment with sodium-carbonate buffer (at the same concentrations as the Tris buffer) and pH of 9.3, 9.8, and 10.3 was also used. The pH during the course of the experiments was not controlled. Three replicate cultures in 18 X 150 ml test tubes with 10 ml of medium were used at each pH and buffer concentration. Small inocula (about 15 cells/ml) were used. Culture conditions were as previously stated.

TABLE II
 PREPARATION OF MEDIUM A-1^a

	Medium Concentration (mg/l)	Molarity
<u>Major ions</u> ^b		
NaH ₂ PO ₄	6.9	5.0 x 10 ⁻⁵
KH ₂ PO ₄	6.8	5.0 x 10 ⁻⁵
KNO ₃	Variable	} up to 212 μM
NaNO ₃	Variable	
<u>Buffer</u> ^c		
Tris-(hydroxymethyl)-aminomethane		5.0 x 10 ⁻³

^aPreparation is as for medium A except for the following differences: (NH₄)₂HPO₄ is omitted and the above listed major ions are added.

^bKNO₃ and NaNO₃ were varied at a constant ratio.

^cMedium A-1 was titrated to pH 8.2 with HCl. A Sargent-Welch model LS pH meter with a Corning ceramic junction electrode #476115 suitable for use with Tris (46) was used for all experiments using Tris buffered medium. Tris standards at pH 7.2 and 9.0 were prepared from dessicated Tris base and Tris HCl (46).

HARDNESS EXPERIMENT

Strain #3 was grown in medium A at $\frac{1}{4}$, $\frac{1}{2}$, normal, twice normal, and four times normal total salt concentration (hardness), measured as mg/l added reagents. Total salt concentration varied from 41 mg/l to 656 mg/l. Cell yield (cells/ml) and growth rates were determined. Two replicate cultures in test tubes with stainless steel closures were counted at various stages of growth until stationary phase was reached. Culture conditions were as previously stated.

NITRATE CONCENTRATION VARIATION EXPERIMENT

Strain #9 was grown in medium A-1 (Table II) containing 6.6 μM , 53 μM , or 212 μM nitrate. Cultures in 250 ml cotton-stoppered Erlenmeyer flasks contained 150 ml of medium and were inoculated with 15 cells/ml. Cell counts were made at intervals until stationary phase was reached. Culture conditions were as previously described.

NITRATE UPTAKE EXPERIMENTS

Strains #3 and #15 were used in these experiments. Cultures were in 250ml, 500ml, or 1 liter cotton-stoppered Erlenmeyer flasks.

Cultures were aerated with filtered air. Air was first passed through a saturated ZnCl_2 solution to remove traces of ammonia and subsequently passed through two successive distilled water baths to insure that there was no ZnCl_2 contamination and to saturate the air with water vapor.

Experiments were conducted with exponentially growing cells fully induced for nitrate uptake in medium A-1. Exceptions to this

were experiments to test the effect of lag phase or synchronous growth. Cultures could be made synchronous above the normal level of approximately 20% by adding nitrate after several days of nitrate depletion.

Cell number and nitrate concentration of the medium were determined for several days. From this data, nitrogen/cell (cellular quota) and total cellular nitrogen of the culture were calculated, assuming total cellular nitrogen to be equivalent to depletion of nitrate from the medium.

Cultures were axenic at the beginning of experiments and bacterial growth during the course of the experiments was minimal. Nitrate was added during the course of the experiments to prevent its depletion. Large inocula were used so that measurable changes in nitrate concentration occurred within a reasonable time. The inocula were prepared by allowing the cells to settle, decanting the medium, washing the cells with sterile nitrate-free medium, and resuspending the cells in fresh medium.

Nitrate and nitrite concentrations were determined by the copper cadmium reduction method (44). Uptake of nitrate was assumed to be equivalent to the depletion of nitrate from the medium. Ethylenediaminetetraacetic acid (EDTA) used in medium A-1 was found to interfere with the reduction of nitrate to nitrite. Experiments to measure nitrate uptake used medium A-1 minus the EDTA and trace metals were reduced to a tenth of the concentrations given in Table I.

Cells were removed from samples for nitrate analysis by filtration through nylon filter floss prior to the nitrate analysis.

Generally, 25 ml were required for nitrate determinations, although at concentrations higher than 20 μM , smaller samples were removed and diluted with distilled water to 25 ml.

Nitrate standards were prepared in distilled water and treated in the same way as the samples. Standard curves were prepared daily as well as periodically during the experiments. Colorimetric determinations of standards conformed well to Beer's law between 2 and 20 μM nitrate. Straight line fits were drawn by eye between at least four absorbance values for nitrate standards. Nitrite concentrations were also determined colorimetrically in the same way as the nitrate, although the prior reduction step is omitted. Nitrite was not detected in any samples, at a limit of detection of about 0.2 μM nitrite.

Direct nesslerization, a simple colorimetric method (45), was used to analyse for ammonium, although none was detected in any sample. The level of detection was about 1.2 μM ammonium.

Culture conditions were otherwise as previously stated.

UPTAKE KINETICS

Nitrate concentration and cellular nitrogen were monitored as a function of time. Cellular nitrogen is again considered equivalent to the depletion of nitrate from the medium. Rate of uptake was calculated as $\mu\text{moles nitrate}/\mu\text{moles cellular nitrogen per hour}$. Duplicate determinations were made and precision was greater than 0.5 μmole . Methods and culture conditions were as mentioned under nitrate uptake.

RESULTS

pH EXPERIMENTS

The initial pH of unbuffered medium A had a strong effect on the eventual yield of strain #9 as shown in Figure 1. It can be seen that the largest cell number was found at alkaline pH. Large changes in pH probably occurred because of the very low buffering capacity of medium A, although this was not investigated.

BUFFER EXPERIMENTS

The yield of strain #9, in cells/ml, with three concentrations of Tris buffer at three pH values is presented in Table III. The yield of strain #9 with three concentrations of sodium-carbonate buffer and three pH values is shown in Table IV. The yields at pH 8.3 and 8.8 with 10^{-2} and 10^{-3} M Tris were significantly higher than at pH 7.8 at the 95% confidence level. The trend indicated that pH 8.8 was preferred, although the difference in yield at pH 8.3 and at 8.8 was not significant at this confidence level.

The results with sodium-carbonate buffer were suspect because in the open system in which these experiments were conducted, equilibration with atmospheric CO_2 causes changes in pH. Since Tris appeared more useful, no further experiments with carbonate buffers were conducted.

In all subsequent culture media in which Tris buffer was used,

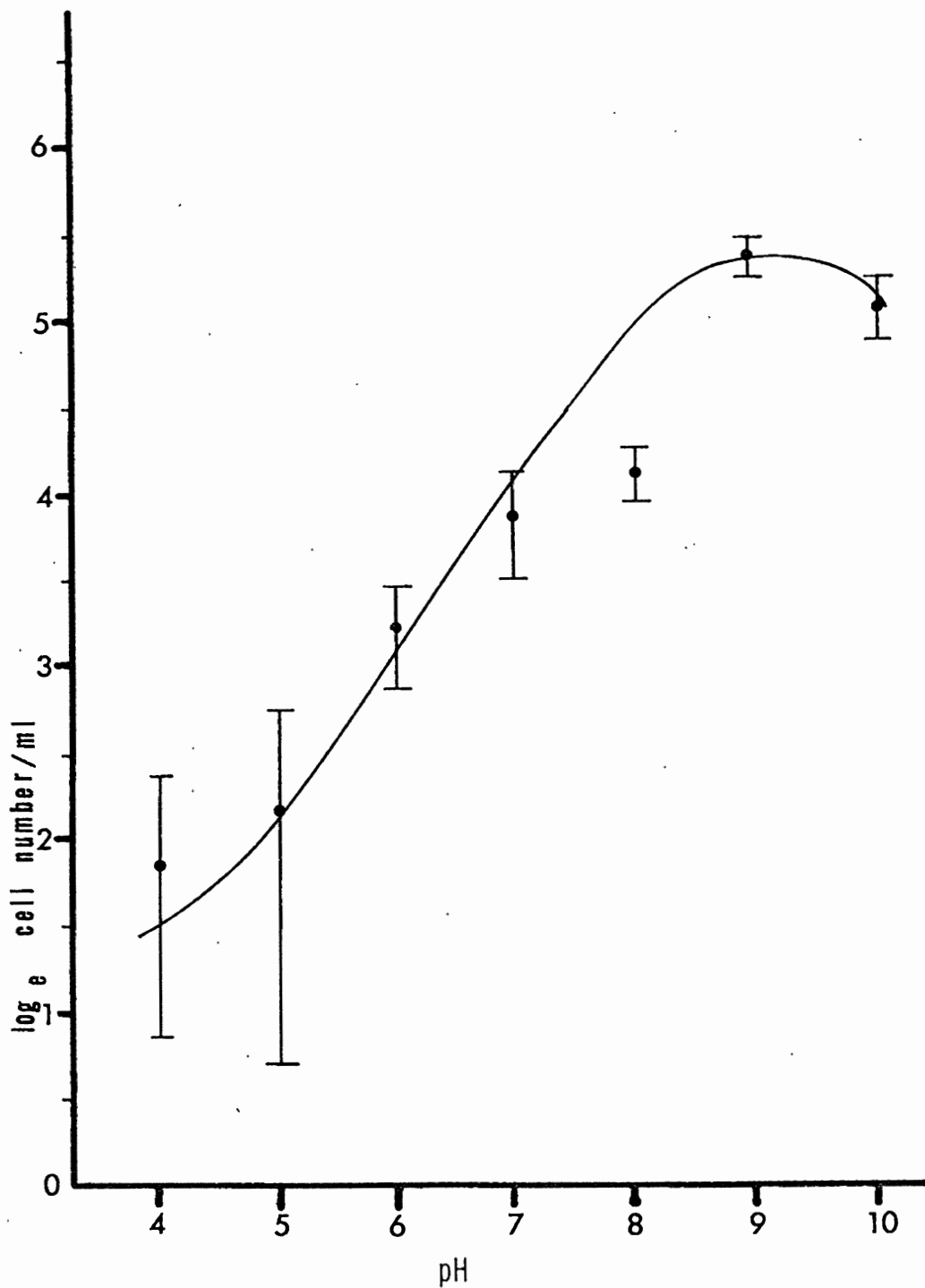


Figure 1. The effect of initial pH on yield of *Closterium ehrenbergii* (#9).



indicates the mean \pm one standard deviation

TABLE III

YIELD OF *CLOSTERIUM EHRENBERGII* #9 ^a
WITH TRIS BUFFER

Tris Concentration	pH		
	7.8	8.3	8.8
10^{-3} M	413 ± 41	640 ± 30	720 ± 43
10^{-2} M	441 ± 30	651 ± 100	761 ± 95
10^{-1} M	toxic ^b	toxic	toxic

a. in cells/ml ± one standard deviation

b. cell death occurs

TABLE IV

YIELD OF *CLOSTERIUM EHRENBERGII* #9
WITH SODIUM-CARBONATE BUFFER

Na-carbonate Concentration	pH		
	9.3	9.8	10.3
10^{-3} M	395 ± 38	416 ± 37	322 ± 18
10^{-2} M	toxic	toxic	toxic
10^{-1} M	toxic	toxic	toxic

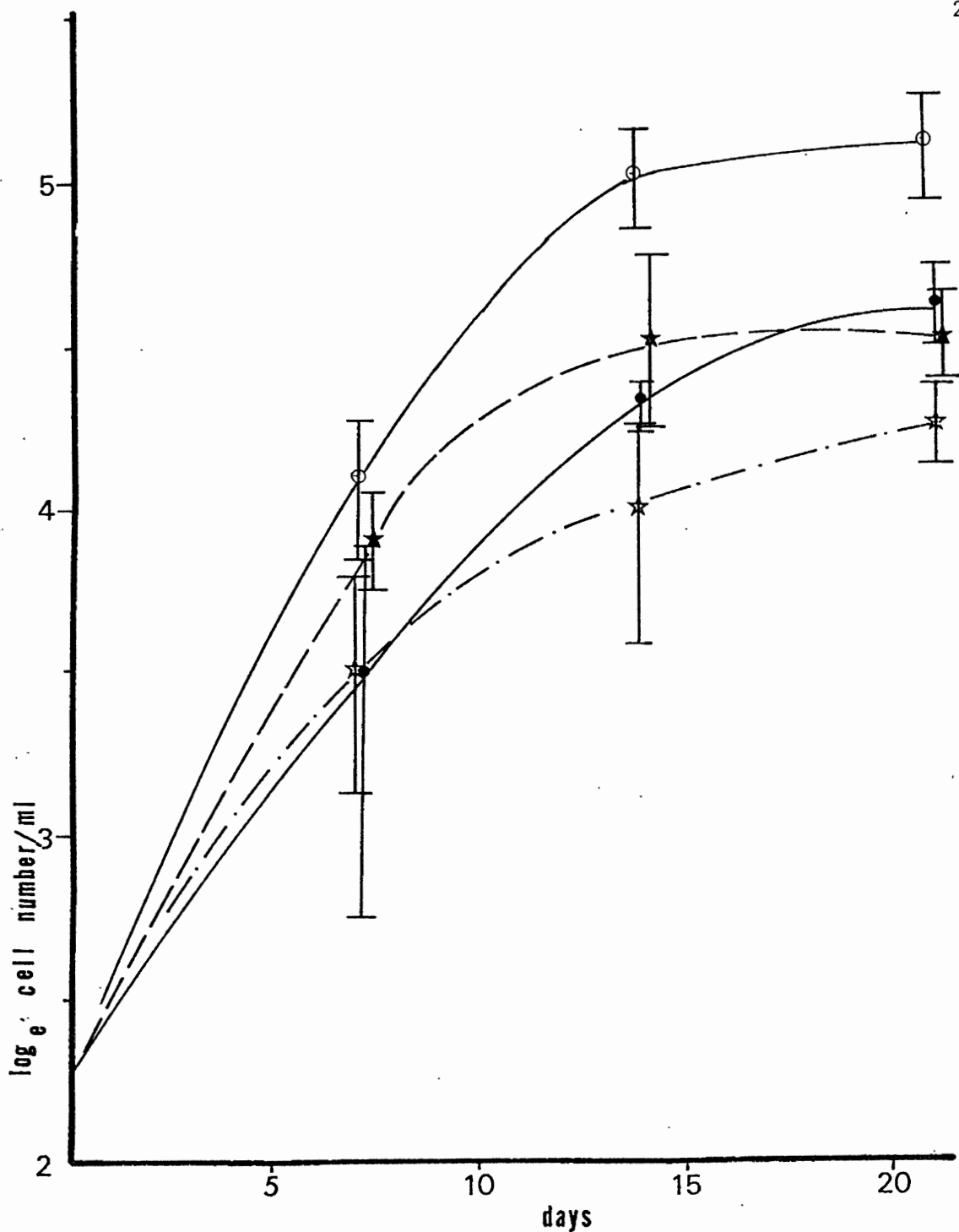
the concentration was .005M and the pH was adjusted to 8.2 with HCl. This pH, which was lower than that which yielded maximal growth, was chosen to increase the buffering capacity of the Tris (pK 8.3). Various measurements of pH during growth experiments using .005M Tris did not vary more than ± 0.30 pH units from the initial pH.

HARDNESS EXPERIMENT

Growth and yield of strain #3 at different total salt concentrations of medium A from 41 mg/l up to 656 mg/l indicated that higher concentrations inhibited final yield (see Figure 2). The dynamics of growth rate were not apparent in these batch culture experiments, especially maximum growth rates. A speculation is that yield is limited by the higher salt concentrations. At 41 mg/l total salts the yield is limited by nitrate, as indicated by later experiments where nitrate alone was varied. However this cannot be the case at the two higher salt concentrations, which have respectively 8 and 16 times the nitrate concentrations as the 41 mg/l total salt concentration. The similarity in the curves at 41 mg/l and 328 mg/l total salts appears to be caused by distinct phenomenon, i.e. nitrate limitation at 41 mg/l and inhibition by high total salt concentration at 328 and 656 mg/l. The results at 82 and 164 mg/l were practically identical and are combined in Figure 2.

NITRATE CONCENTRATION VARIATION EXPERIMENT

Growth rates of strain #9 were not significantly different at the three nitrate concentrations, with possibly a longer lag phase



- 41 mg/l total salts
- 82 " " " and 164 mg/l combined
- ★ 328 " " "
- ☆ 656 " " "

Figure 2. The effect of medium concentration on growth of *Closterium ehrenbergii* (#3).

occurring at the highest concentration [212 μM (see Figure 3)]. If the yield at these nitrate concentrations is plotted versus the nitrate concentration, a relatively straight line results. This line does not intersect the abscissa at zero yield with zero added nitrate. It seems doubtful that ammonium contamination from the atmosphere or excess nitrogen stored in the inoculum cells could account for this discrepancy.

NITRATE UPTAKE EXPERIMENTS

Specific growth rates (k) in these experiments were higher than in previous work, averaging 0.15 to 0.25 divisions/day, instead of 0.05 to 0.10 division/day. Presumably this was due to the aeration by water saturated, ammonia-free air. This probably indicates that growth rates in previous experiments were carbon-limited. No experiments with aeration at higher concentrations of carbon dioxide were conducted to test for this possibility.

Nitrate Uptake Experiment #1 with Strain #3

Data obtained from the second and third days of growth (after nitrate depletion for three days) are presented in Figure 4. The large fluctuation in cellular nitrogen quota during the second night shown in Figure 4 is the result of cell division, which occurs almost exclusively at night. When cells reach stationary phase, the cells are nitrogen depleted. When cultures are inoculated with stationary phase cells the apparent cellular quota rises to a maximum within a few days before assuming more regular fluctuations. This may be due to the fact that cell quota values are averages of all

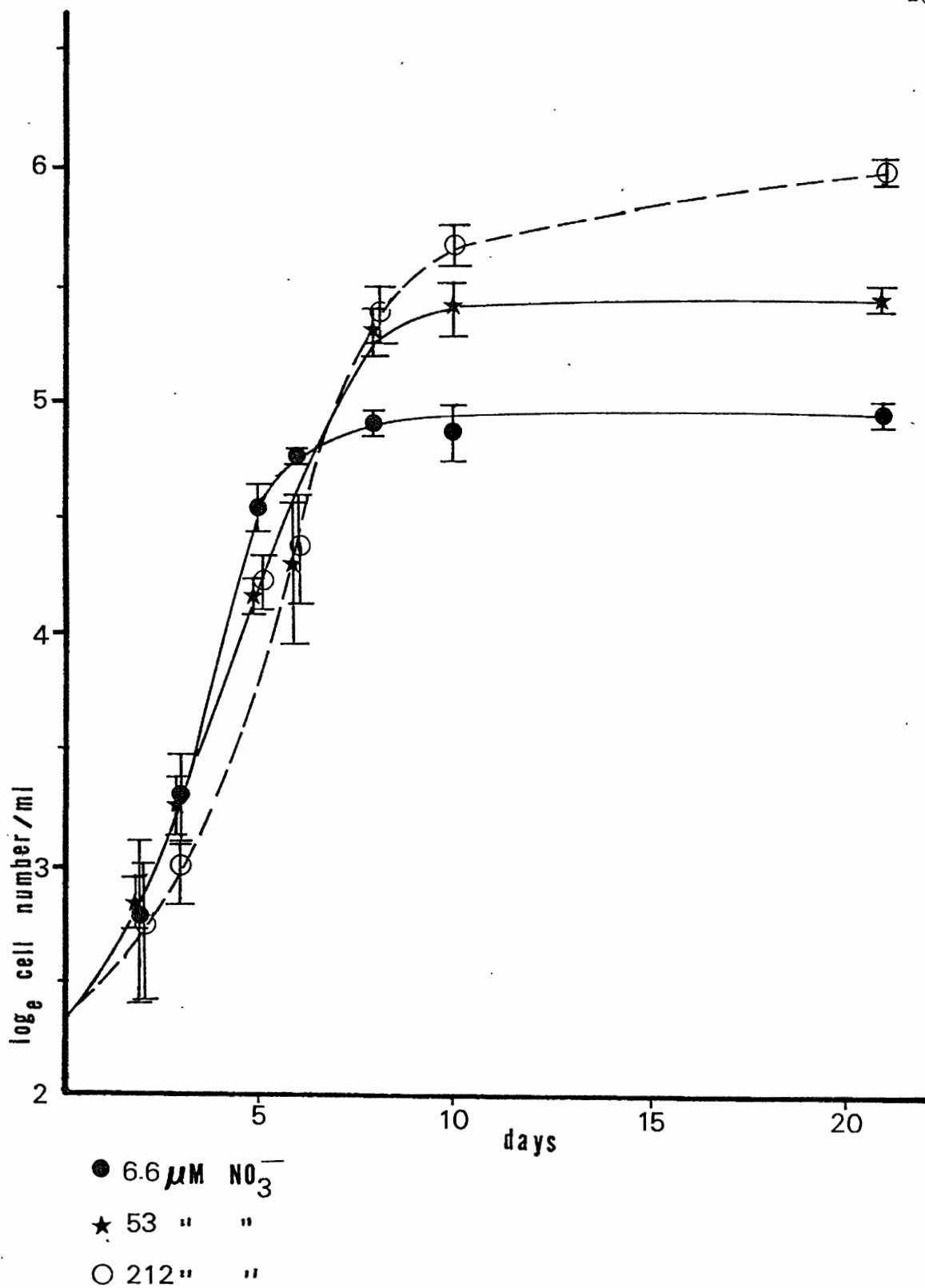


Figure 3. The effect of nitrate concentration on growth of *Closterium ehrenbergii* (#9).

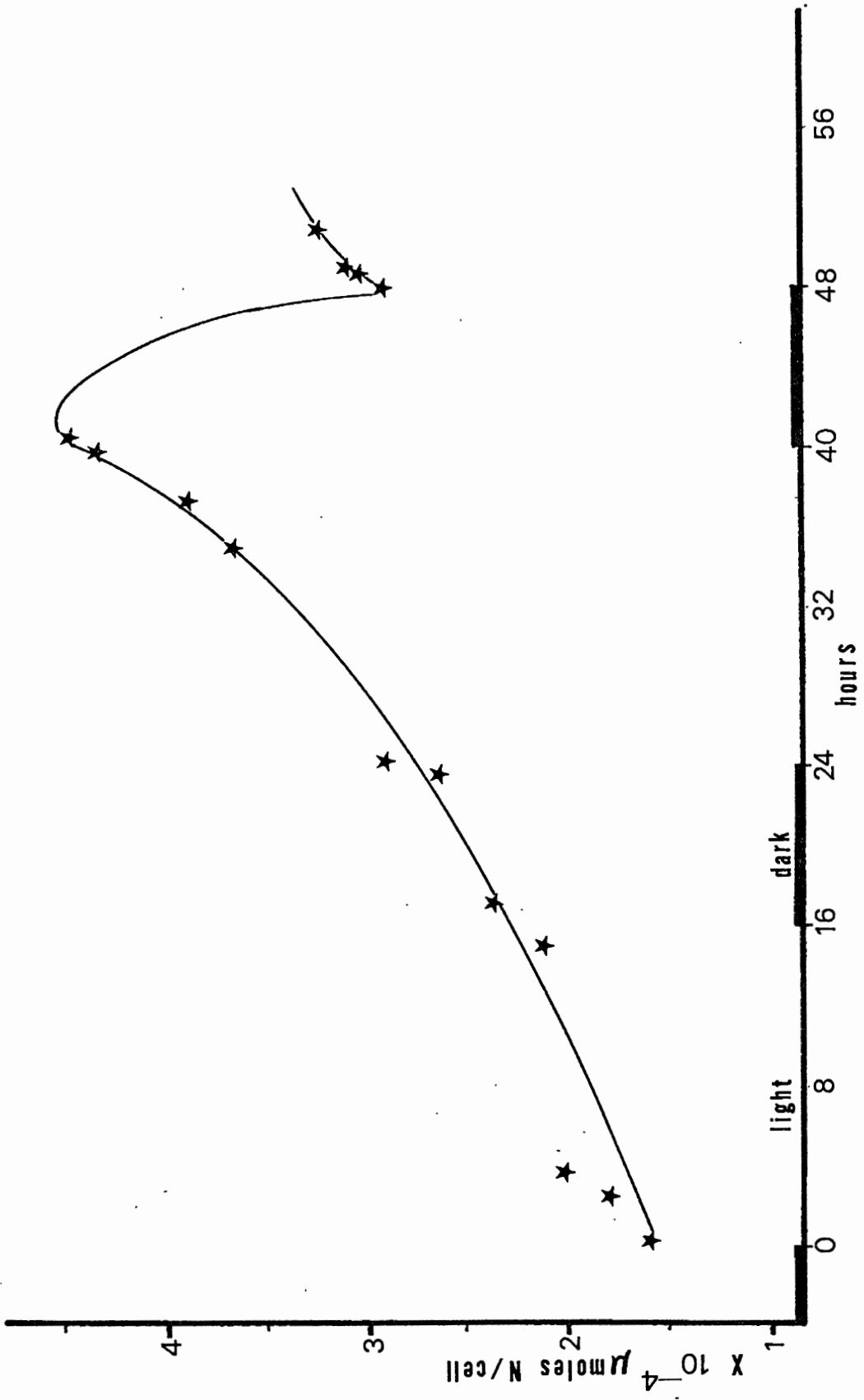


Figure 4. Nitrogen/cell in synchronous cultures of *Clostridium ehrenbergii* (#3).

the cells in the culture. Cultures which have been nutrient deprived become synchronous when nutrients again are available. Because of this synchrony, the large fluctuation shown in Figure 4 is more representative of individual cell quota. About 40% of the cells divided during this dark period (i.e. cell division synchrony is 40%). The lesser fluctuations in cell quota from later cell divisions (not shown in Figure 4) occur because cell division synchrony levels off at about 20%.

Comparisons of the specific growth rate (k), taken as the difference in cell count on successive days with the average cell quota of strain #3 at the beginning of the dark period indicated a possible relationship between cellular nitrogen and cell division which may be independent of external nitrate concentration.

SYNCHRONY

Figure 5 shows a plot of time versus cell number and depicts the establishment of synchronous cell division in *C. ehrenbergii* (#15) by nitrate depletion. An examination of these results indicates:

1. That cell division continues after nitrate is depleted. This partitions out cellular nitrogen to daughter cells, resulting in lower cellular quota. The related results shown in Figure 4 show how cellular nitrogen varies after resumption of growth when nitrate is added.
2. Synchronous cell division occurs when nitrate is provided again. In my experience, this effect occurs most readily if the cells are held in stationary phase for 2 to 5 days.

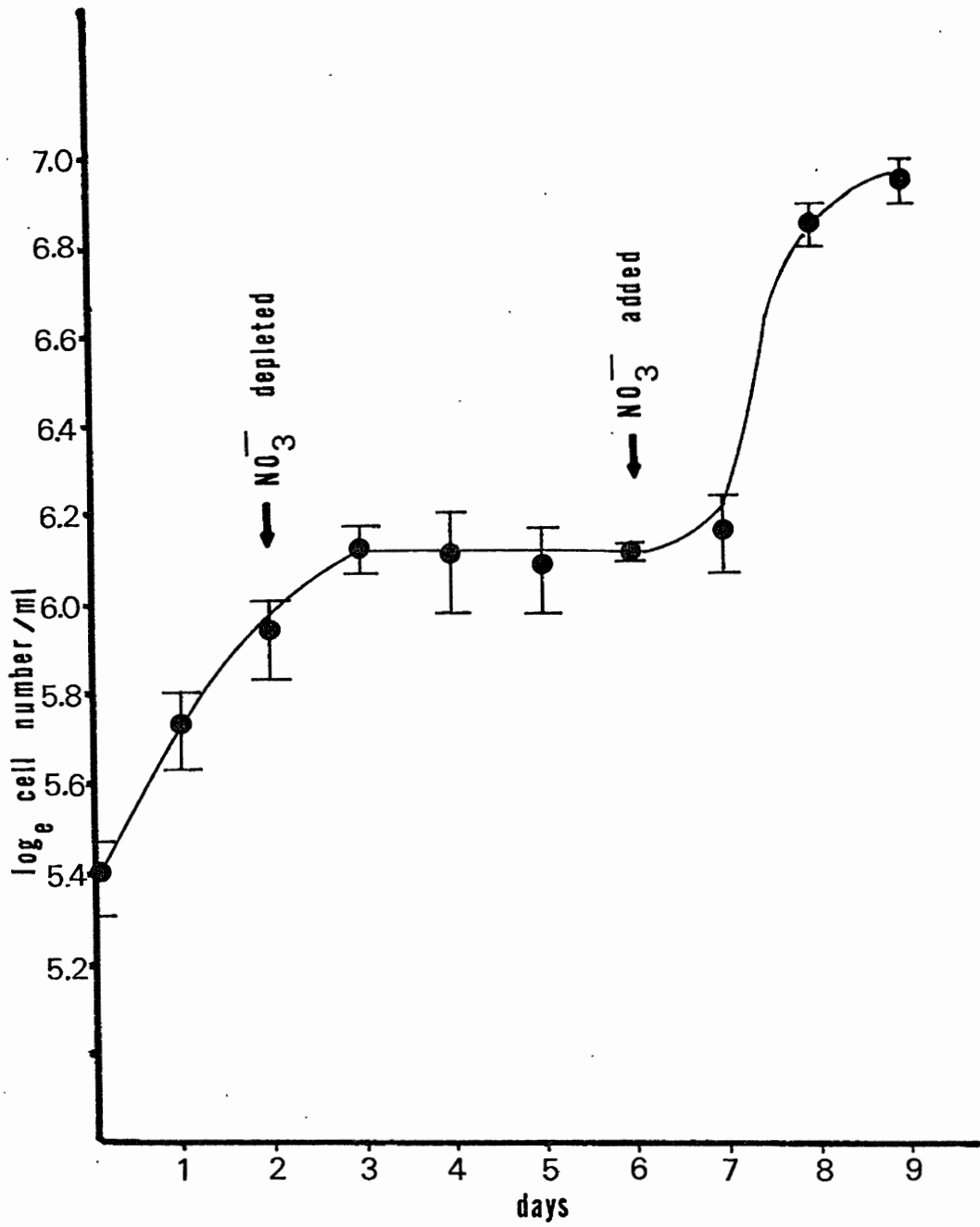


Figure 5. Synchronous cell division of *Closterium ehrenbergii* (#15).

Cells in stationary phase for longer periods of time gradually lose the ability to take up nitrate without long lag periods.

3. Enhanced synchrony of cell division occurs from day 7 to 8, where $k = 0.95$ divisions/day. This apparent growth rate, as determined from cell counts alone, is much higher than the real exponential growth rate. Synchronous cell division by nutrient depletion causes this anomaly in growth rate because cell counts are used as the measure of growth. In this case 65% of the cells divided between day 7 and 8, so cell division is said to be 65% synchronous. The base level of synchronous cell division during exponential growth in this experiment was about 30% (day 0 to 2, Figure 5). Since the generation time is about $2\frac{1}{2}$ days under these conditions, the actual degree of synchrony is difficult to assess. If we assume that all cells in the culture grow at about the same rate, the actual synchrony would be somewhat higher than indicated. Cells growing on medium containing agar, where individual cells may be observed, occasionally divide at close to 100% synchrony for 3 to 4 cells divisions after inoculation of healthy single cells.

UPTAKE KINETICS

Figure 6 shows preliminary results on the rate of nitrate uptake by *C. ehrenbergii* relative to the concentration of nitrate in the medium. As can be seen, nitrate uptake rate is corrected for cellular

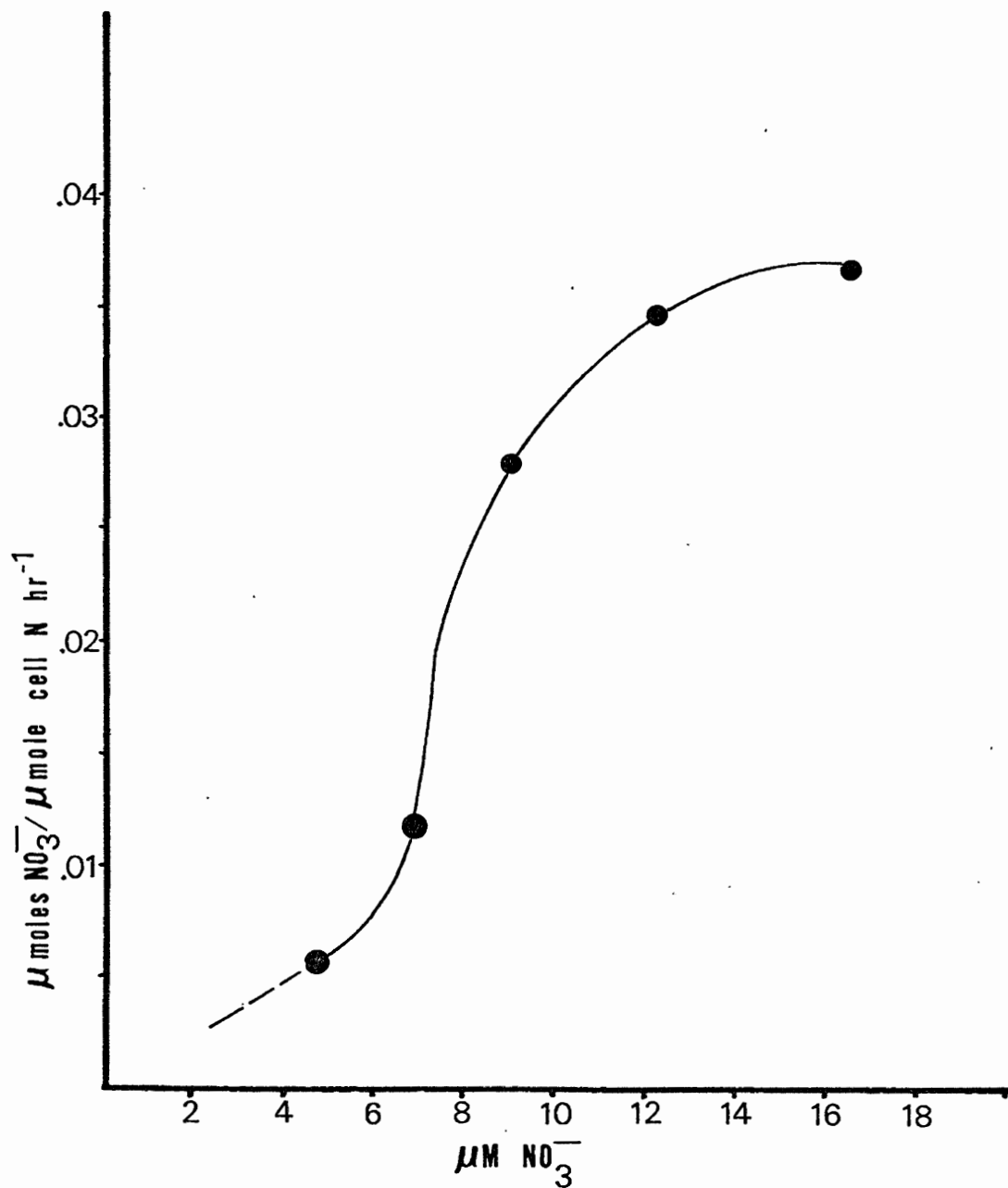


Figure 6. Nitrate uptake by *Closterium ehrenbergii* (#15).

nitrogen to eliminate the effects of growth during the experiment. Since the results appear not to conform to the Michaelis-Menton hyperbola, no determination of a K_m for uptake was attempted.

CONCLUSIONS

These experiments, personal observation of natural distribution, and the results of other workers (25, 27, 28) indicate that *Closterium ehrenbergii* grows well in alkaline waters, even at $\text{pH} > 9$. It is generally considered that the availability of free CO_2 may be the limiting factor for growth of many algae at high pH (28, 29). The ability of some species to continue growth at these pH may be due to their ability to utilize HCO_3^- as a carbon source for photosynthesis. The confirmed presence of carbonic anhydrase in some algae (29), which converts $\text{HCO}_3^- \rightarrow \text{CO}_2$ is strong evidence for this possibility. Another possibility is that these algae can take up sufficient CO_2 from the very low concentrations present at these high pH.

An optimum level of dissolved constituents was indicated by the experiment in which total salt concentration was varied. Since nitrate concentration varied with differences in total concentration of the medium, perhaps variation in nitrate concentration alone can explain the results of the total salt concentration experiment. My results seem to indicate that both an optimal nitrate concentration and an optimal total salt concentration for growth occur but the effects of each cannot be separated on the basis of these experiments. The work of both Moss (23) and Gough (25) is consistent with these results in indicating that the concentration of calcium ion is unlikely to affect the distribution of certain *Closterium* species in natural waters, although *C. ehrenbergii* was not used by these workers. They also

indicated that many species of *Closterium* favor alkaline pH.

The use of Tris buffered medium is not new. Ichimura (41) used 4.1×10^{-3} M Tris at pH 7.5 for growth of *Closterium* (see Appendix for Ichimura's *Closterium* medium). My results indicated that up to 10^{-2} M Tris could be used without toxic effect, but that a higher pH (8.3 to 8.8) produced higher cell yields.

The induction of cell division synchrony by nutrient deprivation is discussed by Lorenzen and Hesse (47). The potential for error in the determination of exponential growth rates of desmids using cell counts for growth measurements, is compounded by synchronous effect. On the other hand, high levels of synchrony may allow a new approach to the effect of cellular nitrogen on cell division and on uptake of nitrate. In a completely synchronous culture, rates of nitrate uptake are indicative of uptake rates of single cells if uptake of nitrate is related to cellular nitrogen.

Experiments in synchronous cultures could be used to investigate the relationship between uptake rate and cellular nitrogen. The present experiments with uptake of nitrate and synchronous growth in a desmid suggest the possibility of such relationships as well as that between cellular nitrogen and cell division.

A survey of the literature did not reveal any reports of nutrient uptake studies with desmids. The kinetics of uptake by marine phytoplankton cultures has been extensively studied by many workers (48, 49, 50) in an ecological context. They have well documented the fact that open ocean species of phytoplankton, where nitrate concentration is very low, typically have lower K_m for nitrate uptake than do estuarine

and sublittoral species where nitrate concentration is usually higher. Differences in ability to take up nutrients at low concentrations are thought to be important variables determining distribution of various species.

Uptake of nitrate by marine phytoplankton typically can be described by the Michaelis-Menton hyperbola. This relationship implies the existence of a substrate-saturable component to the uptake system, like many transport and enzyme systems. Typical K_m values for nitrate uptake by marine phytoplankton are 0.5 to 2.0 μM nitrate. My results with *C. ehrenbergii* indicate a much lower affinity for nitrate as well as a non-hyperbolic expression for uptake at low concentrations. It may be that uptake of nitrate by this organism may be described by sigmoidal kinetics, possibly cause by interaction of the uptake (transport) system and the assimilation of nitrate via nitrate reductase.

Although not reported here, preliminary results indicated that the three strains of *Closterium ehrenbergii* used for these experiments could use urea or ammonia as sole nitrogen source, with approximately the same growth rates and yields as with nitrate nitrogen. Urea was toxic at concentrations greater than about 10^{-2}M .

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APPENDIX

PUBLISHED DESMID MEDIA

Medium Reagents	Medium Concentrations (mg/l)
<u>Chu #10 medium (54)</u>	
Ca(NO ₃) ₂	40
K ₂ HPO ₄	5-10
MgSO ₄ ·7H ₂ O	25
Na ₂ CO ₃	20
Na ₂ SiO ₃	25
FeCl ₃	.8
pH 6.5-7.0	
<u>Pringsheim's <i>Micrasterias</i> medium (21)</u>	
KNO ₃	200
(NH ₄) ₂ HPO ₄	20
MgSO ₄ ·7H ₂ O	10
CaCl ₂ ·6H ₂ O	.5
FeCl ₃	.5
<u>Pringsheim's desmid medium (55)</u>	
MgSO ₄ ·7H ₂ O	10
K ₂ HPO ₄	10
KNO ₃	100
Agar	750-1500
50 ml/l soil-water supernatant	
Kies vitamin solution (23)	
B ₁₂	2 x 10 ⁻⁴
thiamine	1 x 10 ⁻³
nicotinamide	1 x 10 ⁻⁴

PUBLISHED DESMID MEDIA (cont.)

Medium Reagents	Medium Concentrations (mg/l)
<u>Pringsheim's desmid medium (cont.)</u>	
PII trace metals (56)	
Boron	2×10^{-1}
Fe	1×10^{-2}
Mn	1×10^{-2}
Zn	5×10^{-3}
Co	1
<u>Iron sequestrine solution (54)</u>	
FeSO ₄ ·7H ₂ O	5
EDTA	5.2
<u>Waris solution (54)</u>	
KNO ₃	100
MgSO ₄ ·7H ₂ O	20
(NH ₄) ₂ HPO ₄	20
CaSO ₄	50
Adjust pH to 6.0	
<u>M-3 medium (pers. comm. from W. Raymond, Washington State Univ.)</u>	
KNO ₃	100
Na ₂ glycerophosphate	100
MgSO ₄ ·7H ₂ O	48
CaCl ₂ ·2H ₂ O	19
PIV trace metals (44)	
Na ₂ EDTA	4.5
FeCl ₃ ·6H ₂ O	582
MnCl ₂ ·4H ₂ O	.246
ZnCl ₂	.030
CoCl ₂ ·6H ₂ O	.012
Na ₂ MoO ₄	.024

PUBLISHED DESMID MEDIA (cont.)

Medium Reagents	Medium Concentrations (mg/l)
<u>Bold's modified Bristol's solution (54)</u>	
NaNO ₃	250
MgSO ₄ ·7H ₂ O	50
K ₂ HPO ₄	100
KH ₂ PO ₄	150
CaCl ₂	25
NaCl	25
EDTA solution	
EDTA	50
KOH	26
H-Fe solution	
FeSO ₄ ·7H ₂ O	5
(stock solution is made in .1% con. H ₂ SO ₄)	
Boron solution	
H ₃ BO ₃	11.4
H-H5 minor elements solution	
ZnSO ₄ ·7H ₂ O	8.82
MoO ₃	.71
Co(NO ₃) ₂ ·6H ₂ O	.49
MnCl ₂ ·4H ₂ O	1.44
CuSO ₄ ·5H ₂ O	1.57
<u>E.A. George's medium (43)</u>	
KNO ₃	100
K ₂ HPO ₄	10
MgSO ₄ ·7H ₂ O	10
ferric citrate	1
citric acid	1
Medium may be supplemented with 60 ml/l peat extract	

PUBLISHED DESMID MEDIA (cont.)

Medium Reagents	Medium Concentrations (mg/l)
<u>Ichimura's <i>Closterium</i> medium (44)</u>	
KNO ₃	100
Ca(NO ₃) ₂ ·4H ₂ O	150
MgSO ₄ ·7H ₂ O	40
β-Na ₂ glycerophosphate·5H ₂ O	50
vitamin B ₁₂	1 x 10 ⁻⁴
Biotin	1 x 10 ⁻⁴
Thiamine-HCl	1 x 10 ⁻²
PIV trace metals (see M-3 medium)	
Tris buffer	500
Adjust pH to 7.5	
<u>Moss standard culture medium (26)</u>	
Ca(NO ₃) ₂ ·4H ₂ O	40
K ₂ HPO ₄	5
MgSO ₄ ·7H ₂ O	25
NaHCO ₃	20
Trace element mixture	
EDTA, tetrasodium salt	3
FeCl ₃ ·6H ₂ O	.08
MnCl ₂ ·4H ₂ O	.06
ZnCl ₂	.01
CoCl ₂ ·6H ₂ O	.012
NaMoO ₄ ·2H ₂ O	.01
Vitamin mixture	
Cyanocobalamin	3.3 x 10 ⁻⁵
Biotin	3.3 x 10 ⁻⁴
Thiamine-HCl	3.3 x 10 ⁻⁵
Adjust pH to 7.3-7.4	