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THE EFFECTS OF SILICA, NITROGEN, AND PHOSPHORUS LIMITATION ON
THE BIOCHEMICAL COMPOSITION OF CYCLOTELLA MENEGHINIANA KUTZ:
AN EXPERIMENTAL ANALYSIS

Iowa State University

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The effects of silica, nitrogen, and phosphorus limitation on the
biochemical composition of Cyclotella meneghiniana Kütz.:

An experimental analysis

by

David Fred Millie

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1984

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ABBREVIATIONS

ANOVA	- analysis of variance
ATP	- adenosine triphosphate
Chl/Ph	- chlorophyll/phaeophytin
DNA	- deoxyribonucleic acid
FAMES	- fatty acid methyl esters
FAS	- fatty acid synthetase
gj	- gigajoule
ha	- hectare
L/G	- lipid/glucan
LSD	- least significant difference
ml	- milliliter
N	- nitrogen
P	- phosphorus
P/G	- protein/glucan
Si	- silica
Si(OH) ₄	- silicic acid
yr	- year

INTRODUCTION

The biochemical composition of algae has been studied extensively. This information is useful to researchers analyzing the suitability of algae as food sources in aquatic food chains. In addition, biochemical information may aid in the characterization of the physiological condition of natural algal populations and may provide phylogenetic implications (Darley, 1977).

In recent years, the concern over limited energy sources has prompted researchers to investigate algal biochemistry and physiology in a technological perspective. Some algae, particularly diatoms, accumulate lipid as a storage product. This storage product, coupled with the high photosynthetic efficiency of the algal cell, has prompted researchers to consider algae as a potential energy source. The high-energy lipid, when harvested, could be used as is or converted to hydrocarbon fuels (Bergeron et al., 1983). The high-energy content of the lipid-enriched cells may also allow algae to serve as a potential food source.

The effect of nutrient concentration on algal biochemical composition is not well-documented. Researchers have reported that nutrient limitation generally causes a decrease in protein and photosynthetic pigments and an increase in lipid and carbohydrate storage products (Darley, 1977). However, most researchers have used batch cultures where algal cells are limited to a finite amount of nutrients. In batch cultures, cells progressively modify the nutrient concentration of the medium and exhibit exponential, linear, and stationary growth phases.

Any physiological measurements made on such a constantly changing population cannot be identified with a particular growth stage or nutrient concentration (Weete, 1980).

Continuous culture systems maintain a constant rate of nutrient supply coupled with a constant rate of cell removal. Under these conditions, cultures achieve a steady state where cell density, growth rate, and mean biochemical composition are constant. Changes in the rate of nutrient supply will cause changes in the growth rate, biochemical composition, and physiological state of the cells. Physiological measurements made on steady-state cells at different rates of nutrient supply would characterize a population at specific points along a growth curve and/or nutrient gradient.

Little information is available on the effect of nutrient limitation on the biochemical composition of algae under steady state conditions. Richardson *et al.* (1969) analyzed the amount of cellular lipid and chlorophyll of two green algae in response to increasing nitrogen concentration in a chemostat. Myklestad (1974), using continuous culture techniques, reported the amount of cellular carbohydrate and protein of a marine diatom in response to increasing nitrogen and phosphorus concentrations. To my knowledge, the amount of cellular lipid of diatoms under steady state conditions along a nutrient gradient has not been investigated.

The intent of this study was to analyze the effects of nutrient starvation and nutrient stress on the amount of cellular lipid, fatty acid, glucan, protein, and chlorophyll of a diatom. In this study,

the term "nutrient-starved cultures" refers to batch cultures in which the nutrient under investigation is deleted from the growth medium. The term "nutrient-stressed cultures" refers to semi-continuous cultures in which the nutrient under investigation is present within the growth medium, but at lower concentrations than necessary for maximum rates of growth. Silica (Si), nitrogen (N), and phosphorus (P) were chosen for investigation because other researchers have shown these nutrients to frequently limit diatom growth. The centric diatom, Cyclotella meneghiniana Klütz., was selected for study because it can be easily cultured in defined media and produces large amounts of lipid.

LITERATURE REVIEW

The importance of Si, N, and P in algal biochemistry and physiology is well-documented. Diatoms readily absorb Si as silicic acid (Si(OH)_4) and incorporate it in a polycondensed form as the main structural component of the cell wall (Werner, 1977). The amount of Si of diatoms typically comprises 10 to 30% of the cell's dry weight (Paasche, 1980), but may change with variations in the cell's growth rate, life cycle, structural features, and amount of surface area as well as the Si(OH)_4 concentration available for uptake (Eppley, 1977; Werner, 1977). Researchers have indicated that diatoms do not accumulate excess Si early in their development for later use in cell division (Darley, 1969; Paasche, 1980). However, Kilham et al. (1977) reported Diatoma elongatum can store up to 4.4 times more Si than needed for cell function.

The role of Si in diatom physiology is not well-understood. Besides its obvious function as a structural component, Si is also considered to be a required element for certain physiological functions. Metabolic Si has been reported to affect the citric acid cycle (between acetyl coenzyme A and 2-oxoglutarate), ATP oxidative phosphorylation, protein synthesis, respiration, chrysolaminaran utilization, chlorophyll synthesis, and DNA synthesis (Coombs et al., 1967b; Darley and Volcani, 1969; Azam et al., 1974; Werner, 1977; Sullivan and Volcani, 1981).

The role of N in algal physiology has been extensively studied (Morris, 1974; McCarthy, 1980). N is often reported to be the limiting nutrient in marine systems. Along with carbon, hydrogen, and oxygen, N

comprises a major portion of the dry matter of algal cells (Syrett, 1981). It is an essential constituent of important molecules such as amino acids, nucleic acids, and chlorophyll.

Algae have been reported to absorb N in the form of nitrate, nitrite, ammonia and urea (Lewin and Guillard, 1963; McCarthy, 1980). Amino acids have also been reported to be a N source for several diatoms (Hellebust and Lewin, 1977). The preferred order of N utilization appears to proceed from ammonia to urea to nitrate in both natural and culture systems (McCarthy, 1980). Morris (1974) stated that cellular growth rate is usually the same with either ammonia or nitrate as the N source. Paasche (1971), however, reported that Dunaliella tertiolecta, grew 30% faster with ammonia as the N source than with nitrate. Nitrate and nitrite must undergo an energy-requiring reduction prior to their incorporation into glutamate and amino acids (Syrett, 1962; Bidwell, 1974; Morris, 1974). Therefore, cells which can readily assimilate reduced forms of N would be expected to have increased growth rates because energy could then be utilized for cell synthesis rather than for cell maintenance.

The role of P in algal physiology has also been studied extensively (Kuhl, 1974; Nalewajko and Lean, 1980). Researchers often report P to be the nutrient responsible for increased algal productivity in freshwater systems. It is essential to all life forms as it functions in storage and transfer of metabolic energy (adenosine triphosphate) and is required for the synthesis of nucleotides, phospholipids, and other phosphorylated compounds.

Algae assimilate P in an inorganic soluble form such as orthophosphate (Wetzel, 1975). In some algae, organically-bound P appears to be the preferred form for P uptake (Nalewajko and Lean, 1980). Unlike N, however, P does not undergo any valency change during its assimilation and within the cell it forms polyphosphates. Many algae, when provided with sufficient P, can store excess P as polyphosphate bodies in the cytoplasm or vacuole. Stevenson and Stoermer (1982) reported these P "sinks" in algal cells to be related to the P concentration in the water. Tilman and Kilham (1976) reported that the diatom Asterionella formosa can store up to 82 times more P than needed for cell maintenance. These P "sinks" have great physiological and ecological significance for they allow the cell to continue normal metabolic functions even when P in the environment is limited (Kuhl, 1974).

Researchers have proposed that optimum cell nutrient ratios be used to indicate the limiting role of algal nutrients. The optimum cell nutrient ratio is the ratio at which transition from limitation by one nutrient to another takes place (Rhee and Gotham, 1980). Redfield (1958) reported a N:P ratio of approximately 15:1 for algal cells. However, cellular N:P ratios have been reported to range from 5.1 to 25 depending upon the taxon and the environmental conditions to which the cells are exposed (Parsons et al., 1961; Davis, 1976; McCarthy, 1980; Rhee, 1978; Rhee and Gotham, 1980). Generally optimum cellular N:P ratios appear to be high in green algae and low in diatoms (Rhee and Gotham, 1980).

Little information exists describing optimum cellular P:Si ratios

for diatoms. Parsons et al. (1961) reported P:Si ratios in Skeletonema costatum and Coscinodiscus sp. to be 0.19 and 0.02, respectively.

Davis (1976), in a study of Si-limited cultures of Skeletonema costatum exposed to various light intensities, reported values which ranged from 0.27 to 1.2.

Whether lipid or carbohydrate is the photosynthetic reserve product of diatoms has long intrigued algal researchers. For many years, lipid (oil) was considered to be the only storage product of diatoms. The discovery that diatoms accumulated a β -1,3 linked glucopyranoside during photosynthesis led some researchers to believe that carbohydrate was the main storage product. Most likely, diatoms can store both lipid and carbohydrate as photosynthetic reserve products with the exact abundance of each product dependent upon the physiological state of the cells (Barker, 1935; Fogg, 1956; Badour and Gergis, 1965).

Carbohydrate in diatoms is present primarily as reserve polysaccharides and cell wall constituents (Fogg, 1953). The principal monosaccharides are glucose, galactose, mannose, ribose, xylose, rhamnose, and fucose with glucose representing the greatest percent of total carbohydrate (Darley, 1977). Chrysolaminaran (leucosin) is the principal polysaccharide of diatoms and chrysophytes. It was first isolated from diatoms by von Stosch (1951). Beattie et al. (1961) and Ford and Percival (1965) reported that chrysolaminaran consists of approximately 12 β -1,3 and β -1,6 linked glucose units in a ratio of 11:1. Chrysolaminaran is similar to laminaran, the photosynthetic reserve product of brown algae, but possesses a smaller ratio of β -1,3 to

β -1,6 linkages (11:1 vs. 15:1), a smaller glucose chain length (12 vs. 16), and lacks the terminal mannitol residues.

Chrysolaminaran has been identified as a photosynthetic reserve product in diatoms and chrysophytes. It was the radioactive product formed during photosynthesis in chrysophytes after exposure to $^{14}\text{CO}_2$ (Kauss, 1962). Coombs and Volcani (1968) noted that the "main labelled soluble carbohydrate" after $^{14}\text{CO}_2$ uptake by Navicula pelliculosa was a glucan. Handa (1969), in an analysis of carbohydrate metabolism, reported that the β -1,3 glucan of Skeletonema costatum was consumed during the dark.

Chrysolaminaran has been reported to accumulate in membrane-bound vesicles outside of, and directly abutting, the chloroplast (Duke and Reimann, 1977; Lee, 1980). Although chrysolaminaran can comprise 15 to 20% of the mass of the cell (Meeuse, 1962), it has rarely been mentioned in electron microscope studies of diatoms (Duke and Reimann, 1977). This is because chrysolaminaran is dissolved during the fixation and embedding process.

Lipids are broadly defined as materials which are soluble in organic solvents and are essentially insoluble in water (Stryer, 1981). The lipid composition of diatoms resembles that of green algae and higher plants. Lipid components of diatoms include triglycerides, sulfoquinavosyl diglyceride, digalactosyl diglyceride, monogalactosyl diglyceride, lecithin, phosphatidyl inositol, phosphatidyl glycerol, phosphatidyl ethanolamine, brassicosterol, clionosterol, and chondrillosterol (Low, 1955; Kates and Volcani, 1966; Kates and Volcani, 1968;

Tornabene et al., 1974).

Fatty acids are esterified with glycerol (or other alcohols) to form the principal building blocks of polar and nonpolar lipids (Fisher and Schwarzenbach, 1978). Polar lipids, such as phospholipids, sulfolipids, and galactosyl lipids, are the major constituents of cell and organelle membranes. Most non-polar lipids are triglycerides (Otsuka and Morimura, 1966). Non-polar lipids serve as a photosynthetic reserve product (Fisher and Schwarzenbach, 1978) and accumulate in membrane-bound lipid "bodies" (Darley, 1977). Lipid "bodies" have been found in the chloroplast near the pyrenoids, in the central vacuole, and scattered throughout the cytoplasm (Drum, 1963; Stoermer et al., 1964; Stoermer et al., 1965; Crawford, 1973; Dawson, 1973; Duke and Reimann, 1977).

Fatty acid composition varies among algal groups. Diatoms characteristically possess fatty acids that have carbon chain lengths ranging from 12 to 22 (Ackman and Tocher, 1968; Wood, 1974). Diatoms generally possess large amounts of tetradecanoic ($C_{14:0}$), hexadecanoic ($C_{16:0}$), hexadecenoic ($C_{16:1}$), eicosatetraenoic ($C_{20:4}$) and eicosapentanoic ($C_{20:5}$) and small amounts of hexadecadienoic ($C_{16:2}$), hexadecatrienoic ($C_{16:3}$), octadecenoic ($C_{18:1}$), octadecadienoic ($C_{18:2}$), and octadecatrienoic ($C_{18:3}$) acids (Ackman et al., 1964; Brockerhoff et al., 1964; Kates and Volcani, 1966; Ackman and Tocher, 1968; Kates and Volcani, 1968; Pugh, 1971; DeMort et al., 1972; Opute, 1974a; Tornabene et al., 1974; Fisher and Schwarzenbach, 1978).

Several researchers have indicated that the biochemical composition

of algae is variable and dependent on the cellular growth stage. In exponentially growing populations, the main biochemical "product" is protein (Olive et al., 1969). Darley (1977) stated that in "nutrient sufficient cells, protein content almost always exceeds carbohydrate content, which, in turn, usually exceeds the lipid content." Morris (1981) reported the amount of cellular protein in exponentially-growing diatom cultures ranged from 17 to 35% of the cell dry weight. The amount of cellular carbohydrate and lipid ranged from 4 to 20% and 2 to 7%, respectively.

Researchers have reported algal fatty acid composition to vary as cells age. Collyer and Fogg (1955) reported the amount of fatty acid per culture increased or decreased with age depending upon the taxon. Pugh (1971) reported an increase in fatty acids that have carbon chain lengths of 18 as cells age. Dodecanoic acid ($C_{12:0}$) decreased with cell senescence. Ackman et al. (1964) noted that tetradecanoic acid ($C_{14:0}$) increased and eicosapentaenoic acid ($C_{20:5}$) decreased as cells age.

The amount of cellular carbohydrate and lipid is greater in senescent cells than in actively-growing cells (Badour and Gergis, 1965; Handa, 1969; Opute, 1974b; Conover, 1975). The amount of cellular protein remains constant (Badour and Gergis, 1965; Handa, 1969) or decreases (Myklestad and Haug, 1972; Conover, 1975) with cell senescence. The amount of cellular chlorophyll content is lower in senescent cells than in young, actively-growing cells (Antia et al., 1963; Ackman and Tocher, 1968).

Algal physiology and biochemistry have been reported to be affected

by the Si, N, and P concentrations of the growth medium. Diatom cell division is inhibited as Si is depleted from the medium (Eppley, 1977). In a Si-starved culture of Navicula pelliculosa, cell division stopped but cellular morphological development continued until the culture consisted mostly of biprotoplastic cells (two daughter protoplasts contained within a parent frustule, each surrounded by a new plasmalemma and separated by an intercellular space). In these cells, the rate of carbon and P uptake was lower than that in Si-replete conditions (Coombs et al., 1967a,b; Coombs and Volcani, 1968).

Diatoms show a gradual decrease in major metabolic activity as Si becomes limiting (Darley, 1977). Protein, chlorophyll, carbohydrate, and nucleic acid synthesis are partially, or completely, inhibited. Lipid synthesis increases as Si becomes limited (Coombs et al., 1967a,b; Healey et al., 1967; Coombs and Volcani, 1968). Darley (1977) noted a 60 to 70% reduction in glycolysis when Cyclotella cryptica cells were transferred to Si-deficient media. The rate of fatty acid synthesis, however, increased by greater than 100%.

When Si is introduced into Si-starved cultures, a reduction in cell lipid synthesis and an increase in cell carbohydrate, protein, and DNA synthesis occurs. Repletion of Si also causes an increase in cell nucleosidetriphosphate and a resumption of cell division (Coombs et al., 1967a; Coombs and Volcani, 1968; Darley, 1969; Darley, 1977).

The effects of N limitation on algal cells have been well-documented. Eppley and Renger (1974) and Opute (1974b) observed a reduction in photosynthetic efficiency when actively-growing cells were exposed to N

limitation. The amount of cellular lipid of diatoms and green algae also increased as N becomes limiting (Fogg, 1956; Badour and Gergis, 1965; Richardson et al., 1969; Werner, 1977; Shifrin and Chisholm, 1981; Rosen, 1982; Bergeron et al., 1983). Collyer and Fogg (1955) stated that accumulation of lipid is not associated with low cellular N in red and blue-green algae. However, de Vasconcelos and Fay (1974) reported increased lipid content in a blue-green alga during N starvation.

The amount of fatty acids in algal cells has been reported to increase as N becomes limiting (Zhukova et al., 1968; Klyachko-Gurvich et al., 1969). Cells exposed to N limitation predominantly possess large concentrations of saturated or monounsaturated fatty acids that have carbon chain lengths of 16 and 18. Cells exposed to high concentrations of N predominantly possess polyunsaturated fatty acids that have carbon chain lengths of 16 and 18 (Ackman et al., 1964; Matsuka et al., 1966; Klyachko-Gurvich et al., 1969; DeMort et al., 1972; Pohl, 1974).

The amount of cellular carbohydrate in diatoms and green-algae has also been reported to increase as N becomes limiting (Matsuka et al., 1966; Klyachko-Gurvich et al., 1969; Myklestad and Haug, 1972; Werner, 1977). The amount of cellular protein and chlorophyll has been observed to decrease as N becomes limiting (Fogg, 1956; Matsuka et al., 1966; Thomas and Dodson, 1972; Eppley and Renger, 1974; Morris et al., 1974; Myklestad, 1974; Syrett, 1981). Syrett (1981), however, reported a study of Thalassiosira in which N deprivation caused a decrease in

photosynthetic rate but little change in the amount of cellular chlorophyll.

The biochemical composition of algae is also affected by P limitation. Researchers observed an accumulation of cellular lipid and carbohydrate only after all cellular P reserves had been utilized (Kuhl, 1974; Werner, 1977; Rosen, 1982). Rosen (1982) reported a decrease in the amount of cellular chlorophyll of Cyclotella meneghiniana as P becomes limiting. Spoehr and Milner (1949) observed no biochemical differences between P-stressed and P-sufficient cells of Chlorella.

METHODS

Cells of Cyclotella meneghiniana Kütz. were collected from the Des Moines River (Boone County, IA) on 18 May, 1982. Several cells were isolated into soil water extract (Nichols, 1973) using a micropipette. A clonal culture was obtained by washing cells through 4 distilled water baths and reisolating a single cell into a defined medium (Table 1). This clone was used in all subsequent cultures. Diatoms were grown in 350 ml of culture media in 500 ml, polycarbonate, Erlenmeyer flasks. Cultures were maintained in a Percival Growth Chamber (Boone, IA) at $18 \pm 2^{\circ}\text{C}$. Six parallel 40-watt cool white fluorescent lamps alternated with 6 34-watt warm white incandescent lamps provided approximately 450 foot candles of illumination (approximately $0.1 \text{ mMoles photons} \cdot (\text{M}^2)^{-1} \cdot \text{day}^{-1}$). Cultures were exposed to a 16:8 hr light:dark cycle in an attempt to obtain synchronously-dividing cultures and thus minimize cell variability (Cook, 1961). Diatom cultures were shaken daily to keep cells suspended in the medium.

Cyclotella cultures were unialgal, but not axenic. To minimize further contamination of the cultures, sterile techniques and equipment were utilized throughout the study. Glass, double-distilled water was used in the preparation of the culture media. Media solutions minus vitamins were prepared for maintenance cultures by autoclaving the solution 15 minutes at 250°C . Vitamins were added aseptically with a Gelman 0.2 μm mesh Acrodisc filter assembly attached to a syringe. For nutrient-starvation experiments, the treatment media were filtered through a Gelman 0.2 μm mesh Metrice1 membrane filter into a sterilized flask.

Table 1. Composition of culture medium: modified from Guillard and Lorenzen (1972) and Rosen (1982)

Nutrient	mg/liter	Nutrient	mg/liter
Macronutrients:		Micronutrients:	
CaCl ₂ ·2H ₂ O	36.76	Na ₂ ·EDTA	4.36
MgSO ₄ ·7H ₂ O	36.97	FeCl ₃ ·6H ₂ O	3.15
NaHCO ₃	12.60	CuSO ₄ ·5H ₂ O	0.01
K ₂ HPO ₄ ^{a,b}	8.71	ZnSO ₄ ·7H ₂ O	0.022
NaNO ₃ ^{c,d}	85.01	CoCl ₂ ·6H ₂ O	0.01
NaSiO ₃ ·9H ₂ O ^{e,f}	28.42	MnCl ₂ ·4H ₂ O	0.18
NaCl	584.50	Na ₂ MoO ₄ ·2H ₂ O	0.006
K ₂ HCO ₃ ^g	6.91	H ₃ BO ₃	1.0
Vitamins:		Buffer:	
Thiamine·HCl	0.1	Tris(hydroxymethyl)-aminomethane	500.00
Biotin	0.0005		
Cyanocobalamin	0.0005		

^aOmitted from P-deficient cultures in nutrient starvation experiments.

^bUsed at 1/10th strength in P-limited media in nutrient stressed experiments.

^cOmitted from N-deficient cultures in nutrient starvation experiments.

^dUsed at 1/10th strength in N-limited media in nutrient stressed experiments.

^eOmitted from Si-deficient cultures in nutrient starvation experiments.

^fUsed at 1/10th strength in Si-limited media in nutrient stressed experiments.

^gUsed in P-deficient cultures.

Treatment media in nutrient-stressed experiments were prepared and autoclaved in 2.5 liter polycarbonate bottles. Vitamin concentrations in these bottles were increased 2.5-fold over normal strength to overcome degradation of vitamins during autoclaving.

Experimental Design

Diatom cultures were subjected to Si-, N-, and P-starvation and to Si-, N-, and P-stressed experiments. In nutrient-starvation experiments, diatom cells were removed from nutrient-replete batch cultures and re-suspended in nutrient media with either $\text{NaSiO}_3 \cdot 9\text{H}_2\text{O}$, NaNO_3 , or K_2HPO_4 omitted. Since the only potassium source normally in the medium was K_2HPO_4 , P-deficient media would have lacked potassium also. Therefore, an appropriate amount of potassium was added to the P-deficient media in the form of K_2HCO_3 (Table 1).

In nutrient-starvation experiments, a 2^3 factorial experimental design in fractional replication (Cochran and Cox, 1957) was used. In this type of design, the three nutrients in various combinations produced 8 possible nutrient treatments (Table 2). However, due to the number of variables analyzed per flask and the number of flasks considered, all 8 treatments could not be analyzed collectively. Rather, only half of the total treatments were considered at one time. Each pair of 4 treatments (hereafter referred to as a half-replicate) was selected to minimize experimental "confounding" of nutrient effects (Table 2). The 2 half-replicates were then put together to make a single replication of the factorial design.

In a single replication of the 2^3 factorial design, each nutrient

Table 2. Nutrient combinations in media treatments for nutrient-starvation experiments. Presence or absence of a nutrient is indicated by a plus (+) or minus (-). Half-replicates analyzed collectively are indicated by presence or absence of an asterisk (*)

Treatment	Nutrient		
	Silica	Nitrogen	Phosphorus
1	-	-	-
2*	+	-	-
3*	-	+	-
4	+	+	-
5*	-	-	+
6	+	-	+
7	-	+	+
8*	+	+	+

effect was an average over 4 combinations of other factors and had, in effect, 4-fold replication (Cochran and Cox, 1957). Variable means for nutrient deficient effects were calculated using treatments in which the nutrient under consideration was omitted. Variable means for nutrient-replete effects were calculated using treatments in which the nutrient under consideration was present.

A schematic representation of the experimental procedure used in nutrient-starvation experiments is presented in Fig. 1. For each half-replicate, 10 culture flasks containing nutrient sufficient media were inoculated with equal amounts of cells from a maintenance culture. Cultures were placed in the growth chamber and allowed to grow until cell density reached approximately 14,000 cells/ml. At this density, cell division was still occurring, but at a much reduced rate than cells of cultures undergoing exponential growth.

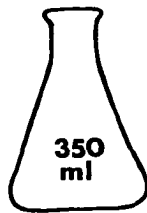
Two of the flasks were then analyzed. The day in which experimental analysis began was designated Day 0. The remaining 8 flasks were randomly divided into 4 pairs and each pair was assigned to 1 of the 4 possible media treatments in the half-replicate considered. The entire volume of each flask was centrifuged at approximately 3,000 rpm for 4 minutes. Centrifuged cells were washed 4 times in their assigned treatment medium and resuspended in flasks containing the appropriate treatment medium. Cultures were replaced in the growth chamber. Cultures were analyzed 3 and 9 days after transfer to treatment media, a time sequence used by Shifrin and Chisholm (1981). On day 3, 1 flask from each of the 4 treatment pairs was removed for analysis. On day 9,

Fig. 1. Schematic representation of the experimental procedure used for each treatment in nutrient-starvation experiments

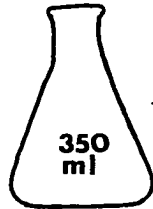
DAY 0

DAY 3

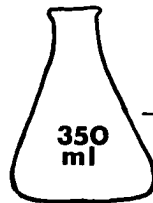
DAY 9



→ ANALYZED



→ CENTRIFUGED &
RESUSPENDED IN → ANALYZED
TREATMENT MEDIA



→ CENTRIFUGED &
RESUSPENDED IN → ANALYZED
TREATMENT MEDIA

the four remaining flasks were analyzed. For the nutrient-starvation experiment, a single replication of the factorial design was completed.

In nutrient-stressed experiments, semi-continuous culture techniques were used. Cultures were diluted by removing a certain percentage of the culture at regular intervals (hereafter referred to as dilution rate) and replacing it with an equal amount of fresh media. Cell densities of semi-continuous cultures are inversely proportional to the dilution rate. A net decrease in cell density occurs when dilution rate is greater than growth rate. If dilution rate exceeds growth rate for a sufficient period of time, the cell population "washes out" of the culture flask. A net increase in cell density occurs when growth rate is greater than dilution rate. Cell density increases until dilution rate equals growth rate. Consequently, a steady-state cell density is achieved. At steady state, the population possesses a constant growth rate and constant mean biochemical characteristics. High dilution rates produce steady state populations possessing low cell densities and, therefore, a high nutrient supply per cell. Low dilution rates, in turn, produce steady-state populations possessing high cell density and a low nutrient supply per cell. By varying the dilution rate, the culture can be "held" at various points along its growth curve (Fogg, 1965). This allows analysis of the physiological and biochemical condition of steady-state populations along a nutrient gradient.

The molar ratio of N:P:Si was 1.0:0.05:0.1 in media used for maintenance cultures. For nutrient-stressed experiments, culture media were prepared with the nutrient under investigation at 1/10th normal

strength (Table 1). Preliminary experimentation indicated that cultures in media with Si, N, and P at 1/10th normal strength were nutrient limited. Cell densities of cultures for all dilution rates were less than similarly diluted cultures using normal strength media. In this manner, the N:P:Si molar ratio was 1.0:0.05:0.01 in Si-stressed media, 0.1:0.05:0.1 in N-stressed media, and 1.0:0.005:0.1 in P-stressed media.

In each of the nutrient-stressed experiments, cells from a maintenance culture growing exponentially were centrifuged and washed 3 times in the appropriate medium. Aliquots of the washed cell suspension were resuspended in 9 flasks containing nutrient stressed media. Cultures were placed in the growth chamber. Cells within the flasks were allowed to divide without interruption for 3 or 4 days, after which the dilution process was initiated. The 9 flasks were randomly divided into 3 groups and a daily dilution rate of 10%, 25%, or 50% assigned to the groups. Dilutions were continued until a steady state cell density was determined by microscopic counting. When steady state conditions were reached, the daily supply of limiting nutrient per cell for flasks within a dilution rate was calculated (Table 3). Flasks were then analyzed.

Sample Analysis

Diatom cultures were analyzed for the amount of cellular lipid, glucan, protein, chlorophyll, and phaeophytin. Samples were obtained by centrifuging a known volume of the cultures at approximately 2,000 rpm for 3 minutes. Examination of centrifuged material by phase contrast microscopy revealed some bacteria, but their biomass was insignificant in comparison to that of diatom cells. Samples for glucan

Table 3. Supply of limiting nutrients per cell per day for dilution rates of media in nutrient-stressed experiments. Means and standard errors (in parentheses) for replicate flasks are presented

Limiting nutrient	Dilution rate (%·day ⁻¹)	Supply rate (mg × 10 ⁻⁷ ·cell ⁻¹ ·day ⁻¹)
Silica	10	0.140 (± 0.009)
	25	0.449 (± 0.051)
	50	1.178 (± 0.147)
Nitrogen	10	0.126 (± 0.012)
	25	0.868 (± 0.012)
	50	3.475 (± 0.137)
Phosphorus	10	0.013 (± 0.002)
	25	0.064 (± 0.014)
	50	0.434 (± 0.019)

analysis were obtained by filtering a known volume of culture directly onto Whatman 934-AH glass microfibre filters. Culture cell densities were determined by microscopic counting techniques utilizing a Palmer-Maloney chamber (Guillard, 1973).

Total lipid was extracted from diatom samples by aqueous alkaline hydrolysis (modified from Hammond et al., 1981). Diatom cells were refluxed for approximately 1½ hours in 10 ml of 12% alcoholic KOH. The digested cells were cooled, adjusted to pH 3 with 1N HCl, and diluted with 50 ml of distilled water. Ten ml of hexane was added to the reflux flask. The solution was mixed thoroughly and filtered through a Whatman GF/C glass microfibre filter to remove diatom frustules. The reflux flask was rewashed with 5 ml of hexane and the filtering repeated. The filtered solution was transferred to a separatory funnel fitted with a teflon stopcock. The hexane layer was allowed to separate, removed from the funnel, and transferred to a tared glass vial. The hexane was evaporated under N gas and the resulting residue and vial were weighed. Total lipid was expressed gravimetrically as lipid per cell.

Total glucan was extracted and determined spectrophotometrically by the sulfuric acid-phenol method presented by Myklestad (1978). Total protein was determined by the "Lowry Method" (Lowry et al., 1951) using bovine serum albumin as a standard. Chlorophyll and phaeophytin values were determined by spectrophotometric methods (Richards with Thompson, 1952) using the equations of Parsons and Strickland (1963). Values for glucan, protein, chlorophyll, and phaeophytin were expressed on a per cell basis. Chlorophyll/phaeophytin (Chl/Ph), protein/glucan (P/G),

and lipid/glucan (L/G) ratios were also calculated.

The fatty acid composition of lipid residues from cells grown in nutrient-stressed experiments was analyzed. The lipid residues for replicate samples within a supply rate were resuspended in hexane, pooled, and re-evaporated under N gas. The fatty acids were converted to fatty acid methyl esters (FAMES) by adding 1 ml of 3% H_2SO_4 in methanol to the residue and heating in a teflon-sealed vial at 55°C for 12 hours. After heating, 3 ml of distilled water and 1 ml of hexane were added to the vial. The solution was mixed and centrifuged. The hexane layer was removed and washed 3 or 5 times with distilled water to remove traces of methanol. The hexane was evaporated to near dryness to concentrate the FAMES. One μl of sample was injected into a Beckman GC-5 gas chromatograph equipped with a 183 x 0.3 cm stainless steel column packed with 15% EGSSX on 100/120 mesh Chromosorb P (Applied Science Laboratories, State College, PA). The carrier gas was N at a flow rate of 50 ml/minute. The column temperature varied between 139° and 176°C , depending upon the date of analysis. Retention peaks of sample FAMES were compared to standard FAME mixtures (Supelco, Inc., Bellefonte, PA) for identification. The fatty acid samples from P-stressed experiments were inadvertently destroyed in preparation.

Statistical Analysis

For nutrient-starvation experiments, values of biochemical variables and the calculated ratios for flasks on days 3 and 9 were compared by a factorial analysis using a split-plot, randomized block design

(Cochran and Cox, 1957). Since only cells that had been cultured in nutrient-sufficient media were used as inoculum for the experimental flasks, no comparison to nutrient-deficient treatments could be made for day 0. Therefore, values for this initial day of experimentation were not included in the statistical analysis. The sources of variation investigated in the factorial analysis are presented in Table 4. Half-replicates were considered blocks. The significance of nutrient effects (Si, N, and P) and their 2-factor interactions (Si·N, Si·P, and N·P) were assessed by a Half-Normal Probability Plot (Daniel, 1959).

For each nutrient-stressed experiment, values of biochemical variables and the calculated ratios were compared in terms of nutrient supply rate by an Analysis of Variance (ANOVA) (Snedecor and Cochran, 1980). The sources of variation investigated in the ANOVA are presented in Table 5. The effect of nutrient supply rate was subdivided to analyze for deviation from linearity. The significance between specific pairs of variable means was analyzed by a Least Significant Difference (LSD) analysis (Snedecor and Cochran, 1980).

Table 4. Sources of variation with their corresponding degrees of freedom investigated in the Factorial Analysis performed on variables of nutrient-starvation experiments

Source of variation	Degrees of freedom
Fractional replicate	1
Limiting nutrient	(3)
Silica	1
Nitrogen	1
Phosphorus	1
Error (A) ^a	3
Days	1
Interaction	(3)
Days·Silica	1
Days·Nitrogen	1
Days·Phosphorus	1
Error (B) ^b	4

^a(Silica·Nitrogen + Silica·Phosphorus + Nitrogen·Phosphorus).

^b(Days·Error (A) + Days·Fractional replicate).

Table 5. Sources of variation and their corresponding degrees of freedom investigated in the Analysis of Variance performed on variables of nutrient-stressed experiments

Source of variation	Degrees of freedom
Nutrient supply rate	(2)
Linear	1
Quadratic	1
Error (replicates)	6 ^a

^aValue expressed is based on 3 replicates per nutrient supply rate.

RESULTS

The biochemical composition of the cells varied little throughout the nutrient-starvation experiments. The amount of cellular lipid (Fig. 2) generally ranged from 2.0 to $4.0 \times 10^{-3} \mu\text{g}\cdot\text{cell}^{-1}$. The amount of cellular lipid initially appeared to increase in cells grown in N-sufficient media and decrease in N-deficient media. The amount of cellular glucan remained fairly constant (approximately 0.6 to $0.8 \times 10^{-3} \mu\text{g}\cdot\text{cell}^{-1}$) in all treatments (Fig. 3). The amount of cellular protein varied greatly between Si-sufficient and Si-deficient treatments (Fig. 4). In Si-deficient treatments, the amount of cellular protein increased from 3.1 to $5.3 \times 10^{-1} \mu\text{g}\cdot\text{cell}^{-1}$ between days 0 and 3. The amount of cellular protein then remained constant until day 9. The amount of cellular protein in N and P treatments remained fairly constant (3.0 to $4.0 \times 10^{-1} \mu\text{g}\cdot\text{cell}^{-1}$) throughout the study. The amount of cellular chlorophyll varied little (1.0 to $1.25 \times 10^{-5} \mu\text{g}\cdot\text{cell}^{-1}$) throughout all nutrient treatments (Fig. 5).

The calculated biochemical ratios also varied little throughout the nutrient-starvation experiments. Chl/Ph ratios remained fairly constant (approximately 1.7 to 2.5) over all treatments (Table 6). L/G ratios (Table 7) ranged from 1.5 to 7.0 with the majority of values approximately 5.0 . P/G ratios ranged from 300 to 1900 among all nutrient treatments (Table 8).

A factorial analysis using a split-plot, randomized block design was used to analyze the effects of half-replicates and nutrient effects on

Fig. 2. Changes in the amount of cellular lipid in silica, nitrogen, and phosphorus-sufficient (solid lines) and deficient (dashed lines) cultures through time. Values represent mean of four replicate flasks. Bars indicate standard error of the means

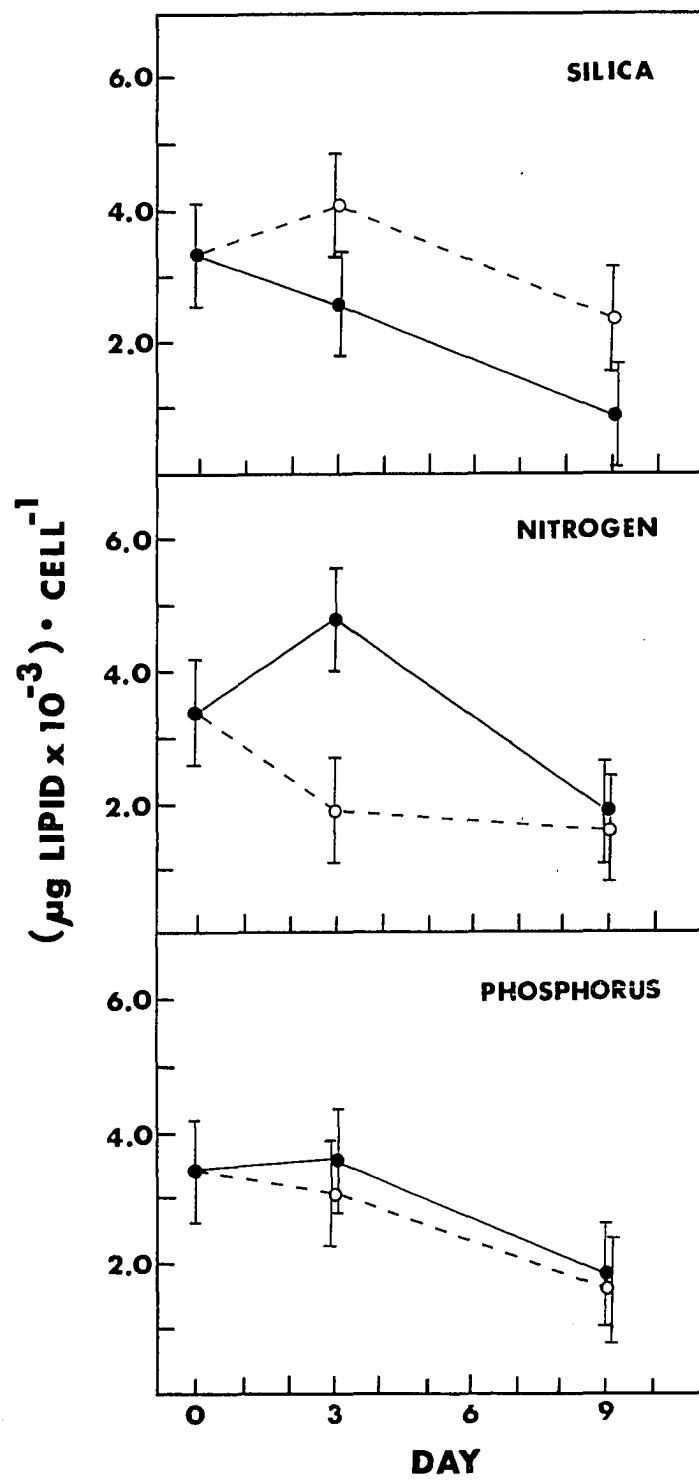


Fig. 3. Changes in the amount of cellular glucan in silica, nitrogen, and phosphorus-sufficient (solid lines) and deficient (dashed lines) cultures through time. Values represent mean of four replicate flasks. Bars indicate standard error of the means

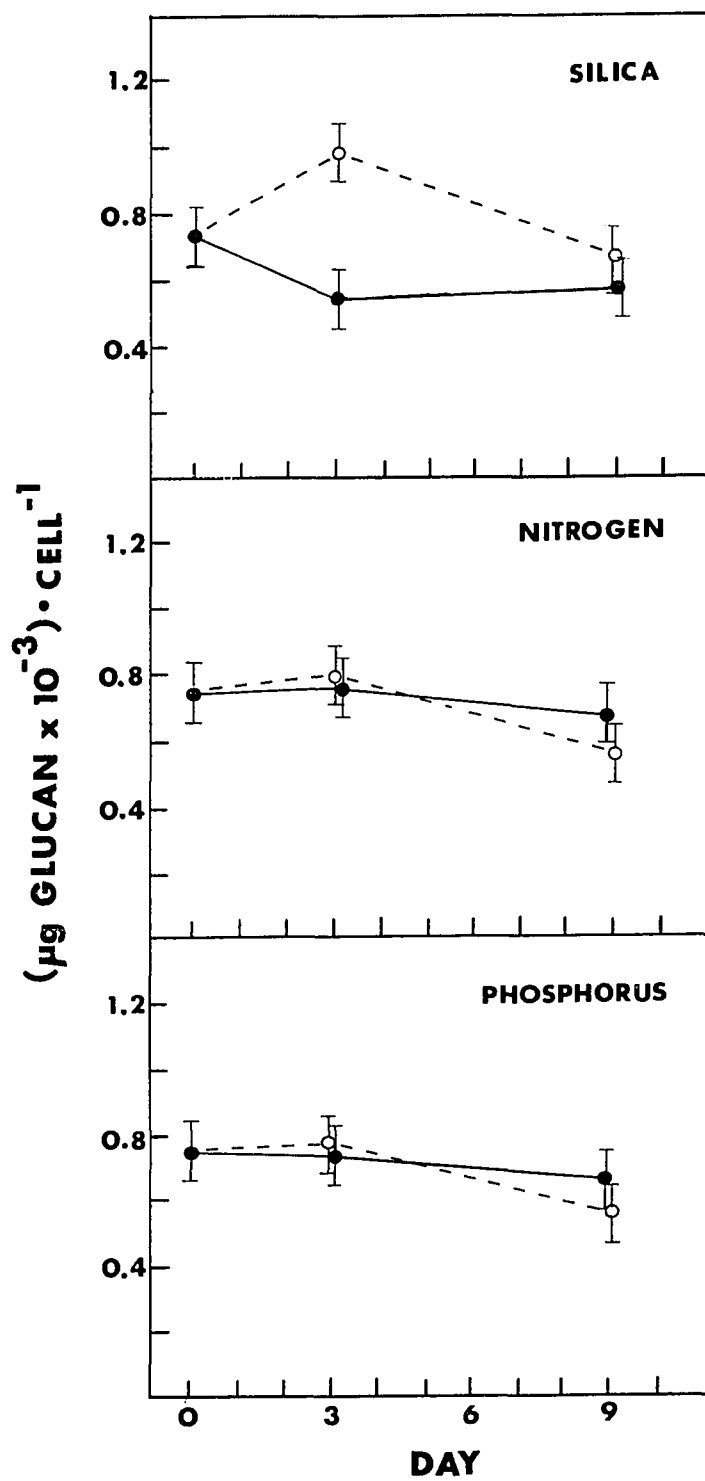


Fig. 4. Changes in the amount of cellular protein in silica, nitrogen, and phosphorus-sufficient (solid lines) and deficient (dashed lines) cultures through time. Values represent mean of four replicate flasks. Bars indicate standard error of the means

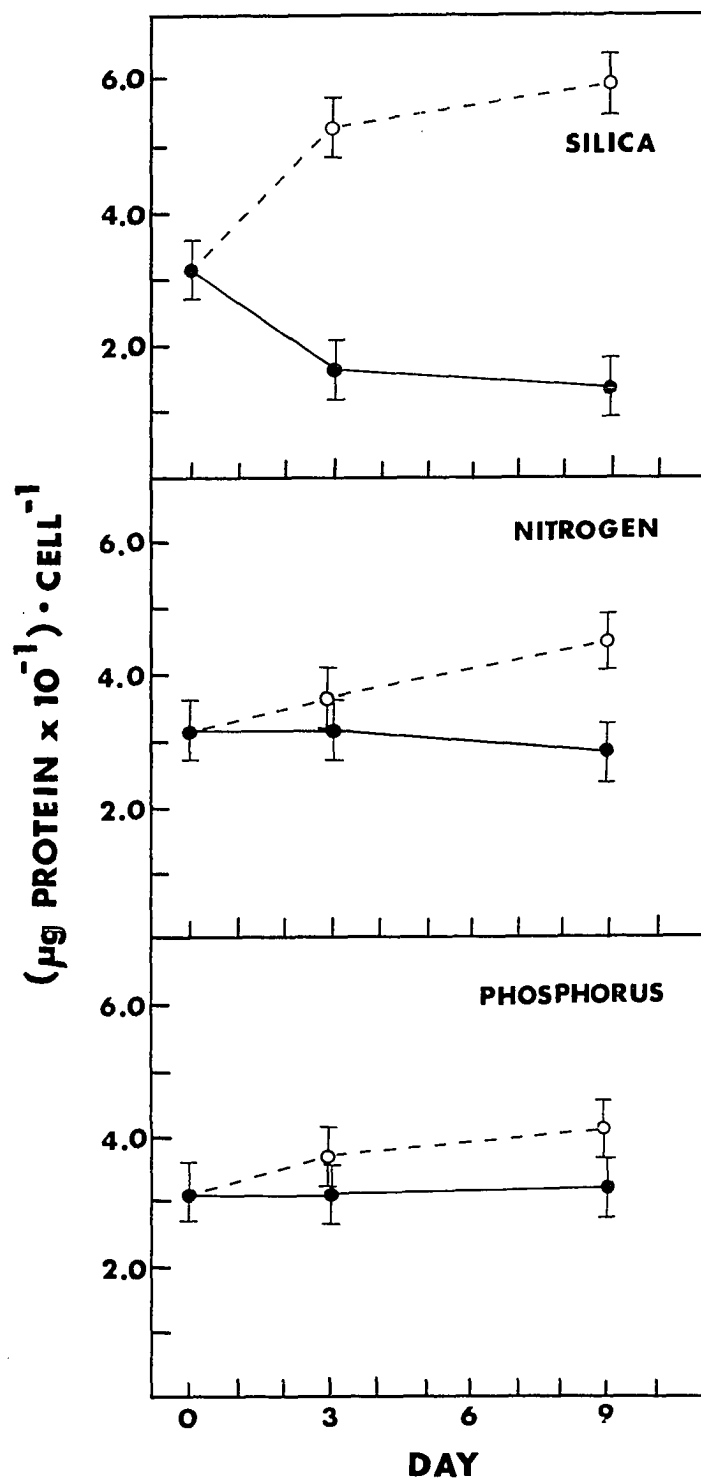


Fig. 5. Changes in the amount of cellular chlorophyll in silica, nitrogen, and phosphorus-sufficient (solid lines) and deficient (dashed lines) cultures through time. Values represent mean of four replicate flasks. Bars indicate standard error of the means

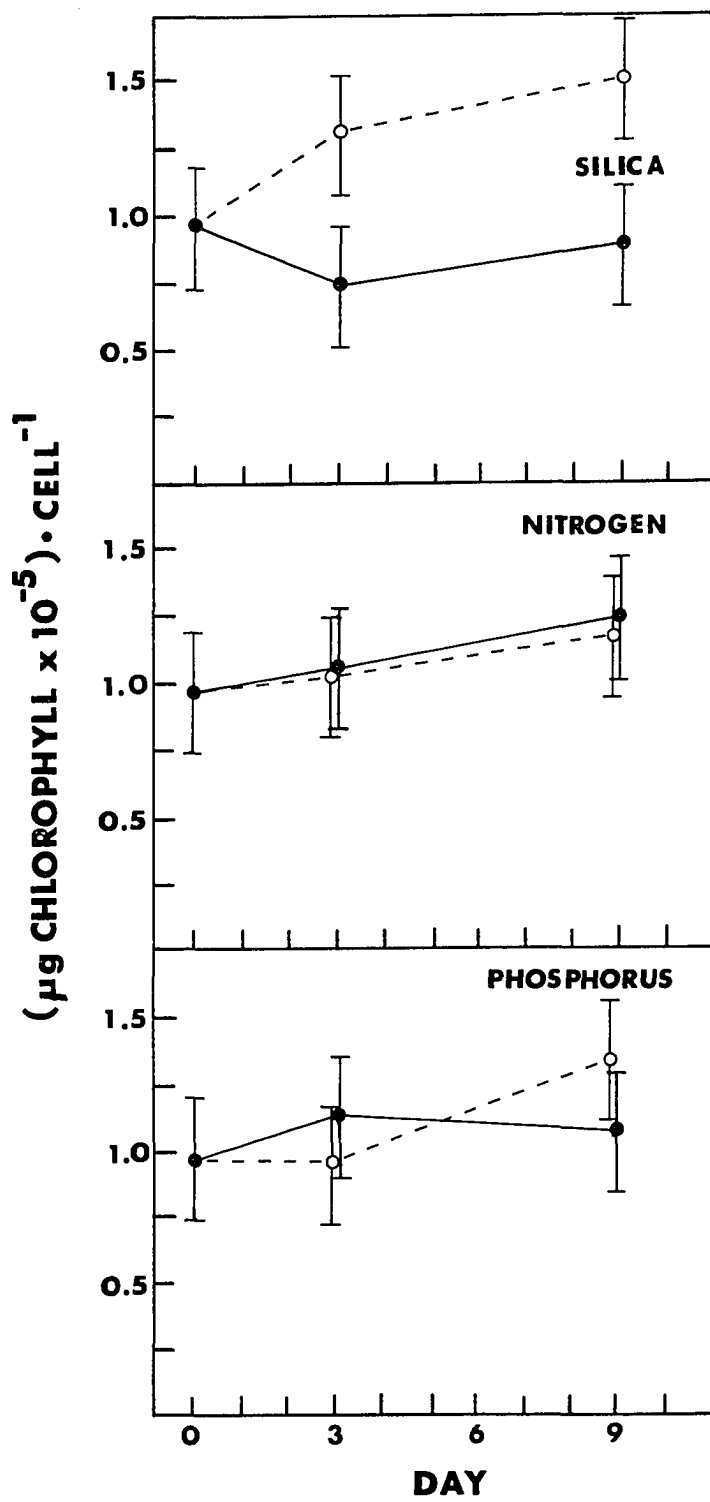


Table 6. Chlorophyll/phaeophytin ratios for replicate flasks in nutrient-starvation experiments. Means of replicate flasks are presented

Nutrient	Nutrient availability	Day		
		0	3	9
Silica	+	2.46	2.08	2.33
	-		1.84	2.16
Nitrogen	+	2.46	1.68	2.49
	-		2.24	2.00
Phosphorus	+	2.46	2.34	1.66
	-		1.57	2.82

Table 7. Lipid/glucan ratios for replicate flasks in nutrient-starvation experiments. Means of replicate flasks are presented

Nutrient	Nutrient availability	Day		
		0	3	9
Silica	+	5.13	5.06	1.51
	-		4.20	6.40
Nitrogen	+	5.13	6.97	2.37
	-		2.29	5.55
Phosphorus	+	5.13	4.46	2.62
	-		4.80	5.29

Table 8. Protein/glucan ratios for replicate flasks in nutrient-starvation experiments. Means of replicate flasks are presented

Nutrient	Nutrient availability	Day		
		0	3	9
Silica	+	377.88	303.27	255.16
	-		527.46	1926.31
Nitrogen	+	377.88	361.21	400.92
	-		434.52	1780.55
Phosphorus	+	377.88	386.32	440.69
	-		444.42	1740.78

biochemical variables and their calculated ratios between days 3 and 9 (Appendix Table A1). The Half-Normal Probability Plot indicated no significant effect of 2-factor nutrient interactions on the biochemical composition of the cells. Therefore, only the effects of individual nutrients were considered in the factorial analysis.

In this factorial analysis, the fractional-replicate effect measured the variability of values between fractional replicates. This effect, in theory, measured the reproducibility of values between half-replicates. Only the values for the amount of cellular protein were significantly different between the 2 half-replicates ($p < 0.05$). The limiting-nutrient effect indicated the variability between the mean of values on days 3 and 9 in nutrient-sufficient treatments and the mean of values on days 3 and 9 in nutrient-deficient treatments. Only Si had a significant effect on a biochemical variable. A significant increase in the amount of cellular protein occurred with Si deficiency. The effect of days measured the variability between the mean of values on day 3 and the mean of values on day 9. Interaction effects measured the variability between the difference of nutrient-deficient and nutrient-sufficient treatments on day 3 and this difference on day 9. Neither day nor day·nutrient interactions significantly affected any biochemical variable investigated.

The biochemical composition of the cells varied in response to nutrient supply rate (dilution rate) within and between nutrient-stressed experiments (Figs. 6 to 8). An ANOVA was used to measure the effect of supply rate on the biochemical composition of the cells (Appendix Table

Fig. 6. The amount of cellular lipid, glucan, protein, and chlorophyll in silica-stressed cultures as a function of silica supply rate. Values represent mean of replicate flasks. Bars indicate standard error of the means

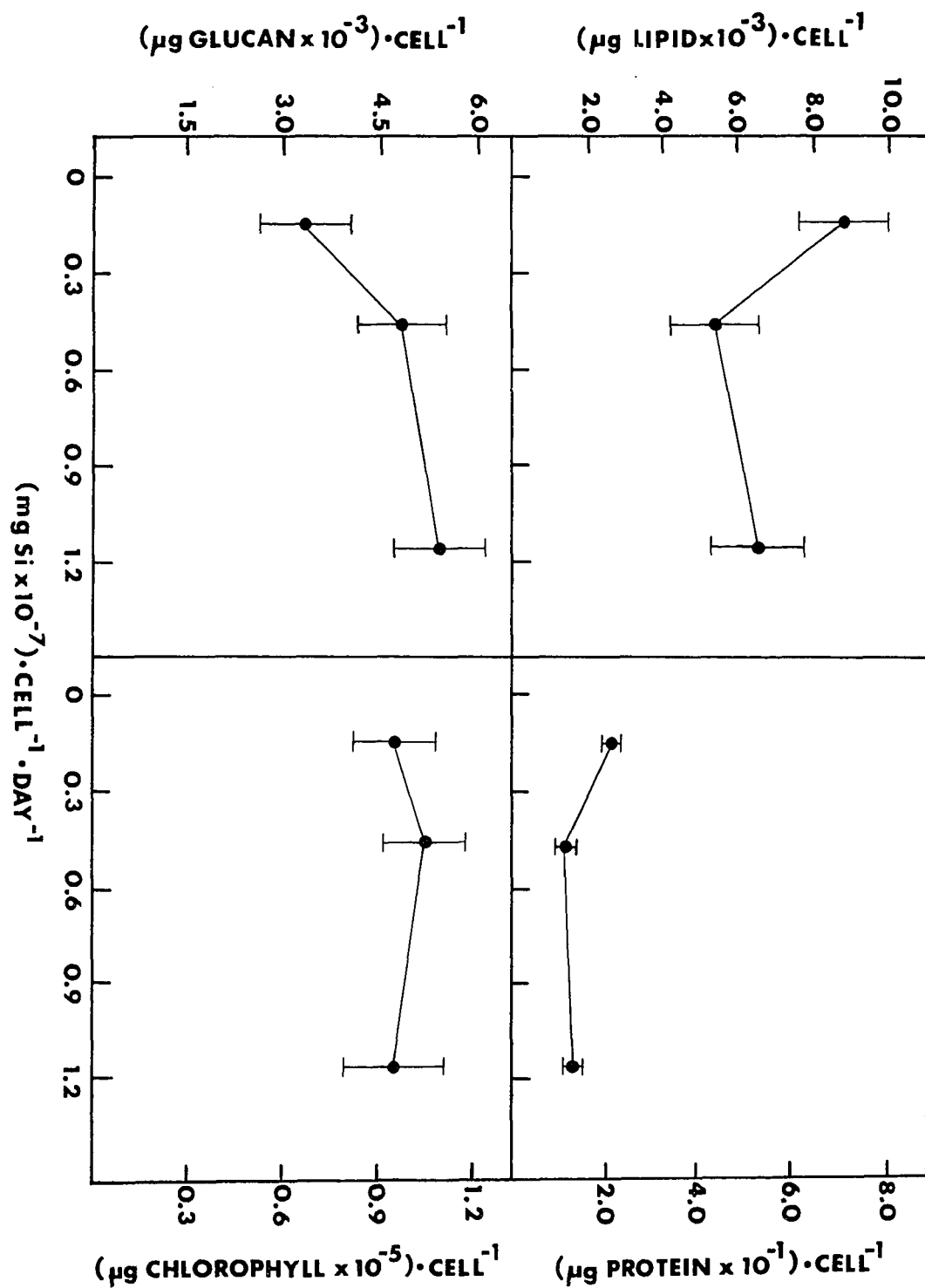


Fig. 7. The amount of cellular lipid, glucan, protein, and chlorophyll in nitrogen-stressed cultures as a function of nitrogen supply rate. Values represent mean of replicate flasks. Bars indicate standard error of the means

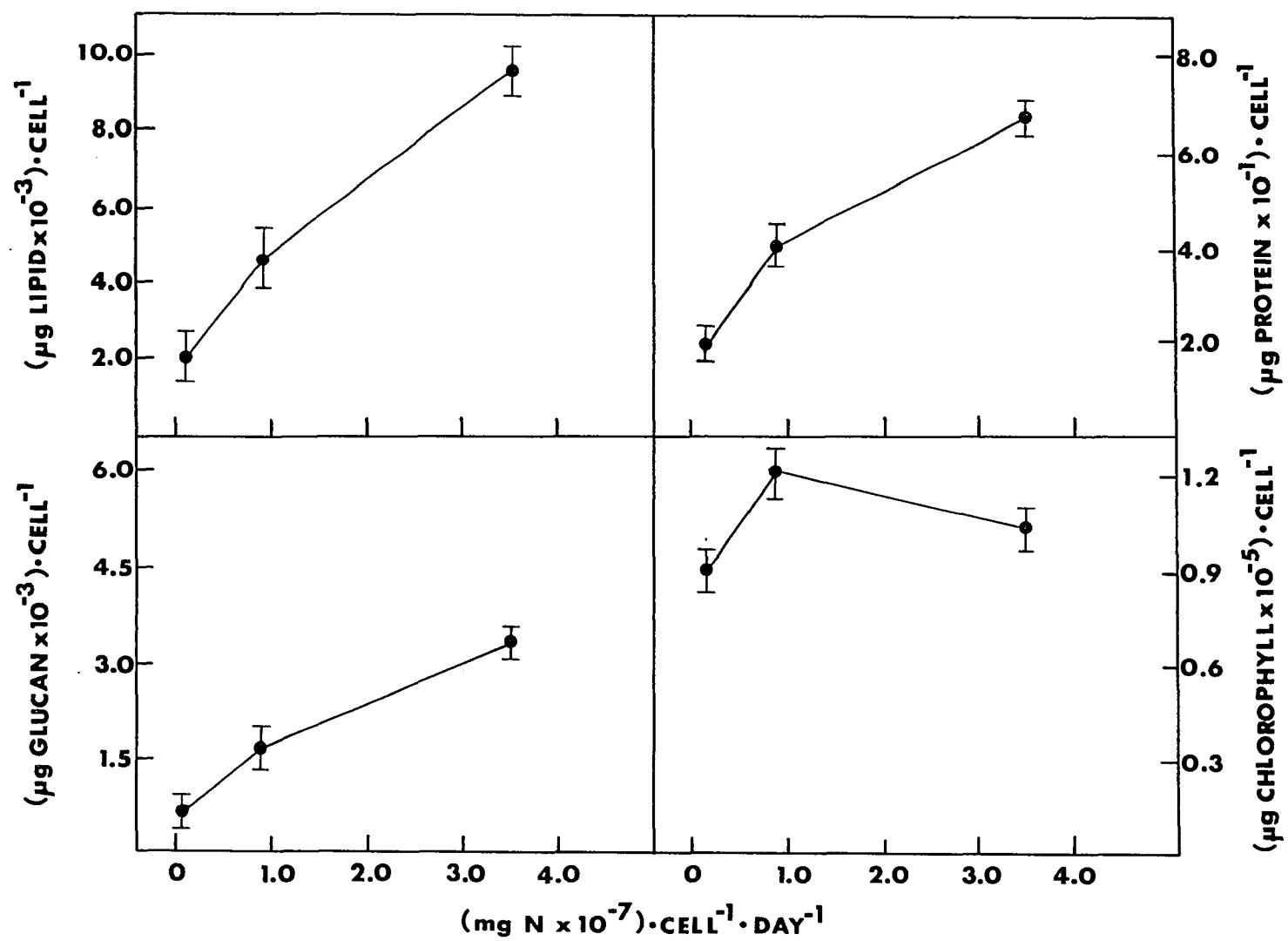
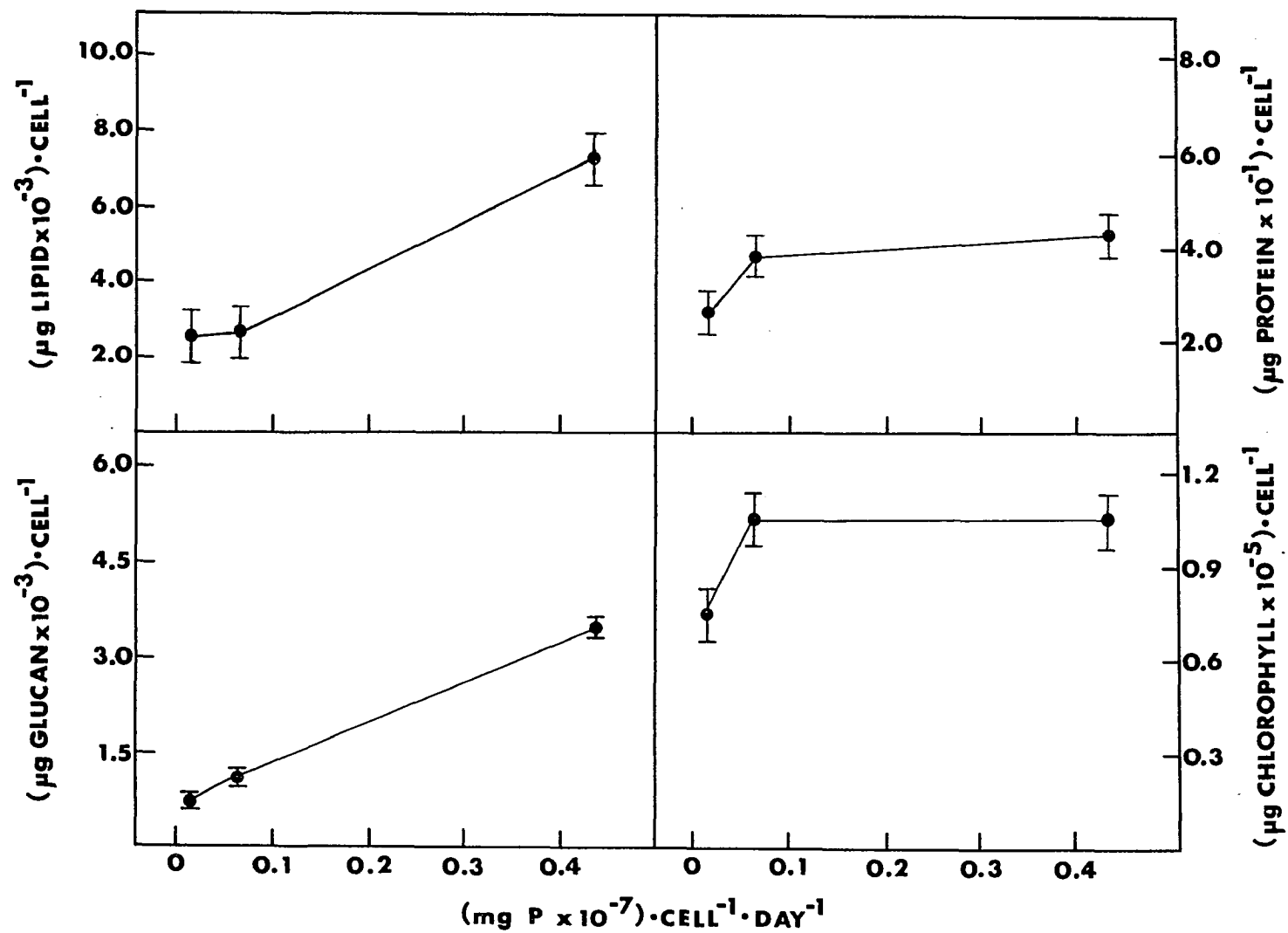


Fig. 8. The amount of cellular lipid, glucan, protein, and chlorophyll in phosphorus-stressed cultures as a function of phosphorus supply rate. Values represent mean of replicate flasks. Bars indicate standard error of the means



A2). A LSD analysis was used to test the significance between specific pairs of variable means within an experiment (Table 9).

The LSD analysis indicated that increasing Si supply rate caused a significant decrease in the amounts of cellular lipid and protein and cellular L/G ratios. However, the ANOVA revealed no significant effects of Si supply rate on any variable except the L/G ratio ($p < 0.05$).

Cellular L/G ratios decreased from 2.6 to 1.2 between 0.140 and $0.449 \times 10^{-7} \text{ mg Si} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$ but remained constant between 0.449 and $1.178 \times 10^{-7} \text{ mg Si} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$.

The LSD analysis indicated that increasing N supply rate caused significant increases in the amount of cellular lipid, glucan, protein, and chlorophyll. The ANOVA revealed significant effects of N supply rate on all of these variables except chlorophyll ($p < 0.001$). The amount of cellular lipid remained constant (approximately $3.4 \times 10^{-3} \mu\text{g} \cdot \text{cell}^{-1}$) between 0.126 and $0.868 \times 10^{-7} \text{ mg N} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$ but increased to $9.6 \times 10^{-3} \mu\text{g}$ at $3.475 \times 10^{-7} \text{ mg N} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$. The amount of cellular glucan remained constant (approximately $1.1 \times 10^{-3} \mu\text{g} \cdot \text{cell}^{-1}$) between 0.126 and $0.868 \times 10^{-7} \text{ mg N} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$ but increased to $3.3 \times 10^{-3} \mu\text{g}$ at $3.475 \times 10^{-7} \text{ mg N} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$. The amount of cellular protein increased from 1.95 to $4.11 \times 10^{-1} \mu\text{g} \cdot \text{cell}^{-1}$ between 0.126 and $0.868 \times 10^{-7} \text{ mg N} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$ to $6.7 \times 10^{-1} \mu\text{g} \cdot \text{cell}^{-1}$ at $3.475 \times 10^{-7} \text{ mg N} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$.

The LSD analysis indicated that significant increases in the amount of cellular lipid and glucan occurred with increasing P supply rate. The ANOVA also indicated significant increases in the amount of cellular lipid ($p < 0.001$) and glucan ($p < 0.001$). The amount of cellular lipid

Table 9. Mean values of the cellular biochemical composition and the cellular biochemical ratios for nutrient supply rates in nutrient-stressed experiments

Limiting nutrient	Supply rate (mg x 10 ⁻⁷ . cell ⁻¹ .day ⁻¹)	Cellular composition				Cellular ratio		
		Lipid (μg x 10 ⁻³)	Glucan (μg x 10 ⁻³)	Protein (μg x 10 ⁻³)	Chloro- phyll (μg x 10 ⁻³)	Chlorophyll/ Phaeophytin	Protein/ Glucan	Lipid/ Glucan
Silica	0.140	8.62 ^a	3.33	2.13 ^a	0.95	No data	68.4	2.6 ^{a,b}
	0.449	5.37 ^a	4.79	1.17 ^a	1.05	No data	28.1	1.2 ^a
	1.178	6.60	5.45	1.33	0.94	No data	30.4	1.3 ^b
Nitrogen	0.126	2.10 ^a	0.66 ^a	1.95 ^a	0.91 ^a	7.1	298.7	3.1
	0.868	4.67 ^b	1.58 ^b	4.11 ^{a,b}	1.20 ^a	5.1	263.4	3.0
	3.475	9.63 ^{a,b}	3.33 ^{a,b}	6.71 ^{a,b}	1.03	5.3	211.7	3.0
Phosphorus	0.013	2.59 ^a	0.72 ^a	2.56	0.75	No data	363.6	3.9
	0.064	2.68 ^b	1.09 ^b	3.73	1.04	No data	363.6	2.6
	0.434	7.35 ^{a,b}	3.49 ^{a,b}	4.24	1.05	No data	121.8	2.1

^{a,b}Variable means within a nutrient category with similar superscripts are significantly different (p<0.05).

remained constant (approximately $2.63 \times 10^{-4} \mu\text{g}\cdot\text{cell}^{-1}$) between 0.013 and $0.064 \times 10^{-7} \text{ mg P}\cdot\text{cell}^{-1}\cdot\text{day}^{-1}$. The amount of cellular glucan remained constant (approximately $0.91 \times 10^{-3} \mu\text{g}\cdot\text{cell}^{-1}$) between 0.013 and $0.064 \times 10^{-7} \text{ mg P}\cdot\text{cell}^{-1}\cdot\text{day}^{-1}$ but increased to $3.5 \times 10^{-3} \mu\text{g}\cdot\text{cell}^{-1}$ at $0.434 \times 10^{-7} \text{ mg P}\cdot\text{cell}^{-1}\cdot\text{day}^{-1}$.

The fatty acids of lipid extracted in N-stressed and Si-stressed experiments were tetradecanoic ($\text{C}_{14:0}$), pentadecanoic ($\text{C}_{15:0}$), hexadecanoic ($\text{C}_{16:0}$), heptadecanoic ($\text{C}_{17:0}$), heptadecanoic-branched ($\text{C}_{17:\text{branched}}$), octadecanoic ($\text{C}_{18:0}$), octadecenoic ($\text{C}_{18:1}$), octadecadienoic ($\text{C}_{18:2}$), octadecatrienoic ($\text{C}_{18:3}$), eicosanoic ($\text{C}_{20:0}$), eicosenoic ($\text{C}_{20:1}$), eicosatetraenoic ($\text{C}_{20:4}$), docosadienoic ($\text{C}_{22:2}$), docosatetraenoic ($\text{C}_{22:4}$), and tetracosanoic ($\text{C}_{24:0}$). Although FAME samples were washed several times with distilled water, there was, probably, a retention of methanol in the sample. This caused a gradual slope off the solvent peak during gas chromatographic analysis which did not allow the area under the FAME retention peaks to be measured. Therefore, the relative percentage of each FAME in the lipid sample could not be calculated. Rather, only the presence or absence of a FAME could be determined.

The fatty acid composition of the extracted lipid differed little among nutrient supply rates within an experiment but was distinctly different between N-stressed and Si-stressed experiments (Tables 10 and 11). In the N-stressed experiment, carbon chain length of fatty acids ranged from 14 to 24. Except for the absence of $\text{C}_{15:0}$, $\text{C}_{17:0}$, and $\text{C}_{24:0}$ at $0.126 \times 10^{-7} \text{ mg N}\cdot\text{cell}^{-1}\cdot\text{day}^{-1}$ and $\text{C}_{20:4}$ and $\text{C}_{24:0}$ at 0.868×10^{-7}

Table 10. Fatty acid composition of *Cyclotella meneghiniana* for supply rates of nitrogen-stressed cultures. Presence of a fatty acid is indicated by a plus (+)

Fatty acid	Supply rate ($\times 10^{-7}$ mg N·cell ⁻¹ ·day ⁻¹)		
	0.126	0.868	3.475
14:0 ^a	+	+	
15:0		+	
16:0	+	+	+
17:branched	+	+	
17:0		+	
18:0	+	+	+
18:1	+	+	+
18:2	+	+	+
20:0	+	+	
20:1 (or 2)	+	+	+
20:4 (or 5)	+		+
22:2	+	+	+
22:4 ?	+	+	+
24:0 ?			+

^aShorthand notation for carbon chain length:number of double bonds.

Table 11. Fatty acid composition of *Cyclotella meneghiniana* for supply rates of silica-stressed cultures. Presence of a fatty acid is indicated by a plus (+)

Fatty acid	Supply rate ($\times 10^{-7}$ mg Si·cell ⁻¹ ·day ⁻¹)		
	0.140	0.449	1.178
14:0 ^a	+	+	+
15:0	+		
16:0	+	+	+
17:0	+	+	+
18:0	+	+	+
18:1	+	+	+
18:2	+	+	

^aShorthand notation for carbon chain length:number of double bonds.

mg P·cell⁻¹·day⁻¹, all of the aforementioned fatty acids were observed at these two nutrient supply rates. At 3.475×10^{-7} mg N·cell⁻¹·day⁻¹, fatty acids with a low carbon chain length (e.g. C₁₄ to C₁₇) were, for the most part, absent.

In contrast, the fatty acids in the Si-stressed experiment were limited to low carbon chain lengths (C₁₄ to C₁₈). In addition, there was a preponderance of FAMES with retention times less than that associated with C₁₄. These peaks were, most likely, FAMES with carbon chain lengths of 10 to 13. Since the standard samples used did not possess FAMES with carbon chain lengths less than 14, no definite identification of these fatty acids could be made. Fatty acids at 0.140 and 0.449×10^{-7} mg Si·cell⁻¹·day⁻¹ ranged from C_{14:0} to C_{18:2}. C_{15:0} was absent in the extracted lipid at 0.449×10^{-7} mg Si·cell⁻¹·day⁻¹. At 1.178×10^{-7} mg Si·cell⁻¹·day⁻¹, C_{15:0} and C_{18:2} were absent from the extracted lipid.

DISCUSSION

Researchers have reported that algae accumulate storage products when exposed to nutrient limitation (Fogg, 1956; Coombs et al., 1967a,b; Darley, 1977; Shifrin and Chisholm, 1981). However, in this study, accumulation of storage products by C. meneghiniana varied with the type and degree of nutrient limitation. In addition, the statistical significance of nutrient-limitation effects on the biochemical composition of the cells varied with the culture design used.

A reduction in the amount of cellular carbohydrate, protein, and chlorophyll and an increase in the amount of cellular lipid has been reported to occur in diatoms with increasing Si limitation (Werner, 1977). In this study, the LSD analysis indicated some increases in the amount of cellular lipid and protein as Si becomes limiting. However, Snedecor and Cochran (1980) suggested that the LSD analysis should be used to claim significance between variable means only if the "F test" is significant. Therefore, the LSD analysis was considered applicable only if the ANOVA indicated a significant difference between variable means among nutrient supply rates. The ANOVA indicated that only L/G ratios were significantly different among Si supply rates.

The significant decrease in cell L/G ratios with increasing Si supply rate indicated a shift from glucan to lipid accumulation as Si becomes limiting. While the ANOVA did not indicate a significant difference in the amount of cellular lipid and/or glucan among Si supply rates, it is possible that the shift from glucan to lipid accumulation occurs at a particular Si concentration. In the nutrient-stressed

experiments, the use of only 3 distinct supply rates may have restricted the ANOVA from redefining lipid differences above and below this threshold concentration. In this study, the threshold concentration appeared to be between 0.140 and $0.449 \times 10^{-7} \text{ mg Si} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$. Future studies should investigate cellular lipid accumulation over this range.

The amount of cellular lipid of C. meneghiniana significantly increased with increasing N supply rate. This is in contrast to results from researchers who observed lipid accumulation with N limitation (Fogg, 1956; Badour and Gergis, 1965; Richardson et al., 1969; Werner, 1977; Shifrin and Chisholm, 1981). However, several researchers have observed results similar to those reported in this study. Collyer and Fogg (1955) stated that N concentration had no direct effect on fat accumulation in certain algae. Fogg (1956) stated that when fat-producing, N-deficient cells of Navicula pelliculosa underwent N repletion, fat accumulation continued. Badour and Gergis (1965) noted fat accumulation in Nitzschia sp. at times when N was not deficient.

The amount of cellular glucan of C. meneghiniana significantly increased with increasing N supply rate. In contrast, Myklestad and Haug (1972) observed a decrease in carbohydrate in Chaetoceros affinis var. willei with increasing N concentration. In this study, no significant differences in the amount of cellular glucan were observed between N-sufficient and N-deficient batch cultures.

Because of the essential role of N in amino acids and chlorophyll, N limitation might be expected to cause a decrease in the amount of

cellular protein and chlorophyll. However, no significant differences in the amount of cellular protein or chlorophyll were noted between N-sufficient and N-deficient batch cultures. However, decreasing N supply rate in the nutrient-stressed experiment did cause a significant decrease in cellular protein.

Decreasing P supply rate caused a significant reduction in the amount of cellular lipid and glucan. This contrasts with reports from several researchers (Kuhl, 1974; Werner, 1977; Rosen, 1982) who observed an accumulation of lipid and carbohydrate in algal cells as P becomes limiting. Rosen (1982) also noted a decrease in the amount of cellular chlorophyll of C. meneghiniana as P becomes limiting. In this study, a significant decrease in the amount of cellular chlorophyll as P becomes limiting was not observed.

Chloroplast ultrastructure has been reported to be differentially affected by Si, N, and P limitation (Butler and Simon, 1971; Duke and Reimann, 1977; Hurkman, 1979; Rosen, 1982). It might be expected that the ratio of cellular chlorophyll to its breakdown product, phaeophytin, would decrease as nutrients become limiting. However, no significant differences in the ratios were noted between nutrient-sufficient and nutrient-limited cultures. This observation agrees with results of a previous study dealing with the effects of nutrient deficiency on C. meneghiniana (Rosen, 1982). Rosen stated that "If adjustments to light intensity and nutrient deficiency were occurring through the degradation of chlorophyll, they did not appear to be through the creation of phaeophytin." In addition, Marker et al. (1980) noted that phaeophorbid,

rather than phaeophytin, is the major naturally-occurring breakdown product of chlorophyll and little is known of its spectral characteristics.

Negative phaeophytin values were calculated for several replicate cultures within supply rates of the N-stressed and P-stressed experiments. These values, in turn, produced negative Chl/Ph ratios which were omitted from further analysis. The negative values might have occurred because of improper acidification procedures prior to spectrophotometric readings or to extremely variable absorbance readings. Cell densities of cultures in the nutrient-stressed experiments were much less than cell densities of cultures in the nutrient-starvation experiments. The low chlorophyll concentrations resulting from the low cell densities produced spectrophotometric readings consistently below 0.1 absorbance. Since absorbance readings are relatively inaccurate at levels below 0.1 (A.P.H.A. *et al.*, 1971), extreme variability among replicate samples could have occurred. Therefore, if these values were substituted into the defined equations for determination of chlorophyll and phaeophytin concentrations, highly inaccurate values could have resulted.

Myklestad and Haug (1972) considered the protein/carbohydrate ratio to be a valuable indicator of the physiological state of the cell. In rapidly-growing cells, most cellular carbon is incorporated into protein. In times of stress, more cellular carbon is incorporated into storage products (lipid and carbohydrate) and protein synthesis is retarded (Cook, 1961; Myklestad and Haug, 1972; Weete, 1980). Therefore,

high P/G ratios would be expected in healthy, nutrient-sufficient cells and low P/G ratios in stressed, nutrient-limited cells (Myklestad and Haug, 1972; Sakshaug and Myklestad, 1973; Myklestad, 1974). In this study, no significant differences in P/G ratios were noted in any experiment.

Carbon chain lengths of fatty acids generally ranged from 14 to 24. This is in agreement with results of previous studies on diatom fatty acids (Ackman and Tocher, 1968; Wood, 1974; Fisher and Schwarzenbach, 1978). No distinct differences in fatty acid composition were noted among supply rates within Si-stressed and N-stressed cultures. However, distinct differences in fatty acid composition were observed between Si-stressed and N-stressed cultures. The cultures exposed to Si stress did not possess fatty acids that have carbon chain lengths greater than 18. The cultures exposed to Si stress also possessed fatty acids that have carbon chain lengths less than 14. The cultures exposed to N stress did possess fatty acids that have carbon chain lengths greater than 18. In addition, a greater amount of unsaturated acids was observed in N-stressed cultures than in Si-stressed cultures.

The synthesis of long chain fatty acids from CO_2 involves the sequential addition of 2 carbons to the growing fatty acid chain utilizing the enzyme complex, Fatty Acid Synthetase (FAS). This elongation process stops at the formation of hexadecanoic acid ($\text{C}_{16:0}$) (Stryer, 1981). Subsequent elongation of the fatty acid chain into longer carbon chain lengths and/or unsaturation involves several enzymatic systems besides FAS. The FAS or the additional enzyme systems may have been

blocked, in some manner, by Si stress, thereby preventing formation of long carbon chain, saturated fatty acids.

Algae are reported to possess large amounts of unsaturated fatty acids (Otsuka and Morimura, 1966; DeMort et al., 1972; MacCarthy and Patterson, 1974a,b; Opute, 1974a; Pohl, 1974; Fisher and Schwarzenbach, 1978). Since long chain unsaturated acids are believed to be involved in photosynthetic reactions and are essential components of diatom cell membranes (Kates and Volcani, 1966; Opute, 1974a; Tornabene et al., 1974), it appears that Si stress caused a greater disruption of C. meneghiniana's lipid composition than did N stress. In addition, a predominance of fatty acids that have carbon chain lengths less than 14 has also been observed in marine diatoms exposed to Si stress (Larry Raymond, Solar Energy Research Institute, personal communication). However, the current study did not quantitate the fatty acids of the extracted lipids and the mere presence or absence of an acid may not provide a realistic appraisal of the actual fatty acid dynamics. In addition, researchers (Ackman and Tocher, 1968; Orcutt and Patterson, 1974) have proposed that changes in the fatty acid composition of a cell reflects changes in the type of lipid. Therefore, "it is really necessary to consider the fatty acids of each individual group of lipids and their changes, rather than the fatty acids in toto, before a proper interpretation of nutrient-linked changes in the fatty acid composition of a cell can be made" (Pugh, 1971).

The exact reason for the biochemical response of C. meneghiniana to Si, N, and P limitation is not known. Coombs and Volcani (1968)

reported researchers who suggested that Si limitation leads to a reduction in Si-containing compounds essential to the metabolism of the cell. However, several researchers (Coombs et al., 1967a; Darley, 1969; Darley, 1977) theorized that the biochemical response to Si limitation results from cessation of growth prior to cell division. Cells of cultures in exponential growth would develop into mature cells or progress to a biprotoplastic stage before Si deficiency inhibited their development. Cellular constituents would accumulate in the biprotoplastic cells since these cells have more of their division cycle to complete than do the older, mature cells (Darley, 1969). A biochemical response associated with Si limitation may, therefore, simply "reflect the percentage of morphologically arrested cells in the culture" (Coombs et al., 1967a). Darley and Volcani (1969) supported this hypothesis as they observed a decrease in the overall energy metabolism and net biosynthetic capacity with the presence of biprotoplastic cells in Si-deficient, diatom cultures. This hypothesis might explain the increase in cellular protein in Si-deficient batch cultures of C. meneghiniana.

Researchers have proposed several theories to explain the effects of N and P concentration on lipid accumulation in algae. Fogg (1956) theorized that N deficiency caused an increase in the proportion of fats to other organic constituents within a cell as a result of the hydrolysis and subsequent loss from the cell of materials other than lipids. De Vasconcelos and Fay (1974) and Rosen (1982) suggested that the formation of lipid droplets in algal chloroplasts results from the degradation

of the chloroplasts' thylakoid membranes. The formation of these lipid droplets would, of course, cause increases in the total amount of cellular lipid. Atkinson (1965) suggested that in N-deficient cells, an increase of ATP occurs due to the inhibition of protein synthesis. The concentration of AMP would decrease and the reduced activity of isocitric dehydrogenase would cause an accumulation of citric acid. Since citrate is the allosteric activator of acetyl CoA carboxylase (Stryer, 1981), the conversion of acetyl CoA to fatty acids would be enhanced. On the other hand, increased photosynthesis caused by increasing N and P supply rates might cause increases in the citric acid intermediates of C. meneghiniana. Beardall et al. (1976) noted photosynthetic fixation of carbon in 2 marine diatoms by reactions similar to those of C₄ plants. More than 70% of the carbon fixed in these 2 taxa was in the form of amino acids or as intermediates of the citric acid cycle (including citric acid). Increased citric acid concentrations would cause an increase in the activity of acetyl CoA carboxylase and, subsequently, an increase in fatty acid synthesis. If C. meneghiniana possesses a photosynthetic mechanism similar to these 2 diatoms, the increase in the amount of cellular lipid with increasing N and P concentrations might be explained.

In addition, N stress could cause a reduction in the cells' protein synthesis and, consequently, in enzymatic activity. Collyer and Fogg (1955) speculated "that N deficiency brings about a change in the proportions of enzyme systems in favor of those concerned in fat synthesis." It may be that the enzymatic systems involved in fatty acid synthesis

are of low "priority" in the metabolism of C. meneghiniana. As N becomes increasingly limited, the low "priority" enzymatic systems might be inhibited while systems more essential for cell survival remain operative. Since the amount of cellular protein appeared to be positively correlated with the amount of cellular lipid of C. meneghiniana, this hypothesis might explain the small amounts of cellular lipid at low N concentrations.

Several factors could have contributed to differences in results between this study and others. The extraction techniques used in this study were chosen for their precision, ease of use, and applicability to algal samples. For example, the "Lowry Method" was used for protein determination because of its ease of use and universal acceptance. However, Coombs et al. (1967a) noted that protein determination by the "Lowry Method" produced values 30% lower than the "Kjeldahl Method." In addition, most researchers have used the chloroform/methanol extraction technique for lipid analysis. Only a few researchers (Otsuka and Morimura, 1966; Fisher and Schwarzenbach, 1978) have used alcoholic KOH to extract algal lipids. The alcoholic KOH techniques used in this study have been shown to be more effective in extracting lipids than chloroform/methanol techniques (Moon and Hammond, 1978). Therefore, differences in protein and lipid results between this study and others may have resulted from the extraction techniques that were used.

No direct comparisons between data in this study and others can be made. The biochemical data in this study were expressed on a "per cell" basis. Other researchers have almost exclusively expressed biochemical

data on a "per dry weight" basis. Data expressed on a dry weight basis are, however, difficult to interpret in diatoms "because of the presence of the silicious cell wall which is a highly variable percentage of the dry weight in different species" (Darley, 1977). Therefore, since data in this study were intended only for comparisons within and between experimental treatments and not for comparisons between studies, expression of biochemical data on a "per cell" basis was judged to be satisfactory.

Most researchers investigating nutrient-limitation effects on algal biochemical composition have analyzed nutrient-sufficient cells resuspended in nutrient-deficient media. The biochemical evaluation of such cells does not take into account the physiological history of the cells. Many algae can carry on normal physiological functions in nutrient-limited conditions as long as sufficient nutrient "pools" are maintained within the cells. For example, diatoms have been reported to store cytoplasmic "pools" of Si and P (Coombs et al., 1967a; Azam et al., 1974; Tilman and Kilham, 1976; Kilham et al., 1977; Paasche, 1980; Sullivan and Volcani, 1981). Therefore, the biochemical response of a culture during the initial period of Si or P limitation might simply be a response to the utilization of the cells' cytoplasmic "pools" rather than the nutrient concentration of the growth medium.

To minimize the variability caused by the luxury consumption of nutrients by diatoms in batch-starvation experiments, Kilham et al. (1977) suggested a 5-day, Si-starvation period and a 3-week, P-starvation period of cells prior to performing batch-growth experiments. In

this study, the biochemical composition of C. meneghiniana was analyzed 3 and 9 days after resuspension of nutrient-sufficient cells in nutrient-deficient media. No consideration was given for luxury consumption of nutrients by C. meneghiniana which may account for some of the differences in results between the nutrient-starvation and nutrient-stressed experiments.

Most researchers investigating diatom lipid production have used marine taxa or a species of the freshwater genera, Nitzschia and Navicula, as a test organism. Physiological characteristics of C. meneghiniana could have caused differences in results between this study and others. For example, Lowe (1974) reported C. meneghiniana to be a "facultative N heterotroph." Therefore, C. meneghiniana might possess different nutrient assimilation mechanisms than taxa used in other studies.

To my knowledge, this is the first study to investigate the amount of cellular lipid of a diatom at steady state along nutrient gradients. The semi-continuous culture technique maintained constant cell growth rates by maintaining a constant rate of nutrient supply to the cells. Changes in the rate of nutrient supply cause changes in the growth rate and the physiological state of the cell. Biochemical measurements made on cells of semi-continuous cultures in different physiological states characterized the population at specific points along a nutrient gradient. On the other hand, the batch culture technique did not maintain constant growth rate or similar physiological states of the cells. Biochemical measurements made on cells of batch cultures cannot be

identified with a particular nutrient concentration. Therefore, results of the nutrient-stressed experiments were more reliable than results of the nutrient-starvation experiments. Future studies should use continuous culture techniques for accurate biochemical and physiological analyses of algae.

BIOTECHNICAL PERSPECTIVE

The application of mass algal cultures to biotechnology has attracted scientific attention since the late 1940s. Mass algal cultures have been proposed for use in wastewater treatment, bioregenerative life support systems, chemical production, water renovation and recycling, and, most recently, hydrocarbon fuel production (Anonymous, 1978; Goldman, 1979; Goldman, 1980; Bergeron et al., 1983). The latter usage has received much attention in the last several years. However, the U.S. Research and Development Administration projected that for biomass "fuels" to have potential in energy production, they must provide 5 to 10% of the total U.S. energy needs (Goldman and Ryther, 1977).

If C. meneghiniana was mass cultured as a biotechnological energy source, the data presented in nutrient-stressed experiments could be used to estimate "conceivable" production of lipid, glucan, and protein. The amount of cellular lipid, glucan, and protein was greatest in N-stressed cultures with the greatest N supply rate. The following hypothetical yields of lipid, glucan, and protein production were estimated using this data set.

Assume that C. meneghiniana could achieve a uniform growth of $25,000 \text{ cells} \cdot \text{ml}^{-1}$ in a 1 hectare (ha) by 50 cm pond. Since the harvesting of unicellular algae is difficult (Goldman and Ryther, 1977), a sampling efficiency of 75% is assumed. An extraction efficiency of 90% (Shifrin and Chisholm, 1980) is also assumed. Using these assumptions, hypothetical yields of $0.78 \text{ metric tons lipid} \cdot \text{ha}^{-1}$, 0.31 metric tons

glucan·ha⁻¹, and 61.87 metric tons protein·ha⁻¹ would result. If harvesting could be completed 3 times per year (yr), the hypothetical yield would be 2.33 metric tons lipid·ha⁻¹·yr⁻¹, 0.94 metric tons glucan·ha⁻¹·yr⁻¹, and 185.61 metric tons protein·ha⁻¹·yr⁻¹. Using the caloric values of 15 gigajoules (gj) per metric ton carbohydrate and protein and 40 gj per metric ton lipid (Dubinsky et al., 1979), the total energy yield would be 2,891 gj·ha⁻¹·yr⁻¹.

The importance and applicability of such an energy yield is difficult to interpret. Based on the projected biomass cost target (i.e., gross income) of 0.95 to 1.40 dollars·gj⁻¹ (Lipinsky, 1978), mass cultures of C. meneghiniana could produce a gross income of 2,602 to 4,047 dollars·ha⁻¹·yr⁻¹. This value is substantially higher than the yearly gross income calculated for algal biomass by Dubinsky et al. (1979) and approximately 5 to 8 times greater than that projected for plant energy "farms" (Lipinsky, 1978). However, these estimated gross incomes do not take into account any harvesting, production, or material costs. Consequently, no comparisons between algal and higher plant net incomes can be made.

The success of algal mass cultures in biotechnology is dependent upon the selection and utilization of taxa which produce optimal yields concurrent with maximum growth. Researchers have indicated that algae produce optimal biochemical yields under conditions that are not conducive to optimal growth. The data generated from nutrient-stressed experiments indicated that C. meneghiniana produced greater biochemical yields with decreasing N stress. The ability of a taxon to produce

high biochemical yields under optimal growth conditions does, therefore, exist. This may be an occurrence specific to a particular taxon or a particular strain of a taxon.

The challenge for scientists in biotechnology is to select organisms capable of producing the desired compounds under specific growth conditions. Such organisms could possibly be obtained in several ways. Certain taxa in Israeli mass algal cultures are the "preferred" taxa with large yields of glycerin and beta carotene (Anonymous, 1978). Additional studies may allow researchers to identify other taxa that can produce large quantities of desired biochemical compounds (e.g., octadecatrienoic acid, a fatty acid of high commercial value). In addition, some researchers (Hall, 1979; Bergeron et al., 1983) have proposed that manipulation of a taxon's genetic structure has great promise in biotechnology. The data from the nutrient-stressed experiments with C. meneghiniana indicate that taxa can produce large amounts of desired compounds under low-stress growth conditions. Genetic engineering would be best used on these "preferred" taxa. Therefore, a thorough screening for these "preferred" taxa should be completed before manipulation of a taxon's gene structure is used to obtain large biochemical yields.

SUMMARY

- (1) The statistical significance of nutrient-limitation effects on the biochemical composition of the cells varied between batch and semi-continuous culture experiments. In batch cultures, Si deficiency caused a significant increase (300%) in the amount of cellular protein. Cellular protein was also significantly different between fractional replicates. In semi-continuous cultures, cellular L/G ratios significantly decreased (50%) with increasing Si supply rate. The amount of cellular lipid, glucan, and protein significantly increased (300%) with increasing N supply rate. The amount of cellular lipid and glucan significantly increased (300 and 400%, respectively) with increasing P supply rate.
- (2) Fatty acids of lipid extracted from cells of semi-continuous cultures generally possessed carbon chain lengths of 14 to 24. Carbon chain lengths of fatty acids from cells in Si-stressed cultures ranged from less than 14 to 18. No distinct differences in diatom fatty acid composition were observed among supply rates within Si-stressed and N-stressed cultures.
- (3) Greater amounts of unsaturated fatty acids were found in lipid extracted from cells of N-stressed cultures than in cells of Si-stressed cultures.
- (4) Discrepancies between results of this study and others were attributed to culture design, extraction techniques, the scaling factor used for expression of biochemical content, and the physiological characteristics of C. meneghiniana.

- (5) An estimated yield for a hypothetical mass culture of C. meneghiniana was calculated to be 2.33 metric tons lipid·ha⁻¹·yr⁻¹, 0.94 metric tons glucan·ha⁻¹·yr⁻¹, and 185.61 metric tons protein·ha⁻¹·yr⁻¹. The total energy yield derived from such a biochemical yield would be 2,891 gj·ha⁻¹·yr⁻¹.

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"...Don't you understand? When you give up your dream,
you die...."

Michael Nouri,
Flashdance (1983)

APPENDIX: MEAN SQUARE VALUES OF THE FACTORIAL ANALYSIS
AND THE ANALYSIS OF VARIANCE

Table A1. Mean square values for the sources of variation investigated in the factorial analysis of nutrient-starvation experiments

Source of variation	Variable						
	Lipid	Glucan	Protein	Chloro- phyll	Chlorophyll/ Phaeophytin	Protein/ Glucan	Lipid/ Glucan
Fractional replicate	8.60	7.60×10^{-2}	6.88×10^4 *	1.21×10^{-6}	3.58	8.86×10^5	6.41×10^1
Limiting nutrients							
Silica	9.17	2.67×10^{-1}	6.87×10^5 *	1.43×10^{-4}	1.68×10^{-1}	3.59×10^6	1.62×10^1
Nitrogen	8.78	5.89×10^{-3}	4.40×10^4	9.03×10^{-7}	3.97×10^{-3}	2.01×10^6	2.25
Phosphorus	7.01×10^{-1}	1.24×10^{-2}	2.33×10^4	8.10×10^{-7}	1.56×10^{-1}	1.84×10^6	9.14
Error (A)	2.43	5.59×10^{-2}	3.28×10^4	3.92×10^{-5}	4.50×10^{-1}	1.66×10^6	9.48
Days	1.12×10^1	9.17×10^{-2}	2.68×10^3	1.16×10^{-5}	3.21×10^{-1}	1.82×10^6	1.83
Interaction							
Days·Silica	1.56×10^1	1.61×10^{-1}	6.60×10^3	2.03×10^{-7}	4.36×10^{-3}	2.09×10^6	3.30×10^1
Days·Nitrogen	6.39	2.43×10^{-2}	1.54×10^4	6.25×10^{-8}	1.08	1.80×10^6	6.17×10^1
Days·Phosphorus	6.38×10^{-2}	2.05×10^{-2}	3.33×10^2	2.11×10^{-5}	3.76	1.54×10^6	5.43
Error (b)	4.02	3.78×10^{-2}	8.89×10^3	2.05×10^{-5}	1.49	1.59×10^6	1.98×10^1

*p<0.05.

Table A2. Mean square values for the sources of variation investigated in the Analysis of Variance for nutrient-stressed experiments

Experiment	Source of variation	Variable						
		Lipid	Glucan	Protein	Chlorophyll	Chlorophyll/Phaeophytin	Protein/Glucan	Lipid/Glucan
Silica	Supply rate	8.09×10^{-6}	3.55×10^{-6}	5.23×10^{-3}	9.51×10^{-3}	--	1.03×10^3	1.75*
	Linear	4.01×10^{-6}	6.22×10^{-6}	4.78×10^{-3}	3.67×10^{-5}	--	1.16×10^3	2.04*
	Quadratic	1.22×10^{-5}	8.70×10^{-7}	5.67×10^{-3}	1.90×10^{-2}	--	8.87×10^2	1.47
	Error	4.37×10^{-6}	1.43×10^{-6}	8.25×10^{-4}	5.23×10^{-2}	--	1.74×10^2	3.44×10^{-1}
Nitrogen	Supply rate	$4.36 \times 10^{-5***}$	$5.40 \times 10^{-6***}$	$1.70 \times 10^{-1***}$	5.26×10^{-2}	3.34	5.73×10^3	1.36×10^{-2}
	Linear	$8.72 \times 10^{-5***}$	$1.08 \times 10^{-5***}$	$3.30 \times 10^{-1***}$	1.40×10^{-2}	4.31	1.14×10^4	1.37×10^{-2}
	Quadratic	1.00×10^{-7}	1.00×10^{-8}	2.08×10^{-3}	9.12×10^{-2}	2.36	1.02×10^1	1.34×10^{-2}
	Error	1.24×10^{-6}	1.90×10^{-7}	4.31×10^{-3}	1.24×10^{-2}	5.38	2.49×10^3	1.45
Phosphorus	Supply rate	$1.48 \times 10^{-5*}$	$4.51 \times 10^{-6***}$	1.49×10^{-2}	6.03×10^{-2}	--	3.89×10^4	1.80
	Linear	$2.58 \times 10^{-5**}$	$8.43 \times 10^{-6***}$	2.59×10^{-2}	7.72×10^{-2}	--	6.72×10^4	3.06
	Quadratic	3.77×10^{-6}	5.80×10^{-7}	3.76×10^{-3}	4.35×10^{-2}	--	1.08×10^4	5.44×10^{-1}
	Error	9.10×10^{-7}	4.00×10^{-8}	4.13×10^{-3}	1.37×10^{-2}	--	1.02×10^4	3.61

*p<0.05.
 **p<0.01.
 ***p<0.001.