J. Phycol. **36,** 510–522 (2000)

EFFECT OF NUTRIENT AVAILABILITY ON THE BIOCHEMICAL AND ELEMENTAL STOICHIOMETRY IN THE FRESHWATER DIATOM *STEPHANODISCUS MINUTULUS* (BACILLARIOPHYCEAE)[1]

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The objective of this study was to examine the differences in the biochemical and elemental stoichiometry of a freshwater centric diatom, *Stephanodiscus minutulus* (Grun.), under various nutrient regimes. *Stephanodiscus minutulus* was grown at μ_{max} or 22% of μ_{max} under limitation by silicon, nitrogen, or phosphorus. Cell sizes for nutrient-limited cultures were significantly smaller than the non-limited cell sizes, with N-limited cells being significantly smaller than all other treatments. Compared with the nutrient-replete treatment, both carbohydrates and lipids increased in Si- and P-limited cells, whereas carbohydrates increased but proteins decreased in N-limited cells. All of the growth-limited cells showed an increase of carbohydrate and triglyceride, and a decrease of cell size and polar lipids as a percentage of total lipids. The non-limited cells also had a significantly higher chl *a* concentration and galactolipids as a percentage of total lipids than any of the limited treatments, and the low-Si and low-P cells had significantly higher values than the low-N cells. The particulate C concentrations showed significant differences between treatments, with the Si- and P-limited treatments being significantly higher than the N- and non-limited treatments. Particulate Si did not show a strong relationship with any of the parameters measured, and it was the only parameter with no differences between treatments. The low-Si cells had a significantly higher P content (about two times more) than any other treatment, presumably owing to the luxury consumption of P, and a correspondingly high phospholipid concentration. The elemental data showed that *S. minutulus* had a high P demand with low optimum N:P (4) and Si:P (10) ratios and a C:N:P ratio of 109:16:2.3. The particulate C showed a positive relationship with POM ($r = 0.93$), dry weight ($r = 0.88$), lipid ($r = 0.87$) and protein ($r = 0.84$, all $P < 0.0001$). Particulate N showed a positive relationship with galactolipids ($r = 0.95$), protein ($r = 0.90$), dry weight ($r = 0.78$), lipid ($r = 0.75$), and cell volume ($r = 0.64$, all $P < 0.0001$). It is evident that nutrient limitation in the freshwater diatom *S. minutulus* has pronounced effects on its biochemical and elemental stoichiometry.

Key index words: **biochemical composition; diatom; elemental stoichiometry; lipid classes; nitrogen; nutrient limitation; phosphorus; silicon; *Stephanodiscus minutulus***

Abbreviations: **AFDM, ash-free dry mass; DG, diglycerides; FA, fatty acids; GL, galactolipids; HC, hydrocarbons; N, nitrogen; P, phosphorus; PL, phospholipids; POM, particulate organic matter; PUFAs, polyunsaturated fatty acids; R*, intrinsic equilibrium resource concentration; Si, silicon; ST, sterols; TG, triglycerides; WE, wax esters; μ_{max}, maximum growth rate**

Biochemical and elemental phytoplankton composition is of considerable interest and is prompted by studies in applied phycology in areas such as aquaculture, the nutritional quality of foods fed to cultured animals, and food quality in aquatic food webs with subsequent trophic level effects (Volkman et al. 1989, Sterner and Hessen 1994, Kilham et al. 1997a). Lake seston has been shown to vary seasonally and among lakes in elemental (Hecky et al. 1993, Urabe 1993, Interlandi et al. 1999) and biochemical (Conover 1975, Barlow 1984, Kreeger et al. 1997) composition. Factors that influence phytoplankton composition include external nutrient availability, intrinsic equilibrium resource concentrations (R*), and culture type.

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Algae grown under nutrient limitation exhibit considerable variation in their biochemical composition depending on the limiting nutrient and the degree of limitation (growth rate as percentage of μ_{max}). Consequently, biochemical constituents of phytoplankton vary in concentration depending upon the nutrient availability (Darley 1977, Healey and Hendzel 1979, Shifrin and Chisholm 1981, Parrish and Wangersky 1990, Kilham et al. 1997b). One biochemical parameter receiving much attention is lipids. Diatoms appear to be good food for many animals, which may be because of high concentrations of lipids, particularly long-chained (>18° C) polyunsaturated fatty acids (PUFAs) (Müller-Navarra 1995, Coutteau and Sorgeloos 1997, Weers and Gulati 1997). These PUFAs are important biochemicals for higher aquatic organisms that cannot synthesize them themselves (Goulden and Place 1990). In general, the physiological condition of algal cells is a critical factor that can have trophic level effects (Kreeger and Langdon 1993, Sterner and Hessen 1994, Gulati and DeMott 1997, Kilham et al. 1997a).

Elemental concentrations and stoichiometry are widely used in both studies of limnology (Lizotte and Sullivan 1992, Hecky et al. 1993, Kreeger et al. 1997, Interlandi et al. 1999) and algal physiology (Rhee 1978, Brzezinski 1985, Sterner et al. 1993, Kilham et al. 1997b). A common practice is to compare elemental data with the Redfield ratio (106 C:16 N:1 P, Redfield 1958), which is considered to be the standard elemental ratio for non-limited algal cultures (McCarthy 1980) and natural phytoplankton populations (Hecky et al. 1993). Rhee and Gotham (1980) found evidence indicating a threshold level for nutrient limitation where an alga switches from being limited by one nutrient to another; however, different species have different switching points. Rhee (1978) suggested that the optimal elemental ratios of any given species of algae could vary from the Redfield ratio substantially. Since that time, there has been much work investigating the competitive ability of algae for varying resources (Rhee 1978, Tilman et al. 1982, Taylor 1994). Hecky et al. (1993) recently showed that freshwater systems have a much greater deviation from the Redfield ratio than marine systems.

Many studies of algal physiology have been done in the past on cells that were either growing at the maximal rate (μ_{max}) or at stationary phase in batch culture. Growth rates of algae measured in lakes are frequently found to be at some intermediate growth rate (Sommer 1985, Sterner 1990), rather than at stationary phase or μ_{max}. Algae grown in continuous or semi-continuous culture have reduced variation in physiological condition, can be easily replicated, can be grown consistently at a specified growth rate, and are more representative of the *in situ* condition. Commonly, in algal studies, only semi-quantitative analyses are presented, that is, biochemical composition as a percentage of dry weight or ash free dry weight or only a few parameters are measured under only one treatment type. It is also likely that because lipids are a heterogeneous group of compounds of varying structures and functions, the measurement of only total lipids may be masking different trends occurring among the individual lipid classes. Algal species vary considerably in cell size and biochemical composition, and semi-quantitative data can give a distorted description of the composition of different species or of the same species under different environmental conditions, making comparisons among studies difficult.

The objective of this study was to present a comprehensive picture of the physiology of a representative freshwater diatom under various nutrient regimes. The diatom *Stephanodiscus minutulus* (Grun.) was grown at 22% of its maximum growth rate under limitation by nitrogen, phosphorus, or silicon. It was also grown under non-limiting conditions at μ_{max} and the resulting biochemical and elemental compositions were then compared. *S. minutulus* was used because it is readily available, easy to grow and, as a small centric diatom, represents a large portion of spring bloom biomass in lakes and rivers around the world (Dorgelo et al. 1981, Kilham et al. 1986, Kobayasi and Kobayashi 1987, Sala 1990, Likhoshway et al. 1996, Kiss 1996, Interlandi et al. 1999).

MATERIALS AND METHODS

Algal cultures. *Stephanodiscus minutulus* was isolated by E. C. Theriot from Yellowstone Lake in Wyoming. Experiments were performed on semi-continuous unialgal cultures of 600 mL grown in 1 L glass flasks. After demonstrating similar growth of non-limited cultures in glass and polycarbonate flasks, Si-limited cultures were maintained in polycarbonate flasks to prevent boro-silicate leaching. Established clean culture techniques were used and, although cultures were not axenic, the bacterial biomass was always orders of magnitude lower than the diatom biomass based on particle size spectrum analyses provided by the Coulter Channelyzer (Coulter Electronics, Inc., Hialeah, FL). Cultures were grown in normal COMBO medium (Kilham et al. 1998) and in low-Si, low-N, or low-P COMBO medium. Normal COMBO medium contains 100 μM Si, 1000 μM N, and 50 μM P, whereas low-Si medium contained 37.5 μM Si, low-N medium contained 50 μM N and 150 μM Si, and low-P medium contained 10 μM P. Previous studies in our laboratory with this species have shown these concentrations to be limiting. Non-limited cultures were grown at μ_{max}, the maximal growth rate, and diluted once per day. Nutrient-limited cultures were maintained at 22% of μ_{max} ($\mu_{max} = 0.84 \cdot d^{-1}$, Kilham et al. 1998) using semi-continuous dilutions (100 mL·d^{-1}) once each day (Kilham 1978), giving a 0.17·d^{-1} exchange rate. Aerated cultures were kept in an environmental chamber at 16 ± 1° C with a 14:10 h LD (light:dark) cycle and with 80 μmol·m^{-2}·s^{-1} of light. To ensure that the biochemical and elemental composition at a particular nutrient-limited growth rate was measured at a steady-state condition, cultures were monitored for 2 weeks before sampling.

Sampling. For each treatment, there were eight replicate cultures from which 100 mL was removed each day. To ensure sufficient sample size of algal material for chemical and biochemical analyses, we combined the daily aliquots from all eight replicates per treatment per day (800 mL) as one composite sample from which subsamples were taken for each parameter. Replication per treatment for each parameter was obtained from composite samples taken on different days. These values were assumed to be independent values for statistical purposes

because cultures were sampled over 11 consecutive days under steady-state conditions. Fluorescence was measured using a Turner Designs Model 10 fluorometer (Turner Designs, Sunnyvale, CA) and cell concentrations and cell size were measured daily with a Coulter Counter and Channelyzer (model ZB 256)(Coulter Electronics, Inc.). Other samples were taken approximately every two of three days.

Triplicate samples were taken for each parameter from each culture type during each sampling. Samples were filtered on ashed (400° C, 4 h) glass fiber filters (GF/F) via vacuum filtration for quantification of carbohydrate, protein, lipid, particulate C, particulate N, particulate P and chl *a* concentrations. Particulate Si samples were filtered on 0.4 μm polycarbonate filters. Chl *a* samples were placed in plastic Petri dishes and immediately wrapped in aluminum foil to prevent light degradation. Lipid filter samples were placed in 2 mL amber vials which were filled with 2:1 (vol/vol) chloroform:methanol and sealed in an N_2 atmosphere. All samples were then frozen at −80° C until analyses were performed. Samples for dry weight and ash-free dry mass (AFDM) were filtered onto pre-weighed ashed GF/F. Samples for dissolved chemistry were collected in conjunction with corresponding particulate samples and were placed in 125 mL polyethylene bottles and stored at 4° C.

Analyses. Algal carbohydrate was determined spectrophotometrically using the Dubois et al. (1956) procedure, which was standardized with potato starch (soluble grade, Baker, 4006-4). Each filter was combined with 2 mL deionized water in a 15 mL borosilicate glass test tube, and the Dubois et al. (1956) reagents were added. Tubes were vortexed for 5 s and a 200 μL subsample was transferred to three replicate wells on a 96 well microplate for spectrophotometric analysis at 490 nm with a microplate reader (Thermomax, Molecular Devices, Sunnyside, CA).

Algal protein was determined spectrophotometrically using a Pierce test kit (BCA 23225) based on the procedure of Lowry et al. (1951), and standardized BSA. Filtered algae were prepared for analysis by first suspending the filter in 4 mL 0.1 M NaOH in a 15 mL polypropylene test tube. The filter was ground in a tissue homogenizer for 60 s and sonicated for 10 s to transform the filter into a slurry. Samples were diluted to 8 mL with 0.1 M NaOH, heated to 60° C for 45 min, vortexed for 10 s, and then centrifuged (800*g*, 5 min). Samples were held on ice whenever possible throughout the procedure. A total of 10 μL of each supernatant was transferred to each triplicate well in a 96 well microplate. BCA reagent (200 μL) was added to each well, and after a 30 min incubation period at 37° C, the microplate was analyzed at 562 nm with a microplate reader.

Concentrations of eight major classes of lipids were measured in each sample and total concentrations of lipids were then calculated by summation. Each filter was ground for 5 min in approximately 4 mL of 2:1 (vol/vol) chloroform:methanol with a tissue grinder (10 mL Potter-Elvehjem with PTFE pestle). During grinding, an internal standard of 2% (wt/vol) 3-hexadecanol (not produced by plants or animals) in 2:1 C/M was added. Each ground suspension was then centrifuged (1000 rpm, 5 min) and the supernatent containing extracted lipid was transferred to a second test tube. A 20% (vol/vol) aliquot of 0.88% (wt/vol) KCl was then added to each sample to effect an aqueous–organic phase separation. Each tube was then centrifuged for 2 min at 1000 rpm and the lipid-containing chloroform layer was then transferred to a 5 mL conical centrifuge tube for rapid drying in a vacuum evaporator (Savant Speedvac; Savant Instruments, Inc., Holbrook, NY). After drying, the lipid was resuspended in 100 μL 2:1 (vol/vol) chloroform:methanol, sealed in an amber 2 mL vial with insert, flushed with N_2, and then stored at −80° C until lipid class analysis.

Lipid classes were quantified by thin layer chromatography–flame ionization detection using an Iatroscan Analyzer (IV) (Bioscan, Inc., Washington, DC) with methods described by Parrish (1987a) and modified by Kreeger et al. (1997). Hydrocarbons (HC), wax esters (WE), triglycerides (TG), fatty acids (FA), diglycerides (DG), sterols (ST), galactolipids (GL) and phospholipids (PL) were quantified in each lipid sample using standards of nonedecane, cetyl oleate, triolein, oleic acid, dipalmitin, cholesterol, digalactocyl diglyceride, and phosphatidyl choline, respectively.

Dissolved nutrients. Nitrogen samples were analyzed with cadmium reduction followed by colorimetric determination of nitrite. Phosphorus samples were analyzed by the ascorbic acid method. Silicon samples were analyzed by the molybdosilicate method (American Public Health Association 1995).

Particulate nutrients. Filters for particulate carbon and nitrogen were dried at 60° C for 24 h and then held in a desiccator until elemental analysis. Subsamples were punched from the filters with a bore tube, packed into pre-cleaned (acetone washed) tin capsules and then combusted and analyzed by gas chromatography on a Carlo Erba 1106 elemental analyzer (Interlandi et al. 1999). Particulate phosphorus filters were digested with 5% (wt/vol) persulfate at 121° C for 30 min and analyzed as dissolved P. Particulate silicon filters were digested with 1.5% (wt/vol) Na_2CO_3, acidified between pH 2–4, and analyzed as dissolved Si (modified from Conway et al. 1977).

Chl a. Filters were extracted using a 90% (vol/vol) acetone solution for 24 h at 4°C and then analyzed on a Turner Designs TD-700 laboratory fluorometer (American Public Health Association 1995).

Statistical analyses. Cell concentrations and volume were examined for steady-state conditions over time using linear-regression analysis. After data were determined to be normally distributed, all parameters were compared statistically for significant differences between culture types with one-way analysis of variance (ANOVA) procedures and least significant difference (LSD) multiple-range analyses (Sokal and Rohlf 1981). No transformations of data were necessary. Select parameters were examined for positive or negative relationships across all treatments using linear regression analysis. The sum of the lipid, protein, and carbohydrate analyses was termed the particulate organic matter (POM), and this value was compared statistically to the AFDM with two-way Student's *t*-test. All statistical analyses were performed with JMP v.3.2 for Macintosh and all ANOVA tests were assumed significant at the $\alpha = 0.05$ level.

RESULTS

Of the three nutrient-limited treatments, low-Si and low-P showed no deviation from steady-state condition for cell concentration (Si: $P = 0.0806$, P: $P = 0.1247$) or volume (Si: $P = 0.0753$, P: $P = 0.1185$). For the low-N treatment, some deviation from strict steady-state conditions was indicated by modest decreases in both cell concentration ($n \cdot mL^{-1}$) ($P = 0.0458$) and volume ($\mu m^3 \cdot mL^{-1}$) ($P = 0.0057$) during the period of sampling. This deviation did not affect most of the physiological parameters of the low-N cells. The only other parameters to show a significant relationship with cell concentration over the 11 d sampling period were cell size ($r = -0.90$, $P = 0.0002$) and Si per cell ($r = 0.93$, $P = 0.0065$).

Cell morphology. The relationship between cell concentration and volume of *S. minutulus* (Fig. 1, Table 1) changed under Si limitation (cell = 0.259 vol − 0.123, $r^2 = 0.98$), N limitation (cell = 0.224 vol + 0.972, $r^2 = 0.93$), and P limitation (cell = 0.228 vol + 0.205, $r^2 = 0.97$) compared with the relationship for non-limited cells (cell = 0.196 vol + 0.327, $r^2 = 0.88$). However, using analysis of co-variance, the slopes of these lines were determined not to be statistically significantly different ($P = 0.728$). Cell sizes for nutrient-limited cultures were significantly smaller than the non-limited cell sizes (Table 1), with N-limited cells being signifi-

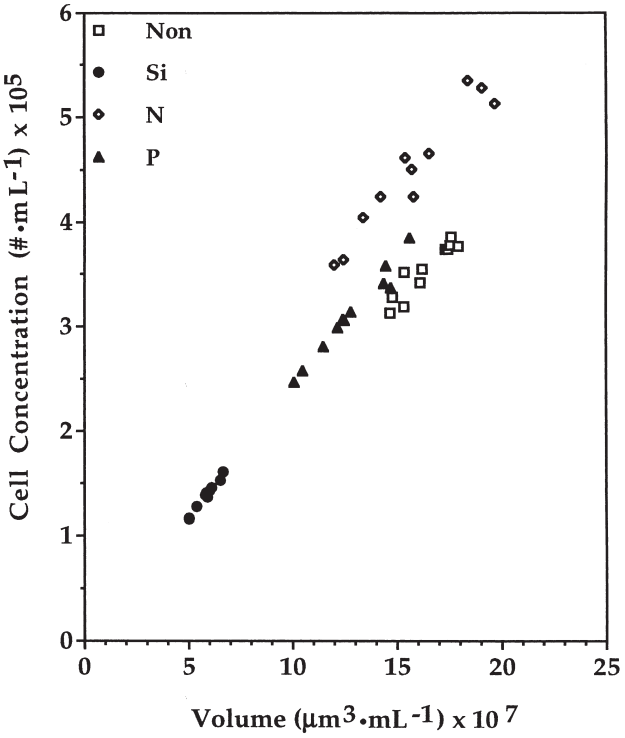

FIG. 1. A comparison of cell concentration and volume for *Stephanodiscus minutulus* growing without limitation at μ_{max} (Non) and at 22% of μ_{max} under limitation by silicon (Si), nitrogen (N), or phosphorus (P).

cantly smaller than all other treatments. The weights and densities of cells showed significant differences between treatments (Table 1). In general, non-limited and N-limited cells had lower cell densities and cell weights than Si-limited and P-limited cells. The non-limited cells also had a significantly higher chl *a* concentration than any of the limited treatments; the low-Si and low-P cells had significantly more chl *a* than the low-N cells (Table 1). No phaeopigments were found in any of the samples, which is expected for exponentially growing cultures (Taguchi et al. 1987).

Biochemical composition. N-limited cells had a significantly lower protein content than any other treatment (Fig. 2A, Table 1). All limited cultures had higher carbohydrate concentrations per cell than the non-limited cells, with N-limited cells being the highest. Si-limited cells had a significantly higher lipid content than any other treatment, and P-limited cells had a higher lipid content than both the non-limited cells and the N-limited cells. Both the Si-limited and P-limited cells had significantly higher POM than the N-limited or non-limited cells (Table 1). The non-limited treatment had a significantly higher protein:lipid ratio (0.86) than any of the limited treatments (Table 1). POM was compared with AFDM for the non-limited ($P = 0.3173$), Si-limited ($P = 0.8293$), N-limited ($P = 0.8227$), and P-limited ($P = 0.4830$) treatments and no significant differences were found for any of the treatments. This validates the biochemical analyses used to determine protein, carbohydrate, and lipid.

Lipid classes. Si-limited cells had the highest polar lipid content (galactolipids and phospholipids) of all the treatments (Fig. 3). Si-limited cells also had higher hydrocarbon, triglyceride, and fatty acid contents than non-limited cells and N-limited cells (Fig. 3A). P-limited cells had a higher triglyceride content than all other treatments and higher sterol and galactolipid contents than non-limited and N-limited cells. N-limited cells had a lower sterol content than Si-limited cells, a higher triglyceride content than non-limited cells and a lower galactolipid content than non-limited cells. The non-limited cells had a significantly higher percentage of galactolipids than did any of the nutrient-limited cells (Fig. 3C). All the nutrient-limited cells had a higher percentage of triglyceride than the non-limited cells, with the N-limited and P-limited cells having a larger percentage than the Si-limited. In all other aspects, the lipid classes were similar, as $\mu g \cdot cell^{-1}$, $\mu g \cdot volume^{-1}$ and as percentages, in the four treatment types. No diglycerides were found in any of the samples and only minute traces of WE were found in a small number of samples. Using a similar lipid analysis, Sicko-Goad and Andresen (1991) also found a lack of diglycerides in three species of freshwater diatoms.

Elemental concentrations. Particulate C concentrations were significantly greater in the Si- and P-limited treatments than the N- and non-limited treatments (Table 2). The C per cell was significantly higher in the non-limited treatment than in the N-limited treatment. Particulate Si concentrations per volume were significantly higher in the low-N treatment than the low-Si or non-limited treatments; the low-P treatment had a significantly higher Si per volume than the non-limited treatment. The particulate Si per cell showed no significant differences between the treatments. The particulate N per cell and volume was significantly higher in the low-Si and low-P treatments than the non-limited and N-limited treatments, with the non-limited treatment having a significantly higher N per cell and volume than the N-limited treatment. The particulate P concentration per cell and volume was significantly higher in the Si-limited treatment than all other treatments. The non-limited treatment had a higher P per cell than the low-P treatment, whereas the N-limited treatment had a higher P per volume than the low-P treatment. As expected, the N-limited treatment had the lowest N per cell and per volume, and the P-limited treatment had the lowest P per cell and per volume.

For all the nutrient-limited treatments, the limiting nutrient had the lowest concentration in the daily effluent aliquots (Table 3). All four treatments depleted the majority of the Si from the media, but the Si-limited treatment had the lowest Si concentration remaining in the media.

Parameter relationships. Cell size showed a strong positive relationship with chlorophyll *a* and particulate N and a moderate positive relationship with protein (Table 4). It showed a strong negative relationship with

TABLE 1. Cell concentration, cell size, cell weight, density, chl *a* concentration, and biochemical composition of *Stephanodiscus minutulus* grown without limitation at μ_{max} (Non) and at 22% of μ_{max} under limitation by silicon, nitrogen, or phosphorus.

Parameter	Non	Si	N	P	ANOVA *P* value
Cell concentration ($n = 11$)					
($\#\cdot mL^{-1}$) $\times 10^5$	3.55	1.38	4.48	3.12	
	(0.08)	(0.04)	(0.18)	(0.13)	
Cell size ($n = 11$)					
($\mu m^3 \cdot cell^{-1}$)	463	421	349	411	
	(3.74)	(2.03)	(5.27)	(3.01)	<0.0001
	A	B	C	B	
Cell weight ($n = 8$)					
($\mu g \cdot cell^{-1}$) $\times 10^{-5}$	8.83	13.0	8.34	11.7	
	(0.33)	(0.54)	(0.38)	(0.76)	<0.0001
	B	A	B	A	
Density ($n = 8$)					
($\mu g \cdot \mu m^{-3}$) $\times 10^{-7}$	1.92	3.09	2.40	2.84	
	(0.07)	(0.13)	(0.13)	(0.19)	<0.0001
	C	A	BC	AB	
Chl *a* ($n = 5$)					
($\mu g \cdot cell^{-1}$) $\times 10^{-7}$	9.37	6.58	1.45	6.08	
	(0.75)	(0.32)	(0.06)	(0.22)	<0.0001
	A	B	C	B	
($\mu g \cdot \mu m^{-3}$) $\times 10^{-10}$	20.2	15.6	4.08	14.8	
	(1.71)	(0.70)	(0.16)	(0.73)	<0.0001
	A	B	C	B	
Protein ($n = 8$)					
($\mu g \cdot cell^{-1}$) $\times 10^{-5}$	2.31	2.92	0.95	2.76	
	(0.16)	(0.17)	(0.09)	(0.27)	<0.0001
	A	A	B	A	
($\mu g \cdot \mu m^{-3}$) $\times 10^{-8}$	5.04	6.92	2.73	6.72	
	(0.35)	(0.41)	(0.26)	(0.67)	<0.0001
	B	A	C	AB	
Carbohydrate ($n = 8$)					
($\mu g \cdot cell^{-1}$) $\times 10^{-5}$	1.51	2.80	3.11	2.76	
	(0.11)	(0.17)	(0.14)	(0.21)	<0.0001
	B	A	A	A	
($\mu g \cdot \mu m^{-3}$) $\times 10^{-8}$	3.26	6.65	9.02	6.77	
	(0.22)	(0.39)	(0.46)	(0.51)	<0.0001
	C	B	A	B	
Lipid ($n = 8$)					
($\mu g \cdot cell^{-1}$) $\times 10^{-5}$	3.01	6.60	2.65	5.18	
	(0.28)	(0.42)	(0.23)	(0.30)	<0.0001
	C	A	C	B	
($\mu g \cdot \mu m^{-3}$) $\times 10^{-8}$	6.47	15.6	7.69	12.7	
	(0.59)	(0.95)	(0.67)	(0.75)	<0.0001
	C	A	C	B	
POM ($n = 5$)					
($\mu g \cdot cell^{-1}$) $\times 10^{-5}$	6.69	12.1	6.75	10.7	
	(0.58)	(0.67)	(0.52)	(0.94)	<0.0001
	B	A	B	A	
($\mu g \cdot \mu m^{-3}$) $\times 10^{-8}$	14.5	28.6	19.9	26.1	
	(1.19)	(1.49)	(1.51)	(2.32)	<0.0001
	C	A	BC	AB	
Protein:lipid ($n = 5$)	0.86	0.51	0.39	0.52	
	(0.07)	(0.05)	(0.05)	(0.01)	<0.0001
	A	B	B	B	
Protein:carbohydrate ($n = 6$)	1.70	1.14	0.33	0.97	
	(0.17)	(0.08)	(0.03)	(0.04)	<0.0001
	A	B	C	B	
Protein:(carbohydrate + lipid) ($n = 5$)	0.57	0.34	0.17	0.34	
	(0.04)	(0.02)	(0.02)	(0.01)	<0.0001
	A	B	C	B	

Values are means (±SE) and rows within each biochemical constituent refer to normalizations per unit cell ($\mu g \cdot cell^{-1}$) and cell volume ($\mu g \cdot \mu m^{-3}$). Same letters designate statistically similar treatments. ANOVA, analysis of variance; N, nitrogen; Non, grown without limitation; P, phosphorus; POM, particulate organic matter; Si, silicon.

carbohydrate and a moderate negative relationship with particulate Si. Cell weight showed a strong positive relationship with POM, lipid, particulate C, particulate N, protein and particulate P and showed a moderate positive relationship with carbohydrate. Chl *a* showed a strong positive relationship with cell size and a moderate positive relationship with particulate N. It showed a moderate negative relationship with carbohydrate.

Protein showed a strong positive relationship with

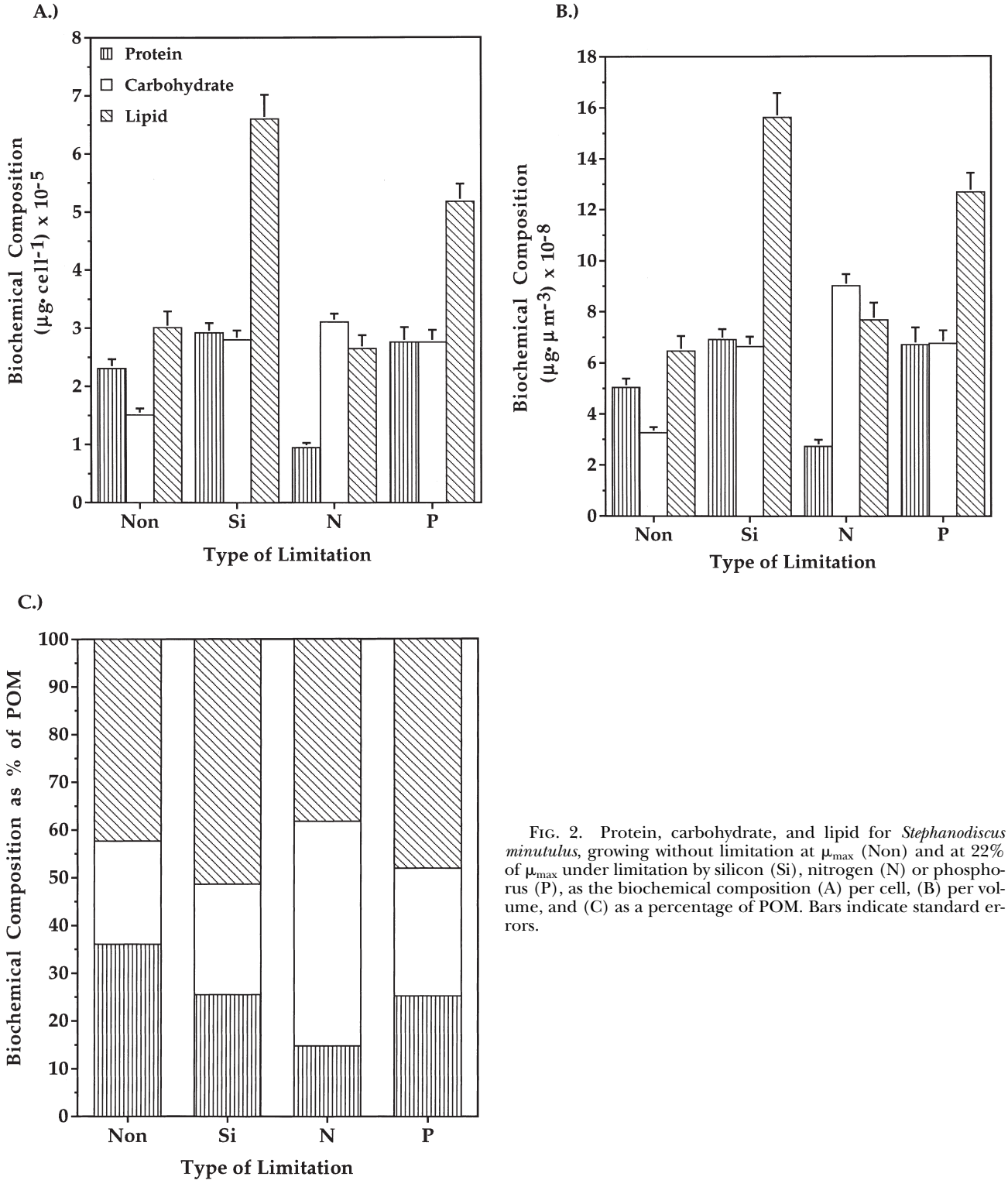

FIG. 2. Protein, carbohydrate, and lipid for *Stephanodiscus minutulus*, growing without limitation at μ_{max} (Non) and at 22% of μ_{max} under limitation by silicon (Si), nitrogen (N) or phosphorus (P), as the biochemical composition (A) per cell, (B) per volume, and (C) as a percentage of POM. Bars indicate standard errors.

particulate N, particulate C, POM, cell weight, and lipid and a moderate positive relationship with cell size. Carbohydrate showed a moderate positive relationship with cell weight. It showed a strong negative relationship with cell size and a moderate negative relationship with chl *a*. Lipid showed a strong positive relationship with POM, cell weight, particulate C and protein, and particulate N and showed a moderate positive relationship with particulate P. POM showed a strong positive relationship with lipid, cell weight, particulate C and protein and showed a moderate positive relationship with particulate N.

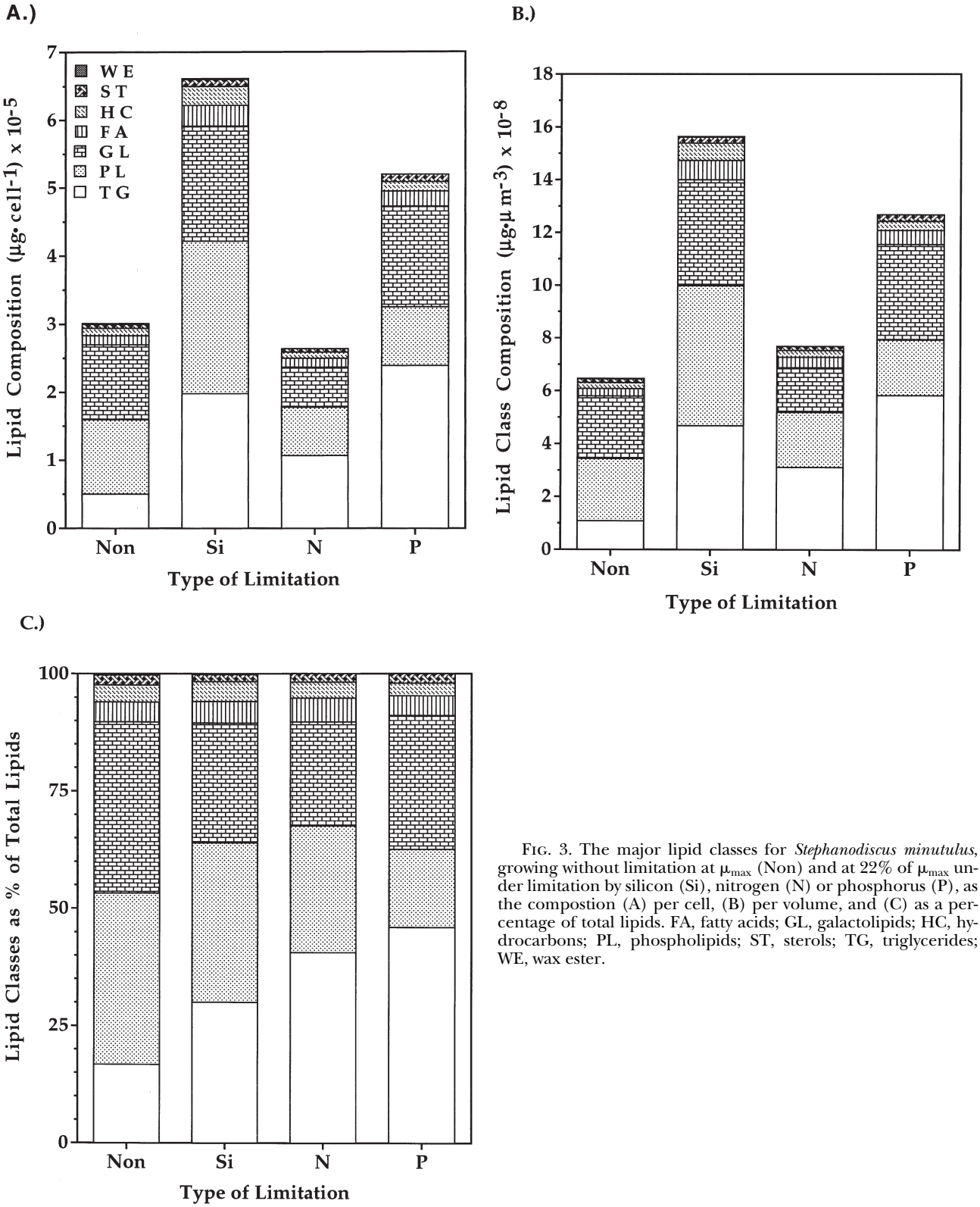

FIG. 3. The major lipid classes for *Stephanodiscus minutulus*, growing without limitation at μ_{max} (Non) and at 22% of μ_{max} under limitation by silicon (Si), nitrogen (N) or phosphorus (P), as the compostion (A) per cell, (B) per volume, and (C) as a percentage of total lipids. FA, fatty acids; GL, galactolipids; HC, hydrocarbons; PL, phospholipids; ST, sterols; TG, triglycerides; WE, wax ester.

Particulate C showed a strong positive relationship with POM, particulate N, cell weight, lipid, and protein and a moderate positive relationship with particulate P. Particulate Si showed a moderate negative relationship with cell size. Particulate N showed a strong positive relationship with protein, particulate C, cell weight, lipid and cell size and a moderate positive relationship with POM, chl *a* and particulate P. Particulate P showed a moderate positive relationship with cell weight, lipid, and particulate C.

TABLE 2. Particulate carbon, silicon, nitrogen, and phosphorus content and ratios of *Stephanodiscus minutulus* grown without limitation at μ_{max} (Non) and at 22% of μ_{max} under limitation by silicon, nitrogen, or phosphorus.

Parameter	Non	Si	N	P	ANOVA *P* value
C ($n = 8$)					
(μmol·cell^{-1}) $\times 10^{-6}$	2.07	3.25	1.64	3.16	
	(0.08)	(0.14)	(0.09)	(0.12)	<0.0001
	B	A	C	A	
(μmol·μm^{-3}) $\times 10^{-9}$	4.45	7.69	4.66	7.70	
	(0.14)	(0.31)	(0.21)	(0.33)	<0.0001
	B	A	B	A	
Si ($n = 7$)[a]					
(μmol·cell^{-1}) $\times 10^{-7}$	2.85	3.08	3.23	3.39	
	(0.10)	(0.16)	(0.15)	(0.20)	0.1051
	—	—	—	—	
(μmol·μm^{-3}) $\times 10^{-10}$	6.14	7.33	9.28	8.27	
	(0.25)	(0.38)	(0.56)	(0.52)	0.0003
	C	BC	A	AB	
N ($n = 8$)					
(μmol·cell^{-1}) $\times 10^{-7}$	3.04	3.95	1.23	3.70	
	(0.09)	(0.23)	(0.05)	(0.14)	<0.0001
	B	A	C	A	
(μmol·μm^{-3}) $\times 10^{-10}$	6.55	9.34	3.51	9.01	
	(0.16)	(0.50)	(0.14)	(0.40)	<0.0001
	B	A	C	A	
P ($n = 6$)					
(μmol·cell^{-1}) $\times 10^{-8}$	4.32	8.86	3.56	3.16	
	(0.15)	(0.32)	(0.20)	(0.14)	<0.0001
	B	A	BC	C	
(μmol·μm^{-3}) $\times 10^{-11}$	9.30	21.0	10.1	7.70	
	(0.36)	(0.78)	(0.65)	(0.40)	<0.0001
	BC	A	B	C	
C:Si ($n = 7$)[a]	7.33	10.6	5.12	9.39	
	(0.41)	(0.51)	(0.32)	(0.37)	<0.0001
	B	A	C	A	
C:N ($n = 8$)	6.79	8.29	13.3	8.56	
	(0.18)	(0.20)	(0.41)	(0.13)	<0.0001
	C	B	A	B	
C:P ($n = 6$)	48.7	37.0	49.4	103.6	
	(2.27)	(1.42)	(3.19)	(0.52)	<0.0001
	B	C	B	A	
Si:N ($n = 7$)[a]	0.94	0.81	2.61	0.91	
	(0.03)	(0.05)	(0.08)	(0.04)	<0.0001
	B	B	A	B	
Si:P ($n = 6$)[a]	6.63	3.59	9.77	11.27	
	(0.14)	(0.15)	(0.16)	(0.57)	<0.0001
	C	D	B	A	
N:P ($n = 6$)	7.06	4.44	3.64	11.94	
	(0.22)	(0.22)	(0.14)	(0.14)	<0.0001
	B	C	D	A	

[a] The N and P treatments each have one less sample number for these parameters (i.e. $n = 6$ or $n = 5$)

Values are means (±SE) and rows within each elemental component refer to normalizations per unit cell (μg·cell^{-1}) and cell volume (μg·μm^{-3}). Same letters designate statistically similar treatments. ANOVA, analysis of variance; N, nitrogen; Non, grown without limitation; P, phosphorus; Si, silicon.

DISCUSSION

Nutrient limitation in the freshwater diatom *S. minutulus* has pronounced effects on its biochemical and elemental stoichiometry. Some of the changes are unique to only a particular nutrient limitation (e.g. N-limitation and protein content), whereas some of the changes appear to be indicators of stress or limited growth in general (e.g. increased triglycerides and carbohydrates).

Cell morphology. The cell size of *S. minutulus* decreased significantly when under nutrient limitation, with N-limited cells being significantly smaller than Si- or P-limited cells (Table 1). Interestingly, the pattern appears to be different in green algae with an increase in cell size under P-limitation for *Ankistrodesmus falcatus* (Kilham et al. 1997b) and *Scenedesmus* sp. (Rhee 1978, Sterner et al. 1993). Cell size was positively correlated with protein and negatively correlated with carbohydrate, making the protein:carbohydrate ratio the best predictor of cell size (Table 4).

Darley (1977) contends that volume is not a valid measure to express biochemical composition; however, this study shows a strong relationship between cells per milliliter and volume per milliliter for all treatment types (Fig. 1). This study also found relatively few significant differences between expressions

TABLE 3. Dissolved silicon, nitrogen, and phosphorus concentrations in the daily influent and effluent aliquots of semi-continuous *Stephanodiscus minutulus* cultures grown without limitation at μ_{max} (Non) and at 22% of μ_{max} under limitation by silicon, nitrogen, or phosphorus.

Parameter		Non	Si	N	P
Si (μM)	I	100	37.5	150	100
	E	0.68 (0.12)	0.56 (0.11)	0.73 (0.16)	0.74 (0.24)
N (μM)	I	1000	1000	50	1000
	E	>800	>800	0.14 (0.07)	>800
P (μM)	I	50	50	50	10
	E	38.7 (1.76)	42.7 (0.47)	39.0 (0.41)	0.13 (0.04)

Values are means (±SD) and $n = 6$. E, effluent; I, influent; N, nitrogen; Non, grown without limitation; P, phosphorus; Si, silicon.

of biochemical or elemental composition per μm^3 (volume) or per cell (Tables 1 and 2). Shifrin and Chisholm (1981) showed a decrease in cell weight for green algae under N limitation, but they got mixed results for the freshwater diatoms. In the present study, it is interesting to note that the cell weight is more closely related to the lipid and protein per cell than to the carbohydrate per cell (Table 4) although, because of the water associated with carbohydrate stores, carbohydrate is usually considered the heaviest of the macromolecules (Lehninger et al. 1993).

The chl *a* was positively correlated with cell size, particulate N, and the galactolipids and was also negatively correlated with carbohydrate (Table 4). The N-limited treatment explicitly shows the relationships described above with a decrease in chl *a*, cell size (Table 1), particulate N (Table 2), and galactolipids (Fig. 3) and an increase in carbohydrate (Table 1), compared to the non-limited cells. Several sources cite chl *a* concentration as an indication of organic biomass (Taguchi et al. 1987, American Public Health Association 1995), stating that chl *a* should account for 1%–2% of the AFDM. Although this relationship held up for the non-limited treatment (1.29%), the nutrient-limited treatments fell well short (Si = 0.55%, N = 0.21%, P = 0.58%).

Taguchi et al. (1987) found that the cellular carbon:chl *a* ratios ranged from 18 to 25 for non-limited cells and gradually increased to over 100 for stationary cells. Steele and Baird (1961) found increasing C:chl *a* ratios for nutrient deficient populations, and Sakshaug (1980) cited several studies on N- or P-limitation of diatoms showing similar results. The C:chl *a* ratios in this study are 29, 60, 146, and 65 for the non-, Si-, N- or P-limited cells, respectively, which is consistent with the trends of increasing ratios with increasing limitation. Nutrient limitation in general affects the photosynthetic apparatus to some degree; however, the effects of nitrogen limitation are the most severe (Harwood 1997).

Biochemical composition. Numerous studies have shown that nutrient limitation has a pronounced effect on the biochemical composition of freshwater algae. It has been well documented that nitrogen limitation leads to decreased total protein production in both green algae (Rhee 1978, Healey and Hendzel 1979, Kilham et al. 1997b) and diatoms (Darley 1977, Harrison et al. 1990, La Roche et al. 1993). Other metabolic changes occurring with nitrogen limitation are the accumulation of carbon storage products (carbohydrates and triglycerides), and a decrease in chl *a* (Rhee 1978, Shifrin and Chisholm 1981, Harrison et al. 1990, Larson and Rees 1996).

Several studies have also shown an increase in carbohydrate under P limitation, both in green algae (Healey and Hendzel 1979, Kilham et al. 1997b) and diatoms (overview in Darley 1977). However, only the N-limited treatment showed a large increase in the carbohydrate concentration, particularly as a percentage of POM (Fig. 2), without the increase in total lipids observed in the P- and Si-limited treatments. All the nutrient-limited treatments had increased triglyc-

TABLE 4. Product–moment correlation coefficients for pairwise linear regressions between parameters for all treatments.

Var 1	Var 2	*n*	*r*
CS	chl *a*	20	0.89[b]
CS	pro	32	0.59[a]
CS	car	32	−0.69[b]
CS	pro:car	24	0.88[b]
CS	Si	26	−0.41[a]
CS	N	32	0.64[b]
CW	pro	28	0.78[b]
CW	car	20	0.45[a]
CW	lip	20	0.89[b]
CW	POM	16	0.96[b]
CW	C	20	0.88[b]
CW	N	20	0.78[b]
CW	P	12	0.78[a]
chl *a*	car	8	−0.76[a]
chl *a*	N	20	0.70[a]
pro	lip	20	0.77[b]
pro	POM	20	0.80[b]
pro	C	20	0.84[b]
pro	N	20	0.90[b]
pro	GL	20	0.84[b]
car	TG:(GL + PL)	28	0.57[a]
lip	POM	20	0.97[b]
lip	C	24	0.87[b]
lip	N	24	0.75[b]
lip	P	16	0.73[a]
POM	C	12	0.93[b]
POM	N	12	0.77[a]
POM	TG	20	0.87[b]
C	N	32	0.90[b]
C	P	24	0.48[a]
N	P	24	0.49[a]
N	GL	24	0.95[b]
P	PL	16	0.73[a]
C:N	pro:car	12	−0.94[b]

[a] Significant at $P < 0.05$.
[b] Significant at $P < 0.0001$.

C, particulate C ($\mu mol \cdot cell^{-1}$); car, carbohydrate ($\mu g \cdot cell^{-1}$); chl *a*, chlorophyll *a* ($\mu g \cdot cell^{-1}$); CS, cell size ($\mu m^3 \cdot cell^{-1}$); CW, cell weight ($\mu g \cdot cell^{-1}$); GL, galactolipid ($\mu g \cdot cell^{-1}$); lip, lipid ($\mu g \cdot cell^{-1}$); N, particulate N ($\mu mol \cdot cell^{-1}$); P, particulate P ($\mu mol \cdot cell^{-1}$); PL, phospholipid ($\mu g \cdot cell^{-1}$); POM, particulate organic matter ($\mu g \cdot cell^{-1}$); pro, protein ($\mu g \cdot cell^{-1}$); Si, particulate Si ($\mu mol \cdot cell^{-1}$); TG, triglyceride ($\mu g \cdot cell^{-1}$).

erides and it has been shown that plants will build up triglycerides as an energy reserve (Harwood 1997), usually for two reasons: (a) triglycerides are highly reduced and yield energy when oxidized; and (b) triglycerides are hydrophobic and do not carry the weight of extra water (Lehninger et al. 1993). Interestingly, carbohydrate, particularly glycerol 3-phosphate, is synthesized into triglycerides and many polar lipids via the Kennedy pathway in both animals and plants (Harwood 1997). If it is a strategy of Si- and P-limited algae to convert excess carbohydrates to lipids, particularly triglycerides, then why do N-limited algae amass carbohydrates and forego the high energy reserves of triglycerides? Unlike animals that use insulin to control the conversion of excess carbohydrates to lipid, lipid synthesis in algae is probably dependent on the activity of acetyl-CoA carboxylase, a key enzyme or protein (Harwood 1997). This may indicate that the N-limited treatment was biochemically unable to efficiently transform excess carbohydrate to lipid stores, owing to the reduced protein content.

The parameter that showed the strongest significant positive relationship with carbohydrate was the triglyceride:polar lipid ratio (Table 4). Triglycerides and polar lipids are comprised of fatty acids, usually saturated and unsaturated, respectively. In higher plant cells, not only does the synthesis of fatty acids require a complex of seven different polypeptides, but the site of synthesis is in the chloroplast stroma (Lehninger et al. 1993). Chloroplasts are markedly different than virtually every other membrane in nature, because they contain protein–glycolipid structures (Harwood 1997). Therefore, fatty acid synthesis could be inhibited in the N-limited treatment, that could result in the reduced capacity to synthesize triglycerides and polar lipids. Whereas most lipids do not have a high N component, the N-limited treatment still had significantly lower lipids than the other growth limited treatments (Table 1 and Fig. 2).

It has been well documented that silica limitation leads to increased lipid production in diatoms, more than any other macronutrient limitation (Shifrin and Chisholm 1981, Taguchi et al. 1987, Roessler 1988). Roessler (1988) found that the increase in lipids appeared to be because of an increase in the neutral lipids, particularly triglycerides, for the diatom *Cyclotella cryptica*. Shifrin and Chisholm (1981) found an increase in lipids in some N-limited diatoms; however, these results are not supported by this study, Harrison et al. (1990), Parrish and Wangersky (1990, 1987), or the studies cited in Darley (1977). One study found that P limitation resulted in an increase in lipids as a proportion of dry weight (Reitan et al. 1994). They contend that lipid accumulation was partially a result of steady lipid synthesis combined with reduced cell division rate and protein synthesis because of availability of nutrients. Lombardi and Wangersky (1991) showed that the increased lipid in *Chaetoceros gracilis* under P limitation was a direct result of the shift of lipid production from polar lipids to neutral triglycerides.

Lipids and lipid classes. Many researchers have found a general trend in lipid classes of a wide range of algae under nutrient limiting conditions. Ahlgren et al. (1992) found an increase in triglyercides under nutrient limitation for 24 species of freshwater phytoplankton grown in batch cultures, consisting of cyanobacteria, green algae, and flagellates. Kilham et al. (1997b) also showed an increased TG:PL ratio under nutrient limited conditions when compared with nutrient replete cultures. Sicko-Goad and Andresen (1991) found that as batch cultures of three freshwater diatoms went from the lag phase (a low growth rate stage) to the exponential growth phase, there were reduced triglycerides and increased polar lipid fractions, especially chl *a*. Parrish and Wangersky (1987) found cells of the marine diatom *Phaeodactylum tricornutum* grown under nitrogen-stressed conditions had more triglyceride than nitrogen-replete cells. They contend that a high percentage of polar lipids was indicative of favorable growth conditions and high growth rates, which can be seen in Fig. 3. The triglyceride:polar lipid (galactolipids and phospholipids) ratios in this study were 0.26, 0.55, 0.98 and 1.1 for the non-, Si-, N- and P-limited treatments, respectively.

The switch of polar lipid production to the production of triglycerides certainly seems to be a useful indicator of nutrient limitation and perhaps general physiological stress. This shift, which has been shown to occur in natural populations at the end of the spring bloom (Parrish 1987b, Palmisano et al. 1988, Kreeger et al. 1997), may be a common feature in diatom metabolism and possibly other algae. Also, an increase in cellular carbohydrates and lipids has been shown for natural phytoplankton blooms as they peak (Conover 1975, Barlow 1984, Kreeger et al. 1997). Larson and Rees (1996) speculated that the increase in carbon storage products (triglyceride and to a lesser extent, carbohydrate) is linked to a decrease in growth rate rather than a specific nutrient deficiency. Carbon storage of triglyceride and carbohydrate occurs in all the nutrient limitations examined here, to varying degrees, and is evidence for the above hypothesis.

Elemental concentrations and ratios. In past studies, the particulate C has been related to the dry weight under the assumption that C is 50% of AFDW (Healey and Hendzel 1979). The results of this study indicate otherwise, suggesting that although the ratio is fairly constant among the treatments, the particulate C is actually only about 30%–38% of the dry weight. This value may be species specific, or perhaps is different for diatoms owing to the siliceous cell walls, but it is obvious that the assumption of such a relationship in natural populations must be made with care. High C:N (>8, Table 2) and low protein:carbohydrate (<1.2, Table 1) ratios correspond to values suggested as indicators of nutrient deficiency in temperate microalgae (Lizotte and Sullivan 1992). This study shows a strong negative relationship between C:N and protein:carbohydrate ratios (Table 4).

Particulate Si, interestingly, did not show a strong

relationship with any of the parameters measured, most probably owing to the fact that it was the only parameter with no differences between treatments. Common sense might say that cell volume should have a positive relationship with Si per cell and although the relationship was significant, it was weak and negative (see Results). It appears that as the cells in the low-N treatment became more N limited, the cell size decreased and the Si per cell increased, indicating the possibility of luxury consumption of Si under severe N limitation. The concept of luxury Si consumption helps to explain the depletion of the majority of Si from the media for all four treatment types (Table 3). The amount of dissolved silica that remained in the media (Table 3), called the R*, indicates the equilibrium resource concentration needed for that growth rate; the lower the R*, the better the competitive ability. Tilman et al. (1982) give a theoretical explanation of the significance of the value in terms of determining the outcome of competition and review the R*s for silicate and phosphorus for a number of freshwater diatom species. At a growth rate of 0.17 d^{-1}, the R* for Si of *S. minutulus* growing at 16° C (0.56 μM, Table 3) falls below over half of the R*s, recalculated for a growth rate of 0.17 d^{-1}, listed for nine species of freshwater diatoms in Table 1 of Tilman et al. (1982). The majority of the R*s that were lower were from different clones of *S. minutulus*.

The particulate P showed a significant relationship with few parameters in this study, one of the highest was the phospholipids. Another was the total lipids, for which Ahlgren et al. (1992) showed no relationship in green and blue–green algae. The low-Si cells had a significantly higher P content (about two times) than any other treatment and a correspondingly high phospholipid concentration. The Si-limited treatment had a relatively low TG:PL ratio (1.4), approaching that of the non-limited treatment (0.96), whereas the N- (3.6) and P- (4.1) limited treatments were much higher. This may be because of luxury consumption of P by the low-Si cells (2.8x), similar to the case of *Asterionella formosa* (Tilman and Kilham 1976) and *Synedra* sp. (Kilham 1984). The phosphorus R* for *S. minutulus* growing at 0.17 d^{-1} at 16° C is 0.13 μM (Table 3). This value expectedly registers very high (highest for the diatoms) against the recalculated R*s for nine species of freshwater diatoms in Table 1 of Tilman et al. (1982) and, coupled with the low silicate R* value (shown previously), distinguishes this clone of *S. minutulus* as a low Si:P specialist.

In the Greater Yellowstone ecosystem, *S. minutulus* is found in abundance in Yellowstone Lake and Jackson Lake, and the mean particulate elemental concentrations from 1996 are given in Interlandi et al. (1999). Overall, the data (C:Si = 5–9, C:N = 9.5–10.5, C:P = 93–112, Si:N = 1.5–3.5, Si:P = 17–19, N:P = 9.5–12.5) correspond well with the results given here (Table 2), especially considering that the two lakes are considered to be predominately N limited throughout the year. The ratio of cell N in the N-limited treatment to cell P in the P-limited treatment (about 4, Table 2), according to Rhee (1978), should give the optimum N:P for a given species. This value is also the N:P ratio found for the Si-limited treatment (4.4) at the same growth rate that was not limited by N or P. Many studies found N:P ratios hovering around 5–13 for nutrient replete conditions while increasing above 22 for P-limiting conditions (Healey and Hendzel 1979, Nalewajko et al. 1981). The optimum Si:N ratio is 2.5, but there are few comparative data for freshwater diatoms. The C:N:P ratio in the non-limited culture should approach the Redfield ratio of 106:16:1 (McCarthy 1980), which it does for C and N (C:N = 6.8, Table 2). However, when P is taken into account there is a distinct variation from the Redfield ratio, 109:16:2.3 (Table 2). Several studies have shown *S. minutulus* clones as having a very low Si requirement and a high P requirement, making this a physiologically low Si:P specialist (Kilham 1984, van Donk and Kilham 1990, Interlandi et al. 1999). Indeed, the N:P ratio of the P-limited treatment (12) and the optimum Si:P ratio (9.7) for *S. minutulus* supports the hypothesis that this species has a high P requirement and confirms the findings of Hecky et al. (1993): freshwater algae can deviate considerably from the Redfield ratio.

These findings demonstrate that *S. minutulus* has a high P requirement, with low optimum N:P and Si:P ratios. The C:N ratio of the non-limited treatment followed the Redfield ratio, whereas the C:P ratio differed substantially. The responses to nutrient limitation were manifested as differences in cell morphology and biochemical and elemental stoichiometry. The potential for significant impacts of variable algal composition on food web dynamics and biomagnification of xenobiotics necessitates future ecological studies of environmentally induced variation of biochemical and elemental stoichiometry in freshwater phytoplankton.

The authors thank the numerous people in our laboratory at Drexel University who assisted with this project: F. Cherian, R. Colau, S. Datta, and H. Roh; D. Velinsky, A.-M. Compton, and H. Hertler at the Academy of Natural Sciences (Philadelphia, PA) provided facilities and technical expertise for analytical work. The authors also thank two anonymous reviewers, who improved several points of discussion. The first author thanks C. Curtis and Sir William. This work was supported in part by a grant from the National Science Foundation (DEB 9220884) to S. S. K.

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