Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake[1]

G-Yull Rhee
Environmental Health Center, Division of Laboratories and Research,
New York State Department of Health, Albany 12201

Abstract

Scenedesmus sp. was grown in chemostats at a fixed growth rate (μ) in an inorganic medium with nitrogen to phosphorus atomic ratios (N:P) ranging from 5 to 80, to investigate the effect of double nutrient limitation. There was no additive or multiplicative effect of the two nutrient limitations: below the optimal cell N:P of 30, growth was determined solely by N limitation and above 30, by P limitation. Cell N remained constant up to the optimal ratio and increased linearly with N:P above it. The level of cell P was high at low N:P (N-limited state) but decreased rapidly until N:P approached the optimal and remained constant at a low level at high N:P (P-limited state).

Protein was the major fraction in which excess N accumulated under P limitation. Cell free amino acids were a constant proportion of cell N at all N:P ratios. RNA concentration was the same regardless of N:P, its level being determined by μ independent of the type of limiting nutrients. Cell carbon (C) concentration was higher in the P-limited than in the N-limited state. The C fixation rate per unit chlorophyll *a*, however, was constant under both P- and N-limited states because the variation in chlorophyll *a* content was similar to that of C.

The apparent maximum uptake rate for nitrate (V) in N- and P-limited cultures decreased with increasing cell N or N:P. In N-limited cultures the half-saturation constant (K_m) also decreased at higher cell N or N:P. The variation of V appeared to be affected by the level of free amino acids.

Transitions between states of nitrogen and phosphorus limitation of phytoplankton growth are common in both lakes and coastal seawaters. Such transitions occur seasonally in eutrophic lakes and may be common in estuarine waters where seawater, which is generally N limited but P sufficient, mixes with freshwater having relatively high N and low P levels. Discharge of wastewaters into a body of natural water may also initiate a transition.

Information on the effects of such a change in nutrient limitation is scarce. Earlier work (Rhee 1974), however, indicated its importance in determining the composition of a phytoplankton community by influencing competition and succession. Although natural phytoplankton contain the two nutrients in an N to P atomic ratio (N:P) of about 15 (Redfield 1958; Ryther and Dunstan 1971), I found the optimal cellular N:P of *Scenedesmus* sp. to be 30; the ratio may vary from species to species.

Baule (1918), Verduin (1964), and Droop (1973) suggested that growth during such transitions was controlled in a multiplicative manner. Later, however, Droop (1974) showed in his experiments on phosphate and vitamin B_{12} limitations with *Monochrysis lutheri* that growth did not follow a multiplicative pattern but was regulated by the single nutrient in shorter supply. In this study, therefore, I have investigated growth during the transition between the N- and P-limited states. Emphasis was placed on changes in cellular composition because the growth rate (μ) of phytoplankton limited by various nutrients is a direct function of the cellular levels of the nutrients (Caperon 1968; Droop 1968; Fuhs 1969; Davis 1970; Rhee 1973; Paasche 1973). Since two nutrient-limited states were involved in this study, the kinetics of N-limited growth and N uptake were also investi-

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gated to provide baseline information to be used with previous findings on P-limited growth and P uptake (Rhee 1973, 1974).

Materials and methods

Organism and culture medium—An axenic culture of *Scenedesmus* sp. was grown in a defined inorganic medium as reported by Rhee (1973, 1974). Nitrate and orthophosphate were the sole sources of N and P. For growth studies at various N:P, the phosphate concentration was kept constant at 3 μM while the nitrate levels were adjusted between 15 and 240 μM. For the investigation of N-limited growth, nitrate and phosphate concentrations were reduced to 21 and 10 μM.

Cells were counted in a hemacytometer. For the double nutrient experiments cell volume was calculated by the geometric formula relating volume to cell shape.

N-limited growth—Four identical chemostats were made from 1-liter (working capacity) Bellco spinner flasks. Each chemostat had a water jacket through which constant temperature water was circulated at 20° ± 1°C by a Neslab cooler-circulator. Cultures were stirred with a magnetic impeller unit on a Bellco nonheat-generating magnetic stirrer. Air was passed first through a saturated zinc chloride solution to eliminate traces of ammonia, then through distilled water to saturate it with moisture, and finally through a series of bottles filled with sterile glass wool. Cultures were aerated at about 1 liter min^{-1}. Continuous illumination of about 0.082 ly min^{-1} was provided by white fluorescent lamps coated with Uvinul D-49 (*see* Rhee 1974). Then cultures were grown at various dilution rates. When a steady state was reached, cells were harvested; the cells and supernatant were stored at −20°C until analysis.

Growth at various N:P—Four Bio-Flo chemostats (model C30), each with a working volume of 600 ml, were used. Continuous illumination at about 0.085 ly min^{-1} was provided. The alga was grown at a constant dilution rate of 0.59 d^{-1}, or relative growth rate of 0.437 (μ/μ_m, where μ_m is the maximum growth rate). The variation in dilution rate was <4%. Aeration was as for the N-limited culture, except that the rate was about 500 ml min^{-1}.

Nitrate uptake experiments—The N uptake study was carried out in a manner similar to the P uptake experiments (Rhee 1973). Aliquots (50 ml) of the steady state culture from N-limited chemostats were delivered into a series of 125-ml Erlenmeyer flasks that contained various concentrations of nitrate. The flasks were shaken on a rotary shaker under the same illumination as for N-limited growth studies. Uptake was measured at 20-min intervals by filtering 10 ml of suspension through a membrane filter (Millipore, pore size 0.45 μm) that had been washed with 30 ml of double-distilled water and determining the amount of nitrate left in the filtrate. Data were analyzed by a nonlinear regression program (Rhee 1973).

The rate of N uptake under P limitation was calculated from the 1973 data according to

$$V = (A - B)D \times \text{cell No.}^{-1}, \quad (1)$$

where V is the apparent maximum uptake velocity; A, the nitrate provided in the inflow medium; B, the residual nitrate level at a steady state; and D, the dilution rate. This study had been done under a 12:12 light-dark cycle, with the light at 0.084 ly min^{-1}.

Carbon (C) uptake was calculated from cell C concentration by the equation

$$V_c = \mu q_c, \quad (2)$$

where V_c is the C fixation rate, and q_c, the cell C content.

Analytical methods—Dissolved organic N was determined by the micro-Kjeldahl method (Strickland and Parsons 1972) and dissolved organic P by the method of Murphy and Riley (1962) after persulfate digestion (Menzel and Corwin 1965). The digestion step was omitted for analysis of orthophosphate. Nitrate, ni-

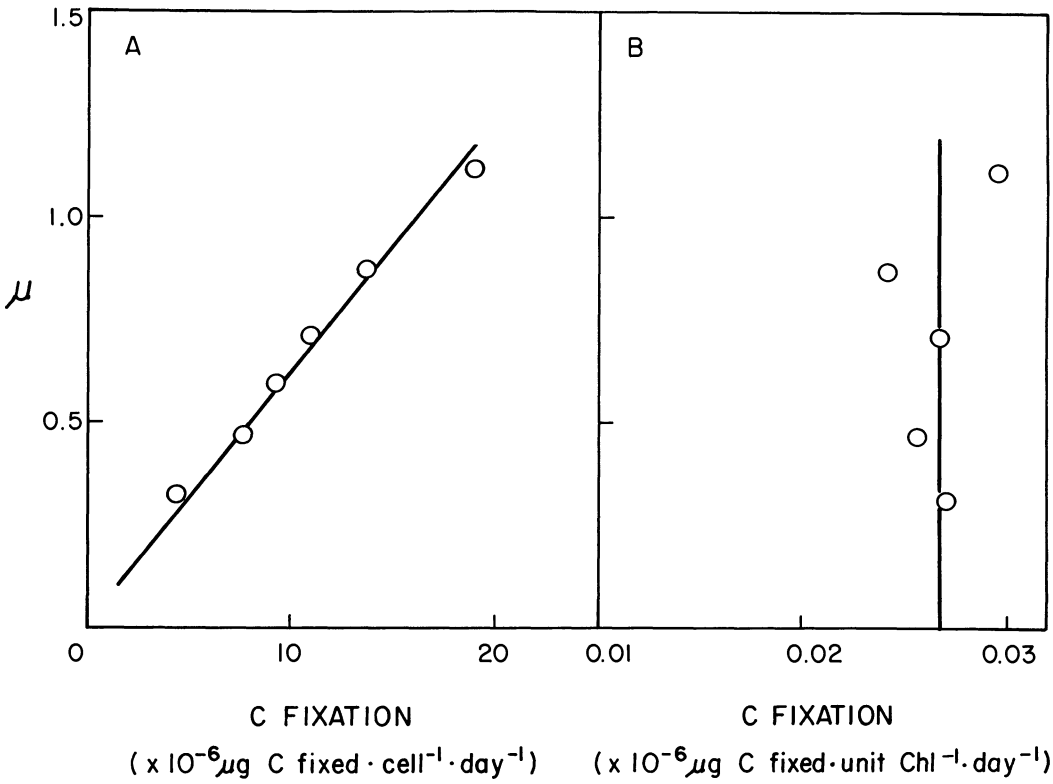

Fig. 1. Carbon fixation rate per cell (A) and per unit chlorophyll (B) as a function of growth rate (μ) in a culture limited in a single nutrient (N). Chlorophyll was measured in arbitrary units representing in vivo fluorescence.

trite, and ammonia were measured in an AutoAnalyzer (Canelli 1976). Total cell N and P were calculated from the amounts utilized by a steady state culture.

Surplus P was determined as described by Rhee (1973) and total cell C by the method of Menzel and Vaccaro (1964). Chlorophyll *a* was measured in vivo using a Turner fluorometer according to Strickland and Parsons (1972) because of difficulty in extracting it with solvents. Its concentration was expressed in arbitrary units.

Samples for free cell nitrate and ammonia were prepared by breaking cells in a French Pressure Cell at 1.4×10^7 kg m^{-2} at 5°C followed by filtration through a membrane filter (Millipore, pore size 0.22 μm). The trichloroacetic acid (TCA) extraction method (Eppley and Coatsworth 1968), performed without breaking cells, was found unsatisfactory.

Free amino acids were extracted with boiling water (Dawson 1965) and assayed by the ninhydrin method of Yemm and Cocking (1955). L-Leucine was used as the standard over the range 0.2–25μg ml^{-1}.

RNA was determined by the orcinol method (Schneider 1960) in a sample prepared as follows: Cells were extracted with 10% cold TCA, and the supernatant was discarded. The residue was suspended in 2 ml of 1 N $HClO_4$ and subsequently heated at 90°C for 30 min. The mixture was centrifuged at 12,100 × *g* for 30 min and washed twice with $HClO_4$. The washings were combined with the supernatant, and the mixture was left overnight at −20°C. The standard was prepared using yeast RNA hydrolyzed in 1 N $HClO_4$.

DNA was extracted with 1 N $HClO_4$ at 70°C for 15 min and measured by the col-

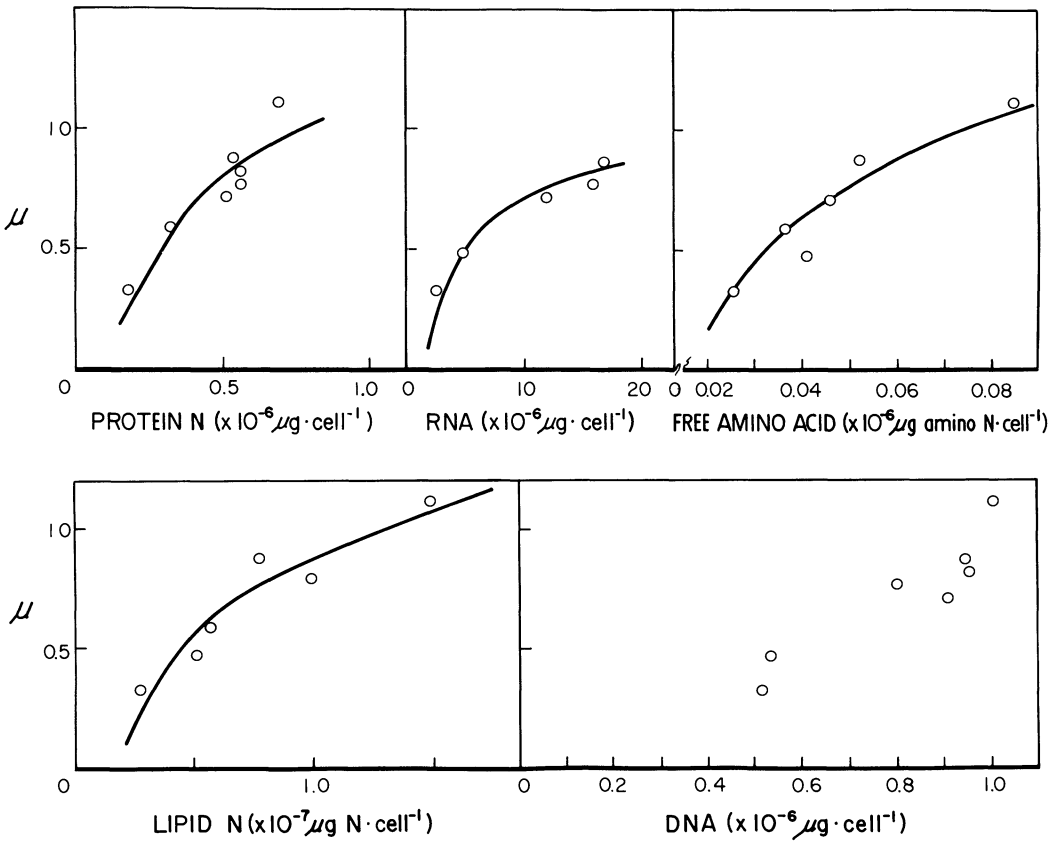

Fig. 2. Concentration of cell protein, RNA, free amino acids, lipid N, and DNA as a function of growth rate (μ) in a N-limited culture.

orimetric method of Burton (1956). Herring sperm DNA was used as the standard. Protein was analyzed by the method of Price (1965) after extraction with hot alkali. The standard was serum albumin. Lipid was extracted sequentially with alcohol, alcohol-ether (3:1), and chloroform-methanol (2:1) and measured for N by the micro-Kjeldahl method.

Results

N-limited growth—Cell numbers at steady states decreased linearly with μ, and no nitrate or dissolved organic N could be detected in the medium. Therefore μ is related to cell N by a saturation curve similar to that found by other investigators (e.g. Caperon 1968).

Cell C concentration was constant regardless of μ and thus the rate of C fixation increased linearly with μ (Eq. 2, Fig. 1A). Since the cellular content of chlorophyll *a* also increased linearly with μ, the C fixation rate per unit chlorophyll *a* (assimilation number) was uniform at all dilution rates (Fig. 1B). Therefore the higher rate of C fixation in faster growing cells is related to an increase in the level of chlorophyll *a*.

Cell protein, lipid N, RNA, and free amino acids measured as amino N were all related to μ by a saturation function (Fig. 2). In P-limited cells the P level in RNA also showed such a relationship (Rhee 1973). DNA, however, appeared to increase linearly with μ (Fig. 2). In studies of P limitation, DNA (measured as P) was invariant with μ when ex-

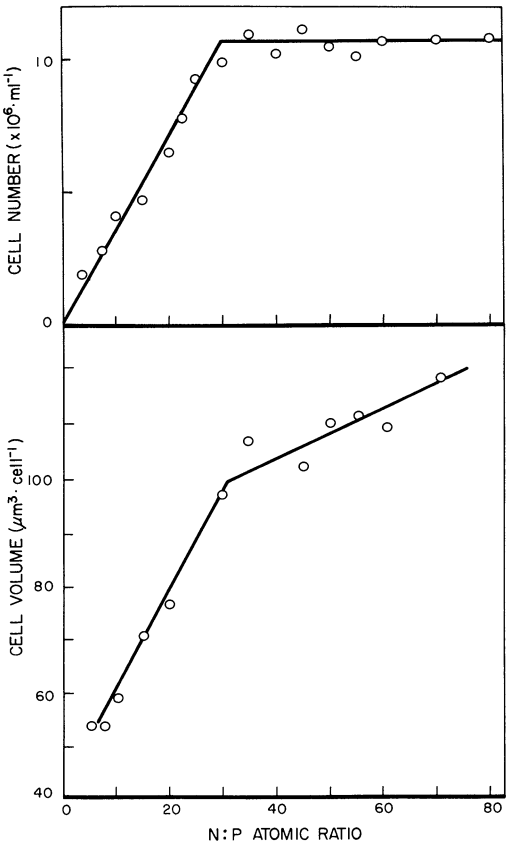

Fig. 3. Steady state cell numbers growing at a fixed growth rate of 0.59 d^{-1} (relative growth rate of 0.437) and changes in cell volume in a medium with varying N:P. (Cell N:P ratios are same as those in inflow medium, because all N and P in culture medium was consumed. N:P ratios in Figs. 5–9 are therefore cell N:P ratios as well as N:P ratios in a chemostat reservoir.)

pressed per unit cell volume (Rhee 1973). In the present study I could not calculate its content per unit volume because of the lack of volume data for the N-limited culture, but microscopic observation during daily cell counts of chemostat samples showed larger cell volumes at higher dilution rates. Since cell nitrate and ammonia levels were too low to be measured accurately because of inadequate sample size, their relation to μ was difficult to ascertain.

Growth at various N:P ratios—The number of cells in steady state cultures grown in media containing varying N:P ratios increased linearly with N:P up to 30 and then abruptly leveled off (Fig. 3). Since N:P was varied by changing only the nitrate concentrations, the proportional increase indicates N limitation below an N:P of 30, and the leveling off shows P limitation above this ratio. This confirms the optimal ratio of 30 found by P uptake kinetics (Rhee 1974). The transition between N- and P-limited states is quite sharp, indicating that there is no multiplicative or additive effect. A test of the single-nutrient limitation and of the multiplicative model with the data for intracellular P and N contents also confirms this.

The multiplicative model is expressed as

$$\frac{\mu}{\mu_m} = \frac{(q_P - q_{0P})}{K_P + (q_P - q_{0P})} \times \frac{(q_N - q_{0N})}{K_N + (q_N - q_{0N})} \quad (3)$$

Growth under single-nutrient limitation is calculated as

$$\frac{\mu}{\mu_m} = \frac{(q_P - q_{0P})}{K_P + (q_P - q_{0P})} \quad (4)$$

when P is limiting, and as

$$\frac{\mu}{\mu_m} = \frac{(q_N - q_{0N})}{K_N + (q_N - q_{0N})} \quad (5)$$

when N is limiting, where K_P and K_N are half-saturation concentrations of cell N and P; q_P and q_N, cell P and cell N levels; and q_{0P} and q_{0N}, minimum cell P and cell N contents.

The hypotheses were tested by calculating μ/μ_m for each of 16 N:P and then determining by a t-test whether the calculated values were significantly different from the experimentally measured value of 0.437 ± 0.016 (mean ± SD).

Since the half-saturation constants are equal to the minimum cell contents of limiting nutrients (Droop 1968; Rhee 1973), the values of q_{0P} and q_{0N} determined in my previous work (1973, 1974) were used as $K_P(1.64 \times 10^{-9}$ μM cell^{-1}) and $K_N(45.40 \times 10^{-9}$ μM cell^{-1}).

The mean value of μ/μ_m was estimated to be 0.286 ± 0.027 with 95% confidence

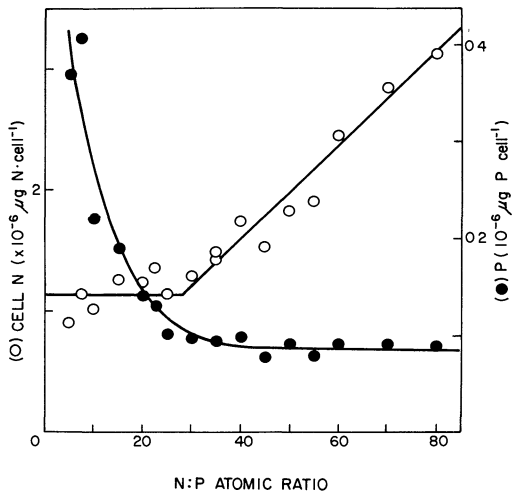

Fig. 4. Total cell N and P concentration as a function of N:P in inflow medium.

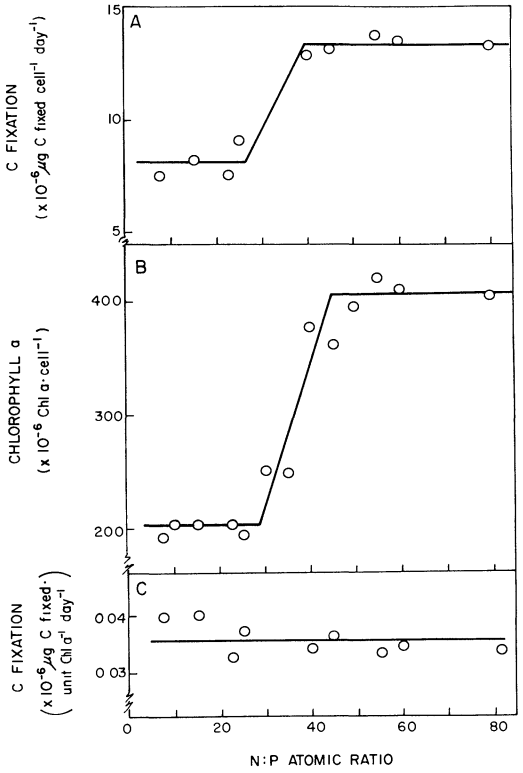

Fig. 5. Carbon fixation rate expressed as $cell^{-1}d^{-1}$(A), chlorophyll *a* concentration per cell (B), and carbon fixation rates per unit chlorophyll *a* (C) as a function of cell N:P.

when the multiplicative model was used. With the single-nutrient limitation equations, 0.428 ± 0.072 was obtained for N:P ≤ 30 and 0.428 ± 0.013 for N:P ≥ 30. The *t*-test indicates the rejection of the multiplicative hypothesis and the acceptance of a single-nutrient limitation model at the 95% confidence level.

K_P and K_N values determined by curve-fitting Eq. 4 and 5 to the data were $1.62 \pm 0.07 \times 10^{-9}$ μM $cell^{-1}$ and $45.70 \pm 5.00 \times 10^{-9}$ μM $cell^{-1}$, not significantly different from the measured q_{0P} and q_{0N} values. This supports the validity of substituting q_{0P} and q_{0N} for K_P and K_N and may also be taken as additional evidence for the single-nutrient limitation model.

Cell volume—Cell volume increased with N:P following two different linear functions, one below the optimal ratio and the other above it (Fig. 3).

Total cell N and P—No residual inorganic or dissolved organic N or P was detected in the medium at any N:P examined, indicating excess accumulation of both nutrients. Since all nitrate and phosphate in the medium was consumed, the cellular N:P must have been identical to the ratio in the medium.

Cell N concentrations in N-limited states with excess phosphate were always the same, about 86×10^{-9} μM $cell^{-1}$ (1.2×10^{-6} μg N $liter^{-1}$ $cell^{-1}$) indicating that in this state cell N concentrations are determined by μ regardless of the N:P of the medium. In P-limited states where N was in excess, the cell P content remained constant at 2.9×10^{-9} μM $cell^{-1}$ (0.09×10^{-6} μg P $liter^{-1}$ $cell^{-1}$), indicating that in these states cell P concentration was dictated by μ without respect to the amount of excess N (Fig. 4). The amounts of cell N and P above these constant levels were therefore considered excess accumulation. The ratio of cell N in N-limited states to cell P in P-limited states was also 30, confirming the optimum N:P found above.

C fixation and chlorophyll a—The calculated rate of C fixation based on cell C contents was constant at low N:P, but as the ratio approached the optimum the rate increased rapidly and then leveled

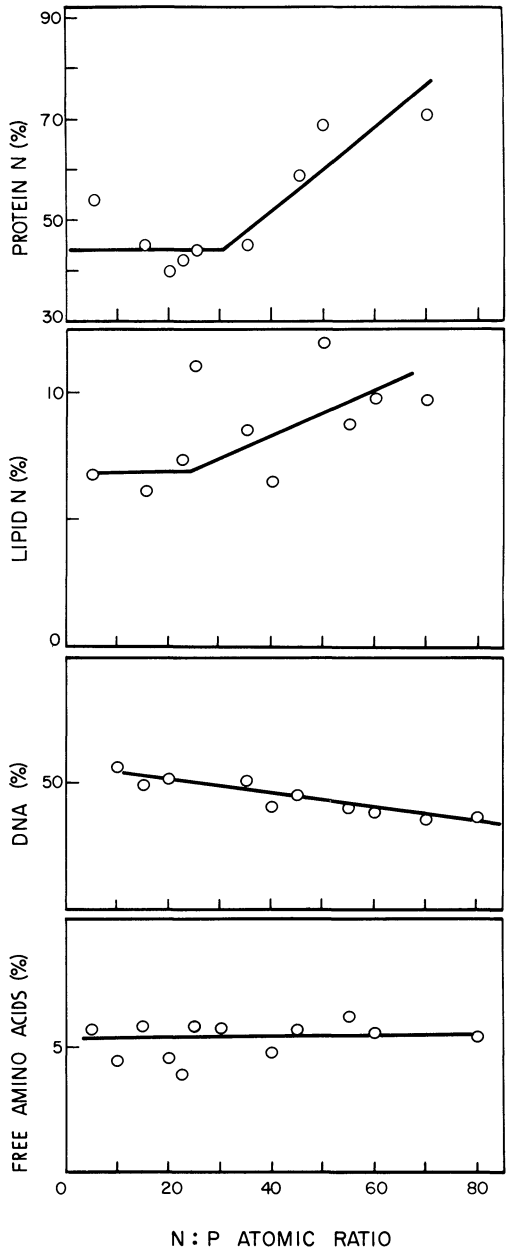

Fig. 6. Percent contributions of cell protein N, lipid N, DNA, and free amino acids to total cell N by weight.

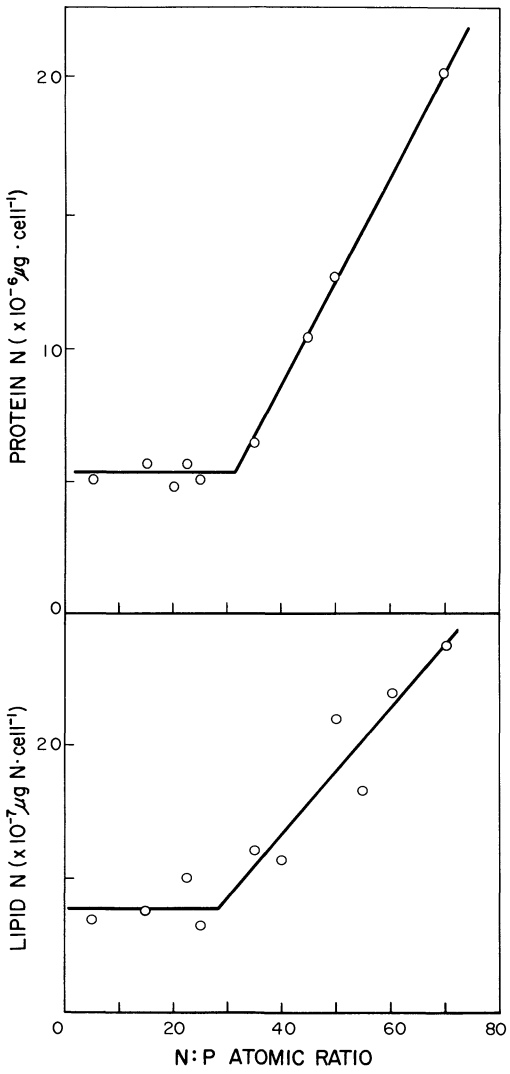

Fig. 7. Protein N and lipid N concentration as a function of N:P.

off at a higher level at high N:P (Fig. 5A). Chlorophyll *a* concentrations show similar sigmoid changes (Fig. 5B). Therefore, when the C fixation rate is expressed per unit chlorophyll *a*, the assimilation number is the same at all N:P (Fig. 5C). As in the N-limited culture experiments, this shows that the faster C fixation rate in the P-limited state is related to the higher concentration of chlorophyll *a*.

Cell composition—Cells grown at various N:P ratios show protein content changing in a manner similar to that of cell N (compare Fig. 6 with Fig. 4). N accounts for about 16% of protein by weight (Conn and Stumpf 1966) and with

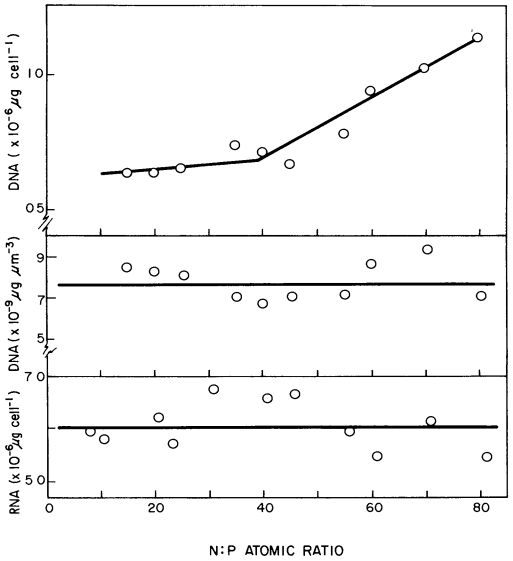

Fig. 8. DNA concentration per cell and per unit cell volume and RNA concentration per cell as a function of cell N:P.

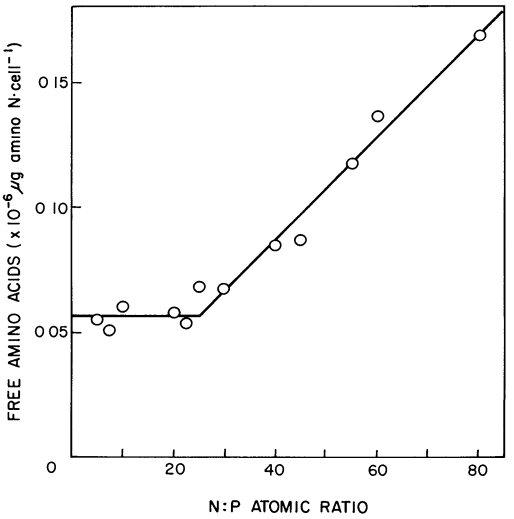

Fig. 9. Free amino acid N as a function of cell N:P.

this factor protein N can be calculated. The proportion of protein N to total cell N averages 44% in the N-limited state, while in the P-limited state, the proportion increases with N:P up to 70% or more (Fig. 6). This indicates that in the P-limited state, most excess N accumulates in the protein fraction. In the N-limited state, protein N concentrations are determined by μ independent of N:P in the inflow medium or of cell P concentrations.

Lipid N remains constant in the N-limited state and increases in the P-limited state in a manner similar to that of protein (Fig. 7). Lipid N, however, accounts for only a small fraction of the total N, its maximum at N:P = 70 being about 10% (Fig. 6).

DNA concentrations in N-limited states increase slowly in proportion to N:P up to the optimum ratio and more rapidly above it (Fig. 8, upper). As seen in Fig. 8 (middle panel) and reported earlier (Rhee 1973), the DNA per unit cell volume is the same at all N:P. Accordingly, as a proportion of the total cell N, it decreases as N:P becomes larger (Fig. 6). RNA levels were the same at all N:P (Fig. 8, lower). This indicates that cell RNA concentration reflects μ independent of the type of limiting nutrient (*see discussion*).

Free amino acid accumulation follows a pattern similar to that of protein, lipid N, and total cell N (Fig. 9). Unlike protein and lipid N, however, it is a constant proportion of total cell N at a given μ at all N:P (Fig. 6). If μ is allowed to vary in N-limited growth experiments, the proportion of N that is free amino acid increases with μ: free amino acid N is only 3.3% of cell N at $\mu/\mu_m = 0.18$ but 6.3% at $\mu/\mu_m = 0.61$. (The generally low values in N-limited culture are due to culture conditions different from those in N:P experiments.)

The changes in free cell nitrate and ammonia could not easily be observed because the levels were too low to be measured accurately, but the level of each seemed to be <0.8% of total cell N.

The accumulation of surplus P is dramatically higher in the N-limited than in the P-limited state. This is consistent with the results obtained from my previous investigation (Rhee 1974). Surplus P decreases rapidly until N:P approaches the optimum. At ratios higher than the optimum, it remains at a uniform level

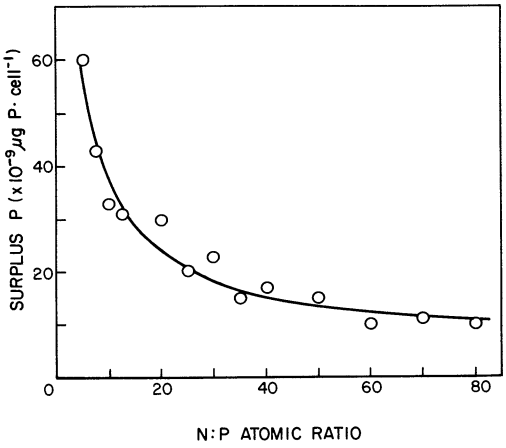

Fig. 10. Surplus P concentrations as a function of cell N:P.

determined by the P-limited growth rate (Fig. 10).

Nitrate uptake—The two maximum uptake velocity curves in Fig. 11 represent the results obtained with N-limited and P-limited cultures. The two curves are not quantitatively comparable because of different growth conditions, as described above. Nonetheless, it is important to note that in both cases the apparent maximum uptake rate (V) is inversely proportional to the cell N:P. The change in N:P in an N-limited culture is caused by higher cell N concentrations at higher μ because the cell P level is the same at all μ. In a P-limited culture, on the other hand, the change in N:P is the result of increasing cell P levels at higher μ, since in this culture the cell N content remains uniform except at very low dilution rates (*see* table 1: Rhee 1974). It is very likely, therefore, that N uptake involves a feedback mechanism by certain N compounds, much as P uptake is affected by acid-soluble inorganic polyphosphates (PP_i) (Rhee 1974). A plot of V against cell free amino acids gives a negative linear slope (Fig. 12), suggesting that free amino acids are directly or indirectly involved in or reflect the regulatory mechanism of nitrate uptake (*see discussion*).

The half-saturation constant (K_m) for nitrate uptake also decreases with N:P

Table 1. Half-saturation constant (K_m) and maximum uptake rate (V) for nitrate uptake in N-limited culture.

μ/μ_m*	Cell N:P	K_m† (µM)	V† (x 10^{-9} µM·cell^{-1} ·min^{-1})
0.35	5.23	8.421±1.570	0.325±0.034
0.44	5.52	5.607±0.350	0.237±0.008
0.51	5.64	3.343±0.479	0.178±0.010
0.57	5.77	2.914±0.286	0.164±0.002

*μ_m=1.35·day^{-1}.
†Values shown are mean ± standard error.

(Fig. 11). In contrast, the K_m for P uptake by the same organism is constant at all μ (Rhee 1973). With the present data it is difficult to determine if K_m values are also related to any intracellular compounds, because of the relatively large standard errors in estimating them (Table 1).

Discussion

N:P and growth—It is clear that there is no growth limitation by N and P simultaneously. Growth is limited by P or N on either side of the optimum N:P. Droop (1974) found no multiplicative effect of phosphate and vitamin B_{12} limitations on the growth of *M. lutheri*, which is strictly controlled by the single nutrient in shorter supply. Droop's experimental approach was different from that used here; he used dilution rates as independent variables in media for four different P:B_{12} ratios. Both his and my results appear to agree with Liebig's law of the minimum.

The optimal cellular N:P may be species-specific. If this is so, the difference in this value would have great ecological importance. For example, in the same nutrient environment the growth of some species may be limited by one nutrient, while others are regulated by another. The severity of each limitation would determine the outcome of competition, and the interval between the ratios would determine the range in which coexistence is possible (Table 2). Competition models based on the kinetic constants for grwoth have also been used successfully to predict competitive exclusion and coexistence between two

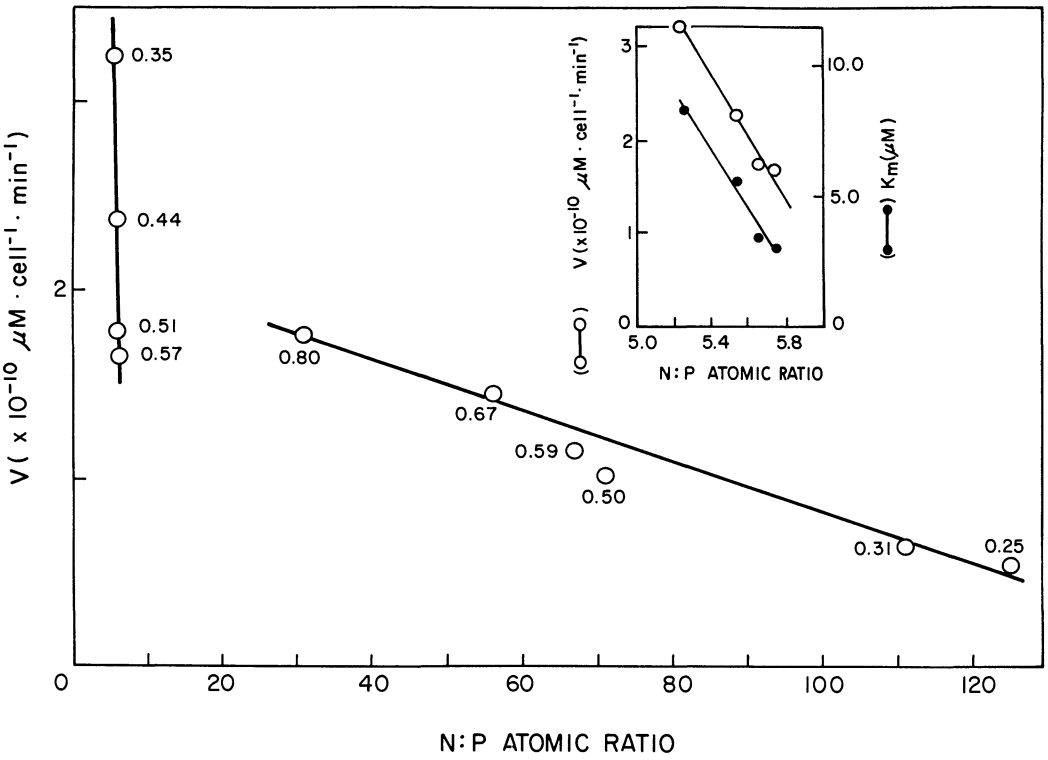

Fig. 11. Change in apparent maximum uptake velocity (V) for nitrate as a function of cell N:P. Values below the ratio 10 were obtained with a N-limited culture and those above it were calculated using data from a P-limited culture (Rhee 1973). Numbers next to open circles are relative growth rate (μ/μ_m). Scale of N:P between 5 and 6 is expanded in inset. Culture conditions for N-limited and P-limited studies were different (*see text*).

species (Titman 1976; Tilman 1977). There are various internal nutrient pools; the size of a pool may vary from species to species, restricting the use of models derived from growth kinetics (as also pointed out by Tilman).

Competition and coexistence based on optimal cellular N:P may explain the seemingly synergistic effects of simultaneous N and P addition frequently encountered in bioassay experiments (e.g. Fuhs et al. 1972). If the mixed populations in a bioassay are in the coexistence range in Table 2, addition of P alone to the medium will shift N:P in favor of organism A, thus competitively eliminating B. The addition of N only, on the other hand, will favor B with the elimination of A. The simultaneous addition of both N and P, therefore, would produce more biomass than the sum of the yields produced by the single addition of P or N. Although there is no proof yet of the species-specificity of optimal N:P, Tilman's data (1977) suggest that the optimal P:Si ratio differs in two competing diatom species.

Determination of optimal N:P may be difficult because of the nutrient pools.

Table 2. Competitive exclusion and coexistence.

N:P	0	10	20	30
	Organism A dominant	P-limited A and N-limited B coexist	Organism B dominant	
		Optimal N:P for A	Optimal N:P for B	

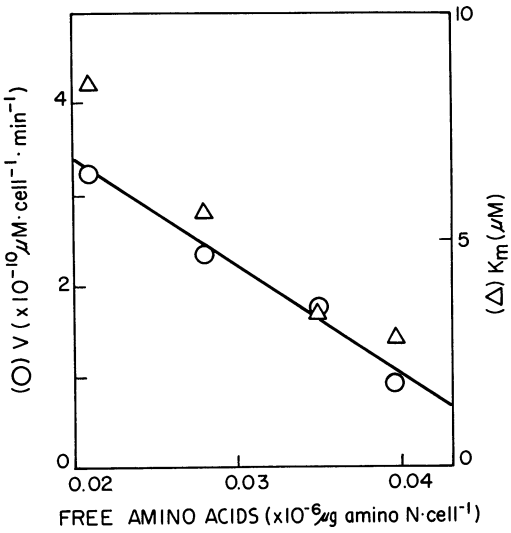

Fig. 12. V and K_m values of nitrate uptake as a function of cell free amino acid concentrations.

For the same reason, the interpretation of N:P ratios found in batch-culture work should be made with extreme care. Optimal N:P ratios of ecologically important species of phytoplankton representing various taxa are being investigated in our laboratory.

N:P and residual N and P—The minimum cellular N:P observed in *Scenedesmus* sp. was 4 under N-limited conditions, and the maximum was 142 or more under P-limited conditions (Rhee 1974). It is therefore not surprising that there was no detectable residual N and P in the culture medium at the inflow N:P used in the present study (5 to 80). It is probably that residual N or P becomes detectable only when N:P in a medium is lower than the minimum or higher than the maximum cellular N:P. The wide range of cellular N:P indicates that cellular nutrient concentrations are better indicators of nutrient limitation than nutrient levels in the surrounding medium when optimum nutrient ratios are known.

Protein—Among the changes in chemical composition measured here, those of protein, RNA, and free amino acids are of special interest. Under conditions of N sufficiency, protein, which accounts for more than 70% of the total cell N, appears to be the major reservoir for excess N. The observation that it is a greater proportion of total cell N in the P-limited state (Figs. 6 and 7) supports this interpretation. Other supporting evidence is that under nutrient stress, or in nongrowing bacteria and fungi, the turnover of protein becomes faster (Mandelstam 1963; Pine 1972), and physiological adaptation and growth become dependent upon the turnover rate. For example, in P-starved cells, amino acids produced as the result of protein turnover or breakdown are reincorporated for the synthesis of alkaline phosphatase by increased differential rate of synthesis of this enzyme (Halvorson 1962). In higher plants such amino acids are largely converted to carbon dioxide (Oak and Bidwell 1970).

RNA—A close correlation between RNA concentration and μ has been well established for many bacteria (Neidardt and Magasanik 1960; Maaløe and Kjeldgaard 1966; Maaløe and Kurland 1963; Smith 1969). In a synchronous culture of *Chlorella*, RNA synthesis was also found to control cell division (Lorenzen 1970). Such findings were made mostly under conditions of single-nutrient limitation or in unrestricted medium. It is therefore very interesting to find that RNA concentrations reflect μ independent of N:P in the medium and therefore irrespective of the limiting nutrient. Data from a study of *Enterobacter aerogenes* under double-nutrient limitation (Cooney et al. 1976) showed similar results, although the investigators failed to point it out. At any given μ in that study, RNA concentration appeared unchanged whether C, N, P, or a combination of C, N, and P was limiting. (The criterion of limitation used was not growth but change in macromolecular composition.) Findings such as these may enable us to use measurements of RNA to estimate physiological conditions of natural populations without precise knowledge of the nature of the limiting nutrient. This could be even more significant in light of the possible species-specificity of optimal N:P and the difficulty of determining it

for a great number of species. RNA can be determined by either biochemical or cytochemical methods. A cytochemical method developed by Fuhs and Chen (1974) may prove valuable for the measurement of RNA in individual species of mixed natural populations.

In using RNA as a measure of physiological conditions, one must remember that it responds in two different ways to environmental changes, such as temperature: by changing the rate of protein synthesis without modifying its cellular levels, or by changing its own cellular concentrations. The second type of response seems to occur under environmental changes which last many generations. In the steady state cells of a chemostat, RNA content varies with temperature and pH (Tempest and Hunter 1965). The first type of adaptation has been observed in batch culture work; when the μ of cells in an unrestricted medium was altered by temperature, the cellular RNA level remained constant (Schaechter et al. 1958; Fuhs and Chen 1974) but the rate of protein synthesis would probably vary.

Free amino acids—The amino acid pool is a sensitive indicator of cellular metabolism, particularly of N supply. It is extremely variable and markedly dependent on the nutritional composition of a medium, the levels of limiting nutrient, μ, and environmental conditions (Cowie 1962; Tempest et al. 1970; Brown and Stanley 1972; Brown et al. 1972, Drozd et al. 1972). In protein synthesis the free amino acids in the pool are obligatory intermediate forms of N (Britten and McClure 1962). Indeed, Cowie (1962) found a precursor-end product relationship between pool amino acids and cell protein. The size of the pool, however, is not related to the rate of protein synthesis (Dawson 1965). The difference in the contribution of protein and free amino acids to the total cell N observed in the present study (Fig. 6) agrees with these findings.

The uniform quantitative relationship of free amino acids to total cell N is of considerable interest because either the amino acid pool or the ratio of free amino N to cell C may be used as a sensitive indicator of N depletion, if N limitation is known to exist. One can also substitute the measurement of free amino acids for that of total cell N. Such substitution would be desirable in cases where detrital N prevents an accurate measurement of the particulate N of living organisms.

In *Escherichia coli* the rate of RNA synthesis has a direct relationship with the availability of free amino acids. One explanation for this mechanism is that transfer RNA (*t*RNA) uncharged with amino acids acts as a repressor of RNA synthesis. In an N-rich medium *t*RNA tends to be saturated with amino acids, more or less completely derepressing RNA synthesis, but in an N-limited medium the relative unavailability of amino acids leaves a large portion of the RNA unadenylated (Stent and Brenner 1961; Maaløe and Kjeldgaard 1966). Under conditions of N limitation, therefore, free amino acid levels reflect μ and are the equivalent of the levels of PP_i or surplus P in P limitation (*see* Rhee 1973).

Carbon fixation—Although cell C concentrations were invariant at all dilution rates in N-limited culture, the C fixation rate per cell had a positive relationship with μ. Such increases in the photosynthetic C fixation rate have also been observed with *Scenedesmus abundans* (Giddings 1975). Assimilation number in these organisms and in *Thalassiosira pseudonana* (Eppley and Renger 1974) showed no variation with respect to μ. A synchronous culture of *Scenedesmus* (Myers and Graham 1975) showed an essentially unvarying size of the photosynthetic unit (the yield of oxygen per chlorophyll per light flash) throughout its life cycle. On the other hand, marked changes of the assimilation number were reported by Thomas and Dodson (1972), Caperon and Meyer (1972*a*), and Caperon and Zieman (1976). Diel variations of the assimilation number have also been found in marine diatoms (Eppley et al. 1971; Eppley and Renger 1974).

Growth studies at various N:P ratios

show identical assimilation numbers, but P-limited cells have higher concentrations of chlorophyll *a* and thus higher cell C contents than do N-limited cells. This indicates that under conditions of N limitation the proportion of N diverted from chlorophyll *a* synthesis to other processes is much greater than the proportion of P diverted under P limitation. Consequently, N limitation impairs chlorophyll *a* synthesis much more than P limitation. Bongers (1958) and Fogg (1959) also reported that in N limitation the amount of chlorophyll per cell decreases faster than the total cell N.

Nitrate uptake—The variation of V seems to be related to free amino acid concentrations (Fig. 12) by way of an amination process, or by the rate of pool amino acids utilization, or both. In *Anabaena cylindrica*, nitrate, nitrite, and hydroxylamine reductase systems are repressed by ammonia and glutamate (Hattori 1962). In certain marine algae, nitrate is quantitatively reduced to ammonia in the absence of carbon dioxide (Brown et al. 1974), and therefore, in the light, the rate-limiting step for nitrate assimilation is ammonia incorporation. In *Chlorella* an exogenous supply of certain amino acids, including glutamate, represses nitrate reductase (Abdullah and Ahmed 1975). Nitrogenase activity in N_2-fixing organisms appears to be regulated by free cell ammonia (Dharmawardene et al. 1972). Indirect but strong evidence of the dependence of V on free amino acid concentration is that sulfur uptake in *Chlorella vulgaris* varies positively with the level of sulfur amino acids in the pool (Passera and Ferrari 1975). Conover (1976) and Eppley and Renger (1974) also suggested that a critical rate-limiting step in N uptake is its incorporation into organic compounds. Lui and Roel (1972) suggested that in ammonia utilization the intracellular ammonium level and carbohydrate reserve regulate uptake. In the present study, cell ammonia levels were too low for accurate measurement, and therefore no meaningful relationship between these levels and nitrate uptake can be ascertained.

Two enzyme systems that mediate amination have been reported in a marine bacterium (Brown and Stanley 1972; Brown et al. 1974) and recently in a marine diatom (Falkowski and Rivkin 1976). The two systems differ markedly in their K_m values. One is glutamic dehydrogenase (GDH) which aminates 2-oxoglutarate to glutamic acid. The other is a two-step process: synthesis of glutamine by glutamine synthetase (GS) and the reductive transfer of amide to 2-oxoglutarate by glutamine (amide)-2-oxoglutarate amino-transferase (GOGAT). In the bacterium the K_m for GDH is 10 mM; the value for GS is about 20-fold smaller. The values in the diatom are 28 mM for GDH and 29 μM for GS. The two enzyme systems thus probably operate at different levels of free cell ammonia. GS has been reported in blue-green algae (Dharmawardene et al. 1973; Haystead et al. 1973) and in green algae (Loomis 1959). Its existence and possible importance in ammonia incorporation were also reported in *C. vulgaris* and *Chlorella pyrenoidosa* (Reisner et al. 1960; Bassham and Kirk 1964) and in some higher plants (Fowden 1965).

It is not possible to elucidate the mechanism of nitrate uptake in *Scenedesmus* sp. using only the present data. Further investigation on enzymatic and cellular levels is under way to find the mechanism regulating its uptake. Solomonson and Spehar (1977), in an important paper that appeared just as this manuscript was being submitted, proposed a model for feedback control of nitrate uptake by free intracellular ammonia and amino acids.

Variations in V and K_m similar to those found here were indicated for *S. abundans* (Giddings 1975), and a decline in V with increasing μ was found in *T. pseudonana* (Eppley and Renger 1974). On the other hand, Caperon and Meyer (1972*b*) reported a positive slope of V with μ in *T. pseudonana*, and Laws and Caperon (1976) found no variation of V in *M. lutheri*. It should be noted, however, that the last two results were obtained by the perturbation technique, which disre-

gards the feedback or regulatory effect of intracellular N compounds.

In summary, I have found the growth of *Scenedesmus* sp. to be limited, not in a multiplicative or additive manner, but in a threshold pattern by the single nutrient in shorter supply. Possible differences in the optimal N:P of various organisms, therefore, may be a basis for coexistence and competitive exclusion. In nutrient limitation not only are the absolute concentrations of limiting nutrients important but also their levels relative to other nutrients. RNA concentrations were independent of which nutrient was limiting, but they were a function of μ and may therefore be a potentially useful parameter in estimating μ of natural populations.

In the P-limited state, most excess N accumulated in the protein fraction and increased linearly with N:P. Free amino acids within a cell also increased with N:P under N-sufficient conditions but were a constant proportion of the total cell N. Thus the level of free amino acids appears to be a good potential indicator of N-limited growth rate and might be used to estimate particulate N concentrations of living organisms in cases where detrital N prevents accurate measurement of total cell N. Levels of cell C were higher in the P-limited than in the N-limited state, but because these levels changed in a manner similar to that of chlorophyll *a*, assimilation number remained invariant with respect to the type of limiting nutrient. Assimilation number was also independent of μ. The K_m for nitrate uptake in N-limited cultures appeared to decrease with μ. V also decreased with μ in both N- and P-limited cultures; the decrease appeared to be related to the level of free amino acids.

References

ABDULLAH, A.-N., AND J. AHMED. 1975. Regulation of nitrate assimilation by amino acids in *Chlorella*. Plant Cell Physiol. **16:** 971–979.

BASSHAM, J. A., AND M. KIRK. 1964. Photosynthesis of amino acids. Biochim. Biophys. Acta **90:** 553–562.

BAULE, B. 1918. Zu Mitscherlichs Gesetz der physiologischen Beziehungen. Landwirtsch. Jahrb. Schweiz **51:** 363–385.

BONGERS, L. H. 1958. Kinetic aspects of nitrate reduction. Neth. J. Agr. Sci. **6:** 70–88.

BRITTEN, R. J., AND F. T. MCCLURE. 1962. The amino acid pool in *Escherichia coli*. Bacteriol. Rev. **26:** 292–335.

BROWN, C. M., D. MACDONALD-BROWN, AND J. L. MEERS. 1974. Physiological aspects of microbial inorganic nitrogen metabolism, p. 1–52. *In* A. H. Rose and D. W. Tempest [eds.], Advances in microbial physiology, v. 2. Academic.

———, ———, AND S. O. STANLEY. 1972. Inorganic nitrogen metabolism in marine bacteria: Nitrogen assimilation in some marine pseudomonads. J. Mar. Biol. Assoc. U.K. **52:** 793–804.

———, AND S. O. STANLEY. 1972. Environment-mediated changes in the cellular content of the "pool" constituents and their associated changes in cell physiology. J. Appl. Chem. Biotech. **22:** 363–389.

BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62:** 315–323.

CANELLI, E. 1976. Simultaneous automated determination of chloride, nitrite, nitrate and ammonia in water and wastewater. Water, Air, Soil Pollut. **5:** 339–348.

CAPERON, J. 1968. Population growth responses of *Isochrysis galbana*. Ecology **49:** 866–872.

———, and J. MEYER. 1972*a*, *b*. Nitrogen-limited growth of marine phytoplankton. 1. Changes in population characteristics with steady state growth rates. 2. Uptake kinetics and their role in nutrient limited growth of phytoplankton. Deep-Sea Res. **19:** 601–618, 619–632.

———, AND D. ZIEMAN. 1976. Synergistic effects of nitrate and ammonium ion on the growth and uptake kinetics of *Monochrysis lutheri*. Mar. Biol. **36:** 73–84.

CONN, E. E., AND P. K. STUMPF. 1966. Outline of biochemistry. Wiley.

CONOVER, S. A. 1976. Partitioning of nitrogen and carbon in cultures of the marine diatom *Thalassiosira fluviatilis* supplied with nitrate, ammonium, or urea. Mar. Biol. **32:** 231–246.

COONEY, C. L., D. I. WANG, AND R. I. MATELES. 1976. Growth of *Enterobacter aerogenes* in a chemostat with double nutrient limitations. Appl. Environ. Microbiol. **31:** 91–98.

COWIE, D. B. 1962. Metabolic pools and the biosynthesis of protein, p. 633–645. *In* J. T. Holden [ed.], Amino acid pools. Elsevier.

DAVIS, A. G. 1970. Iron, chelation and the growth of marine phytoplankton. 1. Growth kinetics and chlorophyll production in cultures of the euryhaline flagellate *Dunalliela tertiolecta* under iron-limiting conditions. J. Mar. Biol. Assoc. U.K. **50:** 65–86.

DAWSON, P. W. 1965. The intracellular amino acid pool of *Candida utilis* during growth in batch

and continuous flow cultures. Biochim. Biophys. Acta **111:** 51–66.

DHARMAWARDENE, M. W., A. HAYSTEAD, AND W. D. STEWART. 1973. Glutamine synthetase of the nitrogen-fixing alga *Anabaena cylindrica*. Arch. Mikrobiol. **90:** 281–295.

———, W. D. STEWART, AND S. STANLEY. 1972. Nitrogenase activity, amino acid pool patterns and amination in blue-green algae. Planta **198:** 133–145.

DROOP, M. R. 1968. Vitamin B_{12} and marine ecology. 4. The kinetics of uptake, growth and inhibition of *Monochrysis lutheri*. J. Mar. Biol. Assoc. U.K. **48:** 689–733.

———. 1973. Some thoughts on nutrient limitation in algae. J. Phycol. **9:** 264–272.

———. 1974. The nutrient status of algal cells in continuous culture. J. Mar. Biol. Assoc. U.K. **54:** 825–855.

DROZD, J. W., R. S. TUBB, AND J. R. POSTGATE. 1972. A chemostat study of the effect of fixed nitrogen sources on nitrogen fixation, membrane and free amino acids in *Azobacter chroococcum*. J. Gen. Microbiol. **73:** 221–232.

EPPLEY, R. W., AND J. L. COATSWORTH. 1968. Uptake of nitrate and nitrite by *Ditylum brightwellii*—kinetics and mechanism. J. Phycol. **4:** 151–156.

———, AND E. H. RENGER. 1974. Nitrogen assimilation of an oceanic diatom in nitrogen-limited continuous culture. J. Phycol. **10:** 15–23.

———, J. N. ROGERS, J. J. MCCARTY, AND A. SOURNIA. 1971. Light/dark periodicity in nitrogen assimilation of the marine phytoplankters. *Skeletonema costatum* and *Coccolithus hyxleyi* in N-limited chemostat culture. J. Phycol. **7:** 150–154.

FALKOWSKI, P. G., AND R. B. RIVKIN. 1976. The role of glutamine synthetase in the incorporation of ammonium in *Skeletonema costatum* (Bacillariophyceae). J. Phycol. **12:** 448–450.

FOGG, G. E. 1959. Nitrogen nutrition and metabolic patterns in algae. Symp. Soc. Exptl. Biol. 13, p. 106–125.

FOWDEN, L. 1965. Origin of the amino acids, p. 361–390. *In* J. Bonner and J. E. Varner [eds.], Plant biochemistry. Academic.

FUHS, G. W. 1969. Phosphorus content and rate of growth in the diatom *Cyclotella nana* and *Thalassiosira fluviatilis*. J. Phycol. **5:** 305–321.

———, AND M. CHEN. 1974. Refractive index of uranyl-treated bacterial cytoplasm as related to ribonucleic acid content and growth rate. Microbial Ecol. **1:** 120–125.

———, S. D. DEMMERLI, E. CANELLI, AND M. CHEN. 1972. Characterization of phosphorus-limited plankton algae. Am. Soc. Limnol. Oceanogr. Spec. Symp. **1:** 113–133.

GIDDINGS, J. M. 1975. Growth and chemical composition of *Scenedesmus abundans* in nitrogen-limited chemostat culture. Ph.D. thesis, Cornell Univ. 89 p.

HALVORSON, H. O. 1962. The function and control of intracellular protein turnover in microorganisms, p. 646–654. *In* J. T. Holden [ed.], Amino acid pools. Elsevier.

HATTORI, A. 1962. Light-induced reduction of nitrate, nitrite and hydroxylamine in a blue-green alga, *Anabaena cylindrica*. Plant Cell Physiol. **3:** 355–369.

HAYSTEAD, A., M. W. DHARMAWARDENE, AND W. D. STEWART. 1973. Ammonia assimilation in a nitrogen-fixing blue-green algae. Plant Sci. Lett. **1:** 439–445.

LAWS, E, AND J. CAPERON. 1976. Carbon and nitrogen metabolism by *Monochrysis lutheri*: Measurement of growth-rate-dependent respiration rates. Mar. Biol. **36:** 85–97.

LOOMIS, W. D. 1959. Amide metabolism in higher plants. 3. Distribution of glutamyl transferase and glutamine synthetase activity. Plant Physiol. **34:** 541–546.

LORENZEN, H. 1970. Synchronous cultures, p. 187–212. *In* P. Haldall [ed.], Photobiology of microorganisms. Wiley-Interscience.

LUI, N. S., AND O. A. ROEL. 1972. Nitrogen metabolism of aquatic organisms. 2. The assimilation of nitrate, nitrite and ammonia by *Biddulphia aurita*. J. Phycol. **8:** 259–264.

MAALØE, O., AND N. O. KJELDGAARD. 1966. Control of macromolecular synthesis. Benjamin.

———, AND C. G. KURLAND. 1963. The integration of protein and ribonucleic acid synthesis in bacteria. Symp. Int. Soc. Cell Biol. **2:** 93–110.

MANDELSTAM, J. 1963. Protein turnover and its function in the economy of the cell. Ann. N.Y. Acad. Sci. **102:** 536–548.

MENZEL, D. W., AND N. CORWIN. 1965. The measurement of total phosphorus in seawater based on the liberation of organically bound fraction by persulfate oxidation. Limnol. Oceanogr. **10:** 280–283.

———, AND R. F. VACCARO. 1964. The measurement of dissolved organic and particulate carbon in seawater. Limnol. Oceanogr. **9:** 139–142.

MURPHY, J., AND J. P. RILEY. 1962. A modified single-solution method for the determination of phosphate in natural waters. Anal. Chim. Acta **27:** 31–36.

MYERS, J., AND J. R. GRAHAM. 1975. Photosynthetic unit size during the synchronous life cycle of *Scenedesmus*. Plant Physiol. **55:** 686–688.

NEIDARDT, F. C., AND B. MAGASANIK. 1960. Studies on the role of ribonucleic acid in the growth of bacteria. Biochim. Biophys. Acta **42:** 99–116.

OAK, A., AND R. G. BIDWELL. 1970. Compartmentation of intermediary metabolites. Annu. Rev. Plant Physiol. **21:** 43–66.

PAASCHE, E. 1973. Silicon and the ecology of marine plankton diatom. 1. *Thalassiosira pseudonana* (*Cyclotella nana*) growth in a chemostat with silicate as limiting nutrient. Mar. Biol. **19:** 117–126.

PASSERA, C., AND G. FERRARI. 1975. Sulfate up-

take in two mutants of *Chlorella vulgaris* with high and low sulfur amino acid content. Physiol. Plant. **35:** 318–321.

PINE, M. J. 1972. Turnover of intracellular proteins. Annu. Rev. Microbiol. **26:** 103–126.

PRICE, C. A. 1965. A membrane method for determination of total protein in dilute algal suspensions. Anal. Biochem. **12:** 213–218.

REDFIELD, A. C. 1958. The biological control of chemical factors in the environment. Am. Sci. **46:** 205–221.

REISNER, G. S., R. K. GERING, AND J. F. THOMPSON. 1960. Metabolism of nitrate and ammonia by *Chlorella*. Plant Physiol. **35:** 48–52.

RHEE, G-Y. 1973. A continuous culture study of phosphate uptake, growth rate and polyphosphate in *Scenedesmus* sp. J. Phycol. **9:** 495–506.

———. 1974. Phosphate uptake under nitrate limitation by *Scenedesmus* sp. and its ecological implications. J. Phycol. **10:** 470–475.

RYTHER, J., AND W. M. DUNSTAN. 1971. Nitrogen, phosphorus and eutrophication in the coastal marine environment. Science **171:** 1008–1013.

SCHAECHTER, M., O. MAALØE, AND N. O. KJELDGAARD. 1958. Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. **19:** 592–606.

SCHNEIDER, W. C. 1960. Determination of nucleic acids in tissues by pentose analysis, p. 680. *In* S. P. Corowick and N. O. Kaplan [eds.], Methods in enzymology, v. 3. Academic.

SMITH, J. M. 1969. Limitation on growth rate, p. 1–13. *In* P. Meadow and S. J. Pirt [eds.], Microbial growth. Cambridge Univ.

SOLOMONSON, L. P., AND A. M. SPEHAR. 1977. Model for the regulation of nitrate assimilation. Nature **265:** 373–375.

STENT, G. S., AND S. BRENNER. 1961. A genetic locus for the regulation of ribonucleic acid synthesis. Proc. Natl. Acad. Sci. **47:** 2005–2014.

STRICKLAND, J. D., AND T. R. PARSONS. 1972. A practical handbook of seawater analysis, 2nd ed. Bull. Fish. Res. Bd. Can. 167.

TEMPEST, D. W., AND J. R. HUNTER. 1965. The influence of temperature and pH value on the macromolecular composition of magnesium-limited and glycerol-limited *Aerobacter aerogenes* growing in a chemostat. J. Gen. Microbiol. **41:** 267–273.

———, J. L. MEERS, AND C. M. BROWN. 1970. Influence of environment on the content and composition of microbial free amino acid pools. J. Gen. Microbiol. **64:** 171–185.

THOMAS, W. H., AND A. N. DODSON. 1972. On nitrogen deficiency in tropical Pacific phytoplankton. 2. Photosynthetic and cellular characteristics of a chemostat-grown diatom. Limnol. Oceanogr. **17:** 515–523.

TILMAN, D. 1977. Resource competition between planktonic algae: An experimental and theoretical approach. Ecology **58:** 338–348.

TITMAN, D. 1976. Ecological competition between algae: Experimental confirmation of resource-based competition theory. Science **192:** 463–465.

VERDUIN, J. 1964. Principles of primary productivity: Photosynthesis under completely natural conditions, p. 221–238. *In* D. F. Jackson [ed.], Algae and man. Plenum.

YEMM, E. W., AND E. C. COCKING. 1955. The determination of amino-acids with ninhydrin. Analyst **80:** 209–213.

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