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Responses of elemental and biochemical composition of *Chaetoceros muelleri* to growth under varying light and nitrate : phosphate supply ratios and their influence on critical N : P

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Abstract

The critical nitrogen-to-phosphorous ratio (N : P) defines the transition between N and P limitation of growth rate and is not a biological constant. To test the effect of environmental conditions on the critical N : P, we cultured the diatom *Chaetoceros muelleri* in chemostats with inflow nitrate : phosphate ratios ranging from 5 to 90 mol N (mol P)$^{-1}$ at two photon flux densities (PFDs; 50 and 700 μmol photons m^{-2} s^{-1}). The nitrate : phosphate ratio marking the transition between N and P limitation increased from between 15–30 mol N : mol P at the high PFD to 45 mol N : mol P at the low PFD. The particulate ratio marking this transition increased from 16–23 mol N : mol P at low PFD to 35 mol N : mol P at high PFD. Cell phosphorus and RNA contents decreased with increasing N : P ratio up to the critical N : P ratio for each PFD, above which they remained stable. In contrast, cell dry weight, chlorophyll *a*, C, N, and protein were not influenced by nitrate : phosphate in the inflow medium, although they were influenced by PFD. Total protein per RNA increased with increasing N : P ratio at the low light conditions, suggesting increased ratio of protein synthesis per RNA. Our results showed the effect of PFD, growth rate, or both on the critical N : P ratio. Agreement was found in the assessment of the transition between N and P limitation on the basis of nutrient enrichment bioassays, cellular elemental (N and P) quotas, and cell RNA content. Our results are consistent with theoretical predictions of higher N requirements under low light conditions due to the coupling of the photosynthetic mechanism with N uptake. In contrast, P-rich cellular components, such as RNA, were dependent on P availability rather than light.

A central issue in phytoplankton ecology and marine biogeochemistry is specification of both the elemental composition of the phytoplankton and the N : P ratio that defines the transition from N limitation to P limitation. The Redfield C : N : P stoichiometry of 106 : 16 : 1 describes the average elemental composition of phytoplankton, although there are wide variations in the particulate C : N : P both spatially and temporally in the ocean (Geider and La Roche 2002). The Redfield N : P ratio of 16 : 1 is often used as a benchmark for differentiating N limitation from P limitation and is thought to set an upper limit on the nitrate : phosphate ratio in the ocean (Falkowski 1997; Tyrrell 1999; Lenton and Watson 2000). The limit of 16 : 1 is often attributed to the P limitation of N_2 fixation (Falkowski 1997).

Recent observations (Ho et al. 2003) and a review of earlier published values (Geider and La Roche 2002) document pronounced interspecific variability in the C : N : P stoichiometry of phytoplankton. Under nutrient-replete conditions, C : P varies from about 200 to 40 mol C : mol P, C : N from about 12 to 5 mol C : mol N, and N : P from about 30 to 5 mol N : mol P (Ho et al. 2003). To these systematic variations among species (Ho et al. 2003) when cultured under nutrient-replete conditions at one temperature and light level must be added the systematic phenotypic variability within species that arises from growth under nutrient-limiting, light-limiting, or both conditions (Goldman et al. 1979; Elrifi and Turpin 1985). Particulate N : P can be as low as 2 mol N : mol P in N-limited cultures or as high as 100 mol N : mol P in P-limited cultures (Elrifi and Turpin 1985). Thus, there is considerable plasticity in the N : P ratio of phytoplankton, although this range is not fully realized in nature (Geider and La Roche 2002). In contrast, there is less scope for variability of the C : N ratio, which is constrained to values of 4 to 20 mol C : mol N under all growth conditions (Geider and La Roche 2002). Although limnologists have long recognized that there is a wide gray area in the N : P ratio between N- and P-limited conditions (Hecky and Kilham 1988), geochemists have often assumed that the Redfield ratio defines the transition between N and P limitation for the ocean in its entirety, regardless of local variations. Despite widespread use of the Redfield N : P of 16 mol N : mol P to differentiate N-limiting from P-limiting conditions, there is growing evidence that the transition point between N and P limitation occurs at a particulate N : P ratio that exceeds 16 : 1 mol N : mol P for phytoplankton cultures in balanced growth (Terry et al. 1985; Wynne and Rhee 1986; Liu et al. 2001).

Optimal and critical N : P—There is some confusion in the literature regarding the use of the terms ''optimal'' and ''critical'' N : P. Both terms are used to define particulate N : P ratios under well-defined reference conditions based on the Droop growth model (Droop 1968). The optimal N : P was originally defined as the ratio of the minimum N to minimum P cell quotas (Rhee 1978; Wynee and Rhee 1986). This is a very convenient ratio to measure operationally but is of less value for assessing the switchover point between N and P limitation when cells are actively growing. Terry et al. (1985) used the term ''critical N : P'' to designate the transition point between N and P limitation at any nutrient-lim-

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ited growth rate. Droop growth theory, under the assumption of a threshold transition between N and P limitation, predicts that the critical N : P will vary with the growth rate. Thus, only at zero growth rate will the critical N : P, as defined by Terry et al. (1985), have the same value as the optimal N : P employed by Rhee and coworkers.

To make matters even more confusing, Klausmeier et al. (2004) defined optimal N : P to be the ratio that maximizes the growth rate under conditions in which all nutrients other than the limiting nutrient are available in excess. Thus, the optimal N : P will differ between P limitation and N limitation (Klausmeier et al. 2004). The critical N : P at which N and P are colimiting will have a value that falls between the optimal N : P ratios for N-limiting/P-replete conditions and P-limiting/N-replete conditions. As with the critical N : P (Terry et al. 1985), the optimal N : P is expected to depend on the growth rate (Klausmeier et al. 2004), as well as other environmental conditions, including temperature and photon flux density (PFD).

Elemental and biochemical composition—As described above, cell quota models of phytoplankton growth, such as the Droop (1968) equations, provide a formalism for defining the critical N : P ratio and determining its dependence on growth rate (Terry et al. 1985). These equations allow observations from N-limited and P-limited cultures to be combined to define the optimal N : P ratios for growth under N- or P-limiting conditions (Klausmeier et al. 2004). They also allow calculation of the minimum cellular requirements of N and P and, thus, the critical N : P ratio that will support a given growth rate (Terry et al. 1985). However, these models do not provide any information on the biochemical basis for the variability of N : P and the critical N : P in microalgae, which is still poorly quantified. This limits the explanatory and predictive ability of the cell quota models to examine the interaction of N and P limitation. In particular, these models suffer because the parameter values depend on environmental factors such as PFD or temperature (Rhee and Gotham 1981*a*,*b*; Wynne and Rhee 1986).

Proteins and nucleic acids are the major macromolecular pools of intracellular N and P, although lipids, low–molecular mass compounds, and chitin (in diatoms) can also contribute significantly (Geider and La Roche 2002). Thus, we expect that critical N : P will be related to cell requirements for protein, RNA, and DNA. Furthermore, we expect that the critical N : P and the protein : RNA ratios will be influenced by PFD because light-harvesting pigment–protein complexes and photosynthetic electron transfer chain components that are nitrogen rich but phosphorous poor account for a large, but variable, fraction of cell mass (Geider et al. 1996). Under low light conditions, a high investment in light harvesting is essential for optimizing energy capture, whereas reductions of cell quotas of light-harvesting components under high light conditions minimize the potential for photooxidative stress (Larkum and Howe 1997). The increase in the proportion of cellular protein required for the light-harvesting apparatus in low light might provide an explanation for the dependencies of the parameters of the Droop cell quota model (Droop 1968) on PFD.

In this study, we used a chemostat–cyclostat system to produce *Chaetoceros muelleri* cells with a stable preconditioning history. Mainly, Liebig, and in one case Blackman, types of limitation were examined under various N : P ratios at two PFDs to identify the limiting nutrient (N vs. P). Elemental composition, along with several key biochemical components, was quantified to partition the nutrients into various biochemical classes. We determined the extent to which biochemical composition (RNA : cell and protein : cell) covaried with elemental composition (P : cell and N : cell). We also found that increased critical N : P under low light was associated with an increased protein requirement that could be accounted for by synthesis of chlorophyll : protein complexes rather than a decreased P requirement for synthesis of RNA.

Materials and methods

Chaetoceros muelleri Lemmermann 1898 (CCMP 1316) was cultured in chemostats with the use of media enriched with f/2 vitamins and trace elements (Guillard and Ryther 1962). NO_3^- concentration was adjusted to 100 μmol L^{-1} in all formulations, and PO_4^{-3} was varied to yield five N : P ratios: 5 : 1, 15 : 1, 30 : 1, 45 : 1, and 90 : 1. Supply (= inflow) N : P and drawdown N : P were calculated as the sum of nitrate + nitrite : phosphate ratio and will be referred hence forth as N : P. Cultures were grown at 25°C under a 14 : 10 light : dark cycle employing two PFDs: 50 μmol photons m^{-2} s^{-1} was the low-light (LL) and 700 μmol photons m^{-2} s^{-1} was the high-light (HL) treatment. The HL chemostats were grown at a fixed dilution rate of 0.6 d^{-1}, whereas the LL chemostats were grown at 0.3 d^{-1}.

Two variations of growth bioassays were used to assess the limiting nutrient. In the first, samples from the chemostat were transferred to culture tubes and left to grow in batch mode under the same light and temperature conditions. When the cultures reached the stationary phase, nutrients (NO_3^-, PO_4^{-3}, or an equal volume of distilled water as control) were added, and cell density was monitored. Nutrient limitation was assigned on the basis of final biomass achieved as a result of cell growth. In the second variation, chemostat samples were used as inocula to a series of batch cultures with the same media plus added nutrients as before. Growth rates and final biomass were monitored as means to differentiate between types of nutrient limitation.

Algal cell concentration was determined daily with an improved Neubauer hemacytometer. Sampling was carried out at the middle of the light phase to avoid diel rhythm effects (Ahn et al. 2002). Samples for protein, RNA, and pigments were collected by centrifugation at 2000 rpm for 20 min at 7°C. The algal pellet was resuspended in 2 ml of sea water and stored at −20°C for subsequent analysis. The supernatant (outflow medium) was collected in high-density polyethylene bottles and stored at −20°C prior to analysis for NO_3^-, NO_2^-, and PO_4^{-3} (soluble reactive phosphate, which will be termed phosphate hereafter) with the methods described by Strickland and Parsons (1972). Dry weight was calculated on the basis of the weight difference of precombusted GFF filters before and after filtering a known volume of known algal density. The filters were rinsed with 2 ml of

Table 1. Assessment of nutrient limitation in *C. muelleri* chemostats from nutrient addition bioassay experiments of varying input nitrate : phosphate ratios from 5 to 90 mol N : mol P. Summarized are the steady-state cell abundance (cells μl^{-1}), growth rate on the first day in batch culture mode before the nutrient additions, cell abundance in stationary phase, and growth rate following addition of the limiting nutrient (in batch cultures originating from chemostat samples). Numbers in parentheses = SD, $n = 3$.

Inflow N : P	Cell abundance in chemostat	Growth rate in day 1 of batch phase (d^{-1})	Stationary phase cell abundance in control	Cell abundance in +N treatment after 1 d	Cell abundance in +P treatment after 1 d	Growth rate when limiting nutrient added (d^{-1})	Limiting factor
Low light							
5	285(70)	0.20(0.00)	350(7)	654(7)	356(11)	0.65(0.01)	light, N*
15	358(78)	0.31(0.05)	544(2)	741(10)	540(4)	0.31(0.05)	light, N*
30	178(27)	0.36(0.02)	472(8)	631(31)	510(8.4)	0.29(0.01)	light, N*
45	248(66)	0.15(0.00)	341(5)	389(11)	371(11)	0.10(0.01)	P
90	249(70)	0.10(0.01)	557(70)	370(33)	791(70)	0.41(0.19)	P
High light							
5	1,020(113)	0.10(0.01)	1,260(9)	2,614(3)	1,239(3)	0.73(0.01)	N
15	1,035(109)	0.00(0.05)	1,108(20)	1,846(113)	1,369(14)	0.51(0.09)	N
30	970(73)	0.10(0.01)	1,213(10)	1,186(15)	1,390(18)	0.14(0.04)	P
45	650(60)	0.21(0.03)	767(25)	785(14)	844(22)	0.10(0.01)	P
90	411(160)	0.18(0.01)	1,280(30)	1,253(24)	2,086(15)	0.49(0.06)	P

* See text for further explanation of the assignment of the limiting nutrient.

1 mol L^{-1} ammonium formate to remove salts and dried at 80°C for 4 h.

Samples for particulate phosphorus, carbon, and nitrogen were collected on precombusted (450°C for 2–3 h) GFF filters under low vacuum. Particulate phosphorus was measured by the method of Solórzano and Sharp (1980), and particulate carbon and nitrogen were analyzed on a 440 Elemental Analyzer (Control Equipment Corporation).

Total protein content was determined by the Bradford (1976) method after resuspension of the algal material in 3 ml of H_2O and incubation at 50°C with 0.2 ml of 10 mol L^{-1} NaOH for 30 min. Calibration was carried out on the basis of hemoglobin standards (Sigma).

Pigments were extracted from the algal pellet with 5 ml of methanol at −10°C overnight and centrifuged at 1,500 rpm for 5 min; 60 μl of the supernatant was injected into the high-performance liquid chromatography (HPLC) system by the method described in Val et al. (1994). Briefly, the HPLC analysis was carried out in a two-solvent system (A = 70 : 30 methanol : ammonium acetate (1 mol L^{-1}) and B = 80 : 20 methanol : ethyl acetate) on a Beckman HPLC system equipped with a Beckman System Gold 168 Diode Array detector and a 25-cm FISONS octadecyl, reverse-phase column. A linear gradient was run from 100% A to 100% B over 5 min, with a further 20 min at 100% B, before returning to 100% A over a linear gradient. Pigments were identified by comparison with known standards (chlorophyll *a* [Chl *a*, Sigma], diadinoxanthin, diatoxanthin, fucoxanthin, Chl *c* [DHI Denmark]) and by comparison of peak absorption spectra from the diode array detector and retention time with the literature (Jeffrey et al. 1997).

RNA was measured by the method described in the Ribogreen Quantitation kit product information sheet (Molecular Probes).

Elemental and biochemical component ratios were calculated from results on the basis of analyses of the same sample. Whenever that was not possible, mean values for each component were used. Mean values were calculated on the basis of at least 6 samples for each condition. To identify the groups that demonstrated significant differences (95% confidence limit), the multiple comparisons between means T′ method was used (Sokal and Rohlf 1995).

Results

Chemostat cell abundance and nutrient addition bioassays—At steady state, all chemostats showed minimal day-to-day cell density fluctuations, typically within 10% of the average cell density (Table 1). The typical cell density was around 800 cells μl^{-1} for the HL chemostats and around 300–400 cells μl^{-1} for the LL chemostat, with no significant differences between the various N : P supply ratios that were employed. An exception was the HL chemostat at N : P = 90, with lower cell densities and greater fluctuation in cell concentration.

In the nutrient enrichment bioassays (Fig 1; Table 1), cell abundance increased by up to a factor of 2.7 before stationary phase was reached in control treatments. A further increase of cell abundance in response to either the addition of nitrate or phosphate to the stationary-phase cultures was used as an indicator of N or P limitation. Under HL conditions, the cell division rate during the first day of the bioassay experiments ranged from 0 to 0.24 d^{-1}, with higher rates at N : P $\geq$ 30 (0.2 d^{-1}) than at N : P $\leq$ 15 (0 d^{-1}). Because the dilution rate of the HL chemostats was 0.6 d^{-1} and the maximum growth rate of *C. muelleri* at the high PFD is 1.7 d^{-1} (Leonardos and Geider 2004), growth rate in these chemostats was clearly nutrient limited. We infer N limitation at N : P $\leq$ 15 and P limitation at N : P $\geq$ 30. Under low light conditions, the cell division rate during the first day of the bioassay experiments averaged 0.36 d^{-1} in the three cultures, with inflow N : P $\leq$ 30. Because these chemostats were operating at a dilution rate of 0.3 d^{-1}, we infer

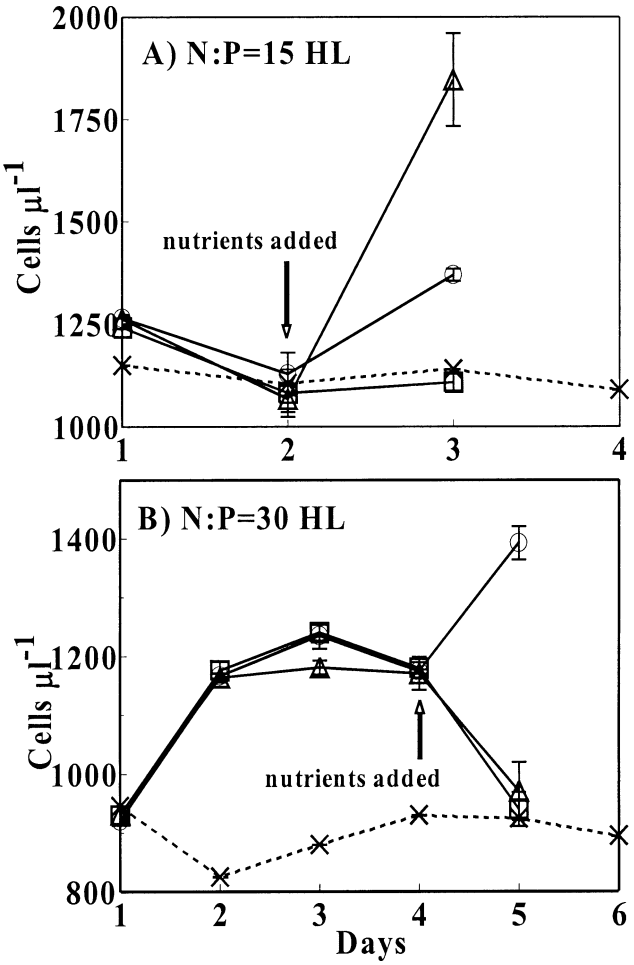

Fig. 1. Nutrient addition growth responses for *C. muelleri* cells sampled from chemostats under high light. Shown are cell abundance in the chemostat (× with dotted line); cell abundance in control treatments removed from the chemostat, but to which nutrients were not added (squares); and cell abundance in experimental treatments to which either nitrate (triangles) or phosphate (circles) was added. Error bars = SD. (A) N:P = 15, high light. (B) N:P = 30, high light.

that they were light limited. In the bioassay from the LL chemostat with inflow N:P = 45, the cell division rate equaled 0.15 d^{-1} during the first day of the bioassay. Combining these results with the residual nutrient concentrations (Table 2) in the N:P = 45 and N:P = 90 chemostats, we infer that growth was P limited in these chemostats.

Nutrient concentrations—Residual phosphate decreased with increasing inflow N:P under both high and low light conditions (Table 2). The phosphate concentration was consistently lower in the HL chemostats at all inflow N:P ratios, except at N:P = 90, where the phosphate concentration was at the detection limit of our method in both HL and LL conditions. Under high light, the phosphate concentration dropped to the detection limit of 0.02 μmol L^{-1} at N:P = 45 and remained low at N:P = 90.

Under high light, residual NO_3^- showed the opposite trend to phosphate, increasing from 0.1 μmol L^{-1} at inflow N:P = 5 to 10 μmol L^{-1} at N:P = 30 and about 30 μmol L^{-1} at N:P = 45 and N:P = 90 (Table 2). Under low light, residual nitrate concentrations in the outflow medium were high at all inflow N:P except N:P = 15 (Table 2).

Cell composition.—LL cells had significantly higher dry weights than their HL counterparts, with no significant effect of N:P ratio (Table 3). This pattern has been demonstrated before for *C. muelleri* (Leonardos and Lucas 2000). A response similar to dry weight was observed for particulate organic carbon (C), and protein content of *C. muelleri* (Table 3). Cells grown under high light contained less protein than their LL counterparts at all N:P ratios ($p < 0.05$). Although cell carbon content was significantly lower ($p < 0.05$) under high light than low light in three treatments (N:P = 15, 30, and 45), there was no significant difference in C content between HL and LL cells at N:P = 5 and 90.

Photoacclimation of pigment content and composition—Light is the primary factor controlling pigment composition in *C. muelleri.* Cells grown at low light contained more Chl *a* than their HL counterparts (Table 3). Supply N:P ratio did not have any consistent significant effect on pigment content or composition ($p < 0.05$). There were no significant differences in either fucoxanthin:Chl *a* or Chl *c*:Chl *a* under different light regimes. Fucoxanthin is the major light-harvesting carotenoid in this diatom, and the average mass ratio was around 1 g fucoxanthin (g Chl *a*)$^{-1}$. The Chl *c*:Chl *a* ratio was also high, averaging about 0.5 g Chl *c* (g Chl *a*)$^{-1}$. In contrast, the ratio of the photoprotective pigments (the sum of diadinoxanthin and diatoxanthin) to Chl *a* was significantly influenced by PFD but not by inflow N:P ratio

Table 2. Residual inorganic nutrient concentrations (in the outflow medium) of the *C. muelleri* chemostats of varying inflow N:P ratio. Numbers in parentheses = SD, $n \geq 4$.

Inflow N:P	Concentration (μmol L^{-1})				
	High light		Low light		
	PO_4^{-3}	NO_3^-	PO_4^{-3}	NO_3^-	Inflow PO_4^{-3}
5	0.68(0.12)	0.10(0.03)	9.97(1.03)	18.7(10.2)	20
15	0.38(0.14)	0.21(0.00)	0.31(0.09)	0.14(0.06)	6.7
30	0.09(0.09)	11.0(11.0)	0.14(0.09)	40.3(3.3)	3.3
45	0.02(0.02)	30.5(1.3)	0.07(0.07)	29.1(3.5)	2.2
90	0.02(0.02)	31.1(0.7)	0.02(0.02)	50.2(1.6)	1.1

Table 3. Cell composition and mass ratios of stedy-state *C. muelleri* under low light and high light conditions. Number in parentheses = SD, n ≥ 4. Fuco = fucoxanthin; Dt + Dd = the sum of diatoxanthin and diadinoxanthin.

	N : P				
	5	15	30	45	90
Low light					
Dry weight (pg cell^{-1})	78(32.3)	58(19.4)	80(16.6)	69(5.9)	61(27.4)
Cellular C (pmol cell^{-1})	1.10(0.26)	1.08(0.04)	1.27(0.20)	1.12(0.18)	0.89(0.11)
Protein (pmol cell^{-1})	7.43(0.39)	8.90(4.57)	14.19(5.38)	8.62(1.30)	9.27(1.40)
Chl *a* (pg cell^{-1})	0.29(0.11)	0.52(0.23)	0.25(0.09)	0.30(0.08)	0.46(0.09)
Chl *c* : Chl *a*	0.47(0.13)	0.56(0.12)	0.55(0.18)	0.39(0.12)	0.54(0.11)
Fuco : Chl *a*	1.15(0.58)	0.97(0.16)	1.15(0.36)	0.80(0.14)	0.97(0.08)
Dt+Dd : Chl *a*	0.14(0.04)	0.24(0.04)	0.20(0.05)	0.16(0.03)	0.24(0.03)
High light					
Dry weight (pg cell^{-1})	45(8.3)	28(3.7)	23(2.1)	32(0.21)	42(9.0)
Cellular C (pmol cell^{-1})	0.90(0.43)	0.75(0.08)	0.59(0.20)	0.73(0.10)	0.86(0.08)
Protein (pmol cell^{-1})	3.15(0.73)	3.51(0.30)	3.57(0.61)	3.42(0.58)	3.10(0.29)
Chl *a* (pg cell^{-1})	0.08(0.00)	0.08(0.03)	0.05(0.01)	0.05(0.01)	0.09(0.03)
Chl *c* : Chl *a*	0.46(0.03)	0.54(0.11)	0.44(0.02)	0.48(0.11)	0.35(0.16)
Fuco : Chl *a*	0.90(0.18)	0.98(0.16)	1.05(0.19)	0.94(0.15)	0.89(0.07)
Dt+Dd : Chl *a*	0.26(0.03)	0.50(0.09)	0.30(0.05)	0.25(0.03)	0.50(0.14)

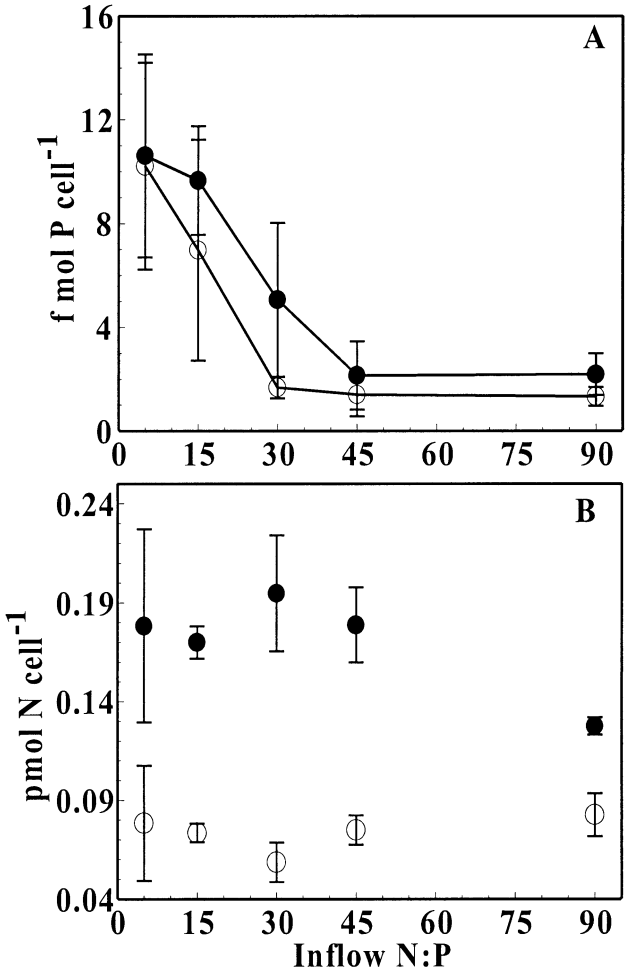

Fig. 2. Dependencies of the (A) phosphorus and (B) nitrogen contents of *C. muelleri* cells on inflow nitrate : phosphate ratios under high light (open symbols) and low light (filled symbols). Error bars = SD.

(Table 3). The photoprotective pigments were found, as expected (Porra et al. 1997), in higher quantities in high light–acclimated *C. muelleri.* In this case, the HL chemostats of inflow N : P = 15 and 90 exhibited a significantly higher ratio ($p < 0.05$) than all the other treatments. The results indicate limited variability of Chl *a* and accessory photosynthetic and photoprotective pigment content with respect to environmental N : P ratio, but greater scope for change in relation to light conditions. Chl *a* : C and Chl *a* : N (data not illustrated) followed the same pattern as the Chl *a* : protein ratio, all three being primarily influenced by light rather than the N : P ratio. Because fucoxanthin : Chl *a* and Chl *a* : Chl *c* ratios did not vary systematically with N : P or light, we were led to conclude that photoacclimation in *C. muelleri* involves a change in the number of photosynthetic units rather than a change in size of the photosynthetic unit (Richardson et al. 1983).

Elemental composition—Cellular P (Fig. 2A) decreased with increasing N : P ratio until N : P = 30 for the HL conditions and N : P = 45 for the LL conditions. Cellular P concentration did not decrease with further increases in the N : P ratio of the inflow medium. The mass content of N per cell was light dependent ($p < 0.05$) but independent of inflow N : P ($p > 0.05$; Fig. 2B). A statistical exception was at N : P = 90 for low light, where cellular N was significantly lower than at any other LL inflow N : P employed.

There was limited variability of the cellular C : N ratio ($p < 0.05$) under the various inflow N : P employed (Fig. 3A), whereas light influenced this ratio to a greater extent. LL cells irrespective of N : P ratio exhibited a C : N (mol mol^{-1}) of around 6.6, which is the Redfield ratio, whereas under high light, C : N increased to around 10.5.

Cellular N : P (mol mol^{-1}) increased with increasing inflow nitrate : phosphate ratio (Fig. 3B). Only at inflow N : P = 45 did light have a statistically significant effect on particulate N : P (HL > LL, $p < 0.05$). The slope of a plot of

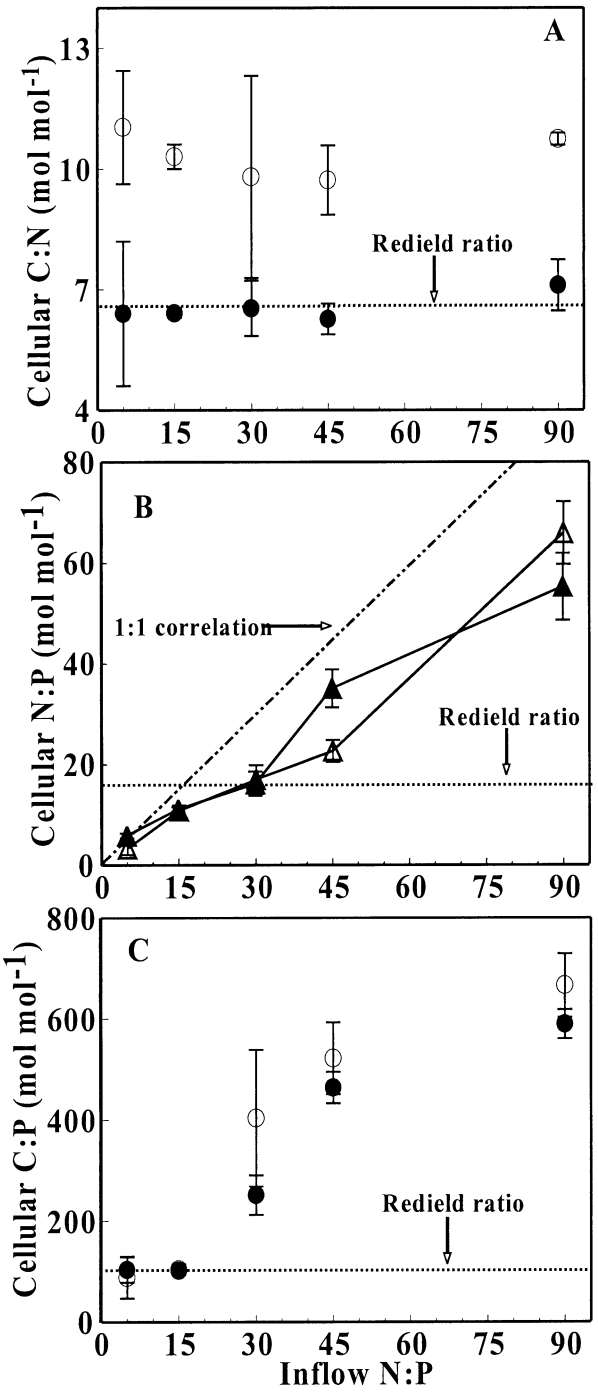

Fig. 3. Dependencies of the molar ratios of (A) C : N, (B) N : P, and (C) C : P of *C. muelleri* cells at a range of inflow nitrate : phosphate ratios under high light (open symbols) and low light (filled symbols). Error bars = SD. The Redfield ratio lines are indicated for visual reference.

the particulate N : P versus inflow nitrate : phosphate was ~0.67. The deviation from the 1 : 1 molar conversion of dissolved to particulate matter was due, in part, to the unassimilated nitrate or phosphate in the phosphorus- or nitrogen-limited chemostats, respectively. There was significant covariation between particulate N : P and the nitrate : phosphate drawdown ratio. Drawdown was calculated as inflow nitrate minus the residual dissolved nitrate divided by inflow phosphate minus the residual dissolved phosphate ($\Delta N : \Delta P$). The slope of a plot of the drawdown ratio versus the particulate (assimilated) N : P was not statistically different from 1 (slope = 0.987, $r^2 = 0.913$). Despite the high covariation of particulate N : P with drawdown N : P, the sum of particulate N plus residual nitrate accounted for about 80% of the nitrate in the inflow medium. Similarly, particulate P plus residual dissolved inorganic phosphate, accounted for about 80% of the phosphate supplied in the dilution water.

The ratio of particulate C : P increased from around the Redfield proportions of 106 : 1 (mol mol^{-1}) for the inflow N : P of 5 and 15, irrespective of light, to around 640 (mol mol^{-1}) at the highest inflow N : P used (Fig. 3C). At a given inflow N : P ratio, PFD did not have a significant effect on particulate C : P ($p > 0.05$).

Relationship between macromolecular and elemental composition—Now we describe how the macromolecular composition of *C. muelleri* is related to its elemental composition. First, we will describe how cell N, P, protein, and RNA varied with light and inflow nitrate : phosphate. Then, to obtain a more quantitative description of the relationship between macromolecular and elemental composition, we calculate the proportion of cell P that can be accounted for by RNA and the proportion of cell N that can be accounted for by protein and RNA. We calculated the N : P ratio of cell protein plus RNA for comparison with the particulate N : P. Finally, we describe the relationship between protein, pigment composition, and cellular carbon.

Cell RNA content (Fig. 4A) followed the same pattern as cellular P content. The mass ratio of protein : RNA (Fig. 4B) increased with increasing inflow N : P, and above N : P = 15, this ratio was significantly higher under low light ($p < 0.05$). The relationship between cellular N and protein (Fig. 5A) depended on light levels but not on inflow N : P. In contrast, cellular P and RNA (Fig. 5B) were correlated with inflow N : P, with light not having a significant effect.

The chemical composition of RNA yields a ratio of about 11 g RNA per gram P in RNA on the basis of the assumption that there are equal numbers of all four bases (Geider and La Roche 2002). Therefore, the proportion of P in RNA was calculated by dividing the RNA : P observed in the cultures by 11. Therefore, the maximum RNA : P that we would expect to find is about 11 : 1 if RNA accounted for essentially all of the P in the cell. This ratio was seen in cells with the lowest cell RNA and P contents (Fig. 5B).

Similarly, for protein, there is about 6.5 g protein per g nitrogen in protein (Geider and La Roche 2002). Thus, the proportion of cell N associated with protein was calculated by dividing the observed protein : N by 6.5. We calculate that the percentage of N associated with protein was between 40% and 70%. Conveniently, the ratio of mass-to-N in RNA is about 6.25 (i.e., similar to that in protein). Thus, we also calculated the proportion of N in RNA by dividing RNA : N by 6.25. The percentage of N associated with RNA was between 3% and 39% (calculations not shown). The amount of N associated with RNA generally decreased with increasing inflow N : P ratio, with a significantly higher proportion

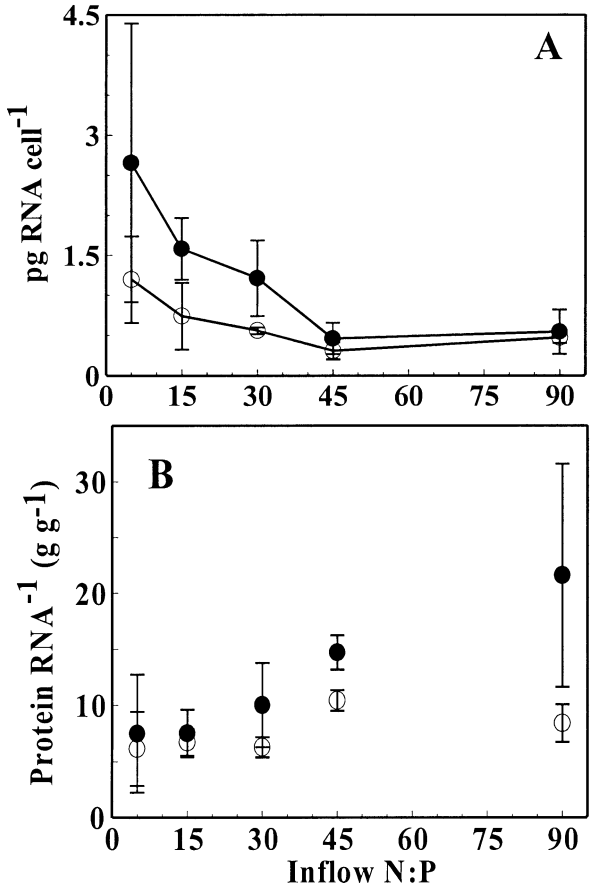

Fig. 4. Dependencies of (A) cell RNA content and (B) the mass ratio of protein : RNA of *C. muelleri* cells at a range of inflow nitrate : phosphate under high light (open symbols) and low light (filled symbols). Error bars = SD.

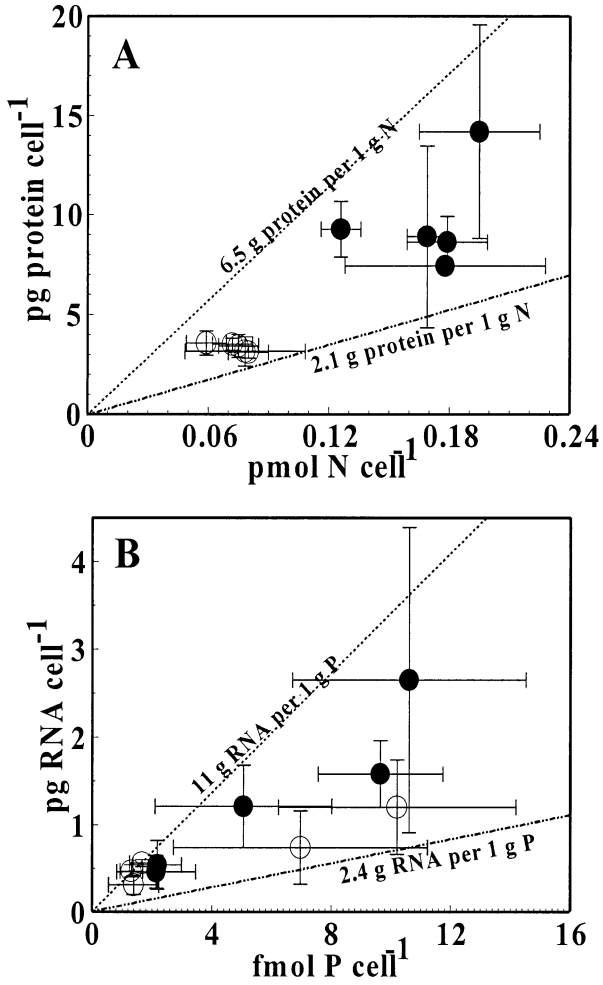

Fig. 5. Relationship between (A) cell protein and cell nitrogen contents and (B) between cell RNA and cell phosphorous contents of *C. muelleri* cells under high light (open symbols) and low light (filled symbols). Error bars = SD. Also shown in panel A is the trend line illustrating the expected relationship between cell protein content and cell nitrogen content if all of the cell N were contained in protein (i.e., 6.5 g protein [g N]$^{-1}$ = 91 g protein [mol N]$^{-1}$). Also shown in panel B is the trend line illustrating the expected relationship between cell RNA content and cell phosphorous content if all of the cell P were contained in RNA (11 g RNA [g P]$^{-1}$ = 340 g RNA [mol P]$^{-1}$).

of N associated with RNA in cultures with inflow N : P = 5 (up to ~40% compared with an overall mean of 14%, calculations not shown; $p < 0.05$).

We found that the protein-to-RNA ratio was largely independent of inflow nitrate : phosphate under high light ranging from 7 to 10 g protein : g RNA (Fig. 4B). The same protein : RNA ratio was observed under high and low light levels when *C. muelleri* was cultured under N-limiting conditions (i.e., at inflow nitrate : phosphate < 30 mol N : mol P). However, the ratio increased to 20 g protein : g RNA under P-limiting conditions (Fig. 4B). Assuming that both protein and RNA are 16% N by mass and that RNA is 9.1% P by mass (Geider and La Roche 2002), we calculate the N : P ratio of protein plus RNA in high light *C. muelleri* equaled about 30 to 40 mol N : mol P. Under the most extreme P-limited condition, the N : P ratio of protein plus RNA was about 80 mol N : mol P, similar to the particulate N : P ratio.

The ~40% increase in cell carbon content between high and low light (Table 3), which was independent of inflow nitrate : phosphate, could be accounted for largely by the almost threefold increase in cell protein content (Table 3), assuming that protein is 53% C by weight (Geider and La Roche 2002). Cell pigment content also increased in low light. We observed that, on average, a 0.30 pg per cell mean increase in Chl *a* between high and low light was accompanied by a 6.35-pg increase in cell protein. Using the observed weight ratio of 1 g Chl *a* to 0.5 g Chl *c* to 1 g fucoxanthin to 0.2 g diadinoxanthin plus diatoxanthin in *C. muelleri* (Table 3) and the molecular weights of these pigments, we calculate a 5.6-kg increase in protein per mol increase in chromophores. This can be compared with the protein and pigment contents of the major light-harvesting complex protein in the diatom *Phaeodactylum tricornutum.* Owens and Wold (1986) found that 3.6 mol of chromophore was associated with an apoprotein doublet of 16.4 + 16.9 kDa in *P. tricornutum.* Thus, they observed a protein : chromophore ratio of 9.3 kg protein : mol chromophore, which is marginally greater than the value that we found for incre-

ment of total protein : mol chromophore in *C. muelleri.* Although the pigment composition of *C. muelleri* differs from that of *P. tricornutum,* we nonetheless conclude that most, if not all, of the increase in cell protein content between high and low light in *C. muelleri* can be attributed to increases in pigment–protein complexes.

Discussion

Concepts and assessment of nutrient limitation—The term nutrient ''limitation'' has been widely used in the literature in a variety of contexts (Beardall et al. 2001), often interchangeably with the terms ''deficiency'' and ''starvation.'' There are two distinctively different types of limitation: one referring to limitation of the maximum population density achieved (*Liebig* limitation) and one referring to limitation of growth rates (Blackman limitation, Perry 1976; Beardall et al. 2001). Measurement of low levels of nutrients in the environment is not sufficient to infer nutrient limitation of growth rate, although it might be sufficient to indicate that there is limited potential to increase cell yield. However, nutrient limitation of growth rate can produce variations in elemental composition (Goldman et al. 1979), macromolecular composition (Lynn et al. 2000), enzyme activity (e.g., increase in alkaline phosphatase activity in P-limiting conditions, Hammed et al. 1999; single cell immunoassay, Dyhrman and Palenik 2001), and cell physiology (e.g., maximum nutrient assimilation and photosynthetic rates, Rhee 1978; Zevenboom et al. 1980). The utility of measurements of elemental or macromolecular composition to diagnose nutrient limitation of growth rate could be compromised by the physiological plasticity of phytoplankton. This is because luxury consumption of nonlimiting nutrients can occur. Internal nutrient storage pools can act as a buffer, allowing the cell to overcome environmental variability (Andersen et al. 1991; Flynn 1998) and introduce a time lag that needs to be accounted for in models of cell growth (Andersen et al. 1991; Flynn 2002). This was illustrated in our experiments by the continuing cell division in subsamples removed from the chemostats before the onset of stationary phase in almost all of the nutrient addition bioassays (Table 1). Cell division continued even when the dissolved inorganic form of the limiting nutrient was present at or near the detection limit (Table 2). Intracellular stores of P might be sufficient for several hours or days of growth, whereas intracellular N pools can be depleted faster (Elrifi and Turpin 1985; Andersen et al. 1991). The potential for cell abundance to increase on addition of the limiting nutrient was greatest at the extreme input nitrate : phosphate ratios (N : P = 5 and N : P = 90) and least near the critical N : P (Table 1).

Variability in elemental and biochemical composition—Our results for *C. muelleri* showing covariation of particulate N : P with the nitrate : phosphate drawdown ratio are consistent with previous observations (Droop 1974; Elrifi and Turpin 1985). Much of the variability in cell P and N contents that we observed could be attributed to covariation of P with cell RNA and N with cell protein contents (Fig. 5A,B). Given the more conservative behavior of the cellular C : N ratio, which ranged from 6 to 11 in our test conditions, it is not surprising that particulate C : P also covaried with particulate N : P.

In *C. muelleri,* the protein-to-RNA ratio, and hence the rate of protein synthesis per unit RNA at a constant dilution rate, increased with increasing P limitation (Fig. 4B), and thus with increasing N : P. Given that RNA accounts for ~30% (Geider and La Roche 2002), or more on the basis of our results (Fig. 5B), of the total P content, increased ribosomal efficiency is a clear mechanism for acclimation to environments with low P availability. RNA content was low under P limitation but increased progressively as P supply increased under N-limiting conditions. Our results are consistent with the assertion that ribosomes represent a significant P repository (Elser et al. 2003) and thus support the hypothesized connection between the C : N : P stoichiometry and growth rate of an organism (e.g., the growth rate hypothesis proposed by Elser et al. [2000, 2003]).

Elemental stoichiometric variability that is induced by changes in environmental conditions is of the same magnitude as measured variability among widely different phytoplanktonic taxa when grown under fixed, presumably optimal, conditions (Quigg et al. 2003). Leonardos and Geider (2004) found a similar range of elemental ratios in different stages of batch cultures of *C. muelleri.* We suggest that intraspecific variability in elemental stoichiometry of phytoplankton might be as important as interspecific variability and thus should be considered explicitly in mechanistic and predictive models of nutrient cycling in the oceans.

Critical N : P exceeds the Redfield ratio—The critical ratio refers to the particulate N : P in the phytoplankton and defines the transition point between N and P limitation (Terry et al. 1985). Under conditions in which inorganic nutrients are converted to particulate matter with no production of dissolved organic matter, the particulate ratios will equal the nutrient (nitrate : phosphate) drawdown ratio. However, unless particulate N : P is measured directly, it is unsafe to assume that dissolved organic nitrogen and phosphorous are not produced (Bronk et al. 1994). Here, we will make a distinction between the dissolved (= supply) critical N : P, which is the dissolved nitrate : phosphate ratio in the dilution water at which a chemostat shifts from N to P limitation, and the particulate critical N : P. These critical N : P ratios will be identical if nitrate and phosphate are converted to particulate matter in equal proportions.

The critical N : P has been determined directly by growing cells at a fixed dilution rate over a range of dissolved nitrate : phosphate for only three species (Terry et al. 1985). In other cases, the so-called optimal N : P has been calculated from the ratio of minimum cell quotas for N- or P-limited growth assuming threshold type limitation (Wynne and Rhee 1986) or the critical N : P from Droop equations for N- and P-limited growth (Liu et al. 2001). The critical N : P has been found to range from about 20 to 90 mol N : mol P and depends primarily on the species under investigation and, to a lesser extent, on the nutrient-limited growth rate (Terry et al. 1985; Liu et al. 2001). Our data from *C. muelleri* show that when the supply N : P was 45, the particulate N : P was 23 at high light and 35 at low light and when the supply N : P was 30, the particulate N : P was 16 at both light intensities

(Fig. 3B). Clearly, the dissolved critical N : P does not equal the particulate critical N : P in *C. muelleri* and is light dependent, growth rate dependent, or both. As in previous investigations of the critical N : P in other species (Terry et al. 1985; Liu et al. 2001), we found that the dissolved critical N : P in *C. muelleri* was greater than the Redfield ratio. This has important implications for both biogeochemistry and the assumptions regarding primary productivity estimations. The critical N : P is significantly greater than both the nitrate : phosphate ratio in the deep sea and the average particulate N : P in surface waters. These observations are consistent with the results from bioassay experiments that show N is more often limiting than P in the oligotrophic North Atlantic (Graziano et al. 1996). Thus, there may be considerable scope for increasing the nitrate inventory of the ocean prior to the onset of P limitation of marine primary productivity.

Light dependence of the critical N : P—The combined results of our measurements of the responses of cell growth to nutrient additions (Fig. 1; Table 1), the residual dissolved nutrient concentrations in the chemostats (Table 2), and the dependence of elemental composition on inflow nitrate : phosphate (Figs. 2, 4) indicate that the supply-critical N : P ratio in *C. muelleri* increased from between 15 and 30 in high light to about 45 in low light.

As far as we are aware, there has been one previous study of the response of both N and P cell quotas to reduced PFD under nutrient-limiting conditions. This study by Wynne and Rhee (1986) found that the optimal N : P ratio calculated from ratios of minimum cell quotas increased in low light in two out of four species, declined in a third species, and was unchanged in the fourth. Wynne and Rhee (1986) determined the minimum cell quotas for stationary-phase N- or P-limited batch cultures, in which growth rate, by definition, is zero and growth is unbalanced. This approach might be flawed because the transition from nutrient-replete to nutrient-starved conditions is abrupt in batch cultures, precluding physiological acclimation. We examined the N and P quotas for *C. muelleri* in balanced growth at fixed dilution rates with a population that was actively growing. This allowed cells to acclimate to light in a way that is not possible in stationary-phase batch culture. We found that both supply and particulate critical N : P increase under low light.

The dependence of critical N : P on light has been asserted to arise from the requirement for increased cell pigment content and associated pigment–protein complexes under low light conditions (Geider et al. 1998; Geider and La Roche 2002), although it has also been suggested to arise from reduced ribosomal RNA contents in slowly growing cells (Schlesinger and Shuter 1981). Consistent with the former suggestion, cell protein levels in *C. muelleri* were much higher under low light than under high light. Much of this increase could be attributed to the increase of pigment–protein complexes under low light (*see Results*).

In contrast to Schlesinger and Shuter (1981), who observed that the contribution of RNA to cell carbon declined under low light in four freshwater chlorophytes grown in light-limited continuous cultures, our data indicate that RNA : C was not reduced in low light. In fact, under P-limiting conditions at inflow nitrate : phosphate of 45 and 90 mol nitrate : mol phosphate, the cell RNA content was slightly (7.3% for these two conditions, 18.6% overall), but not significantly ($p > 0.05$), higher at low light than at high light. Thus, although protein content increased in low light, in large part because of the increased cell pigment content, the RNA content did not decline. In fact, cell RNA content in *C. muelleri* increased about threefold under low light in N-limiting conditions at inflow nitrate : phosphate of 5 and 15 mol N : mol P. Only under P-limiting conditions did the protein : RNA ratio increase in low light (Fig. 4B).

Variability in the elemental stoichiometry of phytoplankton is receiving renewed attention (Ho et al. 2003; Quigg et al. 2003; Klausmeier et al. 2004). Although the variability in phytoplanktonic elemental stoichiometry has been well documented (Geider and La Roche 2002), considerably less attention has been given to the transition point between N and P limitation (i.e., the critical N : P). The typical value of the critical N : P, and the genetic and environmental variability in critical N : P might play important roles in both the evolution of phytoplankton (Klausmeier et al. 2004) and the evolution of marine biogeochemistry (Falkowski 1997). As far as we are aware, we present the first experimental determination for a marine microalga of the light dependence of the critical N : P and its biochemical basis in changing cell protein and RNA contents. Our experimental results show that the critical N : P for *C. muelleri* is greater in low light than in high light. We found that critical N : P was higher than the Redfield N : P ratio and thus that the Redfield does not represent a universal biochemical optimum, at least in *C. muelleri.* The biochemical analyses that we present for *C. muelleri* cells, from steady-state light-acclimated chemostats, also show that allocation to P-rich assembly machinery (ribosomes) is reduced and tightly regulated in P-limited conditions, consistent with the ''growth rate hypothesis'' (Elser et al. 2003).

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