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Differential effects of phosphorus limitation on cellular metals in *Chlorella* and *Microcystis*

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Abstract

We investigated the effect of phosphate bioavailability on cellular metal quotas in two species of freshwater phytoplankton (the eukaryote *Chlorella* sp. UTCC522 and the cyanobacterium *Microcystis* sp. LE3), grown in semicontinuous culture over four controlled levels of phosphate availability, encompassing phosphorus (P) deplete to P replete conditions. P limitation caused reduced growth rate, high C : P (up to 1800 mol mol^{-1}), and increased alkaline phosphatase (APase) activity. Low P availability led to enriched cobalt (Co), cadmium (Cd), and zinc (Zn) in *Chlorella* (up to 2.8-fold, 1.7-fold, and 1.8-fold, respectively, normalized to cellular N, relative to P-replete control) but resulted in enriched Co and nickel (Ni) in *Microcystis* (up to 4.4-fold and 3.0-fold). In contrast, cellular iron (Fe), manganese (Mn) and copper (Cu) were largely unchanged ($\pm\sim$20%) in both organisms. Cd and Co may substitute for Zn in the APase of *Chlorella* while in *Microcystis* the dominant phosphatase may be strictly Co-requiring, as has been reported for other prokaryotes and is consistent with its evolutionary emergence before the oxygenation of the atmosphere, when Co was relatively abundant in natural waters. By extension, the absolute Co requirement of the important marine cyanobacteria *Synechococus* and *Prochlorococcus* may be related in part to widespread depletion of orthophosphate (PO_4^{3-}) in the oligotrophic surface ocean. The enrichment of Ni in *Microcystis* may indicate increased activity of Ni-requiring superoxide dismutase under P limitation or, speculatively, a co-uptake of Ni and Co by a shared transport system. These results shed light on the interaction between trace metals, macronutrient availability, and phytoplankton assemblage composition, and suggest intensified biological cycling of Zn, Cd, Co, and Ni in low-P freshwater and marine systems.

Phosphorus (P) is an essential nutrient that all organisms require for energy transport, construction of membranes, and storage and replication of genetic information. P has been documented to limit community production in marine systems ranging from restricted seas (Krom et al. 1991; Nausch and Wasmund 2004) to the open ocean, including oligotrophic regions of the North Atlantic and the North Pacific (Cotner et al. 1997; Karl 1997; Wu et al. 2000). In addition, P is limiting in many large lakes, as most recently demonstrated for Lake Superior (Sterner et al. 2004). Low P availability has a substantial effect on the biochemistry and physiology of phytoplankton. Severe P limitation makes phytoplankton incapable of producing nucleic acids and leads to a decrease in the rate of protein synthesis, which in turn inhibits cell division and decreases rates of light utilization and carbon fixation (Cembella et al. 1984; Falkowski and Raven 1997). While these physiological effects have been well studied, the effect of P availability on trace metal uptake in phytoplankton is poorly understood. While changes in metalloenzyme activities may result from variations in P availability, there are at present very few published studies of the effects of P bioavailability on intracellular metal concentrations in phytoplankton. Such effects are central to quantifying changes in overall metal-cycling processes that may occur under macronutrient depletion in freshwater and marine systems.

Many phytoplankton have evolved mechanisms for acquiring P from dissolved organic matter when the supply of inorganic P (PO_4^{3-}) is limited, including the globally important marine diazotroph *Trichodesmium* (Dyhrman et al. 2006). Under conditions of potentially limited concentrations of PO_4^{3-}, a subset of dissolved organic phosphorus (DOP) compounds can serve as a reliable growth substrate (Karl and Bjorkman 2002). Many phytoplankton (and bacteria) can produce alkaline phosphatase (APase), a group of enzymes (mono- and di-esterases), that can catalyze the hydrolysis of a broad spectrum of DOP compounds, thereby affording the organism access to the DOP pool, which typically has much higher concentrations

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than (PO_4^{3-}) in low-P waters. APase requires metals as cofactors in the active center. The paradigm for metal ions in APase follows that of Zn and magnesium (Mg) in enzymes isolated from the prokaryotic organism *Escherichia coli*, in which APase is a homodimeric protein containing two 48-kDa subunits, with one Mg atom and two Zn atoms in each of the active sites being essential for catalytic activity (Kim and Wyckoff 1991). However, the metal-binding site in APase is not specific for Zn. It has been observed that Co and Cd can substitute equally well for Zn in *E. coli* APase (Applebury and Coleman 1968; Simpson and Vallee 1969; Gettins and Coleman 1982), following the general pattern of Co–Zn and Cd–Zn substitution seen in antagonistic metal uptake relationships in phytoplankton (Price and Morel 1990; Lee and Morel 1995). In addition to Zn–Cd–Co APase, it has also been reported that some organisms can produce a unique Co(II)-requiring APase, such as the prokaryote *Bacillus subtilis* (Hulett et al. 1991) and the hyperthermophilic bacterium *Thermotoga maritime* (Wojciechowski et al. 2002). The unique Co-requiring APase in these two organisms shows strong homologies with Zn-APase in *E.coli*, both of which are encoded by the PhoA family of APase genes (Hulett et al. 1991; Wojciechowski et al. 2002). Table 1 summarizes the reported metal ions for APase in various microbes, either by Zn–Cd–Co substitution or formation of specific Zn- and Co-APase.

Since metal cofactors are essential to APase activity, it is reasonable to hypothesize that cellular requirements for these metals increase when cells express high APase activity. Very few studies, however, have been carried out to test this hypothesis. Only rather recently, Wang et al. (2001) and Yu and Wang (2004) used radiotracer techniques to investigate the influence of P bioavailability on cellular Zn uptake, Cd uptake, and selenium (Se) uptake in two marine diatoms (*Thalassiosira pseudonana* and *T. weissflogii*) and one freshwater green alga (*Chlamydomonas reinhardtii*). The authors found that P-starved *T. pseudonana* and *T. weissfloggi* exhibited an increased Zn-uptake rate, while P-starved *C. reinhardtii* showed a reduced uptake rate for Zn and Cd but an increased uptake rate for Se. However, considering that P bioavailability has complicated regulatory effects on cell growth rate which in turn affects metal-uptake rate and cell size and that interrelationships between cellular-uptake rate and specific growth rate are uncertain in these studies, the results for metal-uptake rate may not be related directly to cellular metal composition. Moreover, radiotracer methodologies typically do not allow more than a few metals to be measured simultaneously and hence cannot be used to explore comprehensively the relationships between P bioavailability and the full suite of elemental requirements in phytoplankton. Further careful laboratory studies are required to quantify the effects of P availability on cellular composition (e.g., as element : carbon ratios) for a more complete suite of bioactive metals.

In this study, we address this issue by investigating changes in cellular metals in two species of freshwater phytoplankton under increasing degrees of P limitation: a eukaryote (the chlorophyte *Chlorella* sp. UTCC 522) and a prokaryote (the cyanobacterium *Microcystis* sp. LE-3). Both species are isolated from Lake Erie and represent important classes of primary producers that have been documented in the North American Great Lakes (Munawar and Munawar 1982). The current study was carried out to complement ongoing field studies in Lake Superior, an extremely P-deficient aquatic system (Sterner et al. 2004; Anagnostou 2005; Anagnostou and Sherrell 2008) in which Zn cycling has been shown to be particularly intense (Nriagu et al. 1996; Sherrell unpubl.). The principal goals of this work are two fold: 1) to investigate how phosphate bioavailability regulates phytoplankton metal quotas for seven bioactive elements (Fe, Mn, Co, Ni, Cu, Zn, and Cd); and 2) to determine whether this regulation is similar or different in a representative eukaryote vs. a cyanobacterium, examples of groups that evolved in very different periods of Earth's geochemical history (Falkowski et al. 2004) and have different overall mean metal requirements and metal toxicity sensitivities (Saito et al. 2003). Ultimately, the results may shed light on the role of P limitation in the large scale controls on trace metal biogeochemistry in aquatic and marine systems.

Materials and methods

All apparati for preparation of the medium, algal culturing and sampling, and elemental analysis were prepared using rigorous acid cleaning procedures (Cullen and Sherrell 1999) to minimize trace metal contamination.

Growth media—The growth medium was designed to resemble the chemistry of natural water from Lake Superior (Weiler 1978). The metal composition of the growth medium was based on Me′ (the concentration of free metal ions plus that of labile dissolved inorganic speciation) in Lake Superior, estimated from the total metal concentrations in the lake (Sherrell unpubl.) and literature values for typical inorganic metal fractions

Table 1. Reported metal ion cofactors for APase in various organisms.

Organism	Metal ion in active center	Reference
E. coli	Zn, Mg	Kim and Wyckoff (1991)
	Cd, Co substitution for Zn	Gettins and Coleman (1982); Simpson and Vallee (1969); Wojciechowski and Kantrowitz (2002)
Bacillus subtilis, PhoAIV	Co	Hulett et al. (1991)
B. subtilis, PhoAIII	Co	Hulett et al. (1991)
Thermotoga maritime	Co, Mg	Wojciechowski et al. (2002)

($Me' : Me_T$) from other freshwater and marine systems: $Mn' : Mn_T = 100\%$, $Zn' : Zn_T = 1\%$, $Cu' : Cu_T = 0.1\%$, $Co' : Co_T = 10\%$, $Cd' : Cd_T = 10\%$, $Ni' : Ni_T = 1\%$, and $Fe' : Fe_T = 1\%$ (Donat and Bruland 1994). The estimated Me′ concentrations for individual trace metals in Lake Superior were the following: Mn, 2 nmol L^{-1}; Zn, 20 pmol L^{-1}; Cu, 0.1 pmol L^{-1}; Co, 2 pmol L^{-1}; Cd, 7.7 pmol L^{-1}; Ni, 20 pmol L^{-1}; and Fe, 40 pmol L^{-1}. To yield the above Me′ concentrations, total metal concentrations (nmol L^{-1}) in the presence of 5 μmol L^{-1} ethylenediaminetetraacetic acid (EDTA), calculated using the computer program MINEQL+ (version 5.0) were the following: Mn_T, 13; Zn_T, 27.2; Cu_T, 44.3; Co_T, 4; Cd_T, 17.3; Ni_T, 3520; and Fe_T, 116. Metals were added in the form of pure (>99.99%) elemental salts (Alfa Aesar). To prevent nitrogen limitation, we set the nitrate ($NaNO_3$) concentration to 100 μmol L^{-1}. Silicon was omitted from culture media because it is not required for the culture of green algae and cyanobacteria. The growth media was spiked with 0.74 nmol L^{-1} vitamin B_{12}, 4.0 nmol L^{-1} biotin, and 0.6 μmol L^{-1} thiomin, and its pH was titrated to 7.0 with a very small addition of ultrapure ammonium hydroxide. Phosphate (NaH_2PO_4) was added at the desired concentration for each of the four P levels chosen: 50 nmol L^{-1}, 100 nmol L^{-1}, 200 nmol L^{-1}, and 300 nmol L^{-1}, with 10,000 nmol L^{-1} used as the control. The stock solutions (1000× concentrated) of major ions, $NaNO_3$, and NaH_2PO_4 were passed through a Chelex ion exchange resin column to remove contaminant trace metals. Media with these P concentrations were used to dilute the cultures by a factor of 2, hence initial P concentrations immediately following each media exchange were roughly half these values (*see* Semicontinuous culture mode section below).

Culture growth conditions—The algae were grown in semicontinuous mode in 1-L polycarbonate bottles under axenic conditions, initiated by microwave heating of the media in each culture bottle to near 100°C. Replicate culture bottles ($n = 3$) were prepared for each treatment and species, totaling 30 bottles. All experiments were carried out under continuous light at 100 μmol quanta m^{-2} s^{-1} supplied by VHO fluorescent bulbs (comparable in intensity to Lake Superior upper euphotic zone in summer) at 19 ± 1°C. Phytoplankton were maintained in exponential-growth phase throughout the culture period, with cell density monitored daily by measuring the in vivo fluorescence (IVF) of chlorophyll using a fluorometer (Turner 10-AU) and by periodic counting of cells using a hemocytometer under a 400× power microscope. Growth rates were determined from linear regression of the logarithmic-transformed fluorescence vs. time. The maximum quantum yield of photochemistry in photosystem II (PSII; Fv : Fm), a proxy for the physiological status of the cells during the experiments (Falkowski and Raven 1997), was measured using a Fast Repetition Rate fluorometer (FRR; Chelsea Instruments). Because the culture bottles were allowed to exchange CO_2 with the atmosphere during regular openings for withdrawal of monitoring samples, and because the periodic 50% dilutions were made with pH 7.0 media, we assume that pH did not deviate greatly from neutral over the course of the experiment. In addition, accumulation of particulate organic carbon was relatively similar across the treatments because slower growing cells contained more carbon per cell (Table 2).

Semicontinuous culture mode—Cells were maintained at approximate steady state growth in semicontinuous culture mode, with frequent replacement of a fraction of the growing culture with an equal volume of fresh medium. A volume ratio of 1 : 1 was used for culture dilution, i.e., ~50% of culture volume was replaced with fresh media following each doubling of cell density, thus diluting the culture to initial cell density. At this renewal ratio, the phosphate concentration of the culture immediately following each dilution was approximately half that of the stock media at the four selected levels, since phosphate concentrations in the culture bottles were low relative to stock media concentrations immediately prior to each dilution. We refer hereafter to the four P levels by the approximate NaH_2PO_4 concentrations after dilution: 25 nmol P L^{-1}, 50 nmol P L^{-1}, 100 nmol P L^{-1}, and 150 nmol P L^{-1}, with 10,000 nmol P L^{-1} as the control treatment. Media renewal frequency was proportional to cell growth rate so that each culture roughly doubled in cell density between dilutions. Since both organisms showed reduced growth rate with lower [PO_4^{3-}], renewal was less frequent for the lower P treatments: approximately

Table 2. Cellular carbon, nitrogen, and phosphorus content of *Chlorella* and *Microcystis* under individual P treatments. Means of triplicate samples are presented with standard deviation in parentheses.

Organism	Treatment	Carbon (fg $cell^{-1}$)	Nitrogen (fg $cell^{-1}$)	Phosphorus (fg $cell^{-1}$)
Chlorella	25 nmol L^{-1}-P	5110 (590)	320 (40)	7.1 (0.7)
	50 nmol L^{-1}-P	2410 (420)	254 (44)	8.2 (2.1)
	100 nml L^{-1}-P	820 (190)	106 (23)	10.4 (2.3)
	150 nmol L^{-1}-P	580 (50)	81 (14)	12.0 (3.9)
	10,000 nmol L^{-1}-P	600 (110)	94 (12)	18.2 (2.7)
Microcysis	25 nmol L^{-1}-P	6460 (1160)	319 (57)	9.1 (2.4)
	50 nmol L^{-1}-P	3100 (620)	275 (38)	8.4 (1.4)
	100 nml L^{-1}-P	777 (136)	102 (20)	4.4 (0.1)
	150 nmol L^{-1}-P	682 (71)	77 (15)	6.0 (0.1)
	10,000 nmol L^{-1}-P	731 (88)	89 (19)	27.7 (4.0)

once per day for 100 nmol L^{-1}, 150 nmol L^{-1}, and 10,000 nmol L^{-1}; every other day for 50 nmol L^{-1}; and every 3 d for 25 nmol L^{-1}.

Cell harvesting and digestion—Cells were harvested ~1–3 d after the final media dilution by centrifugation (Eppendorf model 5403) using six 50-mL polycarbonate centrifuge tubes (Fisher) at 6500 rpm (3000 × g) for 15 min at 19°C. After removal of supernatant media, cell pellets were rinsed twice with 50-mL Milli-Q H_2O (Millipore) to wash away any residual media. This rinsing procedure removed 99.99% of the residual media volume, thus guaranteeing negligible contribution of metals from residual media in the cell pellet. The process of centrifugation and rinsing concentrated 800 mL of cell culture to a <0.5-mL suspension. Immediately following rinsing, an aliquot of 10% of the homogeneous condensed-cell suspension was pipetted into tin capsules (EMAL Tech) for carbon–hydrogen–nitrogen (CHN) analysis, and the remaining aliquot (90%) was pipetted into 15-mL screw-cap Teflon vials (Savillex) for digestion in preparation for trace metal analysis. The CHN aliquots were desiccated under vacuum with calcium chloride ($CaCl_2$) desiccant for >1 d and analyzed on a Carlo-Erba elemental analyzer (Elemental Microanalysis Ltd.) using acetanilide (71.09% C and 10.36% N) as the reference material, with empty capsules as blanks. The trace-metal samples in Teflon vials were placed with vial lids loosened in doubled open-ended plastic bags to prevent metal contamination and dried in a 60°C oven.

Trace-metal analysis—Prior to trace-metal analysis, the desiccated samples were digested in the same Teflon vials using 0.5 mL of 16 mol L^{-1} nitric acid (HNO_3) (Baseline, SeaStar Chemical) at 120°C on a hotplate for 4 h. Digests of samples and digest blanks (acid only) were analyzed by Magnetic Sector High-Resolution Inductively Coupled Plasma Mass Spectrometer (HR-ICP-MS; Element-1, ThermoFinnigan) fitted with a μFlow all-Teflon self-aspirating nebulizer (Electro Scientific Industries) and a Scott double-bypass spray chamber, following the methods of Cullen and Sherrell (1999). We analyzed a suite of bioavailable metals including Fe, Mn, Zn, Cu, Cd, Co, and Ni as well as P against matrix-matched standard curves (Cullen et al. 2001). Sensitivity and stability of the instrument were adjusted to optimum conditions before sample analysis and monitored using 1 ppb indium added to each sample, blank, and standard. For the following elements, two isotopes were measured to confirm freedom from interferences: Fe, ^{56}Fe and ^{57}Fe; Cu, ^{63}Cu and ^{65}Cu; and Zn, ^{64}Zn and ^{66}Zn. The differences in concentrations calculated using the two isotopes were <5%. Concentrations of Cd, Co, Cu, Mn, P, and Zn in the digestion blanks were always <12% of the sample; blanks were lowest for Ni (0.04%) and highest for Mn (12%).

We interpret the metal concentrations resulting from this rinsing and digestion procedure to represent intracellular metal concentrations, following the findings of Tang and Morel (2006) who demonstrated using chelating rinses and cultures of a coastal diatom that total measured cellular Cu, Zn, Co, Cd, and Mn were essentially unaffected by various rinses even when rinse solutions removed a substantial fraction of the total particulate Fe by dissolving Fe oxyhydroxides that had precipitated on cell surfaces. Since our growth conditions were similar to theirs in terms of metal availability, and because total Fe in our media was near the minimum used in the previous study, we conclude that our measured concentrations represented intracellular metal contents.

APase activity measurement—Measurement of the enzymatic activity of APase in the phytoplankton cultures was carried out using a fluorometor (Model LS-50B, Perkin-Elmer), following published methods (Ammerman 1993). Briefly, the activity was estimated using the compound 4-methylumbilliferyl phosphate (MUF-P) as a substrate analog during timed, temperature-controlled dark incubations, with subsequent analysis of the hydrolysis product 4-methylumbilliferone (MUF) by fluorometry. Incubations were carried out using 10-mL cultures in acid-washed 15-mL polystyrene centrifuge tubes following the addition of 0.1-mL buffered (Tris, pH 8.3) 1 mmol L^{-1} MUF-P. At time zero, and at selected time points thereafter (5 min, 20 min, 60 min), the samples were analyzed for MUF concentration. APase activity was determined from linear regression of the increased MUF signal with time, and the enzyme activity was finally expressed as cell density normalized rates (nmol [MUF] [μg Chlorophyll *a*]$^{-1}$ h^{-1}).

Results

Cell growth and photosynthetic efficiency—Prior to the experiment, cells were acclimated to individual P treatments for several (at least three) generations in semicontinuous mode, i.e., cultures were renewed by ~2-fold dilution with fresh media more than three times. During this acclimation period, growth rates were measured to confirm that acclimation was occurring progressively and completely (Fig. 1). Cells were harvested at a point following the final media dilution when cell density was approaching the maximum observed in the penultimate acclimation cycle. At harvesting point, cell density increased from 5.5 × 10^4 cells mL^{-1} to 4.0 × 10^5 cells mL^{-1} for *Chlorella* and from 1.3 × 10^5 cells mL^{-1} to 3.78 × 10^5 cells mL^{-1} for *Microcystis*, as a positive function of phosphate availability.

The effects of phosphate bioavailability on growth, photochemical quantum yield of PSII (Fv : Fm), phosphate uptake rate, and cellular-metal quotas on *Chlorella* and *Microcystis* were measured in exponential-phase cultures grown under different phosphate regimes. Growth rate provided an early diagnosis of P limitation, which was reduced from 0.85 d^{-1} to 0.22 d^{-1} for *Chlorella* and from 1.06 d^{-1} to 0.15 d^{-1} for *Microcystis* (Figs. 1, 2) as phosphate availability was reduced. *Chlorella* showed a growth rate similar to the control in the 100 nmol L^{-1} and the 150 nmol L^{-1} treatments, and only a small decrease in growth rate at 100 nmol L^{-1}, but *Microcystis* showed this similarity only in the 150 nmol L^{-1} treatment, with a

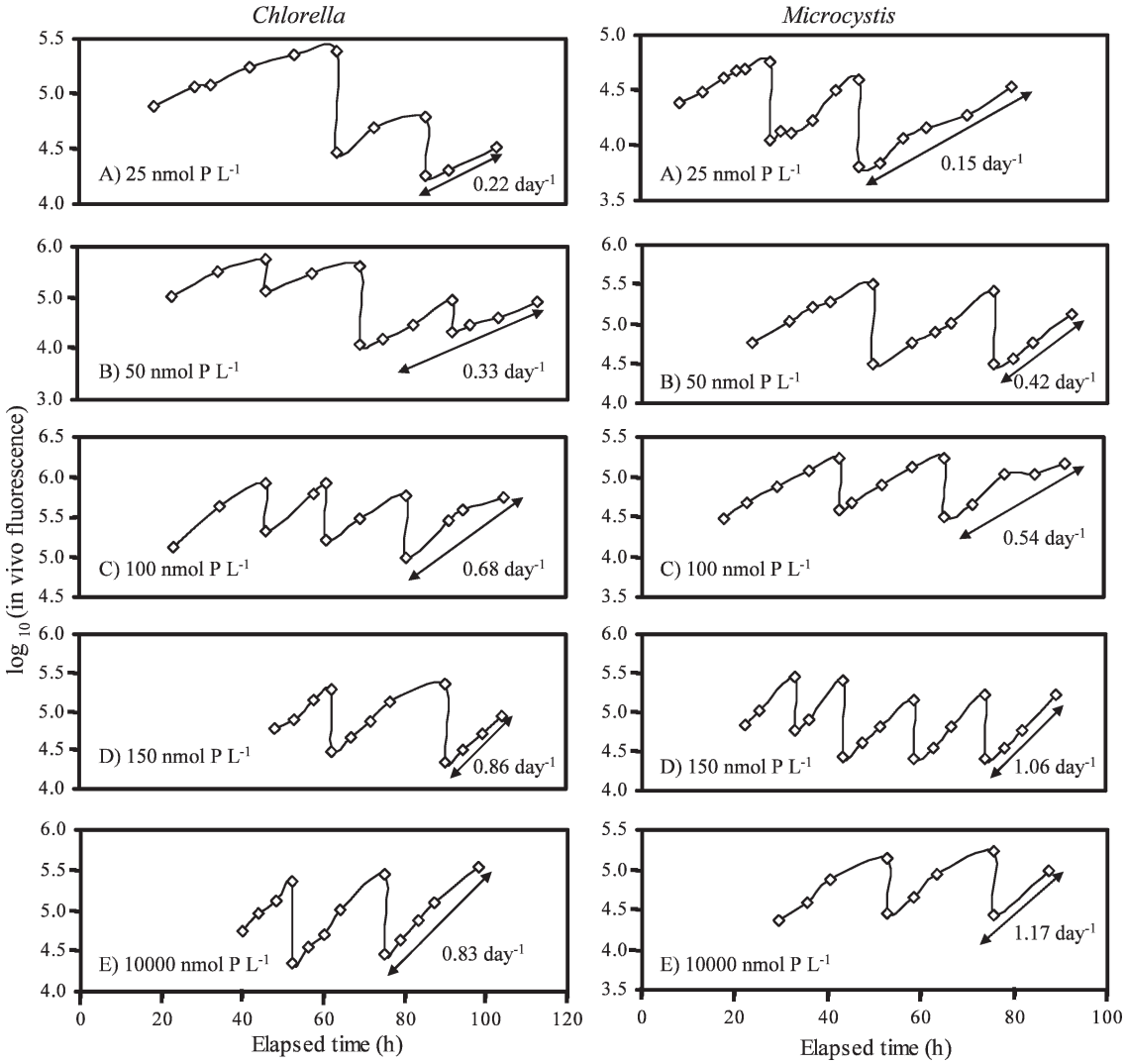

Fig. 1. Acclimation of *Chlorella* and *Microcystis* to varying P treatment in semi-continuous culture, expressed as in vivo fluorescence vs. time. Cultures were inoculated at time zero. After multiple dilutions with fresh media, growth rates (arrows) were calculated for the final exponential growth phase for each treatment. Final point shown for each culture is at harvest for analysis of cellular composition (macronutrient C, N, P, and trace metals) and measurement of photosynthetic efficiency. (A) 25 nmol P L^{-1}, (B) 50 nmol P L^{-1}, (C) 100 nmol P L^{-1}, (D) 150 nmol P L^{-1}, (E) 10,000 nmol P L^{-1}.

sharp, >2-fold drop at 100 nmol L^{-1}. Generally, *Microcystis* displayed a higher growth rate than *Chlorella*, except for the 25 nmol L^{-1} treatment. The photochemical quantum yield of PSII, Fv : Fm, showed no change between the control and the higher two experimental phosphate treatments, but decreased measurably between the 100 nmol L^{-1} and the 50 nmol L^{-1} treatments for both species (Fig. 2). The reduction in Fv : Fm was <20% for the most deplete, relative to the most replete, P treatments.

Cellular C : N : P stoichiometry—Phosphate availability has a substantial systematic effect on cellular C and N content but, perhaps surprisingly, smaller and/or nonsystematic effects on P content per cell (Table 2). The C : P and N : P ratios in both freshwater organisms were similar to Redfield ratios when P was replete, with the exception of *Microcystis*, which showed a 3-fold higher C : P and N : P ratio in the 150 nmol L^{-1} treatment relative to the 10,000 nmol L^{-1} control even though growth rate was similar at the two levels of P availability (Table 3). Reduced P availability induced an enrichment of C-rich compounds but a depletion of P-rich molecules on a per-cell basis, leading to higher C : N, and more markedly, higher C : P and N : P ratios with decreasing P availability. Generally, the correlation of cellular C : N : P stoichiometry with P bioavailability followed the same trend as growth rate for both organisms. The C : N : P stoichiometry was most extreme at 25 nmol L^{-1} for both organisms: 1860 (C : P), 18.6 (C : N), and 78 (N : P) for *Microcystis*, and 1860 (C : P), 23.8 (C : N), and 100 (N : P) for *Chlorella*.

APase activity—*Chlorella* and *Microcystis* demonstrated increased APase activity (Chlorophyll *a* normalized) when phosphate availability was reduced. APase activity was

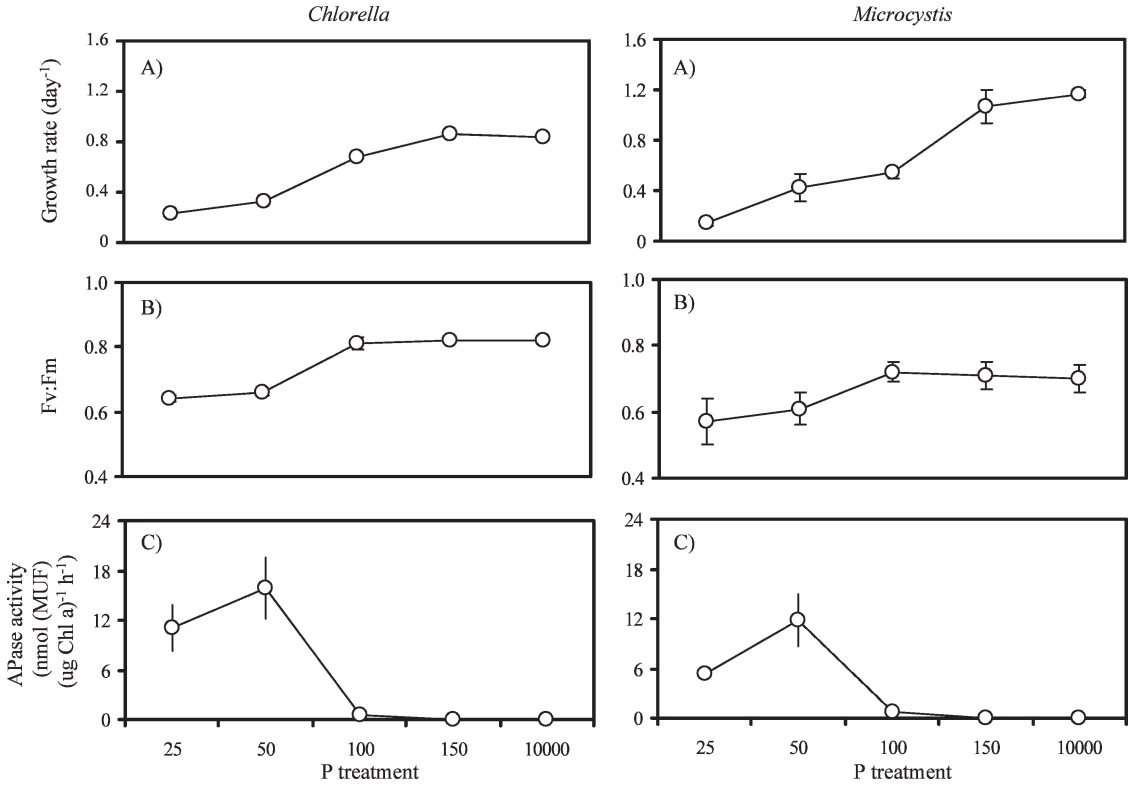

Fig. 2. Growth rate, photochemical quantum yield of PSII (Fv : Fm), and alkaline phosphatase (APase) activity under individual P treatments (nmol P L^{-1}) in and *Chlorella* and *Microcystis*. Error bars represent the standard deviation for replicate samples ($n = 3$). APase activity is undetectable in 150 nmol P L^{-1} and 10,000 nmol P L^{-1} treatments, and is plotted as zero activity. Note *x*-axis is not linear. (A) growth rate, (B) Fv : Fm, (C) APase activity.

Table 3. C : N : P stoichiometry in *Chlorella* and *Microcystis* (molar mean ratio, with standard deviation in parentheses, $n = 3$) under individual P treatments and comparison to stoichiometry reported for marine phytoplankton and natural phytoplankton assemblage in Lake Superior.

Organism	Treatment	C : N	N : P	C : P	Reference
Chlorella	25 nmol L^{-1}-P	18.6	100	1860	This study
	50 nmol L^{-1}-P	11.5 (1.1)	64.4 (24.9)	721 (220)	
	100 nml L^{-1}-P	9.0 (0.5)	23.8 (9.1)	203 (94)	
	150 nmol L^{-1}-P	8.3 (0.7)	15.4 (2.3)	127 (9)	
	10000 nmol L^{-1}-P	7.4 (0.4)	15.4 (5.2)	87 (43)	
Mirocystis	25 nmol L^{-1}-P	23.8 (1.5)	78.2 (3.6)	1865 (149)	This study
	50 nmol L^{-1}-P	13.1 (1.0)	72.9 (3.0)	955 (92)	
	100 nml L^{-1}-P	9.2 (1.2)	63.9 (1.6)	541 (22)	
	150 nmol L^{-1}-P	10.4 (1.0)	28.4 (6.1)	292 (35)	
	10000 nmol L^{-1}-P	8.4 (1.0)	8.1 (0.3)	67 (6.)	
Marine eukaryotic phytoplankton* (lab culture)	Average range	7.7 (2.6) 4–17	10.1 (3.9) 5–19	75 (31) 27–135	Geider and La Roche (2002)
Marine cyanobacteria† (lab culture)	P-replete	5.4 (0.4)	26 (6)	139 (23)	Bertilsson et al. (2003)
	P-limited	7.3 (0.2)	89 (24)	655 (168)	
Lake Superior particulate matter‡	Average range	8.6 (1.4) 6.6–13.3	28.9 (10.2) 14.8–65.7	251 (108) 93–697	Ji (2006)

* P-replete eukaryotic marine phytoplankton cultures.
† Means for three cyanobacteria (*Synechococcus* WH8103, *S.* WH8012, and *Prochlorococcus* MED4).
‡ Seasonally, geographically, and vertically averaged ratios (Ji 2006).

undetectable in either organism for the 150 nmol L^{-1} treatment or the 10,000 nmol L^{-1} control, but was measurable at the lower P levels (Fig. 2). Higher APase activity was generally associated with lower P availability, except that APase activity in the 25 nmol L^{-1} treatment was lower than that in the 50 nmol L^{-1} treatment for both species, possibly attributable to the limitation of photosynthetic capacity or protein synthesis under extremely low phosphate supply. Overall, APase activity, combined with growth rate and C : N : P stoichiometry, suggest that our four phosphate levels spanned the threshold P limitation level for *Chlorella* and *Microcystis*. As P availability decreased, cells in the different treatments exhibited a P-replete state, moderate P stress, and finally severe P limitation.

Trace metal quotas—Cellular metal quotas, typically expressed as the ratio of cellular metal content to cellular C or P (i.e., metal : carbon or metal : phosphorus, hereafter Me : C or Me : P respectively), underwent systematic changes with varying P availability (Table 4). For example, Me : P in *Chlorella* and *Microcystis* showed a substantial increase with reduced phosphate (Table 4). However, normalization to cellular C or P (Table 2) may be an inappropriate or misleading metric of changes in metal composition and cellular requirements as a function of P nutritional status because these variables are themselves strongly influenced by phosphate availability and may not be good representatives of metabolically active cellular biomass. As an example of this complication, we interpret the relatively stable P content per cell over the four P levels (Table 2) to result from the compensating effects of reduced P per cellular biomass and increased cell volume and biomass under low P availability. Unfortunately, cell density (by microscopic counts) was not as accurately determined as cell composition, and cell volume was not determined. Because of the apparent accumulation of cellular C and depletion of P per biomass, we determined that Me : N is the most appropriate normalization that represents the physiological response of the cells with respect to metal uptake under severe P limitation. The justification for this approach is explored further in the Discussion section.

Discussion

Effects of degree of P stress on cellular C : N : P stoichiometry—In a survey of the variability of C : N : P stoichiometry in marine microalgae, Geider and La Roche (2002) reported molar C : P ratios of 27–135 and molar N : P ratios of 7–19 for various nutrient-replete phytoplankton cultures. In the present study, both freshwater phytoplankton growing under P-replete conditions (150 nmol L^{-1} and the control) showed C : P and N : P ratios similar to those in marine phytoplankton (although at 150 nmol L^{-1} ratios in *Microcystis* were still substantially greater than Redfield; Table 3).

The changes we observed in cellular C : N : P stoichiometry (increased C : N, C : P and N : P) under low P availability were consistent with previous research on other taxa (Bertilsson et al. 2003). In our experiment, phytoplankton were grown under a gradient of four phosphate levels rather than under two simple endpoints (P replete and P deplete), typical of experimental designs in previous work. Our design is advantageous for estimating the threshold P-limitation level and for quantifying C : N : P stoichiometry changes within a relatively narrow and low range of phosphate availability. Phytoplankton can adjust the cellular P requirement and re-allocate the cellular P pool to maintain a near-maximum growth rate within a limited range of P availability, avoiding P limitation until P availability drops below a threshold limitation level, a state characterized by a threshold cellular C : N : P stoichiometry (Cembella et al. 1984). For this study, the combined results of growth rate, APase activity, and cellular C : N : P stoichiometry under individual treatments allow us to estimate the threshold cellular C : N : P stoichiometry for *Chlorella* and *Microcystis*. In contrast, it is hard to estimate the threshold P concentration for these two species because we lack accurate measurement of stable SRP throughout the culture. From the growth rate and induced APase, we conclude that the threshold P limitation occurred in the 100 nmol L^{-1} treatment for *Chlorella* but in the 150 nmol L^{-1} treatment for *Microcystis*. For *Chlorella*, the threshold C : N : P stoichiometry is ~203 (C : P) and 24 (N : P); for *Microcystis*, it is ~292 (C : P) and 28 (N : P) (Table 3).

Because *Microcystis* and *Chlorella* were grown under near-identical conditions, this study provides a direct comparison between two phytoplankton species in terms of their capability for sustaining growth under low P availability as well as their species-dependent physiological response to P limitation. Higher threshold C : P and N : P stoichiometry in *Microcystis* than in *Chlorella* suggests that *Microcystis* is less susceptible to P limitation. This result is consistent with previous observations that *Microcystis* has a high P utilization efficiency and can apparently dominate other species for P uptake when external P concentrations are low (Hammer 1964; Bush and Welch 1972). In addition, this result is consistent with recent work on marine cyanobacteria (*Synechococus* and *Prochlorococcus*), which showed that cyanobacteria may have lower P requirements than eukaryotes (Bertilsson et al. 2003).

Effects of degree of P stress on cellular trace metal quotas—Our study is the first, to our knowledge, to explore qualitatively and quantitatively the relationships between phosphate availability and metal quotas for seven bioactive elements (Fe, Mn, Cu, Co, Zn, Ni, and Cd).

As outlined previously in the Results section, we maintain that cellular C and P contents are not appropriate for use as the normalization factors for expressing cellular metal quotas in this study. We suggest that the ~9-fold increases in cellular C from replete P to severe P limitation represents C storage (e.g., carbohydrates or lipids), and the 2–3-fold decrease in P per cell represents the balance of severe P deletion partially ameliorated by larger cell size. Carbon thus appears to accumulate under P limitation, possibly as a result of continued carbon fixation under reduced division rate and protein synthesis, while cellular P

Table 4. Cellular metal quota (Me : C, Me : P, Me : N, and revised Me : C *) under individual P treatment in *Chlorella* and *Microcystis* ($n = 3$). Means are presented with standard deviation in parentheses.

Organism	Treatment (nmol L^{-1}-P)	Metal quota†	Fe	Mn	Cu	Zn	Co	Cd	Ni
Chlorella	25	Me : C	203	17.2	7.09	21.6	0.13	1.34	0.94
		Me : P	332	28.2	11.7	35.4	0.21	2.2	1.53
		Me : N	3778	321	132	402	2.42	25.1	17.5
		Revised Me : C*	514	43.7	18	54.8	0.33	3.41	2.38
	50	Me : C	72.2 (5.7)	6.83 (0.21)	13.9 (2.2)	28.8 (2.0)	0.11 (0.01)	1.92 (0.16)	1.03 (0.17)
		Me : P	67.1 (10.9)	6.3 (0.5)	12.7 (0.8)	26.1 (1.1)	0.10 (0.01)	1.74 (0.03)	0.94 (0.07)
		Me : N	830 (62)	78.6 (2.2)	160 (24)	326 (21)	1.25 (0.15)	21.8 (1.8)	11.8 (1.8)
		Revised Me : C*	113 (8.5)	10.7 (0.3)	21.8 (3.3)	44.4 (2.9)	0.17 (0.02)	2.96 (0.24)	1.60 (0.25)
	100	Me : C	100 (7.5)	10.3 (0.3)	14.5 (1.8)	29.8 (0.8)	0.13 (0.03)	2.00 (0.14)	1.30 (0.04)
		Me : P	36.4 (3.1)	3.49 (0.15)	5.02 (0.59)	9.41 (0.45)	0.04 (0.00)	0.60 (0.01)	0.44 (0.01)
		Me : N	875 (64)	91.1 (2.2)	131 (15)	265 (6)	1.10 (0.22)	17.8 (1.2)	11.5 (0.4)
		Revised Me : C*	119 (8.7)	12.4 (0.3)	17.8 (2.1)	36.0 (0.9)	0.15 (0.03)	2.42 (0.17)	1.56 (0.05)
	150	Me : C	130 (24)	13.8 (1.3)	16.5 (1.2)	28.9 (0.1)	0.11 (0.01)	1.71 (0.18)	1.41 (0.12)
		Me : P	36.2 (4.3)	3.91 (0.24)	4.69 (0.07)	8.09 (0.60)	0.03 (0.00)	0.49 (0.01)	0.40 (0.01)
		Me : N	1058 (196)	114 (11)	136 (10)	234 (1)	0.88 (0.07)	14.0 (1.3)	11.6 (0.9)
		Revised Me : C*	144 (27)	15.5 (1.5)	18.5 (1.3)	31.9 (0.1)	0.12 (0.01)	1.91 (0.18)	1.58 (0.12)
	10,000	Me : C	266 (63)	15.5 (1.9)	18.5 (0.1)	39.9 (1.0)	0.12 (0.07)	1.91 (0.15)	1.58 (0.28)
		Me : P	31.0 (7.8)	3.01 (0.27)	2.32 (0.05)	4.64 (0.04)	0.02 (0.01)	0.21 (0.01)	0.29 (0.04)
		Me : N	1955 (460)	114 (14)	136 (1)	293 (7)	0.88 (0.51)	14.0 (1.1)	11.6 (2.1)
		Revised Me : C*	266 (63)	15.5 (1.9)	18.5 (0.1)	39.9 (1.0)	0.12 (0.07)	1.91 (0.15)	1.58 (0.28)
Microcystis	25	Me : C	59.7 (7.0)	4.72 (0.38)	4.55 (0.47)	4.76 (0.65)	0.13 (0.04)	1.18 (0.07)	3.50 (0.52)
		Me : P	111 (16)	8.77 (0.35)	8.5 (1.2)	8.81 (0.58)	0.24 (0.08)	2.21 (0.23)	6.53 (1.20)
		Me : N	1232 (144)	97.2 (7.5)	93.8 (10.0)	98.0 (13.4)	2.68 (0.75)	24.5 (1.3)	72.3 (10.9)
		Revised Me : C*	147 (17.2)	11.6 (0.9)	11.2 (1.2)	11.7 (1.6)	0.32 (0.09)	2.92 (0.16)	8.63 (1.30)
	50	Me : C	118 (17)	9.92 (0.98)	11.0 (1.2)	8.74 (0.99)	0.26 (0.09)	1.49 (0.10)	9.16 (1.71)
		Me : P	112 (10)	9.43 (0.44)	10.6 (2.1)	8.30 (0.13)	0.25 (0.09)	1.42 (0.12)	8.83 (2.42)
		Me : N	1358 (192)	114 (12)	126 (13)	100 (12)	3.02 (1.09)	17.2 (1.1)	106 (20)
		Revised Me : C*	162 (23)	13.6 (1.4)	15.1 (1.6)	12.0 (1.4)	0.36 (0.13)	2.05 (0.13)	12.6 (2.35)
	100	Me : C	65.9 (2.0)	14.0 (0.1)	8.92 (0.18)	11.0 (0.5)	0.12 (0.03)	2.10 (0.10)	4.62 (1.11)
		Me : P	38.9 (5.7)	7.27 (0.54)	4.79 (0.55)	6.52 (1.01)	0.06 (0.01)	0.96 (0.29)	2.37 (0.42)
		Me : N	511 (15)	108 (1)	69.1 (13.8)	84.6 (3.4)	0.92 (0.25)	16.2 (0.8)	35.9 (8.6)
		Revised Me : C*	61.0 (1.8)	12.9 (0.1)	8.25 (1.65)	10.1 (0.4)	0.11 (0.03)	1.94 (0.09)	4.28 (1.03)
	150	Me : C	97.7 (7.7)	15.5 (0.6)	15.9 (0.7)	13.3 (1.4)	0.08 (0.02)	1.89 (0.18)	3.92 (0.16)
		Me : P	28.3 (1.2)	4.51 (0.37)	4.66 (0.76)	3.84 (0.06)	0.02 (0.01)	0.55 (0.01)	1.14 (0.09)
		Me : N	880 (70)	139 (5)	143 (6)	119 (12)	0.67 (0.17)	17.0 (1.6)	35.3 (1.5)
		Revised Me : C*	105 (8.3)	16.6 (0.6)	17.1 (0.7)	14.2 (1.5)	0.08 (0.02)	2.03 (0.19)	4.21 (0.18)
	10,000	Me : C	125 (14.7)	17.7 (1.0)	8.76 (1.66)	27.0 (1.3)	0.10 (0.05)	2.87 (0.37)	2.42 (0.83)
		Me : P	9.1 (2.1)	1.27 (0.08)	0.64 (0.23)	1.41 (0.16)	0.02 (0.01)	0.21 (0.05)	0.14 (0.01)
		Me : N	1048 (123)	148 (8)	73.4 (13.9)	226 (11)	0.84 (0.42)	24.1 (3.1)	20.3 (7.0)
		Revised Me : C*	125 (14.7)	17.7 (1.0)	8.76 (1.66)	27.0 (1.3)	0.10 (0.05)	2.87 (0.37)	2.42 (0.83)

* revised Me : C obtained by dividing Me : N by the C : N ratio measured under P-replete conditions (7.35 for *Chlorella* and 8.38 for *Microcystis*, Table 3; *see* text).
† Me : C, Me : N and revised Me : C in μmol mol^{-1}; Me : P in mmol mol^{-1}.

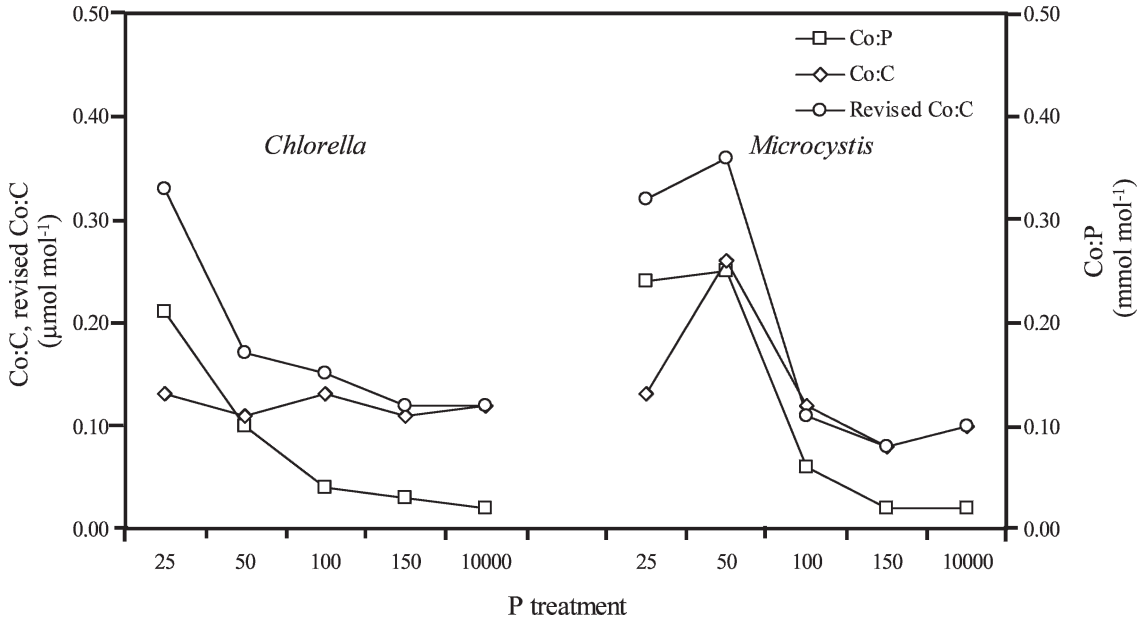

Fig. 3. Cobalt quotas (Co : P, Co : C, and revised Co : C) in *Chlorella* and *Microcystis* under individual P treatments (nmol P L^{-1}), as an example illustrating graphically the effects of various normalization schemes.

is reduced when P availability decreases (even, apparently, between the 150 nmol L^{-1} and the 10,000 nmol L^{-1} treatments, when growth rate was unchanged: Table 3, Fig. 2). Because both cellular C and cellular P appear to be strongly affected by P limitation, we instead use cellular N as an alternative normalization factor (Table 2). The ~3–4-fold increases in cellular N with decreasing P availability represent the increases in cell volume and physiologically active biomass, likely scaling with cellular protein. Presenting metal quotas as Me : N allows us to investigate how metal cofactors for protein change across the P availability gradient, assuming that cellular N is proportional to total protein and that most cellular metals are associated with proteins or polypeptides. However, as Me : C has been the commonly used expression for cellular metal quotas in previous studies (appropriately, since nearly all of these studies were carried out with replete nutrients and therefore approximate Redfield C : N : P ratios) we also present N-corrected Me : C in Table 4 and refer to it as "revised Me : C." The N-corrected Me : C was obtained by dividing Me : N by the C : N ratio measured under P-replete conditions (7.35 for *Chlorella* and 8.38 for *Microcystis*, Table 3) and is assumed to represent normal cellular metal composition neglecting the effect of P limitation on cellular C accumulation. The revised Me : C normalization is thus most appropriate for comparison of metal quotas within this study as well as for comparison to previously reported Me : C quotas. To illustrate graphically the effects of various normalization schemes, we compared Co quotas, which were affected by P limitation for both species, as Co : P, Co : C, and revised Co : C (Fig. 3).

With the decrease of P availability, only specific metals show an increase in cellular quotas, and these combinations of metals differ between the two organisms (Table 4, Fig. 4). The metals Co, Cd, and Zn in *Chlorella* show a progressively increasing ratio to cellular N with reduced phosphate availability; whereas in *Microcystis*, it is Ni and Co that show significant increases. For other elements, cellular quotas are approximately uniform across the four phosphate levels, except for the 25 nmol L^{-1} treatment in *Chlorella*, where we speculate that the exceptionally high quotas of Fe and Mn may be due to anomalous cell breakage as a result of death in some fraction of the cells under the lowest P availability, potentially exposing additional Fe and Mn binding sites. Because Ni in *Chlorella* was also enriched only at this lowest P level, following the pattern for Fe and Mn and in contrast to the progressive enrichments seen for Co, Cd, and Zn, we assume that the 50% Ni enrichment was related to the mechanism driving Fe and Mn enrichment and does not reflect a physiological response to low P availability. This phenomenon needs further investigation, and may be relevant to metal behavior during bloom collapse. To make the comparison of cellular metal responses more apparent, we define the metal quota–enrichment factor as the ratio of metal quotas in the individual P treatment to metal quotas in the 150 nmol L^{-1} treatment (Table 5, Fig. 5). This term thus quantifies the relative changes in cellular metal quotas under varying degrees of P stress below the 150 nmol L^{-1} treatment, making interelement and interspecies comparisons obvious (Fig. 5).

Each species showed clear progressive enrichments for a distinct subset of the metals under increasing P limitation. In *Chlorella*, the Co quota had the largest increase (2.8-fold) under P limitation, followed by Cd (1.8-fold) and Zn (1.7-fold). In *Microcystis*, the Co change again was highest (4.4-fold) and Ni (3.0-fold) was also elevated (Figs. 4, 5). The enrichment occurred to the greatest extent at the

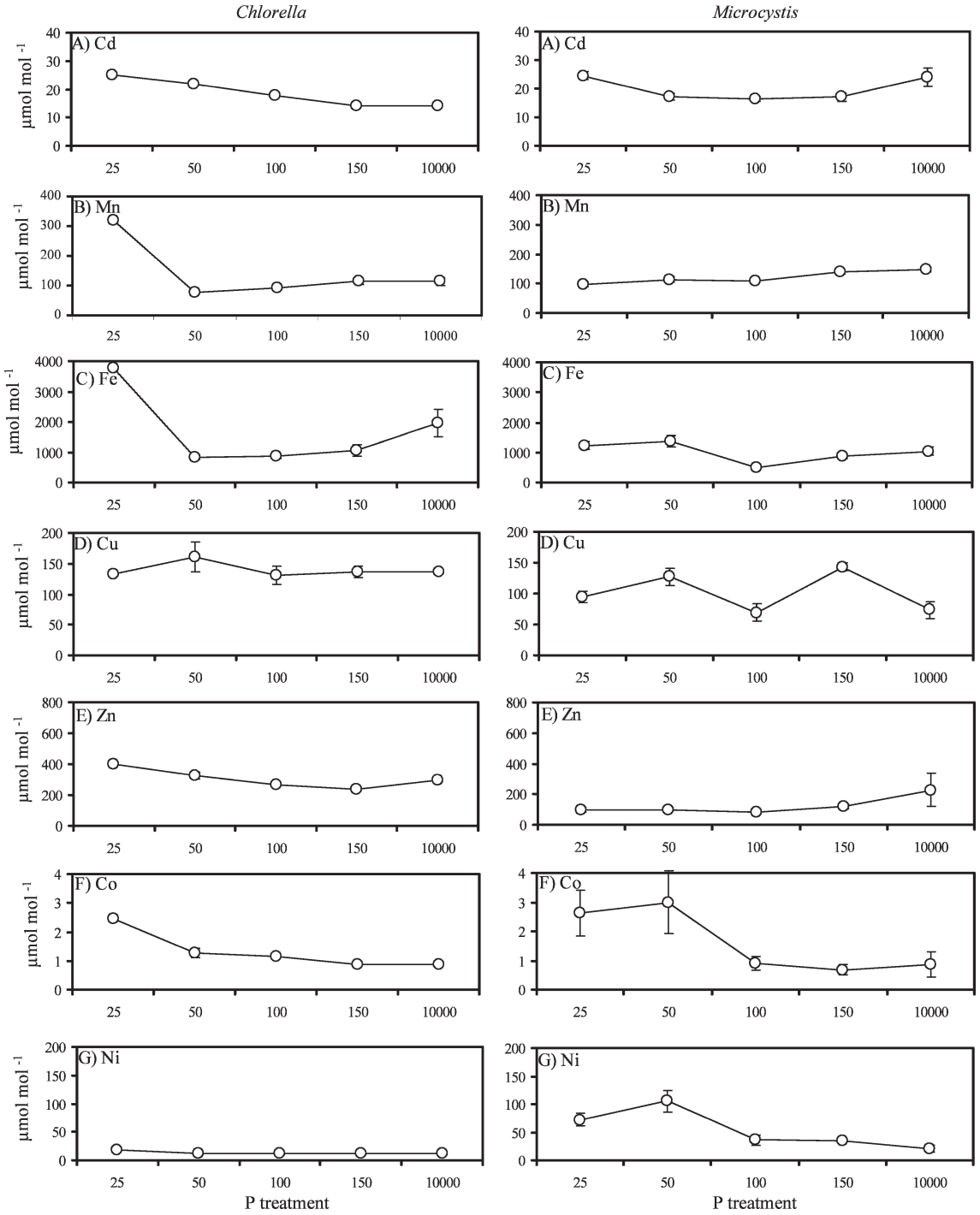

Fig. 4. Metal quotas (Me : N, 10^{-6} mol mol^{-1}) in *Chlorella* and *Microcystis* under individual P treatments (nmol P L^{-1}). Error bars are standard deviations for replicate cultures ($n = 3$). Note x-axis is not linear. (A) Cd, (B) Mn, (C) Fe, (D) Cu, (E) Zn, (F) Co, (G) Ni.

two lowest levels of P treatment (25 nmol L^{-1} and 50 nmol L^{-1}), where organisms were exposed to moderate-to-severe P limitation. Because only certain metals were enriched under P limitation in these two organisms, the enrichment is not readily explained by biodilution (an effect reflecting the inverse relationship between cellular metal quotas and specific cell growth rate at steady state; Sunda and Huntsman 1986), under which the whole suite of metals would be expected to show similar enrichment. In addition, the relative changes in observed metal quotas, normalized by our or any other method (Table 4) do not scale with the relative growth rate changes, either in absolute quantitative terms, or in the relative changes between treatments (Figs. 2, 4).

We also suggest that the metal enrichments are not due to extracellular metal adsorption (a term referring to

Table 5. Enrichment factor of cellular metal quota (Me : N; Table 4) under individual P treatment, normalized to 150 nmol L^{-1}-P treatment.

Organism	Treatment	Fe	Mn	Cu	Zn	Co	Cd	Ni
Chlorella	25 nmol L^{-1}-P	3.58	2.82	0.97	1.72	2.75	1.78	1.50
	50 nmol L^{-1}-P	0.79	0.69	1.18	1.39	1.42	1.55	1.01
	100 nml L^{-1}-P	0.83	0.80	0.96	1.13	1.29	1.26	0.99
	150 nmol L^{-1}-P	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Microcystis	25 nmol L^{-1}-P	1.40	1.01	0.96	0.82	3.85	1.44	2.05
	50 nmol L^{-1}-P	1.54	0.82	0.88	0.84	4.36	1.01	2.98
	100 nml L^{-1}-P	0.58	0.78	0.48	0.71	1.31	0.96	1.02
	150 nmol L^{-1}-P	1.00	1.00	1.00	1.00	1.00	1.00	1.00

noncellular metals that are adsorbed onto extracellular sites and not transported into the cell (Tang and Morel 2006), since by this mechanism we would expect that Fe and Mn would show increased extracellular fractions preferentially to Cd, Co, Ni, and Zn, through formation of hydroxide and oxide phases that could bind to cell surfaces (Morel and Hering 1993; Tang and Morel 2006). Instead, Fe and Mn quotas in both organisms were approximately uniform at the four P levels.

By this reasoning, we propose that the specific metal enrichments reflect increases in the intracellular metal pool, a result of changes in cell physiology in response to severe P limitation. We note, however, that these increases, while relevant to fluxes and cycling important to metal biogeochemistry in the natural environment and triggered by P limitation, may not scale with the immediate physiological requirement for the metals. Cells may strongly upregulate the specific metal-uptake systems, either to increase intracellular stocks for potential future needs, or simply because regulation of metal-uptake rate, while specific to enzymatic elemental requirements, is imprecisely tuned in a quantitative sense to immediate enzymatic requirements.

Mechanisms of metal enrichment under P limitation—The enriched metals under P limitation (i.e., Co, Cd, Zn, and Ni) have Lewis acid properties consistent with their suitability as biologically important metals (Fraústo da Silva and Williams 1991). On the basis of these characteristics, we suggest that the observed specific metal enrichments may be driven by metal requirements for metalloenzymes induced under low P availability. More specifically, since these metals are largely in accord with the potential role of Zn, Co, and Cd in APase as introduced previously, we suggest that their enrichment is related to the increased requirement of these metals to satisfy the upregulated activity of APase under P limitation.

In *Chlorella*, Zn, Cd, and Co are enriched simultaneously under low P availability. While we cannot yet conclude, from the bulk measurement, how this occurs, three possible mechanisms may be postulated. The first is a "metal co-

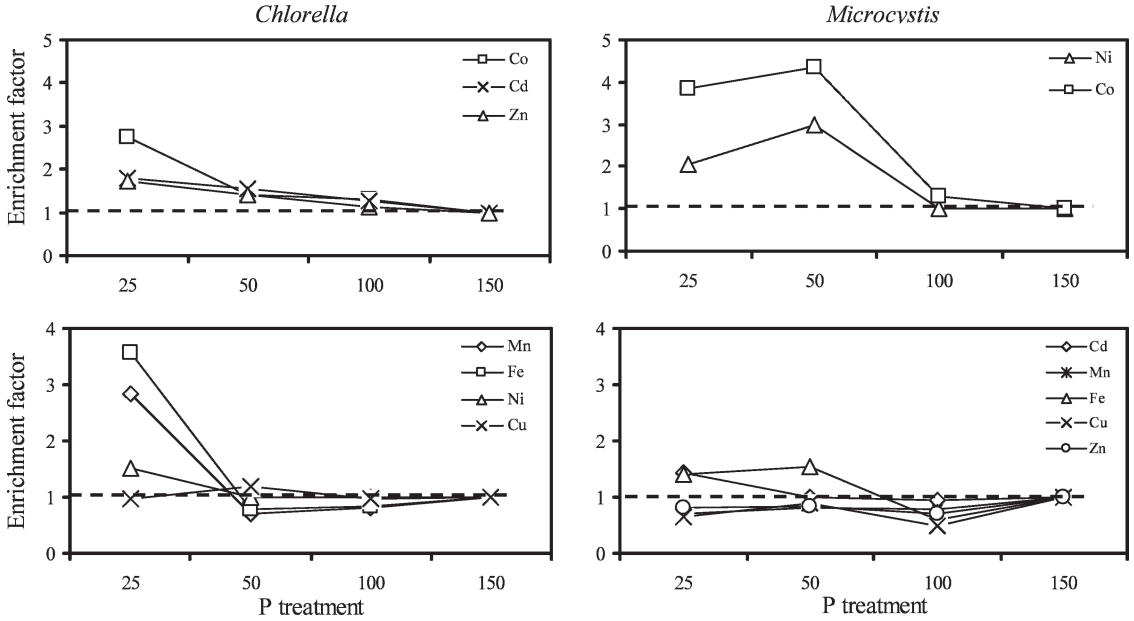

Fig. 5. Cellular metal quota (revised Me : C; *see* text) enrichment factor in *Chlorella* and *Microcystis* under individual P treatments (nmol P L^{-1}), normalized to 150 nmol L^{-1} P treatment, where enrichment factor equals 1.0 by definition. Dashed line indicates level of no enrichment across P treatments. Error bars not plotted for clarity, but uncertainties can be calculated by propagating errors shown in Table 4. Note *x*-axis is not linear.

uptake'' mechanism, under which Co and Cd follow Zn through the up-regulated Zn transporter under P limitation, thereby increasing cellular Co, Cd, and Zn simultaneously. The second is a ''metal substitution'' mechanism. In this process, *Chlorella* might not distinguish strictly among Zn, Co, and Cd for the active center of the induced APase under P limitation, because, in binding and kinetics, their chemical properties are sufficiently similar to afford near-equal activity of the enzyme, regardless of cofactor. The third is the ''multiple APase'' mechanism, i.e., *Chlorella* may produce multiple forms of APase under P stress, each distinct form requiring Zn, Cd, or Co, respectively. It has been suggested that the selected metal for APase is dependent on the specific amino acid residues near the metal-binding positions in the active site. For example, Co-requiring APase has His and Trp at positions 153 and 328 in residues at the active site, while Zn-requiring APase has Asp and Lys at these two positions (Wojciechowski and Kantrowitz 2002; Wojciechowski et al. 2002). For this reason, any changes in the amino acid side chains that are metal-binding ligands at the active site could induce cells to select different metals as cofactors. Although a specific form of Cd-requiring APase has not been reported in living organisms, we cannot rule out the possibility that this form is induced and exhibited in *Chlorella* under P limitation, in analogous fashion to the formation of Cd-carbonic anhydrase under low P_{CO_2} in some diatoms (Cullen et al. 1999; Lane and Morel 2000; Lane et al. 2005).

Unlike the eukaryotic *Chlorella*, the cyanobacterium *Microcystis* exhibits Co and Ni enrichment under P limitation, and its cellular Zn and Cd quotas were approximately uniform across the four phosphate levels (Fig. 5). Because Ni has never been reported to be a cofactor for APase, we suggest that its enrichment under P stress in *Microcystis* may result from another mechanism, discussed below. Therefore, it appears that *Microcystis*, as a representative cyanobacterium, may contain APase that depends only on Co but not on Zn and Cd. This metal specificity in *Microcystis* is consistent with the evolutionary history of cyanobacteria, which arose initially in an anoxic ocean where Co was abundant but availability of free Zn and Cd was low (Saito et al. 2003). Because the rate of gene mutation is very low, the physiology and genomes of modern descendants would be similar to those of their cyanobacteria ancestors. Modern cyanobacteria would retain the requirements for key elements inherited from their ancient origins, despite their continued evolution through the more recent oxygenated ocean and the resultant drastic changes in the bioavailability of metals (Fraústo da Silva and Williams 1991, 2001). Cyanobacteria might have undergone more severe P limitation during early evolutionary stages in the Archaean and early Proterozoic (3.2 Ga to 1.9 Ga), since it has been suggested that ocean phosphate concentrations during this period were probably only 10–25% of present (Bjerrum and Canfield 2002). Under these conditions, ancient cyanobacteria were probably using Co rather than Zn and Cd for constructing APase anaerobically. It is now known that the important marine cyanobacteria *Synechococcus* and *Prochlorococcus* both have absolute Co requirements (Sunda and Huntsman 1995; Saito et al. 2002). Investigations of bulk particulate metals in the Sargasso Sea euphotic zone, where P is severely limited (Wu et al. 2000) and cyanobacteria (*Synechococcus* and *Prochlorococcus*) dominate the primary producer assemblage (Goericke and Welschmeyer 1993), indicate that cellular Co : C in the natural plankton assemblage is enriched 4-fold relative to Co : C in lab-based culture of phytoplankton grown under similar Zn′ and Co′ but high P conditions (Sherrell and Boyle 1992; Sunda and Huntsman 1995). In addition, shipboard incubation studies in this region have revealed that only Co amendment, not additions of Zn and Cd, led to increased cell biomass (Chl *a*) and APase activity (Wisniewski 2006). These results are consistent with the notion of a generalized Co-P connection in prokaryotes.

The enrichment of cellular Ni under P limitation in *Microcystis* is surprising since no Ni function has been reported for APase. We speculate that enhanced Ni uptake is related to increased production of superoxide dismutase (SOD), induced by P limitation. The depletion of P leads to a decrease in the rate of protein synthesis, which in turn may have effects on cellular metabolism and oxidative stress similar to those for inhibition of protein synthesis by N limitation (Bucciarelli and Sunda 2003). If true, the induced SOD in *Microcystis* is probably dependent on Ni as a cofactor, as has been shown in other cyanobacteria (Wolfe-Simon et al. 2005). An alternative explanation for the Ni enrichment is a potential link to enhanced Co uptake through the upregulation of one of several candidate Ni/Co cotransporters identified in prokaryotic genomes (Rodionov et al. 2006). Enhanced Ni uptake under nutrient stress needs further study to reveal and distinguish potential mechanisms.

Implications of this study—Because phosphorus limitation occurs broadly in freshwater and marine systems, our study has implications for the biochemical functions of metals in phytoplankton as well as for the resultant effects on metal biogeochemical cycling, applicable to primary producer assemblages in a variety of natural environments.

Biochemical functions of metals in phytoplankton—The vertical distribution profile of soluble Co and Cd in the ocean is analogous to that of nutrients (Bruland et al. 1978; Saito and Moffett 2002), which results from the uptake of Co and Cd into phytoplankton in ocean surface waters and the subsequent sinking and remineralization at depth. However, we know little about the physiological functions of Co and Cd. The only known absolute requirement for Co in phytoplankton is as a cofactor for vitamin B_{12} (cobalamin; Swift 1981), and the only known absolute requirement for Cd is a unique Cd-carbonic anhydrase (CAase; Cullen et al. 1999; Lane and Morel 2000; Lane et al. 2005). However, it has been estimated that cellular Co and Cd quotas greatly exceed the amounts stoichiometrically required in these enzymes (Fraústo da Silva and Williams 1991; Cullen and Sherrell 2005). This imbalance implies that there may be additional biochemical functions or storage sites for Co and Cd. Our proposed involvement

of Co and/or Cd in P metabolism (APase) constitutes an additional function that can account for maintenance of these metals in the cellular pool and might help to explain the observed variations in total cellular Co and Cd.

Metal cycling in P-limited oligotrophic regions—Our work further implies that it is necessary to consider the effect of P limitation on cellular metal quotas when exploring metal biogeochemistry in natural aquatic systems. For example, in Lake Superior, we observed that the apparent cellular Zn quota (Zn : C) in the natural phytoplankton assemblage is six to eight times higher than the Zn quota obtained from P-replete cultures of representative phytoplankton (Ji 2006). This higher cellular Zn quota leads to the rapid drawdown of soluble Zn and drives intensive Zn recycling in Lake Superior (Sherrell unpubl.). Severe P limitation seems to have a partial role in driving this 6–8-fold enriched Zn in Lake Superior phytoplankton, because P amendment has been observed to drive down Zn : C in the ambient plankton assemblage by a factor of ≤ 3 (Ji 2006).

The effect of P limitation on cellular metal quotas finds further evidence in the tropical Atlantic and Sargasso Sea, where phosphate concentrations are in the low nanomolar range. In the tropical Atlantic, Saito and Moffett (2002) found that the Co uptake relative to P uptake (inferred from the ΔCo : ΔP slope in the upper water column) was more than an order of magnitude higher than in the northeast Pacific where phosphate concentration is in the micromolar range (ΔCo : ΔP = 560 μmol mol^{-1} in the tropical Atlantic vs. $\sim$40 μmol mol^{-1} in the northeast Pacific). In the subtropical North Atlantic, the particulate Co : C ratio in euphotic-zone suspended particles was 1.1–1.5 μmol mol^{-1} (Sherrell and Boyle 1992; Kuss and Kremling 1999). Since Co incorporation into particulate matter in the Sargasso Sea is dominated by phytoplankton uptake rather than by Mn oxidation (Moffett and Ho 1996; Saito and Moffett 2002), bulk particulate Co : C can be interpreted as cellular Co quota for the extant assemblage. Compared to the cellular Co : C of 0.4 μmol mol^{-1} in laboratory culture of *E. huxleyi* under 20 pmol L^{-1} Zn$'$ and 2 pmol L^{-1} Co$'$ (the estimated Zn$'$ and Co$'$ in the ambient Sargasso Sea) but under replete P culture conditions (Sunda and Huntsman 1995), the Co : C in the Sargasso Sea plankton assemblage is enriched $\sim$4-fold. Based on results obtained for freshwater phytoplankton in this study, we hypothesize that the high Co demand in the tropical Atlantic and Sargasso Sea results from persistent P limitation in these regions. In addition, since Co is the only element that shows enrichment in our two species, Co may be enriched in a P-starved plankton assemblage whether eukaryotes or prokaryotes dominate. As a consequence, Co biogeochemistry in low P oligotrophic waters may be influenced strongly by a P limitation effect.

Finally, our study implies a more complex picture of Cd biogeochemistry in P-limited regions that previously envisioned. The biogeochemistry of Cd is of considerable interest, since its broad distribution in the ocean mimics that of PO_4^{3-} (Boyle et al. 1976; Bruland et al. 1978). The utility of Cd as a paleonutrient proxy for ocean surface waters, for example in fossil planktonic foraminifera (Elderfield and Rickaby 2000) requires that we fully understand the processes that control the relative biogeochemical cycling of Cd and PO_4^{3-}. At present, the factors controlling the uptake of Cd relative to PO_4^{3-} and the resulting Cd : P ratio in sinking material are incompletely described. Our study suggests that links between Cd and PO_4^{3-} may depend not only on Zn and CO_2 availability (Cullen and Sherrell 2005) but may be further complicated by increased Cd uptake, in at least some species, when P availability is limited.

References

Ammerman, J. W. 1993. Microbial cycling of inorganic and organic phosphorus in the water column, p. 649–660. *In* B. Kempot [ed.], Handbook of methods in aquatic microbial ecology. Lewis.

Anagnostou, E. 2005. A novel method for detecting nanomolar levels of orthophosphate in freshwater systems: Implications for phosphorus cycling in Lake Superior. M.S. thesis. Rutgers University.

———, and R. M. Sherrell. 2008. A MAGIC method for subnanomolar orthophosphate determination in freshwater. Limnol. Oceanogr.: Methods **6:** 64–74.

Applebury, M., and J. Coleman. 1968. *Escherichia coli* Co(II) alkaline phosphatase. J. Biol. Chem. **244:** 709–718.

Bertilsson, S., O. Berglund, D. M. Karl, and S. W. Chisholm. 2003. Elemental composition of marine *Prochlorococcus* and *Synechococcus*: Implications for the ecological stoichiometry of the sea. Limnol. Oceanogr. **48:** 1721–1731.

Bjerrum, C. J., and D. E. Canfield. 2002. Ocean productivity before about 1.9 Gyr ago limited by phosphorus adsorption onto iron oxides. Nature **417:** 159–162.

Boyle, E. A., F. Sclater, and J. M. Edmond. 1976. On the marine geochemistry of cadmium. Nature **263:** 42–44.

Bruland, K. W., G. A. Knauer, and J. H. Martin. 1978. Cadmium in northeast Pacific waters. Limnol. Oceanogr. **23:** 618–625.

Bucciarelli, E., and W. G. Sunda. 2003. Influence of CO_2, nitrate, phosphate, and silicate limitation on intracellular dimethylsulfoniopropionate in batch cultures of the coastal diatom *Thalassiosira pseudonana*. Limnol. Oceanogr. **48:** 2256–2265.

Bush, R. M., and E. G. Welch. 1972. Plankton associations and related factors in a hypereutrophic lake. Water Air Soil Pollut. **1:** 257–274.

Cembella, A. D., N. J. Antia, and P. J. Harrison. 1984. The utilization of inorganic and organic phosphorus compounds as nutrients by eukaryotic microalgae: A multidisciplinary perspective: Part 1. Crit. Rev. Microbiol. **10:** 317–391.

Cotner, J. B., J. W. Ammerman, E. R. Peele, and E. Bentzen. 1997. Phosphorus limited bacterioplankton growth in the Sargasso Sea. Aquat. Microb. Ecol. **13:** 141–149.

Cullen, J. T., P. M. Field, and R. M. Sherrell. 2001. Determination of trace elements in filtered suspended marine particulate material by sector HR-ICP-MS. J. Anal. At. Spectrom. **16:** 1307–1312.

———, T. W. Lane, F. M. M. Morel, and R. M. Sherrell. 1999. Modulation of Cd uptake in phytoplankton by seawater CO_2 concentration. Nature **402:** 165–167.

———, AND R. M. SHERRELL. 1999. Techniques for determination of trace metals in small samples of size-fractioned particulate matter: phytoplankton metals off Central California. Mar. Chem. **67:** 233–247.

———, AND R. M. SHERRELL. 2005. Effects of dissolved CO_2, Zn, and Mn on the Cd : P ratio in natural phytoplankton assemblages. Limnol. Oceanogr. **50:** 1193–1204.

DONAT, J. R., AND K. W. BRULAND. 1994. Trace elements in the ocean, p. 247–281. *In* B. Salbu and E. Steinnes [eds.], Trace elements in natural waters. CRC Press.

DYHRMAN, S. T., P. D. CHAPPELL, S. T. HALEY, J. W. MOFFETT, E. D. ORCHARD, J. B. WATERBURY, AND E. A. WEBB. 2006. Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. Nature **439:** 68–71.

ELDERFIELD, H., AND R. E. M. RICKABY. 2000. Oceanic Cd/P ratio and nutrient utilization in the glacial Southern Ocean. Nature **405:** 305–310.

FALKOWSKI, P. G., M. E. KATZ, A. H. KNOLL, A. QUIGG, J. A. RAVEN, O. SCHOFIELD, AND F. J. R. TAYLOR. 2004. The evolution of modern eukaryotic phytoplankton. Science **305:** 354–360.

———, AND J. A. RAVEN. 1997. Aquatic photosynthesis. Blackwell Science.

FRAÚSTO DA SILVA, J. J. R., AND R. J. P. WILLIAMS. 1991. The biological chemistry of the elements: The inorganic chemistry of life. Oxford.

———, AND ———. 2001. The biological chemistry of the elements: The inorganic chemistry of life. 2nd ed. Oxford.

GEIDER, J. R., AND J. LAROCHE. 2002. Redfield revisited: Variability of C : N : P in marine microalgae and its biochemical basis, Eur. J. Phycol. **37:** 1–17.

GETTINS, P., AND J. E. COLEMAN. 1982. ^{113}Cd NMR of Cd(II)-substituted Zn(II) metalloenzymes. Fed. Proc. **41:** 2966–2973.

GOERICKE, R., AND N. A. WELSCHMEYER. 1993. The marine prochlorophyte *Prochlorococcus* contributes significantly to biomass and primary production in the Sargasso Sea. Deep-Sea Res. **40:** 2283–2294.

HAMMER, V. T. 1964. The succession of ''bloom'' species of blue-green algae and some causal factors. Verh. Internal. Verein. Limnol. **15:** 829–836.

HULETT, M. F., E. E. KIM, C. BOOKSTEIN, N. V. KAPP, C. W. EDWARDS, AND H. W. WYCKOFF. 1991. *Bacillus subtilis* alkaline phosphatases III and IV. Cloning, sequencing, and comparisons of deduced amino acid sequence with *Escherichia coli* alkaline phosphatase three-dimensional structure. J. Biol. Chem. **266:** 1077–1084.

JI, Y. C. 2006. Effects of phosphorus availability on cellular metals in phytoplankton: Lake Superior as a model system. Ph.D. thesis. Rutgers University.

KARL, D. M. 1997. Temporal variability in dissolved P concentration in the subtropical North Pacific Ocean. Mar. Chem. **56:** 77–96.

———, AND K. M. BJORKMAN. 2002. Dynamics of DOP, p. 246–366. *In* D. Hansell and C. Carlson [eds.], Biogeochemistry of marine organic matter. Elsevier Science.

KIM, E. E., AND H. W. WYCKOFF. 1991. Reaction mechanism of alkaline phosphatase based on crystal structures: Two-metal ion catalysis. J. Mol. Biol. **218:** 449–464.

KROM, M. D., N. KRESS, S. BRENNE, AND L. I. GORDON. 1991. Phosphorus limitation of primary productivity in the Eastern Mediterranean Sea. Limnol. Oceanogr. **36:** 424–432.

KUSS, J., AND K. KREMLING. 1999. Spatial variability of particle associated trace elements in near-surface waters of the North Atlantic (30°N,60°W to 60°N,2°W), derived by large volume sampling. Mar. Chem. **68:** 71–86.

LANE, T. W., AND F. M. M. MOREL. 2000. A biological function for Cd in marine diatoms. Proc. Natl. Acad. Sci. U.S.A. **97:** 4627–4631.

———, M. A. SAITO, G. N. GEORGE, I. J. PICKERING, R. C. PRINCE, AND F. M. M. MOREL. 2005. Biochemistry: A cadmium enzyme from a marine diatom. Nature **435:** 42.

LEE, J. G., AND F. M. M. MOREL. 1995. Replacement of Zn by Cd in marine phytoplankton. Mar. Ecol. Prog. Ser. **127:** 305–309.

MOFFETT, J. W., AND J. HO. 1996. Oxidation of cobalt and manganese in seawater via a common microbially catalized pathway. Geochim. Cosmochim. Acta **60:** 3415–3424.

MOREL, F. M. M., AND J. G. HERING. 1993. Principles and applications of aquatic chemistry. Wiley.

MUNAWAR, M., AND I. F. MUNAWAR. 1982. Phycological studies in Lakes Ontario, Erie, Huron, and Superior. Can. J. Bot. **60:** 1837–1858.

NAUSCH, M., AND N. G. WASMUND. 2004. Phosphorus dynamics during the transition from nitrogen to phosphate limitation in the central Baltic Sea. Mar. Ecol. Prog. Ser. **266:** 15–25.

NRIAGU, J. O., G. LAWSON, H. K. T. WONG, AND V. CHEAM. 1996. Dissolved trace metals in Lake Superior, Erie, and Ontario. Environ. Sci. Technol. **30:** 178–187.

PRICE, N. M., AND F. M. M. MOREL. 1990. Cadmium and cobalt substitution for zinc in a marine diatom. Nature **344:** 658–660.

RODIONOV, D. A., P. HEBBELN, M. S. GELFAND, AND T. EITINGER. 2006. Comparative and functional genomic analysis of prokaryotic nickel and cobalt uptake transporters: evidence for a novel group of ATP-binding cassette transporters. J. Bacteriol. **188:** 317–327.

SAITO, M. A., AND J. W. MOFFETT. 2002. Temporal and spatial variability of cobalt in the Atlantic Ocean. Geochim. Cosmochim. Acta **66:** 1943–1953.

———, J. W. MOFFETT, S. W. CHISHOLM, AND J. B. WATERBURY. 2002. Cobalt limitation and uptake in *Prochlorococcus*. Limnol. Oceanogr. **47:** 1629–1636.

———, D. M. SIGMAN, AND F. M. M. MOREL. 2003. The bioinorganic chemistry of the ancient ocean: The co-evolution of cyanobacterial metal requirements and biogeochemical cycles at the Archean-Proterozoic boundary. Inorg. Chim. Acta **356:** 308–318.

SHERRELL, R. M., AND E. BOYLE. 1992. The trace metal composition of suspended particles in the oceanic water column near Bermuda. Earth Planet. Sci. Lett. **111:** 155–174.

SIMPSON, R. T., AND B. L. VALLEE. 1969. Zinc and cobalt alkaline phosphatases. Ann. N.Y. Acad. Sci. **166:** 670–695.

STERNER, R. W., T. M. SMUTKA, R. M. L. MCKAY, X. QIN, E. T. BROWN, AND R. M. SHERRELL. 2004. Phosphorus and trace metal limitation of algae and bacteria in Lake Superior. Limnol. Oceanogr. **49:** 495–507.

SUNDA, W. G., AND S. HUNTSMAN. 1986. Relationships among growth rate, cellular manganese concentrations and manganese transport kinetics in estuarine and oceanic species of the diatom *Thalassiosira*. J. Phycol. **22:** 259–270.

———, AND ———. 1995. Co and Zn interreplacement in marine phytoplankton: Biological and geochemical implications. Limnol. Oceanogr. **40:** 1404–1417.

SWIFT, D. G. 1981. Vitamin levels in the Gulf of Maine and ecological significance of vitamin B12 there. J. Mar. Res. **39:** 375–403.

TANG, D., AND F. M. M. MOREL. 2006. Distinguishing between cellular and Fe-oxide–associated trace elements in phytoplankton. Mar. Chem. **98:** 18–30.

WANG, W.-X., R. C. H. DEI, AND Y. XU. 2001. The responses of Zn assimilation by coastal plankton to macronutrients. Limnol. Oceanogr. **46:** 1524–1534.

WEILER, R. R. 1978. Chemistry of Lake Superior. J. Great Lakes Res. **4:** 370–385.

WISNIEWSKI, R. 2006. Relating the biogeochemistries of zinc, cobalt, and phosphorus to phytoplankton activities in the Sea. Ph.D. thesis. MIT/Woods Hole Oceanographic Institution.

WOJCIECHOWSKI, C. L., J. P. CARDIA, AND E. R. KANTROWITZ. 2002. Alkaline phosphatase from the hyperthermophilic bacterium *T. maritima* requires cobalt for activity. Protein Sci. **11:** 903–911.

———, AND E. R. KANTROWITZ. 2002. Altering of the metal specificity of *Escherichia coli* alkaline phosphatase. J. Biol. Chem. **277:** 50476–50481.

WOLFE-SIMON, F., D. GRZEBYK, O. SCHOFIELD, AND P. G. FALKOWSKI. 2005. The role and evolution of superoxide dismutase in algae. J. Phycol. **41:** 453–465.

WU, J., W. G. SUNDA, E. A. BOYLE, AND D. M. KARL. 2000. Phosphate depletion in the western North Atlantic Ocean. Science **289:** 759–762.

YU, R. Q., AND W. X. WANG. 2004. Biological uptake of Cd, Se(IV), and Zn by *Chlamydomonas reinhardtii* in response to different phosphate and nitrate additions. Aquat. Microb. Ecol. **35:** 163–173.

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