

Diel variation of molybdenum and iron in marine diazotrophic cyanobacteria

Caroline Tuit¹

MIT/Woods Hole Oceanographic Institution Joint Program in Oceanography, Woods Hole, Massachusetts 02543

John Waterbury

Department of Biological Oceanography, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

Gregory Ravizza²

Department of Marine Geology and Geophysics, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

Abstract

Measurements of Mo:C and Fe:C ratios in cultured cells of two N₂-fixing cyanobacteria, *Crocospaera watsonii* strain WH8501 and *Trichodesmium erythraeum* strain IMS101, agree with estimated metal:carbon ratios based on growth rate and the metal use efficiency of the nitrogenase enzyme. *Crocospaera*, a single-celled nocturnal N₂ fixer, showed two- to eightfold increases in Mo and Fe cellular concentrations in response to nitrogen fixation activity. Mo required for N₂ assimilation can account for almost the entire Mo pool measured in the cells, implying that *Crocospaera* synthesizes its entire nitrogenase pool de novo each night. In contrast, cultures of *Trichodesmium*, a filamentous, diurnal N₂-fixing cyanobacterium, did not show diel variations in Mo or Fe carbon ratios or in cellular metal concentrations. *Trichodesmium* appears to maintain an internal pool of Mo. In *Trichodesmium* cultures, Mo concentrations were up to 30% higher than needed to support measured N₂ fixation. *Trichodesmium* colonies collected from the field had Mo:C ratios 10-fold larger than those measured in culture, far in excess of what is needed to fix N₂ at rates normally measured in the field, despite equivalent Fe:C ratios (66 ± 39 [field samples] and 87 ± 64 [cultures] $\mu\text{mol mol}^{-1}$). The average Fe:C ratio measured in N₂-fixing *Crocospaera* ($16 \pm 11 \mu\text{mol mol}^{-1}$) was equivalent to theoretical estimates of Fe demand based on nitrogenase requirements ($13 \pm 5 \mu\text{mol mol}^{-1}$). These results demonstrate the extremely efficient use of Fe by these organisms and provide support for the use of theoretical estimates of Fe:C ratios to calculate biological Fe demand for N₂ fixation.

Nitrogen fixation, the conversion of N₂ gas into bioavailable forms, is an important source of nitrogen for primary production in the oligotrophic ocean (Capone et al. 1997; Karl et al. 1997). Nitrogen fixation is catalyzed by the nitrogenase complex, a metal-rich enzyme containing 2 mol molybdenum (Mo) and 38–50 mol iron (Fe) mol⁻¹ complex. Recent studies have focused on Fe as a potentially limiting nutrient for both photoautotrophic growth and N₂ fixation in the marine environment (Sunda and Huntsman 1995; Berman-Frank et al. 2001a; Kustka et al. 2002). Although there is an ongoing debate whether the high levels of sulfate (SO₄²⁻) in seawater can competitively inhibit molybdate

(MoO₄²⁻: the predominant form of Mo in seawater) uptake by nitrogen-fixing cyanobacteria (Howarth and Cole 1985), N₂ fixation in the oligotrophic ocean appears unaffected by this competition (Paulsen et al. 1991). Because Mo availability is unlikely to be limiting, its role in marine nitrogen fixation has largely been ignored. To our knowledge, there are currently no published data regarding the Mo content of marine diazotrophs and very few estimates of Mo requirements (Sprent and Raven 1985). We report the results of Mo and Fe analyses of marine cyanobacteria grown exponentially in axenic cultures and collected in the open ocean. These data are used to (1) document changes in cellular metal concentration over diel cycles, and (2) estimate the fraction of cellular Mo and Fe inventories that are bound in the nitrogenase complex. The Mo data were collected as part of a larger effort to evaluate the potential for using Mo concentrations in suspended particulate matter to estimate water column nitrogen fixation rates.

Two cyanobacterial strains were studied, *Crocospaera watsonii* strain WH8501, a single-celled nocturnal diazotroph, and *Trichodesmium erythraeum* strain IMS101, a non-heterocystous, filamentous, diurnal diazotroph that fixes N₂ maximally at the midpoint of the light period, concurrently with photosynthesis. *Trichodesmium* may protect its nitrogenase enzyme from inactivation by photosynthetically derived oxygen by spatially separating N₂ fixation and photosynthesis (Berman-Frank et al. 2001b). In contrast, *Crocospaera* temporally separates photosynthesis and N₂ fixation, fixing N₂ only in the dark. A comparison of the diel

¹ To whom correspondence should be addressed. Present address: Department of Geosciences, Princeton University, Princeton, New Jersey 08544 (ctuit@princeton.edu).

² Present address: Department of Geology & Geophysics, SOEST, University of Hawaii, Manoa, 1680 East-West Road, Honolulu, Hawaii 96822.

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patterns of Mo and Fe cellular concentrations in *Crocospaera* and *Trichodesmium* provides an opportunity to investigate how the different *N*₂ fixation strategies employed by these two marine diazotrophs influence metal cycling.

Trichodesmium has been extensively studied, primarily using field populations, since it was hypothesized to fix *N*₂ (Dugdale et al. 1961). This fragile, difficult-to-quantify organism is found both as single trichomes and macroscopic colonies (0.5–5 mm) that can form large-scale blooms abundant enough to be visible from space (Hood et al. 2002).

Recent research has suggested that single-celled diazotrophic cyanobacteria, such as *Crocospaera*, are potentially significant sources of fixed *N* (Zehr et al. 2000, 2001). There is evidence that unicellular *N*₂-fixing cyanobacteria are widely distributed in the marine environment, with concentrations as high as 10³ cells ml⁻¹ (Neveux et al. 1999; Wasmund et al. 2001). Zehr et al. (2001) showed that, at the HOT station, there are multiple strains of single-celled diazotrophic cyanobacteria (2.0- to 10- μ m size class), several closely related to *Crocospaera* WH8501, which was isolated from the western tropical Atlantic Ocean. They observed maximal levels of nitrogenase messenger RNA, an indicator of enzyme biosynthesis, at night. A similar pattern was observed for nitrogenase activity in isolates, suggesting that nocturnal *N*₂ fixation predominated. The ¹⁵*N*₂ fixation rates were consistent with those measured for *Crocospaera* WH8501 grown on *N*₂ in laboratory culture. By measuring Mo and Fe in *Crocospaera*, we expand our knowledge of a potentially important but otherwise underinvestigated marine diazotroph.

Methods and materials

Bacterial strains—*T. erythraeum* strain IMS101 is the most extensively studied *Trichodesmium* species (Capone et al. 1997; Mulholland and Capone 2000; Berman-Frank et al. 2001a). *C. watsonii* strain WH8501 has been previously described as *Synechocystis* sp. strain WH8501 (Waterbury and Rippka 1989; Rippka et al. 2001; Zehr et al. 2001). Both species were maintained in axenic culture at the Woods Hole Oceanographic Institution and isolated as described elsewhere (Waterbury and Rippka 1989; Paerl et al. 1994). All cultures were verified as axenic by direct microscopic observation and by lack of heterotrophic growth in a marine broth medium as described by Waterbury et al. (1986).

Culture conditions—Stock cultures of *Crocospaera* and *Trichodesmium* were maintained on a 75% Sargasso seawater-based medium. Sargasso seawater, collected on cruises of opportunity and aged in the dark, was successively filtered through 1.0- and 0.22- μ m Millipore membrane filters and then “sterilized” by heating in a microwave oven to boiling in Teflon bottles (Keller et al. 1988). Sargasso seawater was diluted to 75% strength with sterile Milli-Q water. The medium was amended with nutrients and trace metals (Table 1). All cultures were Fe replete, with a final log inorganic iron concentration (log [Fe']) of -7.5. The final Mo concentration was 97 nmol L⁻¹, which is within 10% of the average seawater concentration of 107 nmol L⁻¹. All nutrient and trace metals were purchased from Sigma Chemical. Nu-

Table 1. Culture medium.

	Final concentration (mol L ⁻¹)
Nutrients	
Phosphoric acid	8×10 ⁻⁶
EDTA*	5×10 ⁻⁷
Fe(III)citrate	5×10 ⁻⁸
Citric acid	1×10 ⁻⁵
Trace metals	
MnSO ₄	1×10 ⁻⁷
ZnCl ₂	1×10 ⁻⁸
NaMoO ₄	1×10 ⁻⁸
CoCl ₂	1×10 ⁻¹⁰
NiCl ₂	1×10 ⁻¹⁰
NaSeO ₃	1×10 ⁻¹⁰

* EDTA, ethylenediamine-tetraacetic acid.

trient and trace metal stocks were sterilized either by filtration or autoclaving before being added to the 75% Sargasso seawater base. Stock cultures were grown in polycarbonate (Nalgene) culture bottles that were cleaned in Micro (International Products), then rinsed repeatedly in tap water, followed by several rinses in Milli-Q water. The bottles were then soaked for at least 5 d in 0.5 N trace metal grade HCl and finally rinsed repeatedly with Milli-Q water before use. Cultures were grown on a 14:10 light:dark (LD) cycle using cool white fluorescent lamps at 30 μ mol quanta m⁻² s⁻¹ at a temperature of 28°C. *Trichodesmium* stock cultures were gently stirred continuously, while *Crocospaera* cultures were shaken gently daily to resuspend cells.

Experimental conditions—Growth experiments were conducted in 1.5-liter Nalgene polycarbonate culture chambers with an internally suspended magnetic stir bar. The culture vessel was cleaned with Micro and 0.5 N trace metal grade HCl as described above. As with stock cultures, cells were incubated at 28°C on a 14:10 LD cycle. Cells were grown as batch cultures to late log phase. The *Crocospaera* growth rate was monitored by cell counts using epifluorescent illumination. The *Trichodesmium* cell growth rate was determined by exponential increase in the total filament length per unit volume. Trichome length was measured on at least 30 video fields for 0.5–5 ml of culture and converted to cell counts by a conversion factor of 4.8 cells cm⁻¹ trichome at ×10 magnification. This was possible because the culture grew as single trichomes throughout the experiment rather than forming colonies. Cell counts for both species were determined once a day at the time of maximum *N*₂ fixation and whenever subsamples for acetylene reduction or metal analysis were collected.

Nitrogen fixation rates—Nitrogen fixation rates were measured by the acetylene reduction method (Capone and Montoya 2001) using a ratio of 4:1 for the conversion of acetylene reduction to *N*₂ reduction. Between 10- and 20-ml aliquots of culture were sealed into Micro and 0.5 N trace metal grade HCl cleaned 60-ml Nalgene polycarbonate bot-

tles fitted with Teflon-coated silicone septa. Six milliliters of headspace was removed and replaced with 6 ml acetylene generated from carbide. These subsamples were incubated for 1.5–2 h at growth irradiance and temperature. Ethylene production was measured on a Shimadzu GC-8 gas chromatograph with a Poropak N column and a flame ionization detector and quantified relative to an ethylene standard. Results are reported as nmol N₂ reduced min⁻¹ cell⁻¹.

Fe and Mo cellular concentrations—Fe and Mo concentrations were measured in three *Crocospaera* and two *Trichodesmium* experiments. Cell samples were taken over two to three diel cycles at times of maximum and minimum N₂ fixation as determined by simultaneous acetylene reduction rate measurements. Additionally, samples of field populations of *T. erythraeum* and *Trichodesmium thiebautii* were collected on two cruises in the Atlantic, one at the Bermuda Atlantic Time Series (BATS) site in September 1999 and the other from a survey of the subtropical Atlantic on the RV *Seward Johnson* in May 2001. Colonies in the upper 10 m were collected via trace metal clean plankton tow and hand-picked into sterile Sargasso seawater to rinse and reduce carryover of contaminants. Both *Crocospaera* and *Trichodesmium* cultures and *Trichodesmium* field samples were collected by gently (<5 cm Hg vacuum) filtering a known volume of culture or, in the case of field samples, a known number of colonies onto acid-cleaned (10% Seastar HCl at 60°C overnight, rinsed in Milli-Q and stored in a pH 2 Seastar HCl solution) 47-mm polycarbonate membrane filters (Osmotics) using an acid-cleaned Teflon filter holder. *Crocospaera* was collected using a 0.2- μ m pore-size filter, while the larger size of *Trichodesmium* allowed the use of a 5.0- μ m filter. Prior to use, filters were rinsed in the filter holder with sterile seawater or media to neutralize the filter. Filters were then frozen at -20°C for transport and later analysis.

Filter digestion and metal analyses are described in detail in Tuit (2003). Briefly, metal samples were treated by refluxing with 950 μ l HNO₃ and 50 μ l HF at 120°C for 4 h in a closed Teflon vial to dissolve particulate matter and cells (Cullen and Sherrell 1999). Leachate solutions were diluted 10-fold and stored for analysis. Stored solutions were analyzed on the WHOI Finnigan Element ICP-MS. Fe and Mo concentrations in all samples were determined via isotope dilution (ID). For field samples of picked *Trichodesmium* colonies, Fe, Mo, Al, Rb, and Mn were also determined via standard addition (SA). ID analyses of Mo and Fe had better precision and were used for all calculations. Analyses of Mn, Al, and Rb in the field samples allowed the evaluation of possible additions of Mo and Fe from crustal sources (Al), Mn oxides, and seawater salts (Rb), assuming average mass ratios to Fe and Mo in these sources. Al, Rb, and Mn were not measured in cultures because dust contamination and Mn oxide formation were assumed to be minimal. An Fe:Al ratio of 0.841 g g⁻¹ and a Mo:Al ratio of 23.8 μ g g⁻¹ were used to estimate crustal contributions (Taylor and McLennan 1985). Seawater-dissolved Fe concentrations were assumed to be negligible, but a Mo:Rb ratio of 0.093 g g⁻¹ was used to make a sea salt correction (Spencer et al. 1970). Mn concentrations in the colonies were below detection limits. Con-

tributions from crustal sources were negligible for Mo, and sea salt Mo corrections averaged 1%. Crustal contributions averaged 10% for Fe. This does not account for extracellular authigenic Fe(OH)₃, which may also contribute to the Fe measurements.

Procedural blanks that took into account possible Mo and Fe metal contributions from culture media or seawater and filter apparatus were measured for both cultured and field samples. For *Crocospaera*, Mo media blanks averaged 10% of total analyte (ng Mo filter⁻¹) but were as high as 60% for non-N₂-fixing samples where cellular metal concentrations were very low. Similarly, the Fe media blank for *Crocospaera* averaged 15% but reached as high as 40% for non-N₂-fixing samples. For the cultured *Trichodesmium* experiments, media blanks averaged 12% of total analyte for both Mo and Fe. Average procedural blanks for field samples were 36% of total analyte for Fe and 3% for Mo.

Carbon, hydrogen, and nitrogen analyses: Samples of cultures for carbon, hydrogen, and nitrogen (CHN) analysis were collected by filtering 50–100 ml of culture onto an ashed glass fiber filter (GF/F) or an acid-cleaned polycarbonate filter. Polycarbonate filters were used initially because of concerns that the differences in retention pore sizes of GF/F and polycarbonate filters might make it difficult to compare cellular metal and C/N samples. Polycarbonate filters and cells were transferred to Petri dishes and covered with ethanol. Cells were gently scraped off with a Teflon-coated spatula and then transferred in ethanol to a tin boat and dried down for CHN analysis. This procedure has been shown to be quantitative (Kujawinski 2000). Picked *Trichodesmium* samples were collected by filtering a known number of colonies in a known volume of sterile seawater. Samples were analyzed at the VIMS (Virginia Institute of Marine Science) Analytical Service Center on an Exeter Analytical CE-440 CHN Analyzer. Procedural blanks were determined by filtering 50–100 ml of sterile media or sterile seawater. Procedural blanks were always <10% for C and <5% for N. C and N concentrations in each sample were compared to sample metal concentrations by normalizing all values either to mg cellular dry weight (*Crocospaera* experiments 1 and 2; *Trichodesmium* experiment 1) or to ml of culture filtered (*Crocospaera* experiments 3 and 4).

Results

Crocospaera experiments—Four *Crocospaera* experiments were performed measuring Mo and Fe cellular concentration, Fe:C and Mo:C ratios, and nitrogen fixation rates over two to three diel cycles. Cell counts of late log-phase batch cultures for experiments 1, 2, and 4 gave a growth rate of approximately 0.46 d⁻¹, the maximal growth rate observed for this organism (Fig. 1a,b,d; Table 2). In experiment 3, the cells had entered stationary phase by the end of the experiment (Fig. 1c).

Analytical results from *Crocospaera* culture experiments are presented in Table 3. There was a consistent diel variation in Mo cellular concentrations of three- to eightfold that was in phase with N₂ fixation rates (Fig. 2, left panels). N₂ fixation rates were not measured in experiment 1, and the

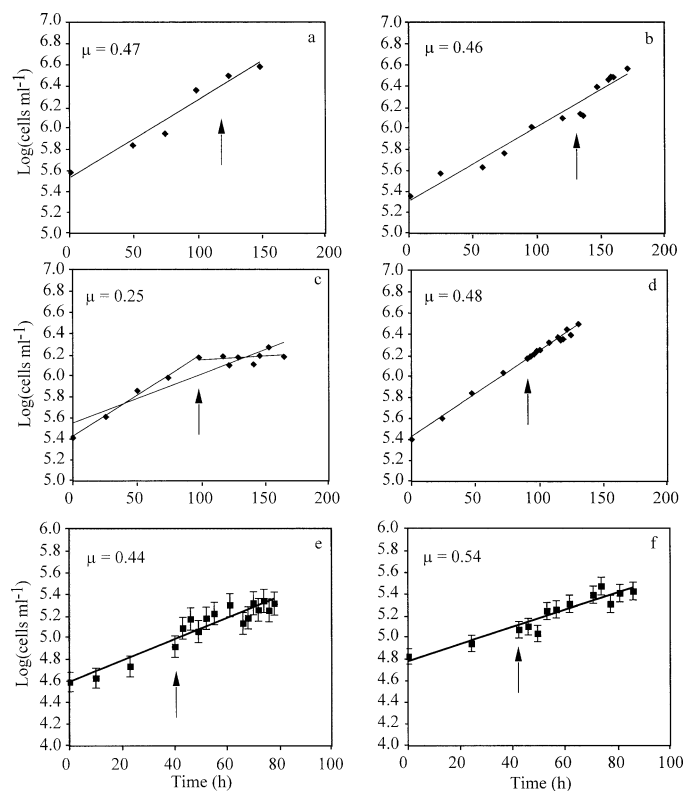


Fig. 1. Growth of *Crocosphaera* in (a) experiment 1, (b) experiment 2, (c) experiment 3, and (d) experiment 4 and growth of *Trichodesmium* in (e) experiment 1 and (f) experiment 2. Lines indicate exponential growth rates, μ (d^{-1}). Cell doubling times were (a) 35.4, (b) 36.2, (c) 66.5, (d) 34.6, (e) 37.5, and (f) 30.5 h. Arrows indicate where acetylene reduction measurements were begun. Growth was exponential in all cultures except for *Crocosphaera* in experiment 3 (panel e), where the culture entered lag phase at the beginning of sampling for acetylene reduction measurements.

low Mo cellular concentrations and Mo:C ratios suggest that the culture was not sampled during the peak of N_2 fixation, but Mo:C ratios also exhibited clear diel variations of a similar magnitude (8- to 10-fold). Fe cellular concentrations also show a 2.4- to 3.3-fold increase during nitrogen fixation (Fig. 2, right panels) with a nine- to fourfold increase in Fe:C ratios. In experiment 4, diel variations are not apparent. Fe:C ratios and Fe cellular concentrations are extremely large compared to the other *Crocosphaera* experiments, suggesting that there was an Fe contamination problem. We suspect that these high Fe levels obscured diel Fe cycling in this experiment. The Fe:Mo ratios of experiments 2 and 3 were lower in the N_2 -fixing samples than in the photosynthesizing samples. The average Fe:Mo ratio (24.1 ± 14.2 mol mol $^{-1}$) is within error of the Fe:Mo molar ratio (19 – 25 mol mol $^{-1}$) predicted by the stoichiometry of nitrogenase complexes.

The difference in magnitude between cellular concentration and the metal:carbon ratio daily variation was due to diel variation of the carbon and nitrogen pool in *Crocosphaera* (Waterbury and Valois unpubl. data). This could be seen in experiment 3 samples 2M–4M (Table 3) by the decrease in the C:N ratio during the dark period, as N was

Table 2. *Crocosphaera* cell-based growth and assimilation rates.

Experiment	μ_{cell} cell-based growth rate* (day^{-1})	N_{cell} (fmol N cell $^{-1}$)	$N_{\text{cell}} \times \mu_{\text{cell}}$ N assimilation rate† (fmol N cell $^{-1}$ day $^{-1}$)
1	0.47	12.4	5.8
2	0.46	6.9	3.2
3	0.25	23.1	5.8
4	0.48	29.6	14.2

* μ_{cell} growth rates were determined directly from cell counts.

† N assimilation rates were calculated from μ_{cell} and N_{cell} .

added by N_2 fixation and glycogen reserves built up during the light period were depleted to fuel N_2 fixation. *Crocosphaera* also has a partially synchronized cellular division; though cell division occurs throughout the light and dark periods, approximately one third of the cells divide 4 h after the start of the light period (Waterbury and Valois unpubl. data). The diel variation in both Fe and Mo, however, was too large to be an artifact of cell division, which could cause at most a 1.3-fold variation in cellular metal content.

Trichodesmium experiments—The growth rates for the *Trichodesmium* experiments were 0.54 and 0.44 d^{-1} (Fig. 1e,f; Table 4). These rates are typical for those observed in the Waterbury laboratory for axenic cultures but are faster than most published growth rates for *Trichodesmium* (Mullolland and Capone 2000; Berman-Frank et al. 2001a).

Neither *Trichodesmium* experiment 1 nor 2 showed a significant correlation of Mo:C molar ratios that was in phase with the N_2 fixation rate (Fig. 3; Table 5). Average C:N molar ratios for both experiments were low and stable at 6.20 ± 0.43 . Average Fe:Mo molar ratios of 34.0 ± 15.7 are, in most cases, within or slightly lower than the 30–58 mol Fe mol $^{-1}$ Mo calculated for the nitrogenase complex. Field samples of *Trichodesmium* did not show a strong diel variation in either Fe or Mo (Table 6). Average C:N molar ratios for the field samples were equivalent to those measured in culture. Fe:Mo ratios of field samples, however, were lower than those observed in culture. This was not driven by a decrease in Fe, since Fe:C ratios of cultured (87.1 ± 64.2 $\mu\text{mol}:\text{mol}$) and field samples (66.1 ± 39.0 $\mu\text{mol}:\text{mol}$) are statistically equivalent within error, but rather, by a large increase in Mo, as seen by the 10-fold increase in the Mo:C ratio compared to culture samples.

The Fe:C ratios reported here are in agreement with the most recently reported ratios. Berman-Frank et al. (2001a) measured the Fe:C ratio of *Trichodesmium* grown at a range of free iron concentrations (Table 7). They report an average Fe:C ratio ($\mu\text{mol}:\text{mol}$) of 168 ± 23 for *Trichodesmium* grown at a free Fe concentration similar to that used in cultures in the present study (pFe' 7.8). However, Berman-Frank et al. (2001a) report that as much as 65% of the Fe associated with *Trichodesmium* cells in these samples is extracellular, which brings their culture Fe:C ratio down to 69 $\mu\text{mol Fe mol}^{-1}$ C. Extracellular Fe was not addressed in this study, but measured values in cultures were more similar to the Berman-Frank et al. (2001a) corrected ratios than to the

Table 3. Fe and Mo cellular concentrations and ratios for *Crocospaera* spp. grown on a 14:10 light:dark schedule.

Sample	Time* (hours)	Cellular concentrations		Ratios			
		Fe (fg cell ⁻¹)	Mo (fg cell ⁻¹)	Fe:C	Mo:C	Fe:Mo	C:N
				(μmol mol ⁻¹)		(mol mol ⁻¹)	
Experiment 1							
C5 N ₂ fixing	30	0.013	0.0013	7.3	0.45	16.2	8.14
C10	42	0.004	0.0004	0.76	0.04	16.9	7.49
Experiment 2							
C9 N ₂ fixing	30	0.040	0.0050	12.0	0.88	13.7	8.65
C2	42	0.016	0.0011			25.3	
Experiment 3							
Mo1 N ₂ fixing	5						9.75
Mo2	17	0.087	0.0016	5.5	0.06	94.1	10.08
Mo3 N ₂ fixing	29	0.244	0.0121	19.9	0.57	34.8	9.56
Mo4	41	0.099	0.0032	6.5	0.10	55.2	10.10
Mo5 N ₂ fixing	53	0.243	0.0133	27.1	0.86	31.5	11.37
Experiment 4†							
2M N ₂ fixing	2.5	0.685	0.0149	51.3	0.65	79.0	9.47
3M N ₂ fixing	5	1.061	0.0170	96.7	0.90	107.4	7.26
4M N ₂ fixing	7.5	0.477	0.0139	44.4	0.75	58.8	5.90
6M	17	0.546	0.0030	43.0	0.14	316.4	7.13
9M N ₂ fixing	28	0.3	0.0136	30.8	0.77	40.1	7.88
12M	41	0.434	0.0027	27.8	0.10	274.5	7.49
Average (SD) N ₂ fixing‡		0.14 (0.12)	0.011 (0.005)	15.8 (11.3)	0.70 (0.27)	24.1 (14.2)	8.6 (1.8)
Average (SD)‡		0.06 (0.05)	0.003 (0.001)	4.2 (3.1)	0.08 (0.04)	54.7 (38.6)	8.8 (1.5)

* Indicates time of sample collection, elapsed time measured from start of the dark period. Periods of N₂ fixation indicated.

† Experiment 4 was possibly contaminated for Fe.

‡ Averages of Fe cellular concentrations and ratios do not include experiment 4.

uncorrected ratios, suggesting that any extracellular Fe makes only a small contribution. For field samples, the total range in Fe:C ratios is from 20 to >500 (Kustka et al. 2002). Berman-Frank et al. (2001a) reported Fe:C ratios of $450 \pm 242 \mu\text{mol Fe mol}^{-1} \text{C}$ for handpicked colonies collected in shelf waters off Australia. These overlap with the earlier ratios of Rueter et al. (1992): $326 \pm 127 \mu\text{mol Fe mol}^{-1} \text{C}$ from the Caribbean. It is likely, however, that both of these field estimates include large proportions of extracellular Fe from Fe oxyhydroxides. *Trichodesmium* collected from the Atlantic more recently (Sanudo-Wilhelmy et al. 2001) had Fe:C ratios ranging from 22 to $72 \mu\text{mol Fe mol}^{-1} \text{C}$, which overlaps with the range in field data from the Atlantic reported here (Table 6).

Discussion

Metal use efficiency of nitrogenase—To our knowledge, this is the first study to present data on the diel variations of Mo and Fe cellular concentrations of a marine diazotrophic cyanobacterium. However, cellular concentration does not indicate how much of the metal in the cell is required by nitrogenase as opposed to the metal required by other enzymes or stored unused by the organism. The fraction of cellular Mo and Fe used by the nitrogenase enzyme can be estimated from the metal use efficiency (MUE) of nitrogenase, or the amount of N₂ reduced min⁻¹ femtogram⁻¹ (fg⁻¹)

of nitrogenase-bound metal. This allows a direct comparison between the total amount of metal measured in the cells and the fraction of that metal required by nitrogenase for growth at a known rate of N assimilation.

The MUE is calculated from the molecular weight, metal requirements, and specific activity of nitrogenase by the following equation:

$$\text{MUE} = \text{SA} \times \frac{1 \text{ mol N}_2}{4 \text{ mol C}_2\text{H}_2} \times \frac{\text{mg}}{10^{12} \text{ fg}} \times \frac{1}{Q} \times \frac{M_{\text{FeMo}}}{M_{\text{M}}} \quad (1)$$

where SA is the specific activity of nitrogenase in nmol C₂H₂ min⁻¹ mg⁻¹ MoFe, M_{MoFe} is the molecular weight of the MoFe protein unit of nitrogenase, Q is the Fe or Mo content of the nitrogenase complex, and M_{M} is the molecular weight of Fe or Mo. The ratio of acetylene to N₂ reduced is approximately the 4:1 mol C₂H₂ mol⁻¹ N₂ ratio that is used for most N₂-fixing bacteria (Capone and Montoya 2001).

This approach has been used previously to estimate the Fe use efficiency of nitrogenase, the mol N₂ reduced min⁻¹ fg⁻¹ nitrogenase-bound Fe (Sprenst and Raven 1985; Raven 1988; Sanudo-Wilhelmy et al. 2001; Kustka et al. 2002, 2003). Using the specific activities of the MoFe protein of nitrogenase isolated from a variety of heterotrophs, including *Azotobacter vinelandii*, Kustka et al. (2003) calculate a range of $5.60\text{--}1.83 \times 10^{-8}$ nmol N₂ reduced min⁻¹ fg⁻¹ enzymatic Fe. We chose to recalculate the Fe use efficiency for several reasons. The Kustka et al. (2003) calculations do not take

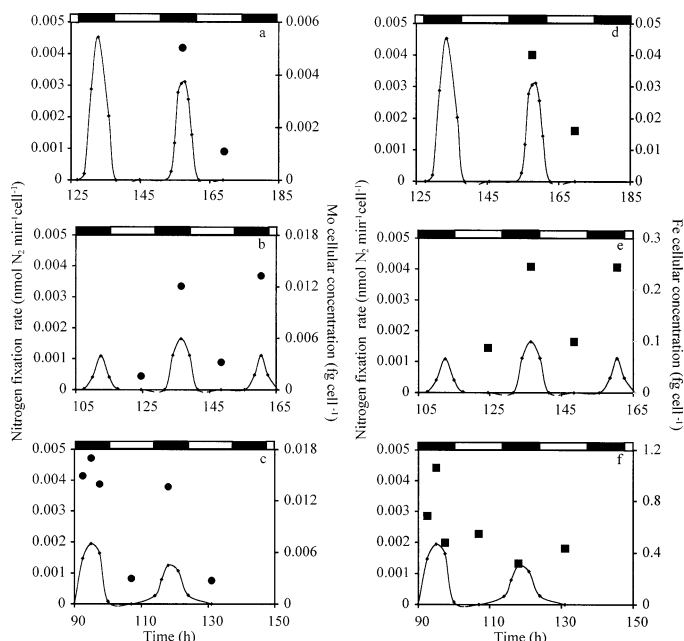


Fig. 2. Diel variations in N_2 fixation rate (lines) from acetylene reduction rate using a conversion factor of 4 compared to Mo (a–c; circles) and Fe (d–f; squares) cellular concentrations in fg cell^{-1} for *Crocosphaera* experiments 2 (a, d), 3 (b, e), and 4 (c, f). N_2 fixation rates are shown on the same scale for all experiments. Dark and light bars indicate the dark and light time periods, respectively. Cellular concentrations are correlated with N_2 fixation in all cases, except for the Fe analysis in experiment 4 (panel f), where high Fe concentrations due to contamination obscure all other variations.

into account the temperature at which the specific activity of nitrogenase was measured. A range of 25–30°C in temperature can lead to a 33% variation in specific activity (Burns 1969). Kustka et al. (2003) also used an older data set for the specific activity. The specific activity of an isolated enzyme is highly dependent on how well the enzyme was purified and how the protein concentration was determined. These techniques have improved with time, generally increasing estimates of specific activity and thus the calculated values of MUE.

To calculate the Fe and Mo use efficiency of nitrogenase, we used data derived from enzymes isolated from the heterotrophic soil bacterium *A. vinelandii*, the most well-studied nitrogenase complex. Comparable data are not available for *Trichodesmium*. However, Zehr et al. (2001) have modeled the Fe protein of nitrogenase from *Trichodesmium nifH* gene sequences and found significant similarity in structural features with Fe proteins isolated from *A. vinelandii*, suggesting that these parameters apply to cyanobacteria as well. The specific activity of the MoFe protein of *A. vinelandii* is 2,200–2,400 $\text{nmol C}_2\text{H}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ MoFe}$ at 30°C (Anderson and Howard 1984). Corrected for the temperature at which these cultures were grown (28°C), this becomes 1,870–2,040 $\text{nmol C}_2\text{H}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ MoFe}$ (Burns 1969).

The nitrogenase enzyme consists of a MoFe protein, which acts as the reaction center, and an Fe protein, which facilitates electron transfer. The 60-kDa molecular-weight Fe protein contains 4 Fe atoms (Howard and Rees 1996) and is

Table 4. *Trichodesmium* cell-based growth and assimilation rates.

Experiment	μ_{cell} cell-based growth rate* (day^{-1})	N_{cell} (fmol N cell^{-1})	$N_{\text{cell}} \times \mu_{\text{cell}}$ N assimilation rate† (fmol N cell^{-1} day^{-1})
1	0.54	528.9	267
2	0.44		

* μ_{cell} growth rates were determined directly from cell counts.

† N assimilation rates were calculated from μ_{cell} and N_{cell} .

encoded by the *Trichodesmium nifH* gene. The MoFe protein is a tetramer with a molecular weight of 230–270 kDa containing a total of 2 Mo and 30 Fe (Anderson and Howard 1984; Howard and Rees 1996) and is encoded by *Trichodesmium nifD* and *nifK* genes. The total Fe content of the nitrogenase complex depends on the ratio of Fe to MoFe protein. This ratio has not, to our knowledge, been measured in *Trichodesmium*, but a ratio of 3 was observed in vivo in the single-celled nitrogen-fixing cyanobacterium *Gloeotheca* ATCC 27152 (Reade et al. 1999). In vitro protein titrations indicate that maximal specific activity for the enzyme occurs at a ratio near 5 for many species (Eady and Smith 1979; Johnson et al. 2000). However, structural analysis of protein interaction based on x-ray diffraction suggests that there are only two possible binding sites for the Fe protein giving a minimum ratio of 2 (Howard and Rees 1996; Schindelin et al. 1997). The contiguous arrangement of the *nifHDK* genes on a tandem operon seems to support the structural analysis, leading to the production of 1 Fe protein to every ½ of a MoFe tetramer for a ratio of 2. However, qualitative analysis of the transcription products of the *Trichodesmium* IMS101 *nifHDK* gene sequence indicates the production of a rela-

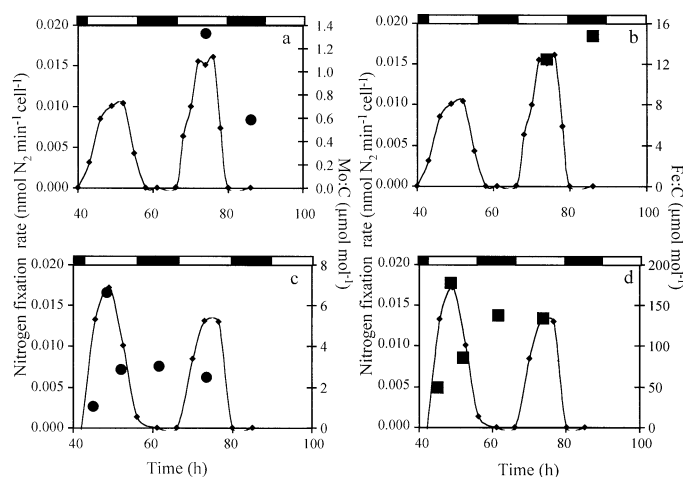


Fig. 3. *Trichodesmium* diel cycles of N_2 fixation (lines) from rates of acetylene reduction using a conversion factor of 4 compared to Mo:C (a, c; circles) and Fe:C (b, d; squares) ratios in $\mu\text{mol}:\text{mol}$ for experiment 1 (a, b) and experiment 2 (c, d). Dark and light bars indicate the dark and light time periods, respectively. Neither the Mo:C nor the Fe:C ratio shows diel variation in phase with nitrogenase activity.

Table 5. Fe and Mo cellular concentrations and ratios for *Trichodesmium* spp. grown on a 14:10 light:dark schedule.

Sample	Time* (hours)	Cellular concentrations		Ratios			
		Fe	Mo	Fe:C	Mo:C	C:N	Fe:Mo
		(fg cell ⁻¹)	(fg cell ⁻¹)	(μmol mol ⁻¹)		(mol mol ⁻¹)	
Experiment 1							
TD N ₂ fixing	34	2.37	0.44	12.4	1.33	6.54	9.3
TL	46	2.94	0.20	14.7	0.58	6.64	25.3
Experiment 2							
2M N ₂ fixing	2.5			49.3	1.04	6.32	47.3
3M N ₂ fixing	6			176.7	6.62	6.06	26.7
4M N ₂ fixing	9.5			84.7	2.87	5.51	29.6
6M	19			138.2	3.02	6.53	45.7
9M N ₂ fixing	31			133.7	2.47	5.79	54.1
Average (1 SD)				87.1 (64.2)	2.56 (2.02)	6.20 (0.43)	34.0 (15.7)

* Indicates time of sample collection, elapsed time measured from start of the light period on day 1. Periods of N₂ fixation indicated.

tively larger concentration of *nifH* transcripts relative to *nifDK*, possibly indicating a larger Fe:MoFe ratio (Dominic et al. 1998). These findings give a range of possible Fe:MoFe protein ratios of 2–5 or a possible Fe content of 38–50 mol Fe mol⁻¹ nitrogenase complex.

By substituting (1) 1,870–2,040 nmol C₂H₂ min⁻¹ mg⁻¹ MoFe, the temperature-corrected nitrogenase-specific activity for *Azotobacter*, (2) the nitrogenase complex Fe concentration of 38–50 mol Fe, and (3) a MoFe protein molecular weight of 230–270 kDa into Eq. 1, we calculated an Fe use efficiency of nitrogenase of $5.17 \pm 1.32 \times 10^{-8}$ nmol N₂ reduced min⁻¹ fg⁻¹ enzymatic Fe. This falls within the $1.83\text{--}5.60 \times 10^{-8}$ nmol N₂ reduced min⁻¹ fg⁻¹ enzymatic range of Sanudo-Wilhelmy et al. (2001) in spite of slightly different ways of making the calculation. The Mo use efficiency given a Mo content of 2 mol Mo mol⁻¹ nitrogenase complex

is $6.46 \pm 1.12 \times 10^{-7}$ nmol N₂ reduced min⁻¹ fg⁻¹ enzymatic Mo.

The MUE can be used to (1) predict nitrogenase-bound metal cellular concentrations that are based on nitrogen assimilation rate, and (2) estimate the nitrogenase-bound metal:C ratio of a cell that is based on the growth rate and C:N ratio. There are several assumptions implicit in using these MUEs to investigate *Trichodesmium* and *Crocospaera* cultures. First, as discussed above, the nitrogenase of the cyanobacteria *Trichodesmium* and *Crocospaera* is assumed to be similar to that of the heterotrophic soil bacterium *A. vinelandii*. Second, all growth is assumed to be supported by N₂ fixation, with no source of combined nitrogen.

The assumption that all growth is supported by N₂ fixation is generally correct for axenic cultures growing exponen-

Table 6. *Trichodesmium* field samples collected in the North Atlantic.

Station and time	Fe:C	Mo:C	C:N	Fe:Mo
	(μmol mol ⁻¹)		(mol mol ⁻¹)	
RV <i>Weatherbird</i> September 1999				
BATS 10:00	45.3	19.8	7.84	2.3
BATS 14:00	79.1	2.6*	7.48	30.5*
BATS 14:00	49.8	17.8	7.81	2.8
BATS 01:00	51.7	8.6	9.66	6.0
RV <i>Seward Johnson</i> May 2001				
Sta. 01 12:00	74.1	22.7	6.26	3.3
Sta. 02 03:00	85.9	8.6	6.59	9.9
Sta. 03 21:00	51.8	53.6	6.55	1.0
Sta. 04 09:00	160.6	38.5	12.64	4.2
Sta. 04 13:00			6.32	
Sta. 07 12:00	1,008.9*	36.4	6.85	27.7*
Sta. 08 12:00			6.8	
Sta. 28 14:00	48.7	24.6	6.66	2.0
Sta. 30 22:00			6.82	
Sta. 32 16:00	13.8	14.7	6.25	0.9
Average field samples (1 SD)	66.1 (39.0)	22.5 (15.1)	7.1 (0.9)	3.6 (2.9)
Average cultures (1 SD)	87.1 (64.2)	2.56 (2.02)	6.20 (0.43)	34.0 (15.7)

* Outlying values not included in the average.

Table 7. Comparison of *Trichodesmium* Fe:C and C:N ratios in culture and field samples from this and other studies. Free Fe concentration of the culture media is shown when known.

Cultures:		Fe:C		C:N		Reference
Growth stage	pFe'	($\mu\text{mol mol}^{-1}$)	1 SD	(mol mol^{-1})	1 SD	
Log	7.5	87	64	6.2	0.4	This study
Log	10.78	13	9.9	12.0	1.0	Berman-Frank et al. 2001a
Log	9.88	30	2.7	8.9	1.0	Berman-Frank et al. 2001a
Log	9.4	33	1.8	9.0	0.3	Berman-Frank et al. 2001a
Log	7.86	48	2.2	16.0	2.0	Berman-Frank et al. 2001a
Log	7.8	168	23	12.0	4.0	Berman-Frank et al. 2001a
Log	Replete			6.2	0.2	Mulholland and Capone 2001
Log	Replete			9.1	1.3	Mulholland and Capone 2001
Stationary	Replete			7.9	2.1	Mulholland and Capone 2001
Field samples:						
Location	Year					
North Atlantic	2000	66	39	7.2	1.0	This study
Australia	1999	450	242	6.1	0.7	Berman-Frank et al. 2001a
Caribbean	1988	326	127			Rueter et al. 1992
Atlantic	1996	36				Sanudo-Wilhelmy et al. 2001
Pacific	1990–1991			6.4	0.4	Letelier and Karl 1996

tially in media with no added N. It may also be a good assumption for field populations of *Crocospaera*, which maintained maximal rates of N_2 fixation in culture despite nitrate and urea additions of up to $20 \mu\text{mol L}^{-1}$ (unpubl. data). *Trichodesmium*, however, has been shown to suppress N_2 fixation in the presence of fixed N sources (Mulholland et al. 2001); thus, estimates of nitrogenase-bound metal:C ratios for field samples may be overestimates.

Ideally, in cultures with no source of fixed nitrogen, N_2 fixation rates, as estimated by acetylene reduction, will equate to cell-based N assimilation rates by a correction factor of 3–4. This correction factor is associated with the relative affinities of nitrogenase for N_2 and C_2H_2 substrates. However, in both the *Trichodesmium* and *Crocospaera* experiments, cellular growth rate exceeds the growth rate calculated from integrated acetylene reduction measurements by up to an order of magnitude, which is far larger than can be accounted for by the standard correction factor. These cultures were growing, rapidly, exponentially, without any source of fixed nitrogen. It seems most likely that the discrepancy between acetylene reduction-based growth rates and cell count-based growth rates is a methodologic problem with our acetylene reduction assay. *Trichodesmium* and *Crocospaera* are fragile organisms, sensitive to physical disturbance and changes in growth conditions. It is possible that the experimental manipulation required to maintain trace metal clean conditions suppresses the rate of N_2 fixation in the assay. Therefore, all subsequent calculations are based on N assimilation rates calculated from the observed cellular growth rate and measured cellular N concentration.

Theoretical estimates of nitrogenase-bound metal:carbon ratios were calculated by dividing the cell-based growth rate by the metal use efficiency of the enzyme and dividing again by the C:N ratio, yielding an estimate of the amount of enzyme-bound metal that is required by each organism. Given a specific growth rate in culture for both *Trichodesmium*

and *Crocospaera* of, on average, 0.5 d^{-1} at 28°C and respective C:N ratios of 6.2 and 9.0, the estimated nitrogenase Mo:C ratios were 0.43 ± 0.05 and $0.29 \pm 0.04 \mu\text{mol Mo mol}^{-1} \text{ C}$, respectively. These ratios are an estimate of the minimum Mo requirement based only on the Mo bound in the nitrogenase enzyme. They are also averaged over a 24 h day rather than the 10- to 12-h period when N_2 fixation takes place, which would raise estimates by about twofold to 0.83 ± 0.05 and 0.58 ± 0.04 , respectively. The average Mo:C ratio for the *Crocospaera* cultures ($0.71 \pm 0.27 \mu\text{mol Mo mol}^{-1} \text{ C}$; Table 3) agrees with the theoretical estimate. The measured *Trichodesmium* Mo:C ratio in culture ($2.56 \pm 2.02 \mu\text{mol Mo mol}^{-1} \text{ C}$; Table 5) is up to fivefold higher than the estimated Mo demand based on nitrogenase requirements, but the average Mo:C ratio of the field *Trichodesmium* samples is almost 50-fold greater than the nitrogenase Mo:C ratios ($22.5 \pm 15.1 \mu\text{mol Mo mol}^{-1} \text{ C}$; Table 6).

A similar estimate made for nitrogenase Fe:C ratios gives $9.6 \pm 2.4 \mu\text{mol Fe mol}^{-1} \text{ C}$ for *Trichodesmium*. This estimate is averaged over the entire day and does not take into account the limited time frame of N_2 fixation. To constrain the total amount of N fixation required for growth at a rate of 0.5 d^{-1} to the 12 h of active N_2 fixation, the ratio must be increased by 2. Because *Trichodesmium* fixes nitrogen and carbon at the same time, the Fe required for carbon fixation by photosynthesis also must be considered. Photosynthetic processes have been estimated to cost approximately $0.9 \text{ mol Fe mol}^{-1} \text{ C s}^{-1}$ (Raven 1988; Sanudo-Wilhelmy et al. 2001). Adjusting the Fe budget to account for photosynthesis and the time limits of nitrogen fixation increases estimates of Fe:C ratios to $29.7 \pm 3.1 \mu\text{mol Fe mol}^{-1} \text{ C}$ for *Trichodesmium*. This estimate is at the low end of the range of Fe:C ratios seen in *Trichodesmium* experiment 2 (Table 5) and the natural populations ($66.1 \pm 39.0 \mu\text{mol Fe mol}^{-1} \text{ C}$; Table 6) sampled for this study, as well as the data available in the literature (Kustka et al. 2002).

Assuming that 0.5 d^{-1} represents the maximum growth rate for *Trichodesmium* and that all of the Fe associated with the cells is available for enzymatic uses, this suggests that both the field populations and *Trichodesmium* experiment 2 are Fe replete. *Trichodesmium* experiment 1, however, had a lower measured Fe:C ratio than the estimate, despite having a growth rate similar to that in experiment 2. This finding can be interpreted two ways. *Trichodesmium* experiment 1 may be down-regulating nitrogenase for some reason, possibly because of the presence of fixed N compounds. We do not believe this to be the most likely explanation, because these cultures are axenic and are growing rapidly and exponentially in media without an added source of combined nitrogen. Nitrogen compounds have only been shown to be released to the media in slow-growing *Trichodesmium* isolates contaminated with heterotrophic bacteria (Mulholland and Capone 1999, 2001). Confirmation of these experiments with axenic cultures is required. Alternatively, *Trichodesmium*, found in the oligotrophic ocean, may have a more Fe-efficient nitrogenase enzyme than the soil bacterium *Azotobacter*. We can back-calculate a nitrogenase-bound Fe concentration of 22–28 mol Fe mol⁻¹ nitrogenase complex for *Trichodesmium* from our lowest measured Fe:C ratio for a nitrogen-fixing *Trichodesmium* culture (12.4 $\mu\text{mol}:\text{mol}$: experiment 1). We speculate that *Trichodesmium* nitrogenase is highly Fe efficient compared to the *Azotobacter* estimates of 38–50 mol Fe mol⁻¹ nitrogenase complex. This theory can be tested by the isolation and analysis of *Trichodesmium* nitrogenase metal concentrations, specific activities, and molecular weights and would greatly increase the reliability of future estimates of enzyme-bound metal concentrations.

Crocospaera temporally separates N₂ fixation (night) and photosynthesis (day). Thus, assuming that nitrogenase dominates Fe demand at night and the photosynthetic apparatus dominates the daytime Fe demand, the enzymatic Fe:C ratio estimates are 13.2 ± 4.7 and $4.8 \mu\text{mol Fe mol}^{-1} \text{C}$ for the N₂ fixation period and light period, respectively. These are within error of measured culture samples for the N₂ fixation period ($15.8 \pm 11.3 \text{ mol Fe mol}^{-1} \text{C}$: Table 3) and the light period ($4.2 \pm 3.1 \text{ mol Fe mol}^{-1} \text{C}$: Table 3). These are extremely low Fe:C ratios, given the Fe-replete nature of these cultures and the relatively fast growth rate of 0.46 d^{-1} . They are similar to average open-ocean Fe:C ratios (Fung et al. 2000). Kustka et al. (2002) have suggested that similarly low estimates of the Fe requirements for heterotrophic diazotrophic growth (Fe:C of 2.3–57 $\mu\text{mol}:\text{mol}$ at 0.06 d^{-1}) preclude Fe limitation. These findings suggest that, depending on growth rate and Fe uptake rate, it will be difficult to cause Fe limitation in *Crocospaera*.

The agreement between theoretical estimates of Fe:C and Mo:C ratios based on metal use efficiency estimates and those measured in culture experiments implies that a very large fraction of the total pool of measured Fe and Mo is bound in active nitrogenase enzyme. This is illustrated graphically in Figs. 4, 5, which show a comparison of measured cellular concentrations of Fe and Mo versus predicted cellular quantities required for the rate of N assimilation, assuming all growth is supported by N₂ fixation. For both *Trichodesmium* and *Crocospaera*, the predicted Mo cellular concentrations during N₂ fixation vary directly with those

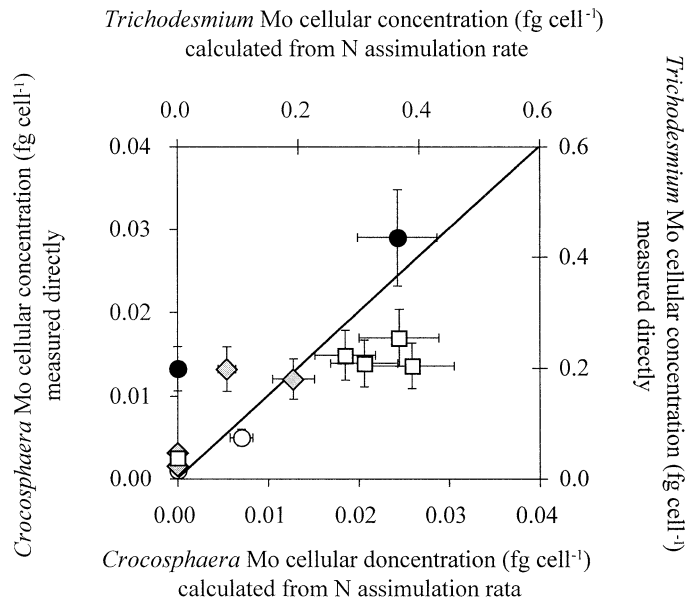


Fig. 4. A comparison of measured Mo cellular concentrations for *Crocospaera* experiments 2 (open circles), 3 (gray diamonds), and 4 (open squares) and for *Trichodesmium* experiment 1 (black circles) with cellular concentrations predicted from the N assimilation rate and Mo use efficiency of nitrogenase. Y-axis error bars are based on measurement error. X-axis error bars are based on the range of Mo use efficiencies calculated for nitrogenase isolated from *Azotobacter vinelandii*. Most samples fall within error of the 1:1 line, implying that most of the Mo measured in the cells is present as nitrogenase. *Crocospaera* cell samples collected during periods of no N₂ fixation all approach detection limits for measured Mo concentrations; however, *Trichodesmium* shows significant Mo concentrations in non-N₂-fixing cells, implying that these cells store Mo or use it in another enzyme system during the dark period.

independently measured in the cells. *Crocospaera* Mo cellular concentrations measured in non-N₂-fixing samples are similar to the predicted concentration, but the *Trichodesmium* Mo measurements are slightly enriched. This implies that while *Crocospaera* releases nearly all of its Mo back into the media, *Trichodesmium* maintains a pool of Mo even when it is not actively fixing N₂. Because of the high solubility of Mo in oxic seawater, it is more likely that this pool of Mo is sequestered internally than adsorbed or bound extracellularly to the trichomes. The only other Mo enzyme known to be present in cyanobacteria is nitrate reductase, but nitrate reductase has a much higher specific activity than nitrogenase and is thought to require less Mo than nitrogenase for similar N assimilation rates (Sprenst and Raven 1985; Raven 1988; Sanudo-Wilhelmy et al. 2001). In a presumably nitrate-free medium, it is even less likely to contribute significantly to the internal Mo pool. Mo is most probably associated either with a preserved pool of nitrogenase enzyme or another storage form.

In *Crocospaera* experiments 2 and 3, measured Fe cellular concentrations are close to the 1:1 line (Fig. 5). Again, this supports the interpretation that much of the cellular Fe inventory in *Crocospaera* is bound in active nitrogenase. However, *Crocospaera* experiment 4 shows Fe enrichment

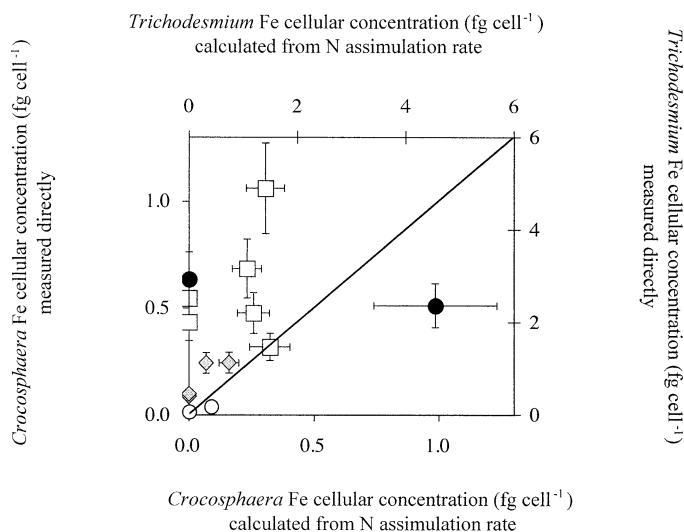


Fig. 5. A comparison of measured Fe cellular concentrations for *Crocosphaera* experiments 2 (open circles), 3 (gray diamonds), and 4 (open squares) and for *Trichodesmium* experiment 1 (black circles) with cellular concentrations predicted from the N assimilation rate and Mo use efficiency of nitrogenase. Y-axis error bars are based on measurement error. X-axis error bars are based on the range of Fe use efficiencies calculated for nitrogenase isolated from *Azotobacter vinelandii*. Most samples fall within error of the 1:1 line, implying that most of the Fe measured in the cells is present as nitrogenase. *Crocosphaera* cell samples collected during experiment 4 have excess measured Fe, likely due to contamination of these samples. *Trichodesmium* has constant Fe cellular quotas, which are lower than the Fe predicted from N assimilation rates. This suggests that the nitrogenase enzyme of *Trichodesmium* is slightly more Fe efficient than that of *A. vinelandii*.

that may be associated with Fe contamination of the sample or extracellular Fe. In *Trichodesmium* experiment 1, as seen previously in the Fe:C data, Fe concentrations are lower than predicted using metal use efficiencies based on *A. vinelandii*. In the absence of a source of fixed nitrogen, this implies that the nitrogenase complex of *Trichodesmium* is more metal efficient.

Metal use by *Crocosphaera*—The strong diel cycling seen in both Fe and Mo that is correlated with the N_2 fixation rate supports the theory that *Crocosphaera* synthesizes nitrogenase de novo each night. Whether nitrogenase degradation during the day is an intentional process that recycles the component metals and proteins for use elsewhere in the cell or is an unintentional by-product of the photosynthetic production of O_2 , which disables nitrogenase, is unknown. Experiments 2 and 3 with reliable Fe data showed Fe cycling as well as Mo cycling. These cultures were Fe replete, and it is uncertain whether this phenomenon would occur at environmentally relevant Fe concentrations. The diel cycling supports theoretical estimates that the nitrogenase enzyme complex requires more Fe than the photosynthetic apparatus active during the light period. Fe:C ratios measured in photosynthesizing samples ($4.2 \pm 0.3 \mu\text{mol Fe mol}^{-1} \text{C}$) are similar to those estimated for photosynthesis with no Fe demand associated with N_2 fixation ($4.8 \mu\text{mol Fe mol}^{-1} \text{C}$).

On the basis of the average size ($2 \mu\text{m}$ diameter) and spherical shape of *Crocosphaera* and the measured cellular metal concentrations, we have estimated cellular metal concentrations on a unit volume basis. The intracellular concentration of Mo is approximately $1.1\text{--}42 \mu\text{mol L}^{-1}$, which is always >10 times higher than seawater concentrations ($0.1 \mu\text{mol L}^{-1}$). The intracellular concentration of Fe is $19\text{--}1,000 \mu\text{mol L}^{-1}$, which is enriched five orders of magnitude above seawater. However, at reasonable cell abundances, $10^6 \text{ cells L}^{-1}$, these calculations suggest that *Crocosphaera* requires only $0.12 \text{ pmol Mo L}^{-1}$ and $2.4 \text{ pmol Fe L}^{-1}$ compared to typical seawater concentrations of $107 \text{ nmol L}^{-1} \text{ Mo}$ and $0.1 \text{ nmol L}^{-1} \text{ Fe}$. Thus, these organisms are likely to account for a relatively small fraction of the total Fe and Mo inventory in seawater.

However, N_2 -fixing organisms may account for a significant fraction of the particulate Mo budget. The metal concentrations of these cells, assuming that the dry weight is about 45% C, average 2.4 ppm Mo when fixing nitrogen compared to 0.2 ppm Mo during photosynthesis. This is similar to the estimated Mo crustal abundance of 2 ppm (Taylor and McLennan 1985). The average dust flux and the particulate Al inventory in the upper 100 m of the Sargasso Sea are approximately $0.5 \text{ g m}^{-2} \text{ yr}^{-1}$ and $6.7 \text{ nmol Al L}^{-1}$, respectively (Jickells 1999). This results in a Mo dust inventory of $0.045 \text{ pmol Mo L}^{-1}$, which is significantly smaller than the $0.12 \text{ pmol particulate Mo L}^{-1}$ calculated above, suggesting that Mo enrichment in suspended particulate matter is a good indicator of N_2 fixation, provided that cellular requirements for other enzymes and contributions associated with Mn oxides are small.

Metal use by *Trichodesmium*—A significant amount of data regarding the elemental composition of *Trichodesmium* is accumulating in the literature, particularly for C, N, and Fe (Table 6). Two recent articles reported C:N ratios in cultures of IMS101 of 5:11 (Mulholland and Capone 2001) and 8:16 (Berman-Frank et al. 2001a). The Berman-Frank et al. (2001a) paper represented cultures grown under a range of Fe concentrations but reported no correlation of the C:N:P ratio with Fe availability or quota. The Mulholland and Capone (2001) paper measured the C:N ratio over the life of a culture. That study witnessed the lowest C:N ratios during log-phase growth and the higher ratios during lag, stationary stages. This suggests that lower C:N ratios are a good indication of the health of a culture. Field measurements of C:N ratios in *Trichodesmium* from the North Atlantic and North Pacific are generally low, ranging from 4.7 to 7.3 (Letelier and Karl 1998; Berman-Frank et al. 2001a; Kustka et al. 2002). Both the culture work and field data presented in this study fell on the low end of the C:N spectrum, which is possibly because our cell count-based growth rates are faster than most reported in the literature (Mulholland and Capone 2000; Berman-Frank et al. 2001a).

The Fe:C ratios for both our field and culture data sets are also at the lower end of the range of reported values and are most similar to the values for North Atlantic colonies reported by Kustka et al. (2002) (Table 7). Our study does not evaluate the possibility of extracellular Fe, but the lowest Fe:C ratios in our combined field and culture data set (Table

6) are comparable with corrected estimates of enzyme requirements, suggesting that any extracellular Fe contribution must be small.

The average Fe:Mo ratios of the cultures were within error of the Fe:Mo ratio of the nitrogenase complex. However, the Fe:Mo ratios of the field samples were much lower (Table 6). This was driven by an increase in the Mo content of field populations. These high Mo:C ratios imply N₂ fixation rates several orders of magnitude higher than current estimates and measurements of marine nitrogen fixation. Additionally, the lack of correlation between N₂ fixation rates and Mo:C ratios in the cultures suggests that particulate Mo cannot be used as a direct proxy for N₂ fixation.

There are several possible explanations for the increased Mo concentration in the field samples. Field-collected *Trichodesmium* colonies are microbial communities, and the colonies may thus contain additional microbes with high Mo requirements. However, any contaminating microbe would contribute only a small fraction of the total carbon measured; therefore, the Mo:C ratio of these unidentified microbes would have to be considerably higher than any currently measured microbial species to produce the observed Mo:C ratios.

Natural populations of *Trichodesmium* colonies may have additional enzymatic uses for Mo. Nitrate reductase is currently the only other Mo enzyme identified in *Trichodesmium*. *Trichodesmium* has been shown to regulate the relative amounts of nitrogenase and nitrate reductase in the cells based on the amount of fixed nitrogen in the growth media at the beginning of the light period (Mulholland et al. 2001). The level of accessible combined nitrogen in the surface waters from which these cells were collected was unknown. As discussed above, the problem with this argument is that the specific activity of nitrate reductase is much higher than that of nitrogenase; thus, growth on nitrate likely requires less Mo than growth on N₂ (Raven 1988; Sanudo-Wilhelmy et al. 2001). Additional, unidentified Mo enzymes are still a possible source of the increased Mo concentration in the field samples.

Alternatively, *Trichodesmium* could be reacting to some form of nutrient stress by taking up more Mo than necessary for growth. The culture, unlike oligotrophic surface waters, is a nutrient-rich environment with plenty of Fe and P. Fe stress is unlikely for these field samples because Fe:C quotas, which have been shown to be a good indicator of Fe stress (Berman-Frank et al. 2001a), do not differ significantly from the Fe-replete cultures. Phosphate limitation has been hypothesized for the North Atlantic (Wu et al. 2000; Sanudo-Wilhelmy et al. 2001) and may be the more important occurrence. Phosphate and molybdate are both oxyanions, although of slightly different size, but if all phosphate channels are wide open, some Mo may be taken up as well. Whatever the cause, these results, unlike those of *Crocospaera*, do not support the use of particulate Mo concentration to estimate the rate of *Trichodesmium* N₂ fixation.

Molybdate is most similar to the sulfate anion, and sulfate channels have been shown to allow Mo uptake in *A. vinelandii* and other heterotrophic soil bacteria. Sulfate has been proposed to limit Mo uptake in estuarine systems and, thus, nitrogen fixation (Howarth and Cole 1985). However, the N₂

fixation of marine diazotrophs appears unaffected by competition with sulfate, despite seawater sulfate concentrations of 28 mM (Paulsen et al. 1991). Evidence from the recently sequenced *Trichodesmium* genome (Department of Energy–Joint Genome Institute) indicates that *Trichodesmium* has a homolog of the modEABC molybdenum transport system identified in *A. vinelandii* (Mouncey et al. 1995) and *Escherichia coli* (Self et al. 2001). This suggests that *Trichodesmium* has a highly selective way to take up molybdenum and is unlikely to be significantly inhibited by sulfate. The diel uptake and release of Mo seen in *Crocospaera* and the very large concentrations of Mo measured in field samples of *Trichodesmium*, up to a 100-fold more Mo than required for N₂ fixation, do not suggest that Mo is difficult for either organism to acquire. These data do not support the hypothesis that sulfate competition limits Mo uptake.

Both *Crocospaera* and *Trichodesmium* use Mo and Fe with remarkable efficiency. Estimates of metal cell quotas based on theoretical metal use efficiency agree with measured cellular concentrations, implying that nitrogenase uses a significant fraction of both Fe and Mo cellular pools. *Crocospaera* shows significant cycling of Mo and Fe in response to N₂ fixation. This suggests that the nitrogenase complex in *Crocospaera* is synthesized de novo each night and broken down each day with no attempt to conserve cellular pools of Fe or Mo. In contrast, *Trichodesmium* appears to maintain an internal pool of Mo during non-N₂-fixing periods and does not release Fe from the cells. The different adaptations employed by these organisms to manage cellular inventories of metals are likely due to their contrasting patterns of N₂ fixation. *Crocospaera* culture data suggest that Mo enrichment of particulates provides a tracer of N₂ fixation, but the large differences in Mo:C ratios of cultured and collected *Trichodesmium* imply that other factors significantly alter Mo cellular concentrations in natural populations. These issues will need to be understood before Mo enrichment can be used to estimate N₂ fixation.

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