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Molybdenum–nitrogen co-limitation in freshwater and coastal heterocystous cyanobacteria

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Abstract

Molybdenum (Mo) is essential for the biological assimilation of inorganic nitrogen (N). We compared Mo requirements for N_2-fixation in two species of filamentous heterocystous cyanobacteria (HC) to test the hypothesis that coastal HC require higher Mo concentrations than freshwater HC. This expectation follows from the fact that Mo is more concentrated in seawater ($\sim$ 105 nmol L^{-1}) than in most freshwaters ($<$ 20 nmol L^{-1}). Contrary to this hypothesis, we found that both strains maintained N_2-fixation for 30 d at 10 nmol L^{-1}. Mo concentrations $<$ 1 nmol L^{-1} induced N-limitation in both species, as indicated by increased C : N ratios and decreased nitrogenase expression and activity. This response took time to induce, likely due to high-affinity molybdate uptake by both species. Measurable N_2-fixation persisted in the coastal strain (*Nostoc* sp. CCMP 2511) for at most 12 d; 3 d were required for chlorophyll *a* concentrations to fall below those of Mo-replete cultures. An additional 7 d and 11 d, respectively, were required for N_2-fixation rates and chlorophyll levels to decline in Mo-limited freshwater cultures (*Nostoc* sp. PCC 7120). When Mo was high ($>$ 1 μmol L^{-1}), the freshwater strain exhibited considerable Mo storage ($>$ 100 μmol mol^{-1} Mo : C) whereas cellular Mo remained $<$ 10 μmol mol^{-1} Mo : C in the coastal strain. The high Mo content and extended time required for N_2-fixation to decrease in the freshwater strain could be due to expression of the gene *mop*, which encodes a putative molybdate-storage protein. This study suggests the importance of Mo storage in freshwater HC.

Molybdenum (Mo) is a key trace nutrient for the biological assimilation of N either as nitrate (NO_3^-) or dinitrogen (N_2) gas. The situation in which acquisition of one nutrient (in this case, N) is dependent upon sufficient supply of another nutrient (in this case, Mo) is an example of "biochemically-dependent co-limitation" (Saito et al. 2008: 284). However, concentrations of Mo vary widely in aquatic environments. In the open ocean, Mo is well-mixed and is present at $\sim$ 105 nmol L^{-1} (Collier 1985), making it by far the most abundant transition metal in seawater. Coastal regions can undergo Mo fluctuations from 30 nmol L^{-1} to 160 nmol L^{-1} (Dellwig et al. 2007). Mo is typically $<$ 20 nmol L^{-1} in freshwaters (Howarth et al. 1988). This study aimed to compare the Mo requirements for N_2-fixation in coastal vs. freshwater cyanobacteria in order to understand how organisms adapt to different Mo abundances in different aquatic environments.

Diazotrophic cyanobacteria from both marine and freshwaters typically use a Mo-dependent enzyme, nitrogenase (Nif), for N_2-fixation. Nitrogenase is composed of a Mo- and Fe-containing multi-subunit protein (NifDK) and a Fe-containing protein (NifH), which perform the energy- and metal-intensive process of N_2 reduction to ammonia (NH_3; Hallenbeck et al. [1979]). Some diazotrophs have one or two additional nitrogenase isoforms with vanadium (V) or iron (Fe) taking the place of Mo. V-containing nitrogenases (Vnf) exist in several freshwater species (Thiel 1993; Boison et al. 2006). No species of cyanobacteria have been found to possess the Fe–Fe nitrogenase (Anf).

In addition to N_2-fixation, Mo is also required for cyanobacterial NO_3^- reduction to NO_2^- via the protein nitrate reductase (Mikami and Ida 1984). Thus, the difference in Mo bioavailability between freshwater and brackish environments may influence expression and activity of Mo-containing enzymes involved in N assimilation from N_2 and NO_3^- (Howarth et al. 1988). Low Mo concentrations in freshwaters could limit the function of Mo-based nitrogenase and nitrate reductase, slowing cyanobacterial growth due to a deficit of cellular N.

The Mo requirement for N_2-fixation by heterocystous cyanobacteria (hereafter, HC) was first recognized by Bortels (1940). Further study showed that the N content of N_2-fixing *Anabaena cylindrica* was positively correlated with the Mo concentration of the growth media (Wolfe 1954). *A. cylindrica* and certain other diazotrophic cyanobacteria isolate nitrogenase in specialized cells called heterocysts to prevent its irreversible destruction by photosynthetically derived oxygen (O_2) and O_2-derived radicals (Fay 1992). Pigment content and nitrogenase activity of *A. cylindrica* declined after 7–10 d of growth in Mo-deficient media ($<$ 5 nmol L^{-1}; Fay and Vasconcelos [1974]; Jacob and Lind [1977]). These physiological changes were reversed by addition of Mo to the growth media (Ter Steeg et al. 1986). Subsequent to these experiments, the Mo content of purified nitrogenase from *A. cylindrica* was determined to be 2 atoms per enzyme complex (Hallenbeck et al. 1979).

More recently, Zerkle et al. (2006) found the Mo concentration threshold in cultures of *A. variabilis* ATCC 29413 to be $\sim$ 5 nmol L^{-1}, below which nitrogenase

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activity measured by acetylene reduction proxy was very low. However, the Zerkle et al. (2006) experiment was run for a short time period in batch culture and the organism studied also contained the *vnf* genes (Thiel 1993). Longer experiments with an organism lacking *vnf* genes are desirable for two reasons: to establish the time required for measurable declines in nitrogenase activity in Mo-limited cultures and to ensure that the only active nitrogenase is Mo-nitrogenase.

Heterocystous cyanobacteria are generally found in freshwater environments, but there are examples of free-living coastal HC. The main basin of the Baltic Sea (salinity of 10–15) harbors HC species such as *Nodularia spumigena* and *Aphanizomenon* sp. (Howarth et al. 1988; Walve and Larsson 2007). Another coastal HC species, *Nostoc* sp. CCMP 2511, was isolated in 2001 from Kaneohe Bay, a coral reef lagoon on the east coast of Oahu, Hawaii (Caperon et al. 1971). There has been no previous laboratory study targeted at Mo–N co-limitation in coastal HC. However, these organisms are ideal models to investigate Mo–N co-limitation because they live at Mo concentrations intermediate between low-Mo freshwaters and high-Mo seawater and contain no *vnf* genes, so we can ensure that all N_2-fixation is performed by Mo-nitrogenase.

In this study, we investigated the physiological and biochemical response of freshwater and coastal HC to changes in Mo concentrations using strains that possess *nif* genes but lack *vnf* and *anf* genes (Kaneko et al. 2001). Experiments were performed for extended periods in semi-continuous batch culture to investigate the time required to induce Mo–N co-limitation. Furthermore, we attempted to amplify genes involved in Mo high-affinity uptake and storage from the coastal strain CCMP 2511 (which lacks a sequenced genome). We also searched sequenced genomes from freshwater, coastal, and marine cyanobacteria to determine if such genes are restricted to freshwater species living in low-Mo environments.

Methods

Culture growth conditions—The freshwater HC *Nostoc* sp. strain PCC 7120 and the coastal HC *Nostoc* sp. strain CCMP 2511 were grown axenically in HCl-washed sterile 250-mL polycarbonate bottles under constant light (150 μmol m^{-2} s^{-1} irradiance) at 25°C for 30 d. Cultures were continuously bubbled with sterile-filtered atmospheric air and were diluted to optical clarity every 3–5 d. Both strains were grown in BG-11 broth media for freshwater cyanobacteria. We modified the media recipe so that it lacked any NH_4^+. The media were made with and without added NO_3^- (18 mmol L^{-1}). Cultures grown on NO_3^- were used as controls. In addition, Ni (170 nmol L^{-1}) and V (200 nmol L^{-1}) were added. Growth-medium salinity was kept constant in order to maintain uniformity of all conditions except the strain being experimented upon. In both experiments, cultures were grown in duplicate at four Mo concentrations (< 0.1, 10, 100, and 1500 nmol L^{-1}, confirmed by inductively coupled plasma mass spectrometry [ICP-MS]).

Metal concentrations—Forty milliliters of each sample culture in exponential growth phase on the last day of the experiment were centrifuged at 10,000 × *g* for 15 min in 50-mL hydrochloric acid (HCl)-washed centrifuge tubes. Biomass pellets were washed three times by resuspension in trace metal-free media containing 50 μmol L^{-1} ethylenediaminetetraacetic acid (EDTA) followed by centrifugation. The biomass was transferred to acid-cleaned, preweighed Teflon vessels, dried at ~ 100°C and weighed. The biomass was then dissolved in concentrated nitric acid, dried, and resuspended in 10 mL of 2% (0.32 mol L^{-1}) nitric acid for ICP-MS analysis (Thermo X Series). An extra H_2O_2 dissolution step was added as necessary.

Chlorophyll a concentrations—Chlorophyll *a* (Chl *a*) concentrations were measured from methanol extracts at wavelength 665 nm on a ThermoSpectronic Genesys 20 spectrophotometer, using the equation Chl *a* (μg mL^{-1}) = 13.42 × $Abs_{665\ nm}$ (P. G. Falkowski unpubl.).

N_2-fixation rates—N_2-fixation rates were measured using the standard acetylene reduction method (Capone 1993). Cultures in exponential growth phase (specific growth rate > 0.5 d^{-1}) were sampled throughout the experiment, ~ 3 d after dilution to optical clarity. Ten milliliters of cyanobacterial culture were removed from each bottle and added to 20-mL HCl-washed serum bottles plugged with rubber septa. Two milliliters of air were removed from each bottle and replaced with 2 mL of acetylene (C_2H_2). A sample at the zero time point was taken to ensure no ethylene (C_2H_4) was present. Bottles were incubated at 150 μmol m^{-2} s^{-1} irradiance. One milliliter of headspace was sampled every hour. C_2H_2 and C_2H_4 were separated and quantified on a gas chromatograph (Hewlett-Packard 5890 Series II) equipped with a thermal ionization detector and a 1.83 m × 0.32 cm SS Porapak N 80/100 column (Ohio Valley Specialty Chemical). Samples were converted to nmol C_2H_4 μg Chl a^{-1} h^{-1} relative to an ethylene standard as described in Capone (1993).

Carbon and nitrogen analyses—Five to fifty milliliters of culture in exponential growth phase on the last day of the experiment were filtered onto preweighed, combusted GF/F filters and dried in an oven at 100°C. Subsequently, filters were weighed to obtain biomass dry weights (ranging from 0.5 mg to 5 mg) and packed into tin capsules for C and N elemental analyses (Costech Elemental Combustion System 4010). Blanks were determined by analysis of an unused filter and were subtracted from all measurements of filtered biomass. The N isotopic composition of each filter was measured on a Thermo Delta Plus Advantage isotope ratio mass spectrometer in line with the elemental combustion system. Glycine, spinach (NIST 1570a), and tomato (NIST 1573) leave standards were used for calibration curves and linearity checks.

Protein concentrations—Fifty milliliters of each culture in exponential growth phase on the last day of the experiment were centrifuged at 4000 × *g* for 10 min. Supernatant was removed and the sample was frozen in liquid N_2 and stored at −80°C. At a later date, the samples

Table 1. Primers used to amplify 16S rRNA, *nifD*, *vnfD*, *mop*, *modA*, and *modBC* genes from heterocystous cyanobacteria.

Gene	Primer name*	Primer sequence†	Reference
16S	CYA106F	5′–CGGACGGGTGAGTAACGCGTGA–3′	Nubel et al. 1997
	CYA781R	5′–GACTACWGGGGTATCTAATCCCWTT–3′	
nifD	*nifD*_F	5′–CGGTTACTGGTCTTGGTCTGGTC–3′	This study
	*nifD*_R	5′–GCGTCGTTAGCGATGTGGTGTC–3′	
vnfD	*vnfD*_F	5′–CTGGTAATTGGTGGCGTACTCA–3′	
	*vnfD*_R	5′–CTTTTGACTACGGCTTTGATGTCG–3′	
mop	*mop*_F	5′–ATGGAARTTAGCGCWCGTAATTYY–3′	
	*mop*_R	5′–TCAACAGCAACTATCACATCTGAKG –3′	
modA	*modA*_F1	5′–TRAAAGABGCAYTRGARGAA–3′	
	*modA*_R1	5′–TCRTCAGCMGCAACTACRAC–3′	
	*modA*_F2	5′–CAAATTGAAMAVGGTGCSCC–3′	
	*modA*_R2	5′–GGYACRCTTCTRGGTTCWCC–3′	
modBC	*modBC*_F1	5′–AAAACKTCMTTRCTTGCYACA–3′	
	*modBC*_R1	5′–GGTAAACTGATRCGCCAAAAGA–3′	
	*modBC*_F2	5′–GAATGAAGCTTGGTTTTGGGC–3′	
	*modBC*_R2	5′–GCTAAGGCTACCCGTTGTTG–3′	

* F at the end of the primer name is the forward primer; R at the end of the primer name is the reverse primer. For *modA* and *modBC* primers, Nos. 1 and 2 correspond to two sets of primers.

† Standard International Union of Biochemistry codes for degenerate bases: B=C+G+T; K=G+T; M=A+C; R=A+G; S=G+C; V=A+C+G; W=A+T; Y=C+T.

were thawed on ice. One milliliter of algal protein extraction buffer (4% sodium dodecyl sulfate (SDS), 0.05 mol L^{-1} sodium carbonate, 30% glycerol, 2.5 mmol L^{-1} phenylmethanesulphonylfluoride, 0.05 mol L^{-1} dithiothreitol) was added to each sample and the sample was sonicated, boiled, and centrifuged for 1 min at 15,000 × *g* at 4°C. Protein concentrations were quantified using the EZQ® Protein Quantification Kit (Molecular Probes, Invitrogen Detection Technologies) and an FLx800 Microplate Fluorescence Reader (Bio-Tek Instruments).

Nitrogenase immunodetection—Western immunoblotting was performed on extracted sample protein and NifDK protein standard (provided by L. Rubio, P. Ludden Laboratory, University of California Berkeley) separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 4–20% gradient gels (NuSep iGels). The gel was transferred to a polyvinylidene fluoride membrane, followed by immunoblotting with antibodies against NifDK (provided by L. Rubio) conjugated to IgG HRP (BioRad) and visualization with Supersignal® chemiluminescent substrate (Pierce). ImageJ (Abramoff et al. 2004) was used to quantify NifDK concentrations per total protein using the NifDK standard calibration curve.

Amplification and sequencing of genes—To establish the phylogenetic identity of the CCMP 2511, *nifD* and 16S ribosomal ribonucleic acid (rRNA) genes were amplified and sequenced. 16S primers were based on a previous study (Nubel et al. 1997). *nifD* primers were designed using the conserved regions of the PCC 7120 gene based on the genome sequence (Kaneko et al. 2001). Genomic deoxyribonucleic acid (DNA) was extracted in 1.2% SDS, 50 mmol L^{-1} Tris (potential hydrogen [pH] = 8.0), 30 mmol L^{-1} EDTA, 50 mmol L^{-1} β-mercaptoethanol, 220 mmol L^{-1} sodium chloride, followed by phenol-chloroform treatments. Polymerase chain reactions were performed with Sigma JumpStart REDTaq on an Eppendorf Gradient Mastercycler. Primer sequences are given in Table 1. Amplified DNA was run on ~ 1% agarose gels, and bands in the correct size range were excised and gel-purified using the QIAquik gel extraction kit (Qiagen) in preparation for sequencing. DNA sequences were edited using Sequencher™ software (Gene Codes) and were deposited into GenBank. Accession Nos. FJ455089 and FJ455090 were assigned to the partial 16S rRNA and *nifD* genes, respectively, from CCMP 2511.

We designed primers to determine whether CCMP 2511 contained the V-nitrogenase (*vnfD*) gene and genes encoding proteins involved in high-affinity molybdate (MoO_4^{2-}) uptake (*modABC*) and storage (*mop*). *vnfD* primers were designed using conserved regions of the *A. variabilis* ATCC 29413 gene based on the genome sequence (http://genome.jgi-psf.org/finished_microbes/anava/anava.home.html). Degenerate *modA*, *modBC*, and *mop* primers were designed by aligning DNA sequences from HC with sequenced genomes: *Nostoc* sp. PCC 7120 (Kaneko et al. 2001), *Anabaena variabilis* ATCC 29413, *Nostoc punctiforme* PCC 73102 (Meeks et al. 2001), and *Nodularia spumigena* CCY 9414 (https://research.venterinstitute.org/moore/) using ClustalW. Because *N. spumigena* CCY 9414 lacks *mop* genes, we used that species only for design of *modA* and *modBC* primers. Primer sequences are given in Table 1.

Genomic search for cyanobacterial mop *and* modABC *genes*—We searched the publicly available genomes of 44 cyanobacteria on the Department of Energy Joint Genome Institute Integrated Microbial Genomics database (Markowitz et al. 2008) using a Basic Local Alignment Search Tool for Proteins (BLASTP) search with Mop, ModA, and ModBC proteins from PCC 7120. The E-value (the number

of hits that one can expect to see by chance) cutoff was set to 1×10^{-5}. BLASTP search results that had similar length (69 residues for Mop, 265 residues for ModA and 601 residues for ModBC) were tagged as positive hits.

Results

The freshwater cyanobacterium (*Nostoc* sp. PCC 7120) required more time to induce symptoms of Mo–N co-limitation than the coastal cyanobacterium (*Nostoc* sp. CCMP 2511). Chl *a* declined in the −N −Mo treatments after Day 3 and Day 14 for CCMP 2511 and PCC 7120, respectively (Fig. 1A). N_2-fixation rates decreased by Day 12 and 19 for CCMP 2511 and PCC 7120, respectively (Fig. 1B). When Mo was added back to −N −Mo treatments on the thirtieth day to PCC 7120, Chl *a* concentrations and growth rates were restored to those of replete conditions in 3 d.

Cellular Mo concentrations increased with Mo in the growth media (Table 2), as shown for Mo : C ratios in Fig. 2. When the freshwater HC PCC 7120 was grown at ~ 1500 nmol Mo L^{-1}, cellular Mo : C was an order of magnitude higher (~ 100 μmol mol^{-1}) than at lower Mo concentrations. At high Mo, N_2-fixing cultures contained 1.5–3 times higher cellular Mo : C than NO_3^--assimilating cultures (Fig. 2). In contrast, cellular Mo : C in the coastal HC CCMP 2511 stayed fairly constant (1–6 μmol mol^{-1}) with and without added N and across the range of media Mo concentrations (Table 2; Fig. 2).

Cultures with < 1 μmol Mo mol^{-1} C had C : N ratios up to ~ 10 mol mol^{-1}, whereas C : N ratios in cultures with cellular Mo > 1 μmol Mo mol^{-1} C were 4–6 mol mol^{-1} (Fig. 3A; Table 2). The higher C : N ratios were driven by a decrease in cellular N at low Mo : C ratios, because there was little change in C content across the range of Mo (Table 2).

In immunoblots, we targeted two nitrogenase subunits, D and K, because they form the core of the nitrogenase complex that contains the Mo co-factor. NifDK protein expression generally increased up to 10 μmol mol^{-1} Mo : C and showed high variability at > 10 μmol mol^{-1} cellular Mo : C, although only two data points were available (Fig. 3B). A similar pattern was observed for dependence of N_2-fixation rates on cellular Mo : C (Fig. 3C), although a clear species difference was also observed. N_2-fixation rates were generally higher in the freshwater HC PCC 7120 than the coastal HC CCMP 2511 with comparable NifDK expression. Nitrogenase activity was suppressed in PCC 7120 when cellular Mo : C ratios were < 100 μmol mol^{-1}. The N isotopic signature of cyanobacterial biomass from −N treatments averaged −2.3 ± 0.2‰ (average ± SD) whereas +N treatments averaged −54.5 ± 0.8‰ (Table 2). −N −Mo treatments of PCC 7120 had extremely depleted biomass $\delta^{15}N$ (averaging −6 ± 2‰) compared to other −N treatments.

We successfully amplified the *mop* gene from three species of freshwater HC with sequenced genomes, but were not able to amplify the gene from the coastal HC CCMP 2511 (Fig. 4A). Using BLAST searches of previously sequenced genomes, we found that the occurrence of *mop* genes in cyanobacterial genomes was limited to coastal and freshwater species; *mop* was not found in any marine cyanobacteria (Table 3). Furthermore, only non-HC in coastal waters contained *mop*, whereas both HC and non-HC in freshwater environments had the gene. Three out of five diazotrophic coastal cyanobacteria contained *mop*, while none of the five coastal nondiazotrophic *Synechococcus* strains possessed it. Every one of the seven freshwater diazotrophic cyanobacteria contained *mop*,

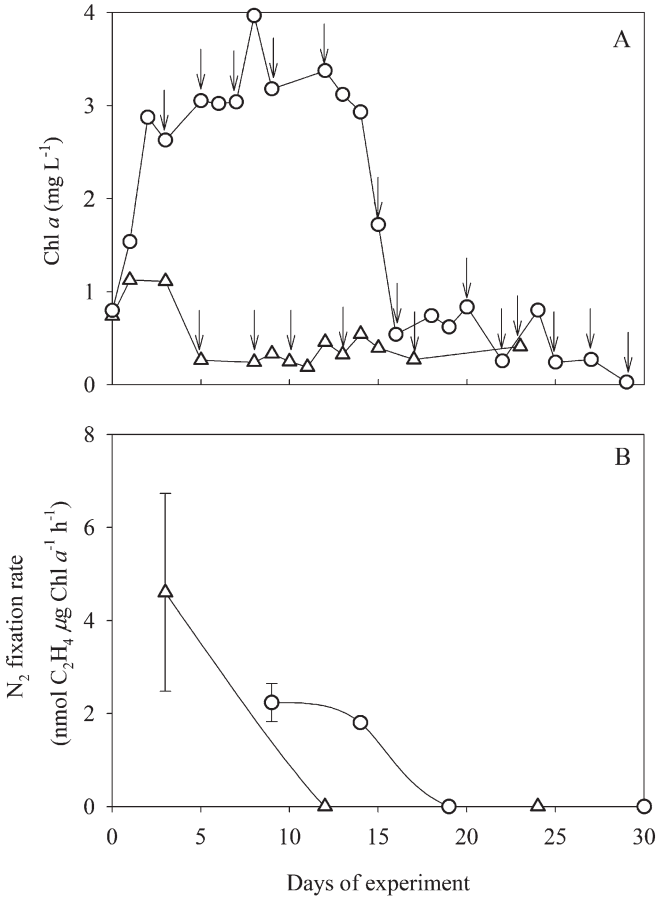

Fig. 1. (A) Average Chl *a* concentrations, and (B) N_2 fixation rates over the course of the month-long experiments for cultures grown in −N −Mo treatments. Circles represent freshwater HC *Nostoc* sp. PCC 7120 and triangles represent coastal HC *Nostoc* sp. CCMP 2511. The cultures were transferred from −N +Mo (1500 nmol L^{-1}) media on Day 0. They were centrifuged and washed three times with −N −Mo media before they were diluted seven-fold into the media for each treatment. Arrows show the days when cultures were diluted to optical clarity, for which pre- and postdilution Chl *a* concentrations were averaged. Maximum rates of N_2-fixation occurred the first day rates were measured in each experiment for each species. For PCC 7120, the maximum N_2-fixation rate was 8 ± 0.5 nmol C_2H_4 μg Chl a^{-1} h^{-1} (Day 9, treatment −N +1500 nmol L^{-1} Mo). For CCMP 2511, the maximum N_2-fixation rates were 10 ± 6 nmol C_2H_4 μg Chl a^{-1} h^{-1} (Day 3, treatment −N +100 nmol L^{-1} Mo) and 8 ± 4 nmol C_2H_4 μg Chl a^{-1} h^{-1} (Day 3, treatment −N +10 nmol L^{-1} Mo).

Table 2. Cellular concentrations of molybdenum (Mo), carbon (C), and nitrogen (N), and cellular N isotopic composition, of *Nostoc* spp. PCC 7120 and CCMP 2511 grown under a range of Mo concentrations with and without NO_3^- in the growth media. Cells were grown for 30 d (and diluted to optical clarity every 3–5 d) before they were harvested for analysis. Numbers in parentheses represent standard deviations.

	Media concentrations		Actively fixing N_2	Mo cellular concentrations					Cellular concentrations			
Nostoc strain	NO_3^- (mmol L^{-1})	Mo (nmol L^{-1})	at time of harvest (Y or N)?	μg Mo g^{-1} dry weight	mg Mo g^{-1} protein	μmol Mo mol^{-1} C	μmol Mo mol^{-1} N	μmol Mo mol^{-1} P	C (mg g^{-1} dry weight)	N (mg g^{-1} dry weight)	C : N (mol mol^{-1})	$\delta^{15}N_{vs.\ air}$ (‰)
7120	18	1650(140)	N	80	2.3	58	220	2335	363(1)	96(1)	4.4(0.1)	−53.5(0.5)
			N	32	0.5	14	265	863	344(62)	91(20)	4.5(0.2)	−54.1
2511	18	1450(50)	N	0.49	0.11	2.5	11	84	287(4)	76(3)	4.4(0.1)	−55.3
			N	8.29	0.11	4.4	19	78	253(13)	69(4)	4.3(0.1)	−55.0(0.1)
7120	0.03	0.2(0.1)	N	0.5	0.01	0.9	7	7	324(1)	41(1)	9.3(0.2)	−7.4(1.1)
			N	0.1	0.02	0.2	0.3	6	379(67)	44(8)	10	−4.4(0.1)
2511	0.03	0.5(0.2)	Y	1.9	0.01	0.5	4	9	396(7)	53(1)	8.5	−2.4(0.1)
			N	1.8	0.04	0.9	8	18	317(15)	39(3)	9.3(0.2)	−2.8(0.3)
7120	0.03	11.2(0.6)	Y	4.1	0.1	2.3	11	87	403(28)	85(6)	5.5	−2.2
			Y	4.9	0.06	1.9	9	90	435(8)	95(1)	5.4	−2.3
2511	0.03	9.8(0.2)	Y	5.1	0.24	2.1	14	69	412(25)	71(4)	6.7(0.1)	−2.3
			Y	5.1	0.06	0.9	6	51	405(49)	73(9)	6.4	−2.0(0.1)
7120	0.03	108(2)	Y	7.4	0.11	3.5	16	159	394(5)	87(2)	5.3	−2.2(0.1)
			Y	9.5	0.1	3.5	16	160	387(49)	87(11)	5.2	−2.2
2511	0.03	100(6)	Y	8.3	0.2	4.4	28	121	376(16)	69(3)	6.5	−2.2
			Y	8.7	0.2	1.8	11	117	395(6)	73(1)	6.4	−2.5
7120	0.03	1650(140)	Y	235	2.1	95	429	3664	309(14)	69(7)	5.3(0.3)	−2.7(0.6)
			Y	485	2.1	132	607	4255	383(26)	83(10)	5.4(0.3)	−2.4(0.1)
2511	0.03	1500(65)	Y	9.6	0.3	4.4	27	136	382(45)	70(7)	6.3(0.1)	−2.4(0.1)
			Y	10.7	0.2	5.8	35	151	372(88)	71(15)	6.1(0.1)	−2.2

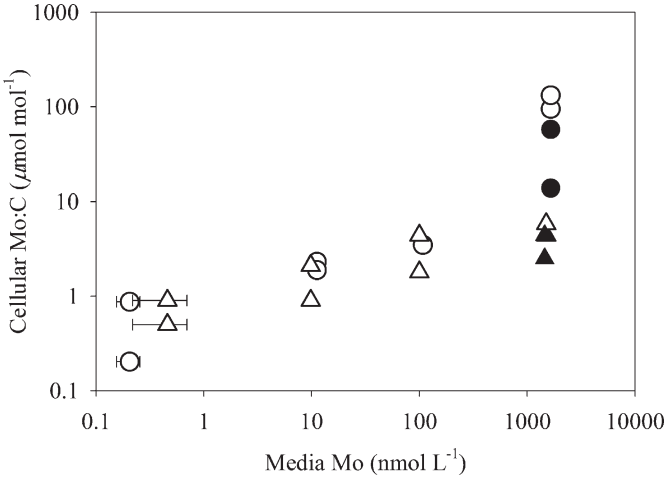

Fig. 2. Cellular Mo : C (taken on the last day of the experiments) over the range of Mo concentrations in the growth media. Circles represent freshwater HC *Nostoc* sp. PCC 7120 and triangles represent coastal HC *Nostoc* sp. CCMP 2511. Filled symbols represent +N (NO_3^--assimilating) treatment, and open symbols represent −N (N_2-fixing) treatments.

while none of the four freshwater nondiazotrophic cyanobacteria sequenced had it.

We were successful at amplifying the *modA* and *modBC* genes from *Nostoc* sp. PCC 7120, *A. variabilis* ATCC 29413, *N. punctiforme* PCC 73102 and *N. spumigena* CCY 9414 with two different primer sets (Table 1), but were unsuccessful at amplifying the genes from CCMP 2511, even after repeated attempts at nested PCR. Both *modA* and *modBC* genes were found to occur in all cyanobacteria with sequenced genomes, with the exception of marine *Prochlorococcus* and *Synechococcus* strains, and two out of five coastal *Synechococcus* strains (Table 3).

Phylogenetic analysis revealed that the freshwater HC PCC 7120 and the coastal HC CCMP 2511 species had 97% and 84% identity in conserved regions of the 16S rRNA and *nifD* genes, respectively. CCMP 2511 was most closely related in 16S rRNA phylogeny (99% identity) to two uncultured samples from the sediments of the saline lake Salar de Huasco in northern Chile (Dorador et al. 2008). Attempts to amplify *vnfD* genes from CCMP 2511 confirmed that the species we studied lacked the V-nitrogenase (data not shown).

Discussion

Mo requirements for N_2-fixation by heterocystous cyanobacteria—Our comparison of Mo requirements for N_2-fixation in the freshwater HC PCC 7120 and the coastal HC CCMP 2511 over 30 d revealed that both strains maintained N_2-fixation at $\geq$ 10 nmol L^{-1} throughout the experiment. This was unexpected, because CCMP 2511 was isolated from a bay where Mo is likely near seawater concentrations ($\sim$ 105 nmol L^{-1}). When Mo was $<$ 1 nmol L^{-1} in the media, both HC species became Mo–N co-limited (Mo : C $<$ 1 μmol mol^{-1} and C : N $>$ 8 mol mol^{-1}; Table 2; Figs. 2, 3A). However, both Chl *a* concentrations and N_2-fixation rates required time periods of weeks to decline (Fig. 1), consistent with previous studies (Fay and Vasconcelos 1974; Jacob and Lind 1977). This lag time suggests HC may maintain adaptive strategies, such as stores of cellular Mo, that allow them to survive on low Mo availability for weeks after Mo concentrations are depleted (*see* below).

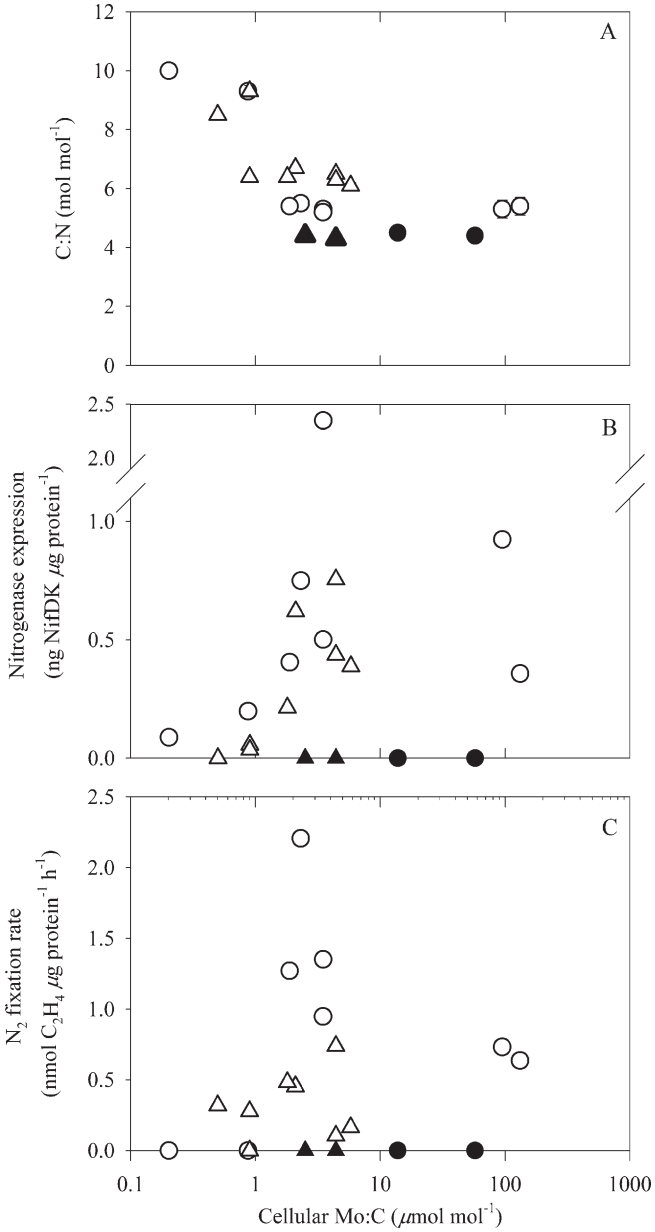

Fig. 3. (A) Cellular C : N ratios, (B) NifDK protein expression, and (C) N_2-fixation rates over a range of cellular Mo : C values, taken on the last day of the experiments (corresponding to the y-axis in Fig. 2). Circles represent freshwater HC *Nostoc* sp. PCC 7120 and triangles represent coastal HC *Nostoc* sp. CCMP 2511. Filled symbols represent +N (NO_3^--assimilating) treatment, and open symbols represent −N (N_2-fixing) treatments. The cells were harvested on the last day of the experiments for measurement of NifDK expression (B) and N_2-fixation rates (C).

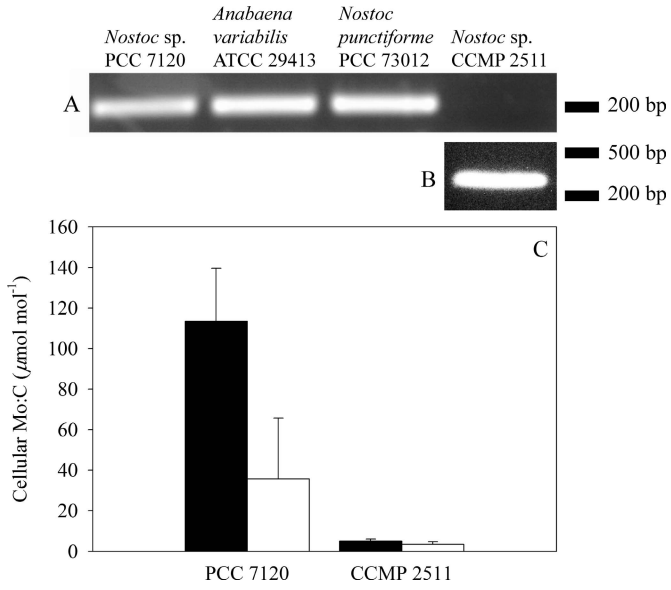

Fig. 4. (A) Degenerate primers were used to amplify *mop* genes (210 base-pairs) from three freshwater HC (*Nostoc* sp. PCC 7120, *A. variabilis* ATCC 29413, and *N. punctiforme* PCC 73102); these primers did not amplify a *mop* gene from *Nostoc* sp. CCMP 2511. (B) A positive control was performed to ensure the CCMP 2511 DNA used for this PCR was of good quality by amplifying part of the *nifD* gene (338 base-pairs) from the same DNA used in (A). (C) Cellular Mo : C was higher in freshwater HC PCC 7120 than coastal HC CCMP 2511 in both +N (NO_3^--assimilating, white bars) and −N (N_2-fixing, black bars) conditions when grown in the 1500 nmol L^{-1} Mo treatment.

Our data indicate that 10 nmol Mo L^{-1} is sufficient for maximal N_2-fixation in the two *Nostoc* species studied, as previously observed for other HC such as *A. cylindrica* (Jacob and Lind 1977) and *A. variabilis* ATCC 29413 (Zerkle et al. 2006). The maximal N_2-fixation rates measured in this study (~ 8 nmol C_2H_4 μg Chl a^{-1} h^{-1}) are comparable to N_2-fixation rates of *A. variabilis* ATCC 29413 and *A. cylindrica* (Attridge and Rowell 1997; Rowell et al. 1998). NifDK expression followed the same trend as N_2-fixation rates; that is, highest expression was observed at intermediate Mo concentrations. However, the immunodetection of NifDK does not necessarily suggest that it is active because inactive NifDK is expressed in freshwater cyanobacteria under conditions of Mo starvation (Nagatani and Haselkorn 1978; Hallenbeck and Benemann 1980; Attridge and Rowell 1997). Accordingly, we found evidence for inactive NifDK in −N −Mo treatments of PCC 7120 because we observed some NifDK expression in cultures lacking nitrogenase activity (Fig. 3B,C).

The N isotopic signature of N_2-fixing biomass was generally −2‰ to −3‰ (Table 2). This is within the range of $\delta^{15}N$ values previously reported for N_2-fixing cyanobacteria, ~ 0‰ to −3‰ (Bauersachs et al. 2009). Cellular $\delta^{15}N$ values of NO_3^--assimilating cultures reflected uptake of isotopically light (−50‰) NO_3^- from the media. Interestingly, the $\delta^{15}N$ isotopic signature of −N −Mo PCC 7120 (−6 ± 2‰, average ± SD) was significantly lighter ($p < 0.01$) than −N treatments with added Mo (−2.3 ± 0.2‰). The mechanism behind the stronger ^{15}N discrimination for Mo-deficient cells is unknown. A previous study of *A. variabilis* ATCC 29413 found more negative N isotopic composition (−3.84 ± 0.85‰, average ± SD) of V-grown than Mo-grown (−1.42 ± 0.40‰) cultures (Rowell et al.

Table 3. Results of a search of 44 publicly available cyanobacterial genomes (*see* Methods) for occurrences of the *mop* and *modABC* genes.

Cyanobacterial species	Diazotrophic? (Y or N)	HC or non-HC?	*mop* gene present? (Y or N)	*modABC* genes present? (Y or N)
Marine				
Trichodesmium erythraeum ISM101	Y	non-HC	N	Y
Crocosphaera watsonii WH 8501	Y	non-HC	N	Y
Prochlorococcus marinus (12 strains)	N	—	N	N
Synechococcus spp. (7 strains)	N	—	N	N
Coastal				
Lyngbya sp. PCC 8106	Y	non-HC	Y	Y
Cyanothece (2 strains)	Y	non-HC	Y	Y
Nodularia spumigena sp. CCY9414	Y	HC	N	Y
Nostoc sp. CCMP 2511 (this study)	Y	HC	?	?
Synechococcus spp. (5 strains)	N	—	N	Y(3) N(2)
Freshwater				
Nostoc sp. PCC 7120 (this study)	Y	HC	Y	Y
Anabaena variabilis ATCC 29413	Y	HC	Y	Y
Nostoc punctiforme PCC 73102	Y	HC	Y	Y
Synechococcus (Octopus Spring, Yellowstone, 2 strains)	Y	non-HC	Y	Y
Cyanothece (5 strains)	Y	non-HC	Y	Y
Synechocystis sp. PCC 6803	N	—	N	Y
Synechoccocus elongatus (2 strains)	N	—	N	Y
Microcystis aeruginosa NIES-843	N	—	N	Y

1998). In ATCC 29413, V-nitrogenase (Vnf) may discriminate more strongly against ^{15}N than Mo-nitrogenase, but this cannot be the case in our study because PCC 7120 does not possess the *vnf* gene (Kaneko et al. 2001). Another study of ATCC 29413 grown on low V found no significant N isotopic variation between cultures grown over a range of Mo concentrations from 0.1 nmol L^{-1} to 100 nmol L^{-1} (Zerkle et al. 2008). Our data suggest that long time periods (weeks) may be required to induce symptoms of Mo–N co-limitation and, thus, production of ^{15}N-depleted biomass. Because N_2-fixation rates were below the limit of detection in ^{15}N-depleted cultures, it is possible that trace amounts of fixed N in the medium were the source of N to the cyanobacteria in −Mo treatments, and contributed to the light isotopic values. An alternative hypothesis is that very slow growth rates of the two −N −Mo treatments of PCC 7120 contributed to the highly depleted isotopic composition, similar to the effect of growth rate and CO_2 concentration on C isotopes in marine phytoplankton (Laws et al. 1997). At low growth rates, gaseous uptake is entirely due to passive diffusion, which imparts greater isotopic fractionation than active gaseous uptake at higher growth rates (Laws et al. 1997). Whatever the mechanism, isotopically depleted N signatures in Mo-limited HC may be a useful isotopic tool to use to understand N-fixation in modern freshwater environments and ancient (possibly Mo-limited) marine environments. Zerkle et al. (2008) found that high Fe concentrations can yield similarly ^{15}N-depleted biomass, so it will be necessary to separate the isotopic influence of these two different metals.

Higher cellular Mo : C was observed in N_2-fixing vs. NO_3^--assimilating PCC 7120 (Figs. 2, 4B), in accordance with the higher Mo content and lower specific activity of nitrogenase compared to nitrate reductase. Nitrogenase contains two Mo atoms and, in HC, has a specific activity of 1.2 μmol C_2H_2 mg NifDK^{-1} min^{-1} (Hallenbeck et al. 1979) corresponding to 4.8 μmol N_2 mg NifDK protein^{-1} min^{-1} using a 1 C_2H_2 : 4 N_2 conversion ratio (Capone 1993). Cyanobacterial nitrate reductase (abbreviated NarB) contains only one Mo atom and has a specific activity of $\sim$ 300 μmol NO_2^- mg NarB protein^{-1} min^{-1} in the diazotrophic cyanobacterium *Plectonema boryanum* (Mikami and Ida 1984). Thus, in terms of N assimilation, NarB is 37 times more efficient per mol N than NifD. This quantitative analysis suggests that significantly less cellular Mo is required to support NO_3^- assimilation than N_2-fixation, consistent with previous calculations by Raven (1988). The high Mo use efficiency of NarB may explain our previous finding that $<$ 1 nmol L^{-1} Mo is sufficient to support high growth rates of NO_3^--assimilating cultures of PCC 7120 over a 1-month experiment (Glass et al. 2009).

Cyanobacterial adaptations to low Mo—Highly specific Mo uptake is one mechanism by which HC can survive on low Mo concentrations ($\leq$ 10 nmol Mo L^{-1}) for extended periods of time. High-affinity ATP-binding cassette-type MoO_4^{2-} transporters, encoded by *modABC* genes, allow cyanobacteria to scavenge trace Mo from growth media (Zahalak et al. 2004). ModA functions as a periplasmic molybdate-binding protein; the ModBC fusion functions as an cytoplasmic membrane protein (ModB) and ATP-binding (ModC) protein (Zahalak et al. 2004). Our BLAST searches showed that *modA* and *modBC* genes are present in numerous cyanobacteria (Table 3) but the gene sequences are dissimilar between different cyanobacterial strains. We attributed the sustained N_2-fixation by CCMP 2511 for $\sim$ 13 d to high-affinity Mo transport through the ModABC uptake system. In order to determine whether coastal HC CCMP 2511 had the ability to express the ModABC uptake system, we attempted to amplify *modA* and *modBC* genes from CCMP 2511, but we were unsuccessful in these attempts. Our lack of success was likely due to the difficulty in designing degenerate primers to amplify genes with very low identity between cyanobacteria strains.

In −Mo treatments, N_2-fixation continued in freshwater HC PCC 7120 for 7 d past coastal HC CCMP 2511 (Fig. 1B). This extended N_2-fixation time under Mo limitation suggests that PCC 7120 has additional mechanisms of coping with low Mo supply beyond those available to CCMP 2511. High cellular Mo : C ($>$ 100 μmol mol^{-1} Mo : C) provides evidence for Mo storage in PCC 7120, but not CCMP 2511 ($\sim$ 5 μmol mol^{-1} Mo : C), which could explain the longer time required to induce symptoms of Mo–N co-limitation in PCC 7120. The extremely high cellular Mo : C ratio of PCC 7120 grown at 1500 nmol L^{-1} Mo suggests the expression of a Mo-containing protein present in greater abundance than nitrogenase. A simple calculation using previously determined values of 2 mol Mo mol NifDK^{-1} and the molecular weight of HC NifDK (220,000 g mol^{-1}; Hallenbeck et al. [1979]) along with our measurements of $\sim$ 1 ng NifDK μg protein^{-1} (Fig. 3B) revealed that, under the highest Mo treatment, $<$ 1% of total Mo could be accounted for by nitrogenase expression alone.

Excess Mo not associated with nitrogenase may be bound to a small (69 amino acid) putative MoO_4^{2-}-binding protein, Mop, which is coded just upstream of the *nif* operon in PCC 7120 (Markowitz et al. 2008). Mop has been shown through two-dimensional proteomic analysis to be one of the most abundant proteins in freshwater HC PCC 7120 cultured with NO_3^- (Sazuka 2003). In other diverse diazotrophic bacteria, Mop proteins are implicated in Mo storage. In anoxygenic photosynthetic bacteria, *mop* transcription is activated only when cellular Mo reaches a critical concentration threshold (Wiethaus et al. 2006). The crystal structure of Mop in *Clostridium pasteurianum* revealed that MoO_4^{2-} is bound to the protein with low affinity, and binding sites are blocked when MoO_4^{2-} is unbound. These observations suggest that this protein only binds MoO_4^{2-} when cellular Mo concentrations are high (Schüttelkopf et al. 2002). Our Mo : C results, in combination with previous studies (Thiel et al. 2002), suggest that Mop may serve a similar role in PCC 7120. Testing this hypothesis would require simultaneous measurement of cellular Mo and *mop* gene expression, which was beyond the scope of this study.

The distribution of *mop* genes appears to be associated with diazotrophy in all freshwater cyanobacteria considered, and three out of the five coastal cyanobacteria (Table 3). Because all cyanobacteria strains in Table 3 can

assimilate NO_3^- as an N source (with the exception of *Prochlorococcus marinus*), this trend is consistent with the higher Mo requirements for N_2-fixation compared to NO_3^- reduction (*see* above and Fig. 4B). It is unclear why three species of coastal non-HC contain *mop* while it is lacking in both HC species (*N. spumigena* CCY 9414 and CCMP 2511). However, the negative *mop* PCR result for coastal HC CCMP 2511 (Fig. 4A) is consistent with the finding that cellular Mo : C in this species is only ~ 10% that of freshwater HC PCC 7120 at high Mo levels (Fig. 4B). Native populations of HC (*Aphanizomenon* sp. and *N. spumigena*) in the Baltic Sea also contain low cellular Mo : C, between 1 μmol mol^{-1} to 2 μmol mol^{-1} (Walve and Larsson 2007), consistent with the lack of the *mop* gene in *N. spumigena* CCY 9414 (Table 3). The cellular Mo : C ratio we measured for N_2-fixing PCC 7120 grown at 100 nmol L^{-1} Mo (3.3 ± 1.1 μmol mol^{-1}, average ± SD) was similar to previous Mo : C measurements of the N_2-fixing marine species *Trichodesmium* ISM 101 (2.56 ± 2.02 μmol mol^{-1}, average ± SD) grown in laboratory cultures at approximately at the same Mo concentration (Tuit et al. 2004). Because *Trichodesmium* lacks the *mop* gene (Table 3), the similarity in Mo content between *Trichodesmium* and PCC 7120 when both are grown at 100 nmol Mo L^{-1} suggests that PCC 7120 does not express Mop until Mo concentrations are higher than 100 nmol Mo L^{-1}.

Our results have potential implications for understanding long-term interactions between the evolution of cyanobacteria and biogeochemical conditions, because Mo–N co-limitation likely influenced the early evolution of cyanobacteria (Anbar and Knoll 2002). Fossil evidence suggests that HC diversified over two billion years ago (Tomitani et al. 2006), when marine Mo concentrations were less than one-tenth of modern values (Anbar et al. 2007; Scott et al. 2008). Thus, there was likely strong selection for cyanobacteria with strategies for Mo acquisition and storage during the early evolution of cyanobacteria. In freshwater HC (*Nostoc* sp. PCC 7120, *A. variabilis* ATCC 29413, and *N. punctiforme* PCC 73102) and non-HC (*Synechococcus* strains isolated from Octopus Spring, Yellowstone National Park), *mop* genes are present upstream of the *nif* operon (Markowitz et al. 2008). Horizontal gene transfer (HGT) of *nif* genes was likely important in the early evolution of cyanobacterial N_2-fixation (Tomitani et al. 2006; Shi and Falkowski 2008); one explanation for the proximity of *mop* and *nif* genes in cyanobacteria is that they were both carried on the *nif* operon during HGT several billion years ago. It is also possible that *mop* genes evolved alongside *modABC* uptake systems because these genes border each other in numerous bacterial species, including the coastal non-HC *Cyanothece* CCY 0110. In either case, *mop* genes would have been lost in marine diazotrophic cyanobacteria and some coastal strains as Mo levels rose in the marine environment ~ 500 million years ago (Collier 1985; Scott et al. 2008), so that Mo storage no longer provided an evolutionary advantage for life in the sea. We suggest that these long-term changes have resulted in a modern-day situation in which freshwater HC must often cope with potential Mo-limitation while marine cyanobacteria rarely face constraints on N_2-fixation due to low Mo supplies.

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