Molybdenum Biogeochemistry in an Evolutionary Context:
Nitrogen Assimilation, Microbial Storage
and Environmental Budgets

by

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A Dissertation Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy

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May 2011
ABSTRACT

Molybdenum (Mo) is a key trace nutrient for biological assimilation of nitrogen, either as nitrogen gas (N_2) or nitrate (NO_3\(^-\)). Although Mo is the most abundant metal in seawater (105 nM), its concentration is low (<5 nM) in most freshwaters today, and it was scarce in the ocean before 600 million years ago. The use of Mo for nitrogen assimilation can be understood in terms of the changing Mo availability through time; for instance, the higher Mo content of eukaryotic vs. prokaryotic nitrate reductase may have stalled proliferation of eukaryotes in low-Mo Proterozoic oceans. Field and laboratory experiments were performed to study Mo requirements for NO_3\(^-\) assimilation and N_2 fixation, respectively. Molybdenum-nitrate addition experiments at Castle Lake, California revealed interannual and depth variability in plankton community response, perhaps resulting from differences in species composition and/or ammonium availability. Furthermore, lake sediments were elevated in Mo compared to soils and bedrock in the watershed. Box modeling suggested that the largest source of Mo to the lake was particulate matter from the watershed. Month-long laboratory experiments with heterocystous cyanobacteria (HC) showed that <1 nM Mo led to low N_2 fixation rates, while 10 nM Mo was sufficient for optimal rates. At 1500 nM Mo, freshwater HC hyperaccumulated Mo intercellularly, whereas coastal HC did not. These differences in storage capacity were likely due to the presence in freshwater HC of the small molybdate-binding protein, Mop, and its absence in coastal and marine cyanobacterial species. Expression of the mop gene was regulated by Mo availability in the freshwater HC species Nostoc sp. PCC
7120. Under low Mo (<1 nM) conditions, *mop* gene expression was up-regulated compared to higher Mo (150 and 3000 nM) treatments, but the subunit composition of the Mop protein changed, suggesting that Mop does not bind Mo in the same manner at <1 nM Mo that it can at higher Mo concentrations. These findings support a role for Mop as a Mo storage protein in HC and suggest that freshwater HC control Mo cellular homeostasis at the post-translational level. Mop’s widespread distribution in prokaryotes lends support to the theory that it may be an ancient protein inherited from low-Mo Precambrian oceans.
DEDICATION

This dissertation is dedicated to my amazing family: my fiancé, Ivan Schustak; my mother, Sara Glass; my father, Geoffrey Glass; my sister, Rachel Glass and our Siamese cats Daisy (née Tofu) and Starkey. Their love and support made the completion of this dissertation possible.
ACKNOWLEDGMENTS

First and foremost, I thank my PhD advisor, Ariel Anbar, for all that he did to make my dissertation research possible. Secondly, I thank the members of my PhD committee: James Elser, Petra Fromme, Hilairy Hartnett, Anne Jones and Everett Shock for their time, advice and support. Thanks to the co-authors of my dissertation chapters for their collaboration and help during the writing of the chapters: Ariel Anbar, Richard Axler, Anthony Chappaz, James Elser, Brooke Eustis, Alan Heyvaert, Amisha Poret-Peterson, David Waetjen and Felisa Wolfe-Simon.

I thank the support and collegiality of the Anbar Laboratory at Arizona State University from 2006-2011, in particular: Ariel Anbar, Gail Arnold, Greg Brennecka, Yun Duan, Gwyneth Gordon, Amy Kelly, Brian Kendall, Brian Majestic, Chris Mead, Jennifer Morgan, Amisha Poret-Peterson, Stephen Romaniello, Laura Wasylenki and Felisa Wolfe-Simon. I thank the laboratory expertise of members of the W. M. Keck Environmental Biogeochemistry Laboratory and Astrobiology Laboratories at Arizona State University, in particular Carina Arrua, Gwyneth Gordon, Marcia Kyle, Anthony Michaud, and Natasha Zolotova. Fellow ASU Ph.D. student Kathryn Noonan (née Alexander) provided a tremendous deal of laboratory assistance. I also thank ASU undergraduate research assistants Eric Hughes, Megan Kelly, Michelle Krieg and Zureyma Martinez who helped with experiments and sample analysis and members of the Castle Lake Long-Term Research Program (Sudeep Chandra, Rene Henery, Jacqie Brownstein, Brooke Eustis, Marcy Kamerath and Lauren
Roaldson) who aided with field work coordination and data collection at Castle Lake during the summers of 2008 and 2009.

I am thankful to the following people outside of the ASU community for helpful discussions pertaining to my dissertation: John Berges, Michael Brett, Robert Blankenship, Christopher Dupont, Charles Goldman, William Hunter, Tatsuo Omata, John Raven, Jason Raymond, Luis Rubio, William Sunda, Teresa Thiel and David Wynne. Thanks to Rene Henery, Mak Saito and anonymous reviewers for critical readings of the manuscripts that compose this dissertation. Thanks to Susan Selkirk for help with figure artwork.

Many people kindly provided samples and standards for this research, including Luis Rubio (anti-NifDK antibodies and protein standards from the Ludden Laboratory at UC Berkeley), Ruth Potrafka, Tanya Do and Ankita Kothari (primers, DNA and cyanobacterial cultures from the Garcia-Pichel Laboratory at ASU), and Brenda Pratte (cyanobacterial cultures and mutants from the Thiel Laboratory at University of Missouri).

This dissertation research was funded by an NSF Graduate Student Research Fellowship (Geosciences-Geochemistry #2006038382), the NASA Astrobiology Institute, the Agouron Institute, the NASA Exobiology and Evolutionary Biology Program, an Arizona State University (ASU) Graduate and Professional Student Association Research Grant, a Grant-in-Aid of Research (#G200810150320) from Sigma Xi The Scientific Research Society, and a Lewis and Clark Astrobiology Field Research Grant from the American Philosophical Society.
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Chapter 1

INTRODUCTION

The chemical evolution of the Earth’s biosphere has been intricately intertwined with the biological evolution of microbial metabolisms. Metals are pervasive in biology and likely have been since life first emerged. Molybdenum (Mo) is a key trace nutrient for biological assimilation of nitrogen, either as nitrogen gas or nitrate, due to its presence in the active sites of enzymes that catalyze nitrogen fixation and nitrate reduction. In the case of nitrogen fixation, Mo is present in the enzyme nitrogenase as an inorganic (Mo-Fe-S) cofactor. This cofactor reduces dinitrogen gas to produce 2 molecules of ammonia, the chemical species of nitrogen that can be converted into biomolecules. In the case of nitrate reductase, Mo is bound to an organic pterin co-factor, which catalyzes the first step in nitrate assimilation: the 2-electron reduction of nitrate to nitrite. The evolution of these Mo enzymes has likely been influenced by changes in the concentration of Mo in earth’s ecosystems through time (Anbar and Knoll, 2002; Dupont et al., 2010).

Early life likely evolved in a low-Mo ocean, and the imprint of Mo limitation may still be present in microbial metabolisms that arose billions of years ago. Before the Great Oxidation Event (GOE) ~2.4 Ga, marine Mo concentrations would have been low (likely 1/100th of modern seawater, which is 105 nM Mo) due to minimal oxidative weathering of continental Mo sulfides (Anbar et al., 2007; Scott et al., 2008b). After the GOE, it is likely that marine Mo concentrations rose to 1/10th of modern seawater, but modern concentrations were
likely not established until ~600 million years ago (Scott et al., 2008b). Today, Mo is the most abundant transition metal in seawater (105 nM), but it remains scarce (<5 nM) in most freshwaters. Therefore, studies of the biological requirements for Mo in microbes from modern freshwaters may be a useful analogue to microbial Mo requirements in ancient marine ecosystems.

Cyanobacteria, in particular heterocystous N2-fixing cyanobacteria that isolate nitrogenase in specialized cells called “heterocysts”, are ideal organisms for studying Mo requirements for microbial nitrogen cycling for several reasons. First, they are fast-growing, with growth rates up to 1-2 doublings per day. Second, they can grow on different N sources, including N2, NH4+ and NO3−, thereby enabling experiments to be conducted to determine the regulation of the Mo storage protein Mop by the availability of different N species. Third, they are likely the best modern analogues available for primary producers in the oceans during much of the Proterozoic and Archean. Fossil evidence for heterocystous cyanobacteria exists as far back as 2.4 billion years ago (Golubic et al., 1995; Tomitani et al., 2006) and genetic evidence suggests that some of the most primitive cyanobacteria were heterocystous (Deusch et al., 2008). The heterocystous cyanobacterium Nostoc sp. PCC 7120 was used as a model organism for laboratory experiments due to the availability of its sequenced genome and its lack of alternative nitrogenases (in which Mo is replaced by iron or vanadium), which complicate studies of Mo requirements for nitrogen fixation.

Castle Lake in the Klamath-Siskiyou Mountains of Northern California is an ideal field locality to investigate the influence of Mo concentration on nitrogen
assimilation because ambient Mo concentrations are very low in the lake water (2-4 nM). Castle Lake has been the subject of intensive limnological monitoring every summer since 1959 at the on-site Castle Lake Limnological Laboratory, managed by University of California Davis and University of Nevada Reno. Molybdenum limitation of primary productivity at Castle Lake was first discovered in 1960 (Goldman, 1960) and follow-up studies with isotope tracers suggested that low-molybdenum specifically limited nitrate assimilation in Castle Lake (Axler et al., 1980). However, no previous studies measured the influence of Mo availability on nitrate assimilation enzyme activity in Castle Lake, nor had there been a quantitative study of Mo cycling, sources and sinks in the lake from a whole-lake perspective.

Main Goals of Dissertation

The goal of this dissertation was to address the following questions:

1. What are the Mo requirements for cyanobacterial nitrogen fixation and cyanobacterial/algal nitrate assimilation?
2. How do these Mo requirements reflect changes in seawater Mo concentration through Earth history?
3. What mechanisms do cyanobacteria possess to cope with Mo limitation and how are these mechanisms regulated by Mo concentration?
4. What are the major sources and sinks of Mo in a modern lake ecosystem?

Overview of methods

This research utilized a combination of geochemical, microbiological, molecular, biochemical and bioinformatic techniques, including (1) metal concentration (and, for Castle Lake sediments, stable molybdenum isotopic composition) analyses of a range of samples (rock, soil, water and biomass) by
inductively-coupled plasma mass spectrometry (with multi-collector ICP-MS for Mo isotopic analyses); (2) enzyme activity assays for nitrogenase (by acetylene reduction proxy), nitrate reductase (by colorimetric assay) and glutamine synthetase (by colorimetric assay); (3) polymerase chain reaction, cloning and sequencing for identification of genes in particular samples; (4) quantitative polymerase chain reaction for quantification of gene expression; (5) immunoblotting with antibodies raised to bind to a specific protein.

Summary of Major Aims of Dissertation Chapters

A note on pronoun usage: second-person pronouns are used for published chapters (Chapters 2 and 3) and first-person pronouns are used for chapters with manuscripts in preparation (Chapters 4, 5 and 6).

Chapter 2 (Coevolution of metal availability and nitrogen assimilation in cyanobacteria and algae) expands on previous studies (Anbar and Knoll, 2002; Zerkle et al., 2006) by integrating geochemical, biochemical and genetic evidence to infer how the use of metals in N assimilation (Fe, Mo Cu and Ni) can be understood in terms of the history of metal availability through time. For instance, the higher Mo content of eukaryotic vs. prokaryotic nitrate reductase proteins may have stalled proliferation of eukaryotes in low-Mo Proterozoic oceans. Methods for testing these evolutionary hypotheses are proposed through more intensive study of the metal requirements in N assimilation and the biological strategies for metal uptake, regulation, and storage.

Chapter 3 (Molybdenum–nitrogen colimitation in freshwater and coastal heterocystous cyanobacteria) compares Mo requirements for N\textsubscript{2} fixation in two
species of filamentous heterocystous cyanobacteria (one freshwater and one coastal). It was predicted that coastal heterocystous cyanobacteria would require higher Mo concentrations than freshwater strains because Mo is more concentrated in seawater (105 nM) than in most freshwaters (~5 nM). Nitrogen fixation, carbon:nitrogen ratios, nitrogenase expression and nitrogenase activity were measured to test this hypothesis. Evidence of Mo storage was found in the freshwater strain, but not in the coastal strain. Mo storage was hypothesized to be due to expression of the gene mop, which encodes a putative molybdate-storage protein.

Chapter 4 (Molybdenum limitation of nitrate assimilation in Castle Lake, California) explores the Mo requirements for NO$_3^-$ assimilation in planktonic microbial communities from Castle Lake. In situ bottle incubations (with Mo and NO$_3^-$ added alone or in combination) were performed at three depths (3, 15 and 25 m) in Castle Lake in 2008 and 2009. NO$_3^-$ assimilation was measured by the activities of nitrate reductase and glutamine synthetase proteins and the rate of incorporation of isotopically-labeled nitrogen. Interannual and depth response differences in nitrate assimilation were explained by preference for NH$_4^+$ over NO$_3^-$ when NH$_4^+$ was available and seasonal succession of plankton species with differing Mo requirements. Both summers a dissolved Mo minimum was observed in the Castle Lake epilimnion, that was likely a result of strong Mo draw-down by N$_2$-fixing periphyton communities in the littoral zone. Laboratory chemostat experiments with a common freshwater green alga, Scenedesmus acutus, confirmed that low Mo (1 nM) severely depressed activity of the Mo-
containing enzyme nitrate reductase when NO₃⁻ was the sole nitrogen source. This chapter lends further support to the theory that low Mo can limit NO₃⁻ assimilation in freshwaters with typically low Mo levels (<5 nM) when NH₄⁺ is scarce.

**Chapter 5 (Identifying major sources of molybdenum to Castle Lake, California: Anthropogenic, historical and natural inputs)** presents a study of Mo cycling in Castle Lake through measurement of Mo concentrations in a suite of bulk solids (lake sediments, soils and bedrock) and aqueous samples (sediment porewaters, soil runoff, spring waters, snow and ice) from Castle Lake and its watershed. Lake sediments were found to be elevated in Mo compared to soils and bedrock. The numerical code PROFILE was used to estimate net reaction rate of Mo in the porewater. The flux of Mo from the sediments to the overlying water was roughly equal to the Mo fluxes from surface inflow and outflow, whereas sediment burial fluxes were significantly higher. The largest source of Mo to the watershed today is likely particulate matter from the watershed, as supported by the light isotopic composition of Mo in the lake sediments. Historical Mo sources include two whole-lake experimental Mo additions in the 1960s, and atmospheric deposition of Mo from extensive copper smelting that occurred south of Castle Lake in the early 1900’s.

**Chapter 6 (Regulation of the molybdate storage protein Mop by molybdenum in Nostoc sp. PCC 7120)** presents an investigation of the influence of Mo concentration on growth rate, cellular Mo content, and transcription and translation of the molybdenum storage protein (Mop) in Nostoc sp. PCC 7120 in
short-term experiments. I expected that Mop transcripts and protein levels would increase with Mo concentration in accordance with Mop’s role as a Mo storage protein. Contrary to that prediction, we found that gene transcription of both mop and nifD was up-regulated after two transfers into low Mo (~1 nM) media. Marked differences in the size distribution of Mop protein bands were observed in immunoblots, suggesting that the subunit composition of the Mop protein changes as a function of media Mo concentration. It is possible that Mop cannot bind Mo in the same manner at ~1 nM Mo that it can at higher Mo concentrations, or that the subunit composition changes facilitate donation of Mo to other enzymes at low Mo concentrations. Either of these explanations lend support to Mop’s role as a Mo storage protein.
Chapter 2

COEVOLUTION OF METAL AVAILABILITY AND NITROGEN ASSIMILATION IN CYANOBACTERIA AND ALGAE

Abstract

Marine primary producers adapted over eons to the changing chemistry of the oceans. Because a number of metalloenzymes are necessary for N assimilation, changes in the availability of transition metals posed a particular challenge to the supply of this critical nutrient that regulates marine biomass and productivity. Integrating recently developed geochemical, biochemical and genetic evidence, we infer that the use of metals in N assimilation – particularly Fe and Mo – can be understood in terms of the history of metal availability through time. Anoxic, Fe-rich Archean oceans were conducive to the evolution of Fe-using enzymes that assimilate abiogenic NH$_4^+$ and NO$_2^-$ . The N demands of an expanding biosphere were satisfied by the evolution of biological N$_2$ fixation, possibly utilizing only Fe. Trace O$_2$ in late Archean environments, and the eventual “Great Oxidation Event” ca 2.3 Ga, mobilized metals such as Mo, enabling the evolution of Mo (or V)-based N$_2$ fixation and the Mo-dependent enzymes for NO$_3^-$ assimilation and denitrification by prokaryotes. However, the subsequent onset of deep-sea euxinia, an increasingly-accepted idea, may have kept ocean Mo inventories low and depressed Fe, limiting the rate of N$_2$ fixation and the supply of fixed N. Eukaryotic ecosystems may have been particularly disadvantaged by N scarcity and the high Mo requirement of eukaryotic NO$_3^-$ assimilation. Thorough ocean oxygenation in the Neoproterozoic led to Mo-rich
oceans, possibly contributing to the proliferation of eukaryotes and thus the Cambrian explosion of metazoan life. These ideas can be tested by more intensive study of the metal requirements in N assimilation and the biological strategies for metal uptake, regulation and storage.

**Introduction**

All living things require N. This element is an essential constituent of amino acids, nucleic acids, pigments such as chlorophyll, and other biomolecules. Although N is the most abundant element in the atmosphere, it is overwhelmingly present as dinitrogen (N\(_2\)), which is not particularly soluble. N is therefore relatively scarce in the oceans, with a total concentration of < 600 micromolar (\(\mu\)M) (Sharp, 1983). The acquisition of N is therefore a major ecological challenge for marine biota.

The dominant primary producers in the modern marine environment are photoautotrophs. Such organisms have probably dominated since the evolution of photosynthesis at least 2.7 billion years ago (Brocks et al., 1999). Today, photoautotrophs include both prokaryotes (cyanobacteria) and eukaryotes (algae). Both types of organisms have evolved a variety of N assimilation pathways to take advantage of the variety of N species in the environment. Today, these N species include:

- **Dissolved N\(_2\)** is the dominant form of N in the oceans, other than particulate N bound up in living and dead organisms. However, it is also highly non-reactive because of the strength of the N-N bond. Breaking this
triple bond is energetically expensive, and so the organisms that can assimilate N\textsubscript{2} only do so when there are no other options.

- **Dissolved ammonium** (NH\textsubscript{4}\textsuperscript{+}), which is the most biologically accessible form of inorganic N because it is already in the chemical form useful for biomolecules; no energetically-expensive reduction reactions are required for its assimilation. However, the concentration of NH\textsubscript{4}\textsuperscript{+} concentration is exceedingly low in present-day oxygenated oceans, typically < 0.1 µM (Sharp, 1983), as it is rapidly converted to other forms of N by biologically-mediated oxidation and other processes.

- **Oxidized N species**, particularly nitrate (NO\textsubscript{3}\textsuperscript{-}), are more abundant than NH\textsubscript{4}\textsuperscript{+}. Collectively, these dissolved inorganic forms of N are known as DIN. Although the concentration of NO\textsubscript{3}\textsuperscript{-} is low in surface waters (0.2 µM) due to biological removal, it can reach up to ~ 40 µM in deep waters (Sharp, 1983). Thus, upwelling events contribute major influxes of NO\textsubscript{3}\textsuperscript{-} to the photic zone. A nitrite (NO\textsubscript{2}\textsuperscript{-}) maximum exists at the base of the euphotic zone in stratified water columns, where the NO\textsubscript{2}\textsuperscript{-} concentration can reach 4.5 µM (Lomas and Lipschultz, 2006).

- **Dissolved organic nitrogen** (DON), excreted by bacteria and zooplankton (Bronk et al., 2007), is the other major form of N. Many nitrogenous compounds compose the DON pool. Urea and amino acids are among the most common constituents of this pool. In the open ocean, these compounds are present at concentrations of ~ 0.1 - 0.5 and 0.01 - 0.03 µM, respectively (Antia et al., 1991). Urea runoff from agricultural lands has
led to elevated urea concentrations in coastal regions, where it contributes to eutrophication (Glibert et al., 2006) and is an important N source for toxic algal species (Dyhrman and Anderson, 2003).

Assimilation of these various forms of N requires a number of complex enzymes (Falkowski, 1983). Many of these enzymes incorporate transition metals such as iron (Fe), molybdenum (Mo), nickel (Ni) and copper (Cu). Cubic clusters of Fe and S constitute a very common type of cofactor in N assimilatory proteins. Chemically similar to pyrite, they occur in Fe:S ratios of 2:2, 3:4 and 4:4, and perform electron transfer and redox reactions. Fe can also be bound at the center of heme in eukaryotic nitrate reductase (as a cytochrome $b_{557}$) and nitrite reductase (as siroheme). Like Fe-S clusters, heme can perform electron transfer and catalysis. Mo is bound to a large Fe-S cluster in nitrogenase, and is at the heart of molybdopterin co-factors in both prokaryotic and eukaryotic nitrate reductase. Ni and Cu are not bound to organic cofactors, but are present as two- and one-atom centers, respectively, at the active sites of DON assimilatory proteins.

Biogeochemical cycling of N therefore depends strongly on Fe, Mo, Ni and Cu (Morel et al., 2003). The abundances of these elements in the environment are typically far lower than those of N species. Transition metals in the open ocean are generally present in trace concentrations, in the low nanomolar (nM) to picomolar (pM) range, due to their low solubilities and rapid removal on particles (Morel and Price, 2003). Fe, Mo, Ni and Cu are present in total concentrations of 0.002 - 1 nM (Johnson et al., 1997), 105 nM (Collier, 1985), 2-12 nM and 0.5-4.5
nM (Bruland, 1980), respectively, in open ocean surface waters. In coastal waters, metal concentrations are typically higher but much more variable. For example, Fe ranges from < 1 to > 10 nM along the California coast (Bruland et al., 2001), coastal Cu concentrations can reach 50 nM (Achterberg et al., 1999; Moffett et al., 1997) and Mo in the North Sea can fluctuate between 30 and 160 nM (Dellwig et al., 2007). Fe and Cu are generally present at higher concentrations in freshwater than seawater (Achterberg et al., 1997; Xue et al., 1995), whereas Ni concentrations show little salinity dependence (Xue et al., 2001). Mo exhibits the opposite trend, with very low concentrations (< 1 - 20 nM) in freshwaters (Bachmann and Goldman, 1964; Cole et al., 1993; Howarth and Cole, 1985).

Because the metals required for N assimilation are scarce, the pathways for N assimilation in the oceans are strongly affected by metal availability. For example, Fe-fertilization experiments reveal that phytoplankton switch to more Fe-intensive N assimilation pathways when Fe is added to ocean regions where Fe scarcity limits primary production (Coale et al., 1996; Martin and Fitzwater, 1988; Price et al., 1991). Marine cyanobacteria have been shown to fix N\textsubscript{2} at higher rates when provided excess Fe (Berman-Frank et al., 2001; Berman-Frank et al., 2007). It has been hypothesized that the availability of other metals may affect N assimilation in the modern oceans. It is possible that Ni scarcity limits urea utilization in the marine environment due to organic complexation and slow uptake kinetics (see references in Dupont et al., 2007). It is unlikely that Mo scarcity limits N assimilation in marine environments, due to its long residence
time and high stability in oxic seawater, but Mo scarcity may limit N uptake, and hence primary productivity, in freshwaters (Axler et al., 1980; Goldman, 1960).

The pathways for N assimilation almost certainly changed over the course of Earth history because the ocean abundances of these bioessential metals are not constant with time. Their abundances have varied in particular as a consequence of secular changes in ocean oxygenation, which affects element delivery to, and residence times in, the oceans. The classic example is Fe. Scarce in the modern oxygenated oceans, geologic evidence indicates that Fe was relatively abundant in the surface and deep oceans during the Archean (3.8 - 2.5 billions of years ago (Ga)) due to widespread ocean anoxia, perhaps reaching concentrations of tens of micromolar (Fig. 1) (Holland, 1973). Strong evidence has emerged recently for comparably large changes in the deep and surface abundance of Mo, but with an opposite sense of direction than Fe (Fig. 1) (Scott et al., 2008a). Analogous shifts probably characterized other elements with redox-sensitive environmental chemistries (Saito et al., 2003), although supporting evidence for these less abundant elements is difficult to extract from the geologic record. However, the connections between historical changes in metal availability and the metal requirements of N assimilation, and their evolutionary consequences, have barely been explored (Anbar and Knoll, 2002; Falkowski, 1997).

One example of such a connection can be seen in the marked differences in N assimilation pathways between prokaryotes and eukaryotes. Prokaryotes can fix N\textsubscript{2} whereas eukaryotes cannot; eukaryotes assimilate fixed forms of N from the environment or (in the case of heterotrophs) from their food. As a
consequence, prokaryotes should respond differently than eukaryotes to changes in metals and N budgets. It has therefore been suggested that the diversification of eukaryotes in marine ecosystems ~ 1.25 Ga was dictated by a shift from Mo-poor to Mo-rich oceans at that time (Anbar and Knoll, 2002).

In this paper, we summarize present knowledge of the metal requirements for N assimilation, and their biochemical basis, in both eukaryotes and prokaryotes. For each N assimilation pathway, we discuss the metals involved in the enzymes performing the reaction and discuss phylogenetic evidence for its evolutionary origin. We then synthesize this information with the emerging understanding of changes in marine metal availability over geologic time to develop hypotheses that may guide future research.

**Metals and nitrogen assimilation**

Bioavailable nitrogen is found in four redox states in nature: +5 (NO$_3^-$), +3 (NO$_2^-$), 0 (N$_2$) and -3 (NH$_4^+$, or in gaseous form, NH$_3$). To assimilate NO$_3^-$, NO$_2^-$ or N$_2$, electrons must be donated to reduce N to the -3 redox level of NH$_4^+$, because only the most reduced form is used in biomolecules. In the following sections, we review the function and metal content of the enzymes responsible for the redox reactions involved in N assimilation: N$_2$ fixation (N$_2$ $\rightarrow$ NH$_3$), NO$_3^-$ reduction (NO$_3^-$ $\rightarrow$ NO$_2^-$), NO$_2^-$ reduction (NO$_2^-$ $\rightarrow$ NH$_4^+$) as well as of the enzymes used for assimilating DON and NH$_4^+$ (summarized in Table 1). In addition, we summarize what is known about the evolution of these enzymes based on gene sequences, which tells us whether eukaryotic proteins were inherited during endosymbiosis or whether they evolved separately in prokaryotes.
and eukaryotes. Analysis of such evolutionary relationships enables relative
dating of N assimilation pathways and, hence, examination of the coevolution of
metal availability in the oceans and photosynthetic N assimilatory enzymes.

*Nitrogen fixation*—Modern industry uses the Haber-Bosch process at
extreme pressures and temperatures to fix N\(_2\) into NH\(_3\) over an Fe catalyst (Smil, 2004). Certain prokaryotes (“diazoorganisms”), on the other hand, can perform this
reaction at ambient pressures and temperatures. Heterotrophic and photosynthetic
diazoorganisms are prominent contributors to both the marine and terrestrial
biospheres because they supply the major input of fixed N (Arrigo, 2005). The
only oxygenic photosynthetic diazoorganisms are cyanobacteria (Capone and
Carpenter, 1982; Capone et al., 1997; Stal and Zehr, 2008). Conversion of N\(_2\) gas
into NH\(_3\) requires electrons, ATP and the metal-rich enzyme nitrogenase
(abbreviated Nif), following the reaction:

\[
N_2 + 8e^- + 8H^+ + 16 \text{ATP} \rightarrow 16 \text{ADP} + 16 P_i + 2 \text{NH}_3 + H_2 \quad \text{(Eq. 1)}
\]

The eight electrons required for each catalytic cycle are supplied by
ferredoxin, a small [2Fe-2S]-cluster protein which accepts electrons from the
photosynthetic electron transport chain (Schrautemeier and Bohme, 1985). The
product NH\(_3\) gas is rapidly converted into the soluble NH\(_4^+\) and directed into
glutamine and glutamate biosynthesis (Carpenter et al., 1992; Meeks et al., 1978;
Wolk et al., 1976).

Nitrogenase is an oxygen-sensitive protein complex composed of two
subunits: dinitrogenase (“Fe protein”, or NifH) and dinitrogenase reductase (“Mo-
Fe protein”, or NifDK) (Berman-Frank et al., 2003). Although cyanobacterial
NifH has never been purified to homogeneity, cysteines that bind a [4Fe-4S] cluster from heterotrophic bacterial strains are conserved in cyanobacterial NifH sequences (Zehr et al., 1997) (Fig. 2A). This suggests that cyanobacteria also contain a [4Fe-4S] cluster in NifH (Fig. 3A, Table 1). Electrons from reduced ferredoxin travel through this cluster to NifDK, which contains the Fe-Mo cofactor (“FeMoco”), the active site of N₂ reduction (Fig. 3A, Table 1). Numerous enzymes are involved in the biosynthesis of FeMoco, including two proteins, NifE and NifN, which serve as the scaffold upon which FeMoco is built (Rubio and Ludden, 2005).

The purified NifDK dimer from a freshwater cyanobacterium contains 2 Mo atoms and 20 Fe atoms (Hallenbeck et al., 1979). This Fe content is lower than the ~25-38 Fe atoms found in NifDK dimers from heterotrophic bacteria (Howard and Rees, 1996; Kustka et al., 2003a), although all Fe-S cluster-binding cysteine residues align to identical positions in cyanobacteria (Fig. 2B,C). The ratio of NifH:NifDK in cyanobacteria is higher (3:1) than observed in other bacteria (2:1) (Reade et al., 1999), suggesting that the overall Fe requirement may be similar between different organisms. The high Fe requirement for marine N₂ fixation is evident from experiments that have shown a steep drop-off of growth rate and nitrogenase activity below ~1 nM for Trichodesmium sp (Berman-Frank et al., 2001). Further biochemical characterization of nitrogenase proteins from marine diazotrophic cyanobacteria is crucial to characterize the metal quotas of photosynthetic N₂ fixation in the sea.
The importance of Mo to N\textsubscript{2} fixation has been well-established since the 1930’s (Bortels, 1930, 1940). Nitrogenases that utilize alternatives to Mo were discovered more recently (Bishop et al., 1980; Burns et al., 1971). These alternative nitrogenases are distinct proteins encoded by separate genes (Chisnell et al., 1988; Pau et al., 1989; Robson et al., 1986) which are expressed when Mo concentrations fall below 100 nM (Joerger and Bishop, 1988). Both vanadium-Fe (Vnf) and Fe-Fe (Anf) nitrogenases exist (Eady, 1996); however, only three species of cyanobacteria contain vnf genes. All three known Vnf-containing species are strains of the freshwater cyanobacterium \textit{Anabaena} sp. (Boison et al., 2006; Kentemich et al., 1988; Thiel, 1993). No alternative nitrogenase genes have been found in the genomes of marine cyanobacteria, and a BLAST search of the J. Craig Venter Global Ocean Sampling database with the \textit{Anabaena} sp. VnfD protein (587 amino acids) returns marine peptides with only 34% identity at most. These are likely NifD proteins hits, since Nif and Vnf proteins share \textasciitilde30\% sequence similarity (Raymond et al., 2004). No cyanobacteria have been found to contain anf genes.

\textit{Nitrate reduction}—Most, though not all, phytoplankton can assimilate NO\textsubscript{3}\textsuperscript{−}. Prokaryotes and eukaryotes express biochemically different proteins with distinct evolutionary origins (Stolz & Basu, 2002), for this purpose, but regardless both kinds of nitrate reductases require Mo. NO\textsubscript{3}\textsuperscript{−} assimilation has been extensively reviewed for cyanobacteria, eukaryotic algae and plants (Berges, 1997; Campbell, 1999; Guerrero et al., 1981; Moreno-Vivian et al., 1999). In all cases, NO\textsubscript{3}\textsuperscript{−} assimilation begins with the two-electron reduction to NO\textsubscript{2}\textsuperscript{−}:
\[ \text{NO}_3^- + 2e^- + 2H^+ \rightarrow \text{NO}_2^- + H_2O \quad \text{(Eq. 2)} \]

The protein responsible for the reduction of \( \text{NO}_3^- \) to \( \text{NO}_2^- \) in cyanobacteria is nitrate reductase, abbreviated NarB. This enzyme, like nitrogenase, depends on the donation of electrons from ferredoxin for its activity (Hattori and Myers, 1967; Hirasawa et al., 2004; Manzano et al., 1976; Mikami and Ida, 1984). Biochemical studies of NarB have shown that this protein binds one \([4\text{Fe}-4\text{S}]\) cluster and one molybdopterin cofactor with cysteines (Rubio et al., 1999, 2002) (Fig. 2D, 3B, Table 1). Two electrons travel from ferredoxin through the \([4\text{Fe}-4\text{S}]\) cluster to the Mo atom in molybdopterin (Fig. 3B), which is reduced from the 6+ to the 4+ redox state by a two-electron reduction. \( \text{NO}_3^- \) then binds to the reduced Mo and is reduced to \( \text{NO}_2^- \), returning Mo to the 6+ state (Jepson et al., 2004).

Eukaryotic algae express a biochemically-different nitrate reductase that arose through convergent evolution to serve the same role as NarB. This enzyme, abbreviated NR, utilizes nicotinamide adenine dinucleotide (phosphate) ((NAD(P)H)), an organic cofactor which accepts electrons from ferredoxin, as its electron donor (Zumft et al., 1969). NR in algae differs from NarB in cyanobacteria, in that NR contains four identical protein subunits, each containing one atom of Mo, whereas NarB contains only one subunit. Each NR subunit contains three prosthetic groups: flavin adenine dinucleotide (FAD) (Zumft et al., 1970), cytochrome \( b_{557} \) (Ahmed and Spiller, 1976) and molybdopterin of a slightly different structure than in NarB (Solomonson et al., 1986; Solomonson et al., 1975) (Fig. 3C, Table 1). Cysteines that bind molybdopterin and cytochrome in NR from yeast (Fischer et al., 2005) and corn (Lu et al., 1995) align to
conserved position in NR from diatoms, and primary green and red algae (Fig. 2E), suggesting that the metal-containing cofactors of NR are likely conserved among these evolutionary distant organisms. During catalysis, electrons donated by NAD(P)H reduce FAD, which transfers the electrons to cytochrome \( b_{557} \). Electrons then tunnel to the active site Mo atom in the molybdopterin, where \( \text{NO}_3^- \) is reduced to \( \text{NO}_2^- \) (Skipper et al., 2001) (Fig. 3C, Table 1).

The Fe content of NR is well documented (Aparicio et al., 1971; Cárdenas et al., 1972). Four Fe atoms, corresponding to four cytochrome molecules, are present in all described eukaryotic phytoplankton NRs (de la Rosa, 1983; de la Rosa et al., 1981; Solomonson et al., 1986). Although the physiological requirement of Mo for nitrate reduction has been established for fungi (Nicholas and Nason, 1954; Nicholas et al., 1954; Steinberg, 1937) and green algae (Aparicio et al., 1971; Arnon et al., 1955; Cárdenas et al., 1971; Vega et al., 1971), the exact number of atoms in NR is still unclear. It was expected that each of the four NR subunits contain one molybdopterin cofactor, and thus one Mo atom, as has been found for NR purified one green algal species (Solomonson et al., 1986). However, another green algal species was found to contain only two Mo atoms in NR (de la Rosa, 1983; de la Rosa et al., 1981). Further study is necessary to determine whether Mo content is in fact variable in eukaryotic NR.

\( \text{NO}_3^- \) reduction also occurs in anaerobic bacteria, which utilize \( \text{NO}_3^- \) as a terminal electron acceptor during dissimilatory \( \text{NO}_3^- \) reduction in anoxic waters and sediments. Dissimilatory nitrate reductases that contain no Mo or Mo-alternatives (heme Fe and V) have been discovered, but only in organisms from
extreme environments (Antipov et al., 2005; Antipov et al., 2003; Antipova et al., 1998).

Nitrite reduction—Following NO$_3^-$ reduction, a six-electron reduction of NO$_2^-$ to NH$_4^+$ is essential for N incorporation. NO$_2^-$ assimilation can also occur independently from NO$_3^-$ assimilation. Nitrite reductase (NiR) is an iron-rich protein, containing one [4Fe-4S] cluster and one heme (Fig. 3D, Table 1). The [4Fe-4S] cluster transfers electrons from ferredoxin to Fe in siroheme, where six electrons reduce NO$_2^-$ to N$\text{H}_4^+$ (Kuznetsova et al., 2004), following the overall reaction:

$$\text{NO}_2^- + 6e^- + 8H^+ \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O} \quad \text{(Eq. 3)}$$

Biochemical and phylogenetic evidence suggests that NiR in eukaryotic algae was inherited from cyanobacteria. This inference follows from the finding that cysteine residues bound to the [4Fe-4S] cluster and heme in the crystal structure of spinach nitrite reductase (Swamy et al., 2005) are conserved in the NiR sequences from cyanobacteria and algae (Fig. 2F). Hence, NiR's Fe cofactors appear to be bound in a similar fashion in all photoautotrophs. This suggests that the origin of NiR in eukaryotic algae may originally derive from the endosymbiont that lead to the modern plastid. In addition to their biochemical similarities, phylogenetic analysis of the NiR-encoding nirA gene shows that cyanobacteria group near the base of the tree, with higher plant and eukaryotic algal sequences branching out from the cyanobacterial clade (Wang et al., 2000).

DON assimilation—Dissolved organic nitrogen (DON) occurs in many chemical forms; here, we discuss only urea and amino acid assimilation. The
enzyme urease, found in cyanobacteria and most eukaryotic algae and plants, catalyzes the hydrolysis of urea, producing two molecules of ammonia and one molecule of carbonic acid (Fig. 2E, Table 1):

\[ \text{H}_2\text{N-CO-NH}_2 + 2 \text{H}_2\text{O} \rightarrow 2\text{NH}_3 + \text{H}_2\text{CO}_3 \]  
(Eq. 4)

Urease isolated from cyanobacteria and diatoms contains three subunits, usually two small and one large (Fig. 3E) (Argall et al., 1992; Carvajal et al., 1982; Collier et al., 1999; Jahns et al., 1995; Palinska et al., 2000; Rai, 1989). Nevertheless, the same amino acids (four histidine residues, one lysine and one arginine) that bind Ni in the large subunit of urease from the heterotrophic bacterium *Klebsiella aerogenes* (Jabri et al., 1995) are conserved in cyanobacterial and diatom ureases (Fig. 2G) suggesting that Ni plays a similar role in the ureases of these species as in other bacteria. Addition of Ni is essential for the growth of coastal diatoms (Egleston and Morel, 2008; Oliveira and Antia, 1984, 1986a, b) and marine cyanobacteria *Synechococcus* (Dupont et al., 2007) on urea as the sole N source. Urease activity has been shown to increased with rising Ni concentration (Mackerras and Smith, 1986; Price and Morel, 1991). Urea assimilation represents one of the few biological uses of Ni in photoautotrophs, besides Ni-superoxide dismustase (Dupont et al., 2007) and hydrogenase (Tamagnini et al., 2002). Interestingly, an alternative urea assimilation pathway exists in certain green algae, which do not require Ni for urea assimilation (Rees and Bekheet, 1982). Instead, ATP is used in a two-step hydrolysis of urea.
The relative age of urease is unknown. This enzyme is member of an ancient and diverse protein superfamily with conserved 3-D structure (Holm and Sander, 1997), but the timing of urease evolution within this family remains unclear. The phylogeny of ureC, which encodes the large, Ni-binding subunit of urease, suggests that cyanobacteria and eukaryotic ureC genes evolved independently after an early gene duplication event (Koper et al., 2004). Very little phylogenetic work has investigated alternative non-Ni containing ureases in green algae (Syrett and Alhouty, 1984). These proteins deserve further research attention, as they may afford an evolutionary advantage for more efficient green algal assimilation of urea in low-Ni aquatic systems.

Eukaryotic algae can also assimilate N from free amino acids by the action of a Cu-containing cell-surface amine oxidase (CuAO) (Fig. 3F). This is the only N assimilatory protein yet discovered that works at the outside surface of the cell, and functionally parallels alkaline phosphatase, the enzyme that strips phosphate groups from nucleic acids during P assimilation. CuAO oxidizes amino acids to produce hydrogen peroxide (H$_2$O$_2$), α-keto acids and extracellular ammonia, following the reaction:

$$RCH_2NH_2 + O_2 \rightarrow H_2O_2 + RCHO + NH_3$$  \hspace{1cm} (Eq. 5)

The NH$_3$ is subsequently taken up through specific transporters and assimilated by the organism. Amine oxidase activity has been observed for phytoplankton with secondary plastid symbionts derived from red algae including coccolithophorids and dinoflagellates (Palenik and Morel, 1990a, b, 1991). Although the Cu content of algal CuAO has not been confirmed, it is likely that that they resemble other
amine oxidases, which contain two atoms of Cu (one in each of the two subunits) because experiments have shown that growth of the marine coccolithophorid *Pleurochrysis carterae* on amines requires 10 nM Cu (Palenik and Morel, 1991). However, no other studies looking at the effect of amine sources on CuAO activity or intracellular Cu of algae have yet been performed. The crystal structure of pea CuAO shows that Cu is coordinated at the active site by three histidine residues (Kumar et al., 1996). Histidine residues are conserved in phytoplankton CuAO as predicted by the gene sequence of green algae (Fig. 2H), suggesting that the active site, and perhaps the metal requirement, of algal CuAO may be similar to that of higher plants. CuAO is present in numerous eukaryotes (Cona et al., 2006), but only a small fraction of prokaryotic genome sequences analyzed contain AO-encoding genes (Andreini et al., 2008). However, the genomes of numerous marine and freshwater cyanobacteria contain putative CuAO-encoding genes. Further phylogenetic and biochemical characterization of this protein is essential to evaluate its Cu content and thus the ecological and evolutionary consequences of this pathway.

*Ammonium assimilation*—The last step in N assimilation is conversion of NH$_4^+$ to glutamate. Regardless of the N source, all N assimilation pathways end with NH$_4^+$ assimilation through the glutamine synthetase-glutamate synthase (GS-GltS) pathway, also known as the GS-GOGAT pathway (Muro-Pastor et al., 2005). First, NH$_4^+$ is incorporated into glutamate, yielding glutamine, and then N is transferred as an amide group to 2-oxoglutarate, producing an additional molecule of glutamate to be used for amino acid synthesis (Fig. 4A,B, Table 1).
The first enzyme in the GS-GltS pathway is the ATP-dependent enzyme glutamine synthetase (GS), which catalyzes the formation of glutamine (Fig. 4A, Table 1):

\[
\text{Glutamate} + \text{ATP} + \text{NH}_4^+ \rightarrow \text{Glutamine} + \text{ADP} + \text{P}_i \quad (\text{Eq. 6})
\]

GS is most often a large, multi-subunit protein (Beudeker and Tabita, 1985; El Alaoui et al., 2003; Florencio and Ramos, 1985; Merida et al., 1990; Orr et al., 1981; Sampaio et al., 1979; Stacey et al., 1977; Sumar et al., 1984; Yuan et al., 2001) present in thylakoid membranes (Lopez-Ruiz et al., 1991) and requires divalent cations, such as Mg or Mn, for activity (Blanco et al., 1989). The wide distribution of GS-encoding genes throughout the three domains of life suggests that this gene is ancient and has likely been horizontally transferred between the bacterial and archaean domains (Raymond, 2005).

The second enzyme in the GS-GltS pathway is glutamate synthase (GltS, also known as GlsF or GOGAT) (Suzuki and Knaff, 2005). GltS catalyzes the conversion of glutamine and 2-oxoglutarate (a product of the tricarboxylic acid cycle (TCA cycle)) into two moles of glutamate, via a 2-electron reduction:

\[
\text{Glutamine} + 2\text{-oxoglutarate} + 2\text{e}^- + 2\text{H}^+ \rightarrow 2\text{Glutamate} \quad (\text{Eq. 7})
\]

In cyanobacteria, electrons for this process travel from ferredoxin through a [3Fe-4S] cluster in GltS to the organic molecule flavin mononucleotide (FMN) at the active site of glutamate formation (Marques et al., 1992; Merida et al., 1990; Navarro et al., 2000; Ravasio et al., 2002) (Fig. 4B,C). The crystal structure of GltS from *Synechocystis* PCC 6803 reveals that the [3Fe–4S] cluster is coordinated by three cysteines (van den Heuvel et al., 2002; van den Heuvel et al.,
which are conserved in GltS sequences from other cyanobacterial and
eukaryotic algae (Fig. 2I), supporting the essential role for Fe in phytoplankton
 glutamate synthesis. However, although eukaryotic algae possess genes encoding
GltS like cyanobacteria, many express a larger two-protein complex, abbreviated
GltBD, for glutamine assimilation (Cullimore and Sims, 1981; Marquez et al.,
1984) (Fig. 4C, Table 1). Like NR, GltBD accepts electrons from NADH instead
of ferredoxin (Clayton and Ahmed, 1986). Sequence similarity to the crystallized
subunit GltB of the greater GltBD complex from a diazotrophic bacterium
(Azospirillum brasilense) (Binda et al., 2000) (Fig. 2I), suggests that algal GltB
also binds one [3Fe-4S] cluster. The additional subunit (GltD) in A. brasilense
contains two [4Fe-4S] clusters (Vanoni and Curti, 1999; Vanoni et al., 2005)
(Table 1), and the cysteines that bind these clusters are conserved in algal GltB
sequences (Fig. 2J). Thus, GltBD contains eleven Fe atoms per enzyme complex,
compared to only three in GltS. Why would utilizing NADH instead of ferredoxin
as an electron donor for glutamate synthesis require a nearly four-fold increase in
Fe? This evolutionary question remains unanswered.

Phylogenetic analyses suggest that GltS-encoding genes are ancient,
perhaps already present in the last universal common ancestor, and have
diversified to use different electron donors (Raymond, 2005). The gltS gene (also
known as glsF) has been found encoded in the chloroplast genome of numerous
primary red algae, suggesting that this gene, like nirA, had an endosymbiotic
origin (Valentin et al., 1993). However, this gene is not present in the chloroplast
genome of primary green algae, suggesting either an early transfer to the nucleus
or an non-endosymbiotic origin (Grzebyk et al., 2003). Despite these differences, both primary red and green algae now rely primarily on Fe-rich GltBD enzymes for NH$_4^+$ assimilation (Clayton and Ahmed, 1986).

**Nitrogen assimilation and metals through earth history: co-evolution and competition**

The redox state of the oceans has changed dramatically through time. The O$_2$ content of seawater has, in turn, likely influenced the speciation and availability of surface and deep-water metal concentrations (Fig. 1). Our understanding of these ancient variations is developing rapidly, particularly as regards the abundances of Fe and Mo (Anbar et al., 2007; Anbar and Rouxel, 2007; Rouxel et al., 2005; Scott et al., 2008a), both metals that are important in N assimilation enzymes. The relationships between O$_2$, N speciation and metal availability create selection pressures that could have shaped the evolution of metals in N assimilation enzymes. If so, the metal requirements of N assimilation pathways found in organisms today may be a legacy of evolution in ancient oceans.

We consider this possibility below, focusing in particular on Fe and Mo. Aside from their importance in N assimilation, these metals are especially interesting in an evolutionary context because their ocean abundances change in an opposite sense to each other in response to changes in ocean oxygenation. Continental Mo sulfides are only weathered into rivers in the presence of O$_2$, whereas Fe hydrolyzes into insoluble Fe oxides. Hence, we propose a sequence
for the co-evolution of Fe- and Mo-containing N assimilation pathways cognizant of historical changes in ocean metal availability and environmental redox conditions during approximately the first half of Earth history. We then explore the implications for the ecological competition between prokaryotes and eukaryotes in the face of subsequent changes in ocean oxygenation.

**Co-evolution of nitrogen assimilation and metal availability**—The early oceans contained a very different suite of bioavailable elements than today. The most primitive N assimilatory enzymes would have evolved to assimilate reduced N species, instead of the oxidized N present in the modern environment (Falkowski and Godfrey, 2008). In order to perform redox chemistry, metals were required. Fe, in the form of Fe-S clusters and heme, was both bioavailable and catalytically useful for these reactions. As O₂ began to rise, new enzymes utilizing more oxidized N substrates and metals made more bioavailable by oxygenation evolved.

**The anoxic Archean**—Molecular O₂ was absent or scarce in the Archean atmosphere and oceans (Holland, 2006). Under these conditions, the most abundant form of bioavailable N was probably NH₄⁺, rather than NO₃⁻ as today (Beaumont and Robert, 1999; Boyd and Philippot, 1998; Papineau et al., 2005). Some N may also have been present as NO₂⁻. However, then, as now, the vast majority of N in the oceans and atmosphere was present as N₂ (Kasting and Catling, 2003), which is not readily bioavailable.

Before the evolution of biological N₂ fixation, the supply of fixed N to the oceans would have been limited by the rate of abiotic N₂ fixation. This rate is
unknown, but could have been significant. As in biology, metal catalysts were probably important for abiotic $N_2$ fixation. For example, iron-sulfide minerals likely fixed some $N_2$ into $NH_4^+$ at Archean hydrothermal vents (Schoonen and Xu, 2001). Some $N_2$ was probably also fixed without metals, by atmospheric reactions driven by lightning. These reactions convert $N_2$ into nitric oxide (NO), which dissolves into the ocean as precipitable $HNO_2$ and $HNO_3$ (Mancinelli and McKay, 1988; Navarro-Gonzalez et al., 2001; Navarro-González et al., 1998; Yung and McElroy, 1979). Bolide impacts and subaerial volcanoes could have supplied additional fixed N as N-oxides (Mancinelli and McKay, 1988; Mather et al., 2004). In the ocean, $NO_2^-$ could have been abiotically reduced to $NH_4^+$ by abundant ferrous Fe (Summers and Chang, 1993).

Thus, it is reasonable to hypothesize that both $NH_4^+$ and $NO_2^-$ were present in the Archean oceans and hence that the first N assimilatory enzymes to evolve were those involved in $NO_2^-$ and $NH_4^+$ assimilation: nitrite reductase (NiR) and glutamine synthetase (GS)/glutamate synthase (GltS(BD)), respectively (Fig. 5). The genetic sequence, protein structure and cofactor assemblage of these enzymes are highly conserved across all photoautotrophs (Luque et al., 1993; Raymond, 2005; Valentin et al., 1993), supporting their great antiquity. Further evidence of their early evolution comes from phylogenetic evidence suggesting that NiR and GltS proteins were transferred from cyanobacteria to a eukaryotic host during endosymbiosis (Temple et al., 1998; Wang et al., 2000). For these reasons, the early evolution of GS and GltS(BD) has previously been proposed by Papineau et al. (2005).
The early Fe requirement for assimilation of NO$_2^-$ and NH$_4^+$ through the action of Fe-containing NiR and GltS(BD) proteins is fully consistent with our understanding of metal availability in Archean oceans. The high Fe content of Archean banded iron formations suggests that Fe was present in micromolar concentrations in the Archean oceans, compared to sub-nanomolar quantities in the modern sea (Holland, 1973). Iron isotopic compositions from sulfides in black shales paint a picture of high Fe content in the Archean oceans compared to the oceans of the Proterozoic and Phanerozoic (Johnson et al., 2008; Kump, 2005; Rouxel et al., 2005). Fe content may have fluctuated in response to relative input from hydrothermal vents vs. export as Fe oxides in banded iron formations (Rouxel et al., 2005). The question of whether N assimilation first evolved at deep-sea hydrothermal systems or in the open ocean remains unanswered. The Archean oceans were low in S (Canfield et al., 2000), however, NiR and GltS(BD) contain Fe-S clusters, so evolution at mineral-water interfaces in deep sea has some compelling logic. It is also clear that oxyanions such as Mo and V are absent from NiR and GltS(BD), consistent with accumulating evidence of very low concentrations of these metals in the Archean ocean (Anbar et al., 2007; Scott et al., 2008a).

Abiotic production of NH$_4^+$ and NO$_2^-$ was probably not sufficiently rapid to support a biosphere comparable to today’s, creating a selection pressure that favored the evolution of biological N$_2$ fixation pathways (Navarro-Gonzalez et al., 2001). Fossil and phylogenetic evidence suggest that some cyanobacteria had acquired the ability to fix N$_2$ by the early Paleoproterozoic (Tomitani et al., 2006),
but there is reason to suspect that \( \text{N}_2 \) fixation evolved much earlier. The high similarity between \textit{nif}D, \textit{nif}K, and \textit{nif}E and \textit{nif}N genes scattered throughout bacteria and archaeal genomes implies that these genes duplicated before the last universal common ancestor, presumably early in the Archean (Fani et al., 2000). This suggested antiquity is consistent with the \( \text{O}_2 \)-sensitivity of all forms of nitrogenase, which implies that they evolved before \( \text{O}_2 \) was pervasive in the environment.

The existence of three forms of nitrogenase using different metals (Mo, V and Fe) has led to the suggestion that Anf – the Fe-Fe nitrogenase – evolved in the Archean, when Fe was plentiful but before Mo (and V) was present in any significant quantities (da Silva and Williams, 1991) (Fig. 1). Intriguingly, if Anf is ancestral it is conceivable that this enzyme complex developed for reasons other than \( \text{N}_2 \) fixation. Anf is the least effective nitrogenase of the three, but is the most effective at \( \text{H}_2 \) production (Schneider et al., 1997). Thus, Anf may have initially evolved in Fe-rich oceans for \( \text{H}_2 \) production and subsequently been adopted for nitrogenase function (Fani et al., 2000; Normand and Bousquet, 1989; Silver and Postgate, 1973). However, the phylogenetic record does not present unambiguous evidence of an evolutionary sequence among the three alternative nitrogenases, probably because of lateral gene transfer and gene loss (Raymond et al., 2004). Thus, although the high Fe content of Anf is consistent with Archean geochemistry, its place in evolutionary history is obscure.

\textit{The oxygen transition}—Oxygenic photosynthesis may have evolved by 2.7 Ga (Brocks et al., 2003a; Brocks et al., 2003b; Brocks et al., 1999; Eigenbrode
and Freeman, 2006), based on the interpretation of molecular biosignatures and stromatolite paleoenvironments (Buick, 1992, 2008), but these interpretations are not undisputed (Kirschvink et al., 2000; Kirschvink and Kopp, 2008; Rasmussen et al., 2008). However, few dispute that by 2.3 Ga, O₂ in the atmosphere rose above ~10⁻⁵ PAL (Holland, 2006). The atmosphere and at least the surface oceans were pervasively oxidized by 1.8 Ga, while the deep sea remained euxinic (Fig. 1) (Poulton et al., 2004). This rise of O₂ would have stabilized NO₂⁻ and then NO₃⁻ in the ocean (Fig. 5) (Beaumont and Robert, 1999; Papineau et al., 2005).

The reason for the delay of at least 300 million years between the origin of oxygenic photosynthesis and the oxygenation of the environment, assuming it occurred, is unclear. Several processes could have conspired to maintain a low-O₂ condition (Catling and Claire, 2005; Fennel et al., 2005; Holland, 2002; Kump and Barley, 2007). Among these were weathering reactions with iron sulfide (pyrite) and other reduced minerals, some of which are host phases of Mo and other bioessential metals. Once mobilized by oxidative weathering, Mo was stabilized in oxygenated waters as the oxyanion molybdate (MoO₄²⁻), making it widely available for the first time for use in N-cycle enzymes such as Nif and nitrate reductase (cyanobacterial NarB and eukaryotic NR). Hence, the use of Mo in N-cycle enzymes probably evolved during a time when biogenic O₂ was commonly produced but not yet pervasive.

During this period, the residence times of both O₂ and Mo in the oceans were short, and hence their concentrations variable. Therefore, it is possible to imagine that both Fe and Mo were sporadically available in near-shore
environments before the rise of a more thoroughly oxygenated environment. Recent geochemical findings suggest that such conditions did, indeed, occur after 2.7 Ga in the form of at least one “whiff” of O\(_2\) to ppm levels, and associated marine Mo enrichment, at 2.5 Ga (Anbar et al., 2007). It is even possible that such events were common between 2.7 and 2.3 Ga but never predominant as Mo concentrations in late Archean shales are generally very low (Cameron and Garrels, 1980; Davy, 1983; Yamaguchi, 2002).

It was presumably during this time that Nif became the dominant N\(_2\) fixation enzyme in aerobic microbial communities due to the rise of marine Mo concentrations (Fig. 5) (Anbar et al., 2007; Scott et al., 2008a). The key advantage of Mo is that Nif is more ~15-times more efficient at \textit{in vivo} N\(_2\) fixation than the alternative forms of nitrogenase (Schneider et al., 1997). Sporadic availability of both Mo and Fe would have facilitated the incorporation of Mo at the enzyme active site while still maintaining heavy dependence on Fe for the rest of the Fe-S clusters. Numerous microbes, namely methanogenic Archaea and anoxygenic photosynthetic Bacteria (likely the predecessors of cyanobacteria (Blankenship, 1992, 2001; Burke et al., 1993)), possess all three sets of nitrogenase genes (Madigan, 1995; Masepohl et al., 2004; Oda et al., 2005), allowing them to inhabit both high- and low-Mo environments. Intriguingly, Anf in some anoxygenic photoautotrophs can incorporate a Mo-Fe cofactor when Mo is available (Gollan et al., 1993). The ability to repurpose a different cofactor for the same gene product suggests that primitive nitrogenases were more flexible in
metal use, consistent with evolution in a world with variable Fe and Mo availability.

Nitrate reductase enzymes may have also evolved during this period of low, possibly transient, levels of $O_2$ and Mo in the environment. The assimilatory form of prokaryotic nitrate reductase shares a common ancestor with the dissimilatory (or respiratory) nitrate reductase utilized in the first step of denitrification (Stolz and Basu, 2002). If denitrification evolved before $NO_3^-$ assimilation, it is possible to constrain the timing based on geologic evidence. Isotopic signatures of denitrification are found in ~ 2.1 Ga sediments (Beaumont and Robert, 1999; Boyd and Philippot, 1998; Papineau et al., 2005), providing a minimum age for the origin of this metabolism.

However, the prokaryotic nitrate reductase family most likely evolved much earlier than 2.1 Ga. Phylogenetic evidence suggests an origin of the gene family before the divergence of eubacteria and archaea (Petri and Imhoff, 2000; Philippot, 2002) between 3-4 Ga (Feng et al., 1997), with subsequent passage of the gene among prokaryotes through horizontal gene transfer (Stolz and Basu, 2002). The apparent antiquity of nitrate reductase is puzzling, given that sources of $NO_3^-$ and Mo would have been limited in the anoxic Archean. It is possible that different metals – perhaps V and Fe – were utilized in place of Mo in early nitrate reductases, but few modern examples of alternative nitrate reductases exist. Alternatively, the antiquity of NarB may tell us that slightly oxidizing conditions, such as those documented at 2.5 Ga (Anbar et al., 2007; Kaufman et al., 2007), occurred episodically through the Archean.
Competition between prokaryotes and eukaryotes in metal-starved marine ecosystems—Once the N assimilation metabolisms became established, the evolutionary focus likely shifted increasingly towards competition between marine organisms for dominance. Cyanobacteria prevailed during the first third-quarters of Earth history, whereas eukaryotic phytoplankton rose to prevalence in the Phanerozoic. In this section, we discuss the progression of marine primary producers with regard to metal requirements for N assimilation.

After the first oxygenation event—The Great Oxidation Event (GOE) ushered in a new period of Earth’s chemical history after 2.3 Ga. Paradoxically, this dramatic rise in atmospheric O$_2$ likely led to intensified anoxia and the generation of H$_2$S in deep ocean waters (Canfield, 1998). This counterintuitive consequence arises from the enhanced influx of sulfate due to oxic weathering of sulfide minerals on the continents, stimulating the action of sulfate-reducing bacteria in the oceans (Canfield, 1998). If the partial pressure of O$_2$ in the atmosphere was still significantly lower than today’s – as generally believed – then the rate of H$_2$S generation could have exceeded the rate of O$_2$ supply to deep waters in many places, resulting in an “H$_2$S maximum” analogous to the “O$_2$ minimum” in modern oceans. This hypothesis is supported by mounting evidence for widespread deep-water anoxia and high sulfide (“euxinia”) by 1.84 Ga (as compared to today), persisting until the Neoproterozoic (Arnold et al., 2004; Brocks et al., 2005; Poulton et al., 2004; Scott et al., 2008a; Shen et al., 2003).

The advent of this euxinic “Canfield Ocean” would have had important consequences for metal availability and hence for N assimilation. Sulfide in the
water column would have facilitated rapid removal of Mo (and some other sulfide-reactive transition metals) to sediments, limiting the accumulation of Mo in the oceans despite the enhanced influx from oxic continental weathering. At the same time, the rise of surface-water oxygen coupled with deep-water sulfidic conditions would have precipitated a crash in Fe concentrations compared to those common in the Archean.

Under these conditions, \(\text{N}_2\)-fixing prokaryotes would have struggled to obtain sufficient Mo and Fe for Nif and Anf, potentially resulting in persistent N-limitation in the oceans (Anbar and Knoll, 2002). Anf and Vnf do not exist in marine cyanobacteria today, so it is possible that Mo was absolutely necessary for cyanobacterial \(\text{N}_2\) fixation throughout Earth history. Experiments testing this hypothesis have shown that the rates of growth and \(\text{N}_2\) fixation by certain cyanobacteria drop when the concentration of Mo is < 5 nano-molar (nM) and when Fe is less than <1 nM in the growth media (Berman-Frank et al., 2001; Zerkle et al., 2006). Measurements of Mo in ancient sediments together with simple mass balance models suggest that Mo concentrations were likely 10-20 nM in the Proterozoic, and even lower in the Archean (Anbar et al., 2007; Scott et al., 2008a). Combined, these experiments and observations suggest that Mo availability in mid-Proterozoic oceans was close to the levels that are problematic for \(\text{N}_2\) fixation by extant cyanobacteria, and at or below that level in the Archean.

Would Proterozoic oceans have contained sufficient Mo for \(\text{NO}_3^-\) assimilation? We sought to determine the cyanobacterial Mo requirements for \(\text{NO}_3^-\) assimilation by growing the filamentous, heterocystous cyanobacterium
*Nostoc* sp. PCC 7120 with \(\text{NO}_3^-\) as the sole N source, with (1500 nM) and without (~0.5 nM) Mo in semi-continuous batch culturing experiments to maintain a growth rate of ~1 doubling per day. After one month, we found no difference in growth rate or intracellular N between Mo-deplete and replete cultures (Fig. 6). It appears, then, that cyanobacteria may be specially adapted to grow on \(\text{NO}_3^-\) with minimal Mo. This could be accomplished through the effective uptake of low quantities of Mo by high-affinity molybdate-uptake systems (Thiel et al., 2002; Zahalak et al., 2004), long-term Mo storage, or expression of nitrate reductases containing alternative metal centers. Thus, cyanobacteria could have used \(\text{NO}_3^-\) as an alternative N source if \(\text{N}_2\) fixation was Mo-limited in the mid-Proterozoic.

Cyanobacteria were the major marine primary producers throughout the Proterozoic (Knoll, 2007), perhaps in part due to their ability to assimilate \(\text{NO}_3^-\) even at low Mo concentrations.

Phylogenetic evidence suggests that NR arose soon after the origin of eukaryotes (Stolz and Basu, 2002) in the late Archean (Brocks et al., 2003a; Brocks et al., 2003b; Brocks et al., 1999; Hedges et al., 2001). The Mo requirement for eukaryotic nitrate reductase (NR) may be considerably higher than that of the cyanobacterial NarB enzyme. NR requires 4 times as much Mo per enzyme as NarB per enzyme complex, due to NR’s tetrameric structure. However, NR is up to 100x more active than the cyanobacterial NarB (Table 2), and thus the Mo requirement of NR may actually be lower than that of NarB. This alternative hypothesis is not supported by preliminary studies, which show that under low Mo, prokaryotes actively cycle \(\text{NO}_3^-\) while eukaryotes show little \(\text{NO}_3^-\).
uptake (Axler and Reuter, 1996). Assuming that the Mo requirement for NR is higher than that of NarB, eukaryotic growth may have been N-limited in the Mesoproterozoic ocean before the major increase in marine Mo ~0.6 Ga (Scott et al., 2008a; Wille et al., 2008) especially since eukaryotes lack the ability to directly utilize N$_2$. The scenario of Mo limitation of eukaryote proliferation fits well with the fossil record of eukaryotic phytoplankton, which displays a radiation of green algae in the Cambrian (Knoll, 2007; Revill et al., 1994). These green algae could have fueled the food web that emerged after the Cambrian Explosion.

The transition to the (mostly) oxic Phanerozoic—Neoproterozoic marine life was influenced by widespread glaciations, the recurrence of banded iron formations and finally the oxygenation of the deep sea between ~0.58-0.56 Ga (Fike et al., 2006; McFadden et al., 2008; Scott et al., 2008a; Shen et al., 2008). Ferruginous anoxic deep water conditions may have prevailed from ~0.7-0.55 Ga (Fig. 1) (Canfield et al., 2008) and could have alleviated Fe stress during upwelling events. At the same time (~0.6 Ga), Mo concentrations were rising (Fig. 1) (Scott et al., 2008a). Concurrent Fe and Mo fertilization of surface waters could have fueled primary productivity in the oceans, supplying food with a high N content to nourish early multicellular organisms in the Ediacaran ocean.

Scattered throughout the early Phanerozoic were warm intervals when the ocean reverted back to deep-water anoxia or euxinia. These periods are thought to have been characterized by N limitation of primary productivity (Saltzman, 2005). Ocean anoxic events (OAEs) of the Paleozoic could have reduced Mo concentrations to 30-50% of modern levels (Algeo, 2004), and ocean Mo during
Mesozoic OAEs may have been drawn down to as little as ~1% of modern levels (Anbar and Gordon, 2008; Pearce et al., 2008). At the Permian-Triassic (P/T) boundary there are indications that euxinia reached the photic zone in much of the oceans (Grice et al., 2005; Kump et al., 2005), which may have vastly depleted transition metal concentrations. Eukaryotic algal growth and evolution may have been slowed during these periods (Falkowski et al., 2004). Biomarker and isotopic evidence shows that cyanobacterial populations surged at the P/T boundary (Xie et al., 2005) and during Cretaceous OAEs (Junium and Arthur, 2007; Kuypers et al., 2004), consistent with the hypothesis that cyanobacteria are particularly well-adapted to cope with metal draw-down in euxinic oceans. If these low-Mo periods limited eukaryotic N metabolism, cyanobacteria would have had an opportunity to flourish.

During most of the Phanerozoic, however, the ocean was fully oxygenated, resulting in high Mo and low Fe concentrations. Organisms with plastids derived from secondary red algal symbiosis, especially diatoms, are also well-adapted to cope with low Fe during NO$_3^-$ assimilation in the modern ocean (Maldonado and Price, 1996, 2000; Marchetti et al., 2006). As shown in Fig. 7, the intracellular Fe content is lower in eukaryotic algae than prokaryotic phototrophs even when the expected Fe demand based on the N assimilation pathway being used is similar. This suggests that modern eukaryotic algae can subsist on lower Fe quotas than cyanobacteria, and may partially explain the success of eukaryotic algae (especially diatoms) in the modern ocean.

Metal-N limitation likely affected not only competition between
eukaryotes vs. prokaryotes but also competition between eukaryotic phytoplankton divisions (e.g. red vs. green algae). The differences in metal concentrations between open ocean and coastal environments likely contributed to the shift from green to red algal dominance after the P/T boundary. Red algae, requiring higher concentrations of Mo, could have survived in oxic coastal environments (Quigg et al., 2003), allowing their radiation in the Mesozoic and continued domination of the marine environment to the present day (Falkowski et al., 2004). Their current prevalence may in part reflect their ability to utilize Cu to assimilate NH$_4^+$ from amino acids. Cu would have been scarce before the oceans became fully oxygenated (Saito et al., 2003). Thus, in part the success of the modern phytoplankton population may reflect their adaptation to low Fe, and high Mo and Cu concentrations.

**Conclusions and future directions**

By coupling geochemical, biochemical and genetic evidence, we have shown that the use of metals in N-assimilation metalloenzymes maps well to the emerging record of changing metal availability in the oceans. In the Archean, abundant Fe and small amounts of bioavailable NH$_4^+$ and NO$_2^-$ led to the evolution of NH$_4^+$ and NO$_2^-$ assimilation pathways in cyanobacteria (Fig. 5). These were insufficient to support the N demands of the growing microbial community, favoring the evolution of N$_2$-fixing nitrogenase enzymes. Fe-Fe and Fe-V nitrogenases may have existed in ancient cyanobacteria, but a “whiff” of O$_2$ (and Mo) at the Archean-Proterozoic boundary (2.5 Ga), and perhaps in earlier times as well, may have spurred the evolution of Mo-bearing nitrate reductase
enzymes. Eventually, as Mo became available in the oceans, Fe-Fe and Fe-V nitrogenases were supplanted by the more efficient Mo-Fe version. It is, therefore, likely that most N assimilation proteins evolved by the time of the GOE. Thereafter, the intersection of metal availability with the differential metal requirements of different organisms may have contributed strongly to competition between prokaryotes vs. eukaryotes. For example, cyanobacteria, which fared better than eukaryotic algae throughout the Proterozoic, are able to grow even at very low Mo concentrations on either N$_2$ or NO$_3^-$. This ability could have been a competitive advantage at a time when oceans are now known to have been low in Mo compared to today (Scott et al., 2008a). If this ecological history is in fact related to the history of metal abundances then the rise of Mo in the Neoproterozoic could have contributed to the switch of the dominant phytoplankton community from prokaryotic cyanobacteria to eukaryotic green algae.

Future efforts should aim to more precisely determine the metal concentrations of ancient oceans, particularly before and after major evolutionary transitions, as well as the intracellular metal quotas and environmental metal abundances required to support N$_2$ fixation and eukaryotic NO$_3^-$ assimilation. Better understanding of metal concentrations through time, coupled to laboratory experiments to explore the physiological changes that organisms experience at representative metal concentrations, are needed to determine how biological pathways coevolved with changing Earth environments. Such an integrated geobiological approach has been quite informative in revealing the connections
between changes in $PO_2$, ocean sulfur abundance and microbial evolution through time (Canfield et al., 2000; Farquhar et al., 2003; Habicht et al., 2002; Johnston et al., 2005; Shen et al., 2001). That precedent should serve as a model for further study of the evolutionary consequences of changing metal abundances.

Below, we highlight examples of a larger set of themes that should drive future geochemical and geobiological studies. We touch on several crucial future directions in geochemical research and then focus on geobiological investigations.

• *Ocean Biogeochemical Evolution*. We now have a crude but expanding picture of how marine Fe and Mo concentrations have changed through time (Fig. 1) (Anbar et al., 2007; Anbar and Knoll, 2002; Rouxel et al., 2005; Scott et al., 2008a), but little is known about the way that other metals have varied in the past. Emerging data indicate that Ni may have been higher in the Archean than in the Proterozoic and Phanerozoic oceans (Pecoits and Konhauser, 2008). Theoretical arguments suggest that Cu was lowest during the Proterozoic (Buick, 2007; Saito et al., 2003; Zerkle et al., 2005). Future geochemical studies and data compilations are needed to explore these and other such possibilities.

Further work is also required to gain greater knowledge on N geochemistry through time, where knowledge remains rudimentary. We do not currently have a clear picture of when certain metabolisms appeared (e.g. denitrification). Geochemical methods such as measurement of N isotopic compositions in sedimentary rocks have been useful in the past (Beaumont and Robert, 1999); more efforts are needed as analytical abilities advance.
Previous studies have explored how marine inorganic N concentrations varied through ocean history (Beaumont and Robert, 1999; Papineau et al., 2005). Much less is known about changes in DON (especially urea and amino acids) through time. This has important implications for metal requirements for N assimilation, as DON assimilation requires Ni and Cu, whereas DIN assimilation requires only Fe and Mo. Both modeling and geochemical studies are required to explore the marine speciation of N through time.

**Microbial Metal Processing.** Better understanding of the strategies for uptake and regulation of metals that were scarce in ancient oceans can provide insight into the selection pressures exerted by metal scarcity on ancient ocean ecosystems, and consequent adaptations. We now know that uptake and regulation mechanisms can be diverse and complex. Today, marine photoautotrophs maximize their ability to acquire Fe by excreting siderophores and by expressing of high-affinity uptake systems to acquire Fe at minute quantities from seawater (Hutchins et al., 1999; Kraemer et al., 2005; Maldonado and Price, 2001). Marine diazotrophic cyanobacteria cycle Fe between nitrogenase and photosynthetic metalloproteins on a diurnal cycle, thus minimizing overall Fe requirements (Berman-Frank et al., 2001; Saito et al., 2008a; Tuit et al., 2004). Fe and N are also linked through regulatory networks that coordinate Fe uptake in accordance with requirements for N assimilatory enzymes (López-Gomollón et al., 2007; Maldonado and Price, 2000). Additionally, some cyanobacteria store Fe in ferritin as a hedge against Fe scarcity (Castruita et al., 2007; Castruita et al., 2006). Other adaptations
involve enzyme substitutions. For example, flavodoxin, an alternative to ferredoxin that does not incorporate Fe, is expressed by numerous phytoplankton when Fe concentrations are low (La Roche et al., 1996).

Analogous intricate metal processing mechanisms could have evolved in ancient oceans starved of Mo, Cu or other metals, and may have been inherited by modern cyanobacteria. This evolutionary legacy should be explored in order to understand how metals required for N assimilation (especially Mo, Cu and Ni) are taken up and allocated intracellularly when scarce in the environment. For instance, a Mo storage protein has recently been discovered in a soil bacterium (Fenske et al., 2005). Such a protein may also be present even in the marine cyanobacterial diazotrophs which possess higher Mo quotas than can be accounted for if nitrogenase is the sole Mo-protein (Tuit et al., 2004). Recent work has shown that molybdenum can be complexed by siderophores released by soil bacteria, allowing these organisms to scavenge trace amounts of Mo (Bellenger et al., 2008). Further research is required to understand the intracellular allocation of metals other than Fe in representative organisms from both modern and ancient environments.

• Secondary Metal Requirements. As N and metal metabolisms are explored with ever increasing detail, utilization of metals for purposes secondary to direct N assimilation have been discovered. These metalloenzymes likely contribute to even higher metal requirements for N assimilation than previous recognized. For example, multi-Cu ferroxidases are used for oxidation of
Fe(II) to Fe(III) in Fe-limited diatom cultures containing organically-bound Fe (Maldonado et al., 2006). Thus, Cu is required in order for diatoms to access low levels of Fe required for acquisition of N in any form. Cu is also required for the biosynthesis of molybdopterin cofactors used in NR in higher plants (Kuper et al., 2004). It is unknown whether cyanobacterial and algal molybdopterin biosynthesis also requires Cu. This represents an important future research direction, since an additional Cu requirement for NO₃⁻ assimilation would likely have had impacts on biological N acquisition in ancient oceans.

• **Metal Quotas.** The first comprehensive study of intracellular metal quotas in photoautotrophs provided an average trace element stoichiometry (Ho et al., 2003). However, these stoichiometries can change under different nutrient regimes. This allows their use as a measure of trace element demand. As shown in Fig. 7, intracellular Fe quotas map well to expectations of Fe demand based on N-assimilation pathways, but show overall higher Fe demand for prokaryotes vs. eukaryotes. Such studies of intracellular Mo, Ni and Cu demand and partitioning are just beginning. Intracellular Mo has been shown to be higher in N₂-fixing vs. NO₃⁻-grown cyanobacteria (Tuit et al., 2004). Early studies showed that several species of eukaryotic algae had an absolute requirement for Mo when grown on NO₃⁻ as the sole N source (Arnon et al., 1955; Loneragan and Arnon, 1954; Walker, 1953; Wallen and Cartier, 1975). Future studies could investigate the Mo requirements for NO₃⁻ assimilation for eukaryotes compared to those of cyanobacteria in order to test
the hypothesis that eukaryotic acquisition of NO$_3^-$ requires more Mo than prokaryotic assimilation. The Cu requirements for amine oxidation in eukaryotic photoautotrophs could be measured in order to ascertain whether such low Cu concentrations could have occurred in ancient oceans.

Finally, a fundamental question in geobiology is whether global environmental changes – like the rise of O$_2$ - trigger evolutionary innovation, or whether such changes enable pathways that evolved earlier, in unusual niches, to radiate. Such distinctions remain unclear in the multi-billion-year history of N assimilation protein evolution, especially in the case of the different nitrate reductases. More precise phylogenetic dating of the evolution of these enzymes, coupled with more detailed geochemical knowledge of the Archean biosphere, will help elucidate this question.

This chapter was previously published (Glass JB, F Wolfe-Simon, AD Anbar. 2009. Coevolution of metal availability and nitrogen assimilation in cyanobacteria and algae. Geobiology 7(2): 100-123). All co-authors have provided consent for inclusion of this paper in the dissertation (Appendix A).
Table 1

Characteristics of the key enzymes involved in nitrogen assimilation in cyanobacteria and algae.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Metal co-factors</th>
<th>Substrates</th>
<th>Products</th>
<th>Distribution</th>
<th>Species from which protein has been purified or overexpressed</th>
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<tbody>
<tr>
<td>Nitrogenase (Nif, 2-3 (?) subunits per enzyme)</td>
<td>4Fe-4S (NifH), 8Fe-8S, Mo(V/Fe)-7Fe-9S (NifDK)</td>
<td>N₂ , 8H⁺, 8e⁻, 16ATP</td>
<td>2NH₃, H₂, 16ADP, 16 P₁</td>
<td>Cyanobacteria</td>
<td>Anabaena cylindrica (Hallenbeck et al., 1979)</td>
</tr>
<tr>
<td>Ferrodoxin-nitrate reductase (NarB)</td>
<td>4Fe-4S, sulfite oxidase-type molybdopterin (Mo)</td>
<td>NO₃⁻, 2 e⁻, 2H⁺</td>
<td>NO₂</td>
<td>Cyanobacteria</td>
<td>Plectonema boryanum (Ida and Mikami, 1983; Mikami and Ida, 1984); Synechococcus sp. PCC 7942 (Rubio et al., 2002); Aphanothece halophytica (Thaiwanich and Incharoensakdi, 2007)</td>
</tr>
<tr>
<td>NAD(P)H-nitrate reductase (NR, 4 subunits per enzyme)</td>
<td>Cytochrome b₅₅₇ (Fe), molybdopterin guanine dinucleotide (Mo)</td>
<td>NO₃⁻, 2 e⁻, 2H⁺</td>
<td>NO₂</td>
<td>Eukaryotic algae</td>
<td>Chlorella vulgaris (Solomonson, 1975); Ankistrodesmus braunii (de la Rosa et al., 1980; de la Rosa et al., 1981)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Metal cofactors</td>
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<tr>
<td>Ferredoxin-nitrite reductase (NiR)</td>
<td>4Fe-4S, siroheme (Fe)</td>
<td>NO$_2^-$, 6 e$, 8$H$^+$</td>
<td>NH$_3$</td>
<td>All</td>
<td><em>Anabaena</em> sp. 7119 (Mendez and Vega, 1981); <em>Chlamydomonas reinhardtii</em> (Romero et al., 1987); <em>Phormidium laminosum</em> (Arizmendi and Serra, 1990)</td>
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<tr>
<td>Glutamine synthetase (GS)</td>
<td>Mn (?)</td>
<td>NH$_4^+$, Glutamate, ATP</td>
<td>Glutamine (C$<em>2$H$</em>{10}$N$_2$O$_3$)</td>
<td>All</td>
<td><em>Anabaena</em> sp CA (Stacey et al., 1977); <em>Nostoc</em> sp. PCC 7120 (Orr et al., 1981); <em>Chlorella kessleri</em> (Sumar et al., 1984); <em>Anacystis nidulans</em> (Florencio and Ramos, 1985); <em>Chlorella sorokiniana</em> (Beudeker and Tabita, 1985); <em>Phormidium laminosum</em> (Blanco et al., 1989); <em>Calothrix</em> sp. PCC 7601 (Merida et al., 1990); <em>Synechocystis</em> sp. PCC 6803 (Garcia-Dominguez et al., 1997; Merida et al., 1990); <em>Prochlorococcus</em> spp. (El Alaoui et al., 2003)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Metal co-factors</td>
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<td>Products</td>
<td>Distribution</td>
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<tr>
<td>Ferredoxin-glutamate synthase (GltS)</td>
<td>3Fe-4S</td>
<td><strong>Glutamine</strong> (C$<em>2$H$</em>{10}$N$_2$O$_3$), 2-oxoglutarate, 2e^−, 2H^+</td>
<td><strong>2Glutamate</strong> (C$_3$H$_9$NO$_4$)</td>
<td>All (mainly used by cyanobacteria)</td>
<td><em>Chlamydomonas reinhardtii</em> (Gotor et al., 1990); <em>Synechococcus sp.</em> PCC 6301 (Marques et al., 1992); <em>Synechocystis</em> PCC 6803 (Ravasio et al., 2002)</td>
</tr>
<tr>
<td>NADH-glutamate synthase (GltBD)</td>
<td>4Fe-4S (GltD), 4Fe-4S, 3Fe-4S (GltB)</td>
<td><strong>Glutamine</strong> (C$<em>2$H$</em>{10}$N$_2$O$_3$), 2-oxoglutarate, 2e^−, 2H^+</td>
<td><strong>2Glutamate</strong> (C$_3$H$_9$NO$_4$)</td>
<td>Some cyanobacteria, mainly used by eukaryotes</td>
<td><em>Chlamydomonas reinhardtii</em> (Marquez et al., 1984)</td>
</tr>
<tr>
<td>Urease (Ure, usually 3 subunits per enzyme)</td>
<td>2Ni (di-nuclear)</td>
<td><strong>Urea</strong></td>
<td><strong>NH$_3$, H$_2$CO$_3$</strong></td>
<td>Cyanobacteria/red algae</td>
<td><em>Spirulina maxima</em> (Carvajal et al., 1982); <em>Anabaena doliolum</em> (Rai, 1989); <em>Anabaena cylindrica</em> (Argall et al., 1992); <em>Leptolyngbya boryana</em> (Jahns et al., 1995); <em>Anabaena</em> sp. PCC 7120 (Jahns et al., 1995); <em>Synechococcus</em> sp. WH 7805 (Collier et al., 1999)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Metal cofactors</td>
<td>Substrates</td>
<td>Products</td>
<td>Distribution</td>
<td>Species from which protein has been purified or overexpressed</td>
</tr>
<tr>
<td>------------------------------</td>
<td>----------------</td>
<td>------------</td>
<td>----------</td>
<td>------------------------</td>
<td>----------------------------------------------------------------</td>
</tr>
<tr>
<td>Urea amidolylase</td>
<td>---</td>
<td>Urea</td>
<td>Allophanate</td>
<td>Green algae</td>
<td>No purification has been published.</td>
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<tr>
<td>Allophanate hydrolyase</td>
<td>---</td>
<td>Allophanate</td>
<td>NH₃</td>
<td>Green algae</td>
<td><em>Chlamydomonas reinhardtii</em> (Maitz et al., 1982)</td>
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<tr>
<td>Cu-amino acid oxidase</td>
<td>Cu (mononuclear)</td>
<td>Amines</td>
<td>NH₃, H₂O₂, RCHO</td>
<td>Dino-flagellates/ coccolithophores/ cyanobacteria/ green algae</td>
<td>No purification has been published.</td>
</tr>
<tr>
<td>(CuAOD, 2 subunits per enzyme)</td>
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<td>(RCH₂NH₂), O₂</td>
<td></td>
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</table>
Table 2

Comparison of the specific activities of prokaryotic (P) and eukaryotic (E) nitrate reductase enzymes.

<table>
<thead>
<tr>
<th>Specific activity (µmoles NO₂ formed min⁻¹ mg enzyme⁻¹)</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>72-80</td>
<td><em>Ankistrodesmus braunii</em></td>
<td><em>De la Rosa et al.</em>, (1981)</td>
</tr>
<tr>
<td>83-86</td>
<td><em>Chorella vulgaris</em> (green alga, E)</td>
<td><em>Solomonson et al.</em>, (1975)</td>
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<td>2,050</td>
<td><em>Thalassiosira pseudonana</em> (diatom, E)</td>
<td><em>Amy &amp; Garrett</em> (1974)</td>
</tr>
</tbody>
</table>
Chapter Two Figure Captions.

**Figure 1.** Estimated surface (dotted lines) and deep (solid lines) concentrations of Fe (red), Mo (blue) and \( \text{O}_2 \) (black) in seawater through time, modeled after Zerkle et al. (2005) with data updated for Mo (Anbar et al., 2007; Scott et al., 2008a), Fe (Canfield et al., 2008) and \( \text{O}_2 \) (Holland, 2006).

**Figure 2.** Schematic of N assimilatory protein alignments. Each horizontal shaded gray square represents a sequence of amino acid that folds into a protein. Each vertical colored bar represents the position of a conserved (present in the same position in the same proteins from different organisms) metal-binding amino acid in the protein sequence. It is clear that not all proteins are present in all organisms. For example, eukaryotes lack nitrogenase and possess different nitrate reductase proteins than prokaryotes, and most green algae do not possess urease nor copper-containing amine oxidase. The metal cofactors that are bound to the amino acids are shown above the sequences. Note the scale bar of 100 amino acids. Cysteines (abbreviated with a small "C") are shown in yellow, histidines ("H") in red, serines ("S") in blue, lysines ("K") in dark green, asparates ("D") in light green and glutamates ("E") in purple. See text for protein abbreviations.

**Figure 3.** N assimilation pathways plotted vs. redox state of N with metal cofactors colored (Fe: red, Mo: blue, Ni: green, Cu: purple, yellow atoms are S atoms), including \( \text{N}_2 \) fixation (A), prokaryotic \( \text{NO}_3^- \) reduction (B), eukaryotic \( \text{NO}_3^- \) reduction (C), \( \text{NO}_2^- \) reduction (D), urea hydrolysis (E) and amine oxidation (F). All assimilation pathways lead to \( \text{NH}_4^+ \), which is assimilated following the
pathways in Fig. 4. Enzyme and cofactor abbreviations are labeled and the N atom being assimilated is highlighted in teal in each pathway. AA stands for amine acid. Numbers in parentheses (i.e. x2) stands for the number of subunits in each enzyme.

**Figure 4.** The NH$_4^+$ assimilation pathway in primary producers with Fe colored in red and S colored in yellow. NH$_4^+$ taken up from the environment or reduced via the pathways in Fig. 3 is converted to glutamine (A) and subsequently to two molecules of glutamate with either ferredoxin (B) or NADH (C) supplying electrons for this process. One molecule of glutamate is recycled to make sure glutamine and the other is converted into biomolecules requiring N, such as other amino acids, heme or chlorophyll.

**Figure 5.** Trends in the relative abundance of metals involved in N assimilation (Fe, Mo, Ni and Cu) and N species (N$_2$, NO$_3^-$, NO$_2^-$, NH$_4^+$, urea and amino acids) through time. Color darkness represents relative average seawater concentration, with darker colors representing higher concentrations, and lighter colors representing lower concentrations. Note that very little data exists on the abundance of organic N (urea and amino acids) through time. Solid blue bars indicate periods when it has been fairly well-established that N assimilation enzymes had evolved, whereas question marks indicate periods when they may have existed. Anf = Fe-Fe nitrogenase; Vnf = V-Fe nitrogenase; Nif = Mo-Mo nitrogenase, NarB = cyanobacterial nitrate reductase; NR = eukaryotic nitrate
reductase; NiR = nitrite reductase; Ure = urease; AO = amine oxidase; GltS = glutamate synthase.

**Figure 6.** Results of an experiment in which the heterocystous cyanobacterium *Nostoc* sp. PCC 7120 was grown for one month and diluted every 3-5 days with new media containing NO$_3^-$ (15 mM) and either no Mo (“-Mo”, blank was 0.5 nM) or 1500 nM Mo as sodium molybdate. (A) Photographs of one replicate culture bottle from each condition on the final day of the experiment, which appear similar in chlorophyll concentrations (A vs. B). (C) Physiological measurements taken on the final day of the experiment. Intracellular Mo was ~ 4x higher in the +Mo bottle than the –Mo bottle, but this intracellular difference had no effect on the growth rate (dark circles), nor the intracellular N content of the cultures (open circles). These results show that cyanobacteria are adapted to cope with very low environmental Mo levels when they are grown with a source of nitrate.

**Figure 7.** Comparison of Fe requirements for assimilation of different N sources for photosynthetic (A) prokaryotes (cyanobacteria) and B) eukaryotes (secondary red algae). Bar graphs show the distribution of Fe atoms present in enzymes used to assimilate N$_2$ (prokaryotes only; Nif and GltS), NO$_3^-$ (NarB in prokaryotes/NR in eukaryotes, NiR and GltS in prokaryotes/GltBS in eukaryotes), NO$_2^-$ (NiR and GltS in prokaryotes/GltBD in eukaryotes), and NH$_4^+$ (GltS in prokaryotes/GltBD in eukaryotes). Note that the total Fe atoms used to assimilate inorganic N is greater in eukaryotes than prokaryotes for all N sources (aside from N$_2$) due to the
11 Fe atoms in GltBD, versus only 3 in GltS. Red data points represent intracellular Fe normalized to C for growth on different N sources at growth-limiting concentrations of Fe. Data sources: A) N$_2$-supported growth of cyanobacterium *Trichodesmium* IMS 101 (Kustka et al., 2003b); NO$_3^-$-supported growth of cyanobacterium *Synechococcus* sp. (Kudo and Harrison, 1997); NH$_4^+$-supported growth of species cyanobacterium *Trichodesmium* IMS 101 (Kustka et al., 2003b) and *Synechococcus* sp. (Kudo and Harrison, 1997). B) NO$_3^-$ and NH$_4^+$-supported growth of marine diatoms *Thalassiosira pseudonana* and *T. weissfloggi* (Maldonado and Price, 1996). C) Intracellular Fe vs. Fe atoms per N assimilation pathway for prokaryotes and eukaryotes. Note that intracellular Fe increases with greater numbers of Fe atoms per pathway, and that for a given numbers of Fe atoms in a pathway, prokaryotes have higher intracellular Fe quotas. This may reflect eukaryotic adaptation to the Phanerozoic ocean, where Fe levels are low, versus cyanobacterial adaptation to the Precambrian ocean, where Fe levels were higher.
Figure 1
Figure 4

Fig. 3

$\text{NH}_4^+$

$\text{GS} \times 12$

$+ \text{Mn, Mg}$

$\text{GltD}$

$\text{GltS}$

$\text{GltB}$

NADH

$\text{2-oxo-glutarate}$

$\text{glutamine}$

$\text{2-glutamate}$

$\text{amino acids, heme, chlorophyll}$
Figure 5

- **Metals**
  - Fe
  - Mo
  - Ni
  - Cu

- **N-Sources**
  - N₂
  - NO₃⁻
  - NO₂⁻
  - NH₄⁺

- **Enzymes**
  - Nif
  - NarB
  - NR
  - NiR
  - Ure, AO
  - GltS

The figure illustrates the presence and activity of various metals, nitrogen sources, and enzymes over billions of years ago. The x-axis represents billions of years ago, with the y-axis showing the presence or activity level of the elements and enzymes. The bands indicate the timeline of their appearance or activity, with different colors representing different levels of activity or presence.
Figure 6
Figure 7

A) Prokaryotes

B) Eukaryotes

C

Fe atoms per assimilation pathway

Intracellular Fe:C (µmol mol⁻¹)

N₂  NO₃⁻  NO₂⁻  NH₄⁺  NO₃⁻  NO₂⁻  NH₄⁺

NiF  NarB  NiR  GlnS  NR  NiR  GlnBD

Intracellular Fe:C (µmol mol⁻¹)

Fe atoms per assimilation pathway

- prokaryotes
- eukaryotes
Molybdenum (Mo) is essential for the biological assimilation of inorganic nitrogen (N). We compared Mo requirements for N\textsubscript{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 (100 nmol L\textsuperscript{-1}) than in most freshwaters (~5 nmol L\textsuperscript{-1}). Contrary to this hypothesis, we found that both strains maintained N\textsubscript{2} fixation for 30 days at 10 nmol L\textsuperscript{-1}. Mo concentrations <1 nmol L\textsuperscript{-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\textsubscript{2}-fixation persisted in the coastal strain (\textit{Nostoc} sp. CCMP 2511) for at most 12 days; 3 days were required for chlorophyll \textit{a} concentrations to fall below those of Mo-replete cultures. An additional 7 and 11 days, respectively, were required for N\textsubscript{2} fixation rates and chlorophyll levels to decline in Mo-limited freshwater cultures (\textit{Nostoc} sp. PCC 7120). When Mo was high (>1 \textmu mol L\textsuperscript{-1}), the freshwater strain exhibited considerable Mo storage (>100 \textmu mol mol\textsuperscript{-1} Mo:C) whereas cellular Mo remained <10 \textmu mol mol\textsuperscript{-1} Mo:C in the coastal strain. The high Mo content and extended time required for N\textsubscript{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.

**Introduction**

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 colimitation’ (Saito et al., 2008b). However, concentrations of Mo vary widely in aquatic environments. In the open ocean, Mo is well-mixed and is present at ~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 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 fresh waters 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 an 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 (Boison et al., 2006; Thiel, 1993). No species of cyanobacteria have been found to possess the Fe-Fe nitrogenase (Anf).

In addition to N\textsubscript{2} fixation, Mo is also required for cyanobacterial NO\textsubscript{3}\textsuperscript{-} reduction to NO\textsubscript{2}\textsuperscript{-} 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\textsubscript{2} and NO\textsubscript{3}\textsuperscript{-} (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\textsubscript{2} fixation by heterocystous cyanobacteria (hereafter, HC) was first recognized by Bortels (1940). Further study showed that the N content of N\textsubscript{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\textsubscript{2}) and O\textsubscript{2}-derived radicals (Fay, 1992). Pigment content and nitrogenase activity of *A. cylindrica* declined after 7-10 days of growth in Mo-deficient media (<5 nmol L\textsuperscript{-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 approximately 5 nmol L\(^{-1}\), below which nitrogenase 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 measureable 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 colimitation in coastal HC. However, these organisms are ideal models to investigate Mo-N colimitation 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 colimitation. 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 \( \mu\)mol m\(^{-2}\) s\(^{-1}\) irradiance) at 25°C for 30 days. Cultures were continuously bubbled with sterile-filtered atmospheric air and were diluted to optical clarity every 3-5 days. 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 x g for 15 minutes 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 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 milliliters of 2% (0.32 mol L\(^{-1}\)) nitric acid for ICP-MS analysis (Thermo X Series). An extra H\(_2\)O\(_2\) 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 x 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, approximately three days 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 two milliliters of acetylene (C₂H₂). A
sample at the zero time point was taken to ensure no ethylene (C₂H₄) was present.
Bottles were incubated at 150 µmol m⁻² s⁻¹ irradiance. One milliliter of headspace
was sampled every hour. C₂H₂ and C₂H₄ were separated and quantified on a gas
chromatograph (Hewlett-Packard 5890 Series II) equipped with a thermal
ionization detector and a 1.83 m x 0.32 cm SS Porapak N 80/100 column (Ohio
Valley Specialty Chemical). Samples were converted to nmol C₂H₄ 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 pre-
weighed, combusted GF/F filters and dried in an oven at 100°C. Subsequently,
filters were weighed to obtain biomass dry weights (ranging from 0.5-5 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 4,000 x g for
10 minutes. Supernatant was removed and the sample was frozen in liquid N\textsubscript{2} and stored at -80°C. At a later date, the samples were thawed on ice. One milliliter of algal protein extraction buffer (4\% sodium dodecyl sulfate (SDS), 0.05 mol L\textsuperscript{-1} sodium carbonate, 30\% glycerol, 2.5 mmol L\textsuperscript{-1} phenylmethanesulphonylfluoride, 0.05 mol L\textsuperscript{-1} dithiothreitol) was added to each sample and the sample was sonicated, boiled, and centrifuged for one minute at 15,000 x g at 4°C. Protein concentrations were quantified using the EZQ\textsuperscript{®} 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, P. Ludden Laboratory, University of California Berkeley) conjugated to IgG HRP (BioRad) and visualization with Supersignal\textsuperscript{®} 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 (pH 8.0), 30 mmol L$^{-1}$ ethylenediaminetetraacetic acid, 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 Corp.) and were deposited into GenBank. Accession numbers 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. Since 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 3.

*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 $1 \times 10^{-5}$. 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 colimitation 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. 8A). N₂-fixation rates decreased by day 12 and 19 for CCMP 2511 and PCC 7120, respectively (Fig. 8B). When Mo was added back to –N –Mo treatments on the 30\textsuperscript{th} day to PCC 7120, Chl a concentrations and growth rates were restored to those of replete conditions in three days.
Cellular Mo concentrations increased with Mo in the growth media (Table 4), 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 \(\mu\)mol 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. 9). In contrast, cellular Mo:C in the coastal HC CCMP 2511 stayed fairly constant (1-6 \(\mu\)mol mol\(^{-1}\)) with and without added N and across the range of media Mo concentrations (Table 4; Fig. 2).

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

In immunoblots, we targeted two nitrogenase subunits, D and K, because they form the core of the nitrogenase complex that contains the Mo-cofactor. NifDK protein expression generally increased up to 10 \(\mu\)mol mol\(^{-1}\) Mo:C and showed high variability at >10 \(\mu\)mol mol\(^{-1}\) cellular Mo:C, although only two data points were available (Fig. 10B). A similar pattern was observed for dependence of N\(_2\)-fixation rates on cellular Mo:C (Fig. 10C), 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 4). –N –Mo treatments of PCC 7120 had extremely depleted biomass δ¹⁵N (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. 11A). 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 5). 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 non-diazotrophic Synechococcus strains possessed it. Every one of the seven freshwater diazotrophic cyanobacteria contained mop, while none of the four freshwater non-diazotrophic 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 3), 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 5).

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 (Dorado 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 N₂ fixation by heterocystous cyanobacteria*—Our comparison of Mo requirements for N₂ fixation in the freshwater HC PCC 7120 and the coastal HC CCMP 2511 over 30 days revealed that both strains maintained N₂ fixation at ≥10 nmol L⁻¹ throughout the experiment. This was unexpected, since CCMP 2511 was isolated from a bay where Mo is likely near seawater concentrations (100 nmol L⁻¹). When Mo was <1 nmol L⁻¹ in the media, both HC species became Mo-N colimited (Mo:C <1 µmol mol⁻¹ and C:N >8 mol mol⁻¹; Table 4, Figs. 9, 10A). However, both Chl a concentrations and N₂-fixation rates required time periods of weeks to decline (Fig. 8), 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 low Mo availability for weeks after Mo concentrations are depleted (see below).

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\(_2\)H\(_4\) 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 (Attridge and Rowell, 1997; Hallenbeck and Benemann, 1980; Nagatani and Haselkorn, 1978). 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. 10B, C).

The N isotopic signature of N\(_2\)-fixing biomass was generally -2‰ to -3‰ (Table 4). This is within the range of δ\(^{15}\)N values previously reported for N\(_2\)-fixing cyanobacteria, ~0‰ to -3‰ (Bauersachs et al., 2009). Cellular δ\(^{15}\)N values of NO\(_3^-\)-assimilating cultures reflected uptake of isotopically light (-50‰) NO\(_3^-\) from the media. Interestingly, the δ\(^{15}\)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., 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 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 colimitation, and thus production of $^{15}$N-depleted biomass. Since 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. 9, 11B), 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 $\mu$mol C$_2$H$_2$ mg NifDK$^{-1}$ min$^{-1}$ (Hallenbeck et al., 1979) corresponding to 4.8 $\mu$mol N$_2$ mg NifDK protein$^{-1}$ min$^{-1}$ using a 1 C$_2$H$_2$: 4 N$_2$ conversion ratio (Capone, 1993). Cyanobacterial nitrate reductase (abbreviated NarB) contains only one Mo atom and has a specific activity of ~300 $\mu$mol 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 one-month experiment (Chapter 2).

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 5) but the gene sequences are dissimilar between different cyanobacterial strains. We attributed the sustained $\text{N}_2$ fixation by CCMP 2511 for ~13 days 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, $\text{N}_2$ fixation continued in freshwater HC PCC 7120 for 7 days past coastal HC CCMP 2511 (Fig. 8B). This extended $\text{N}_2$ 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 $\mu$mol mol$^{-1}$ Mo:C) provides evidence for Mo storage in PCC 7120, but not CCMP 2511 (~5 $\mu$mol mol$^{-1}$ Mo:C), which could explain the longer time required to induce symptoms of Mo-N colimitation 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 ~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 2-D 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 5). Since all cyanobacteria strains in Table 5 can assimilate NO\textsubscript{3} as an N source (with the exception of Prochlorococcus marinus), this trend is consistent with the higher Mo requirements for N\textsubscript{2}-fixation compared to NO\textsubscript{3} reduction (see above and Fig. 11B). 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. 11A) 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. 11B). Native populations of HC (Aphanizomenon sp. and N. spumigena) in the Baltic Sea also contain low cellular Mo:C, between 1 to 2 µmol mol\textsuperscript{-1} (Walve and Larsson, 2007), consistent with the lack of the mop gene in N. spumigena CCY 9414 (Table 5). The cellular Mo:C ratio we measured for N\textsubscript{2}-fixing PCC 7120 grown at 100 nmol L\textsuperscript{-1} Mo (3.3 ± 1.1 µmol mol\textsuperscript{-1}, average ± SD) was similar to previous Mo:C measurements of the N\textsubscript{2}-fixing marine species Trichodesmium ISM 101 (2.56 ± 2.02 µmol mol\textsuperscript{-1}, average ± SD) grown in laboratory cultures at approximately at the same Mo concentration (Tuit et al., 2004). Since Trichodesmium lacks the mop gene (Table 5), the similarity in Mo content between Trichodesmium and
PCC 7120 when both 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, since Mo-N colimitation 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., 2008b). 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 (Shi and Falkowski, 2008; Tomitani et al., 2006); 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 since 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., 2008b), 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\textsubscript{2} fixation due to low Mo supplies.

This chapter was previously published (Glass JB, F Wolfe-Simon, JJ Elser, AD Anbar. 2010. Molybdenum-nitrogen colimitation in freshwater and coastal heterocystous cyanobacteria. Limnology & Oceanography 55(2): 667-676). All co-authors have provided consent for inclusion of this paper in the dissertation (Appendix A).
Table 3

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name¹</th>
<th>Primer sequence²</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYA781R</td>
<td>5’ – GACTACWGGGTATCTAATCCWTT – 3’</td>
<td></td>
</tr>
<tr>
<td>nifD</td>
<td>nifD_F</td>
<td>5’ – CGGTACTGCTTGGTCTGGTC – 3’</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>nifD_R</td>
<td>5’ – GCGTCGTTAGCGATGTGGTGTC – 3’</td>
<td></td>
</tr>
<tr>
<td>vnfD</td>
<td>vnfD_F</td>
<td>5’ – CTGGTAATTTGGTGGCGTACTCA – 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vnfD_R</td>
<td>5’ – CTTTTCATACGGCTTTGGTATCG – 3’</td>
<td></td>
</tr>
<tr>
<td>mop</td>
<td>mop_F</td>
<td>5’ – ATGGAARTTAGCWCAGTAATYY – 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mop_R</td>
<td>5’ – TCAACAGCAACTATCACATCTGAKG – 3’</td>
<td></td>
</tr>
<tr>
<td>modA</td>
<td>modA_F1</td>
<td>5’ – TRAAAGABGCAYTRARGAA – 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>modA_R1</td>
<td>5’ – TCRTCAAGCMGCAACTACRAC – 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>modA_F2</td>
<td>5’ – CAAATGAAMAVGGTGCSCC – 3’</td>
<td></td>
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<tr>
<td></td>
<td>modA_R2</td>
<td>5’ – GGYACRCTTCTRGTTCCWCC – 3’</td>
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</tr>
<tr>
<td>modBC</td>
<td>modBC_F1</td>
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<tr>
<td></td>
<td>modBC_R1</td>
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<td></td>
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<tr>
<td></td>
<td>modBC_F2</td>
<td>5’ – GAATGAAGCTTTGGTTGGGC – 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>modBC_R2</td>
<td>5’ – GCTAAAGGTACCCGTTGGTG – 3’</td>
<td></td>
</tr>
</tbody>
</table>

¹F 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, numbers 1 and 2 correspond to two sets of primers.

²Standard 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
Table 4

Cellular composition of *Nostoc* spp. PCC 7120 and CCMP 2511 grown on a range of Mo concentrations.

<table>
<thead>
<tr>
<th>Nostoc strain&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Media concentrations&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Actively fixing N₂ at time of harvest (Y or N)?</th>
<th>Mo cellular concentrations&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Cellular concentrations&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO₃⁻ (mmol L⁻¹)</td>
<td>Mo (nmol L⁻¹)</td>
<td>µg Mo g⁻¹ dry weight</td>
<td>mg Mo g⁻¹ protein</td>
</tr>
<tr>
<td>7120</td>
<td>15</td>
<td>1650 (140)</td>
<td>N</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>32</td>
</tr>
<tr>
<td>2511</td>
<td>1450 (50)</td>
<td></td>
<td>N</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>8.29</td>
</tr>
<tr>
<td>7120</td>
<td>0.03</td>
<td>0.2 (0.1)</td>
<td>N</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>0.1</td>
</tr>
<tr>
<td>2511</td>
<td>0.5 (0.2)</td>
<td></td>
<td>N</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>1.8</td>
</tr>
<tr>
<td>7120</td>
<td>11.2 (0.6)</td>
<td></td>
<td>Y</td>
<td>4.1</td>
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<td></td>
<td></td>
<td>Y</td>
<td>4.9</td>
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<tr>
<td>2511</td>
<td>9.8 (0.2)</td>
<td></td>
<td>Y</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Y</td>
<td>5.1</td>
</tr>
<tr>
<td>7120</td>
<td>108 (2)</td>
<td></td>
<td>Y</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Y</td>
<td>9.5</td>
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<tr>
<td>2511</td>
<td>100 (6)</td>
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<td></td>
<td>Y</td>
<td>485</td>
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<tr>
<td>2511</td>
<td>1500 (65)</td>
<td></td>
<td>Y</td>
<td>9.6</td>
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<td></td>
<td></td>
<td></td>
<td>Y</td>
<td>10.7</td>
</tr>
</tbody>
</table>
Table 4 cont.

1 Cells were grown for 30 days (and diluted to optical clarity every 3-5 days) before they were harvested for analysis.
2 Numbers in parentheses represent standard deviations
<table>
<thead>
<tr>
<th>Cyanobacterial species</th>
<th>Diazotrophic? (Y or N)</th>
<th>HC or non-HC?</th>
<th>mop gene present? (Y or N)</th>
<th>modABC genes present? (Y or N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichodesmium erythraeum ISM101</td>
<td>Y</td>
<td>non-HC</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Crocosphaera watsonii WH 8501</td>
<td>Y</td>
<td>non-HC</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Prochlorococcus marinus (12 strains)</td>
<td>N</td>
<td>--</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Synechococcus spp. (7 strains)</td>
<td>N</td>
<td>--</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Lyngbya sp. PCC 8106</td>
<td>Y</td>
<td>non-HC</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Cyanothecae (2 strains)</td>
<td>Y</td>
<td>non-HC</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Nodularia spumigena sp. CCY9414</td>
<td>Y</td>
<td>HC</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Nostoc sp. CCMP 2511 (this study)</td>
<td>Y</td>
<td>HC</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Synechococcus spp. (5 strains)</td>
<td>N</td>
<td>--</td>
<td>N</td>
<td>Y(3) N(2)</td>
</tr>
<tr>
<td>Freshwater</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nostoc sp. PCC 7120 (this study)</td>
<td>Y</td>
<td>HC</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Anabaena variabilis ATCC 29413</td>
<td>Y</td>
<td>HC</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Nostoc punctiforme PCC 73102</td>
<td>Y</td>
<td>HC</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Synechococcus (Octopus Spring, Yellowstone, 2 strains)</td>
<td>Y</td>
<td>non-HC</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Cyanothecae (5 strains)</td>
<td>Y</td>
<td>non-HC</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Synechocystis sp. PCC 6803</td>
<td>N</td>
<td>--</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Synechococcus elongatus (2 strains)</td>
<td>N</td>
<td>--</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Microcystis aeruginosa NIES-843</td>
<td>N</td>
<td>--</td>
<td>N</td>
<td>Y</td>
</tr>
</tbody>
</table>
**Chapter Three Figure Captions**

**Figure 8.** (A) Average Chl a concentrations and (B) N₂ 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 3 times with –N –Mo media before they were diluted 7-fold into the media for each treatment. Arrows show the days when cultures were diluted to optical clarity, for which pre- and post-dilution Chl a concentrations were averaged. Maximum rates of N₂ fixation occurred the first day rates were measured in each experiment for each species. For PCC 7120, the maximum N₂ fixation rate was 8 ± 0.5 nmol C₂H₄ µg Chl a⁻¹ h⁻¹ (day 9, treatment –N +1500 nmol L⁻¹ Mo). For CCMP 2511, the maximum N₂ fixation rates were 10 ± 6 nmol C₂H₄ µg Chl a⁻¹ h⁻¹ (day 3, treatment –N +100 nmol L⁻¹ Mo) and 8 ± 4 nmol C₂H₄ µg Chl a⁻¹ h⁻¹ (day 3, treatment –N +10 nmol L⁻¹ Mo).

**Figure 9.** 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 (NO₃⁻-assimilating) treatment, and open symbols represent -N (N₂-fixing) treatments.

**Figure 10.** (A) Cellular C:N ratios, (B) NifDK protein expression, and (C) N₂ 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 day 30 for measurement of NifDK expression (B) and N$_2$ fixation rates (C).

**Figure 11.** (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.
Figure 9

[Graph showing the relationship between Cellular Mo:C (μmol mol⁻¹) and Media Mo (nmol L⁻¹).]
Figure 10

A

C:N (mol mol$^{-1}$)

B

Nitrogenase expression (ng NiDFK/µg protein$^{-1}$)

C

$N_2$ fixation rate (nmol C$_2$H$_4$/µg protein$^{-1}$ h$^{-1}$)

Cellular Mo:C (µmol mol$^{-1}$)
Figure 11
Chapter 4
MOLYBDENUM LIMITATION OF NITRATE ASSIMILATION IN CASTLE LAKE, CALIFORNIA

Abstract

Molybdenum (Mo) is an essential micronutrient for biological assimilation of nitrogen gas (N\textsubscript{2}) and nitrate (NO\textsubscript{3}\textsuperscript{-}). In order to explore the Mo requirements for NO\textsubscript{3}\textsuperscript{-} assimilation in freshwater planktonic microbial communities, \textit{in situ} bottle incubations (with Mo and NO\textsubscript{3}\textsuperscript{-} added singularly or in combination to 100 nmol L\textsuperscript{-1} and 100 µmol L\textsuperscript{-1}, respectively) were performed at three depths (3, 15 and 25 m) in late July 2008 and late June-early July 2009 in Castle Lake, California. Ambient Mo in Castle Lake was 2-4 nmol L\textsuperscript{-1}, typical of Mo concentrations from a literature review of ~200 globally distributed lakes (range: 0.03-13 nmol L\textsuperscript{-1}). Addition of Mo stimulated NO\textsubscript{3}\textsuperscript{-} assimilation in the Castle Lake hypolimnion in 2008 and in the epilimnion in 2009. Interannual and depth response differences were explained by preference for NH\textsubscript{4}\textsuperscript{+} over NO\textsubscript{3}\textsuperscript{-} when NH\textsubscript{4}\textsuperscript{+} was available and seasonal succession of plankton species with differing Mo requirements. In both summers, a dissolved Mo minimum was observed in the Castle Lake epilimnion that likely was in part due to Mo draw-down by N\textsubscript{2}-fixing and nitrate-assimilating periphyton communities in the littoral zone. Laboratory chemostat experiments with a common freshwater green alga, \textit{Scenedesmus acutus}, confirmed that low Mo (1 nmol L\textsuperscript{-1}) severely depressed activity of the Mo-containing enzyme nitrate reductase when NO\textsubscript{3}\textsuperscript{-} was the sole nitrogen source.
This study lends further support to the theory that low Mo can limit NO$_3^-$ assimilation in freshwaters with typically low Mo levels (<5 nmol L$^{-1}$) when NH$_4^+$ is scarce.

**Introduction**

Molybdenum (Mo) is an essential micronutrient for all life and is particularly important for nitrogen (N) assimilation due to its presence in nitrate reductase, the enzyme that performs the first step in NO$_3^-$ assimilation, and in nitrogenase, the enzyme that performs N$_2$ fixation. Early laboratory studies showed that removal of Mo from growth media induced symptoms of N-limitation in freshwater green algae (Arnon et al., 1955; Loneragan and Arnon, 1954; Walker, 1953) and diatoms (Wallen and Cartier, 1975) grown with NO$_3^-$ as their sole N source. Despite the fact that Mo concentrations are very low (<5 nmol L$^{-1}$) in most freshwaters (Archer and Vance, 2008; Cole et al., 1993; Sugawara et al., 1961), few studies have addressed the question of whether N assimilation in freshwater ecosystems is limited by Mo bioavailability. This question is of global relevance because Mo limitation of N assimilation may limit the ability of both aquatic and terrestrial environments to serve as sinks for anthropogenic carbon dioxide emissions (e.g., Hungate et al., 2004). However, the importance of Mo in aquatic habitats remains particularly understudied.

One of the few lakes in which Mo limitation has been studied is Castle Lake, a small (0.2 km$^2$) subalpine lake in the Klamath Mountains of northern California that has been the site of intensive ice-free season limnological
monitoring every year since 1959 (Goldman et al., 1989; Jassby et al., 1990; www.castlelake.ucdavis.edu). Castle Lake is representative of meso-oligotrophic lakes with low levels of dissolved inorganic N and Mo. A pioneering paper by Goldman (1960) demonstrated that low Mo concentrations limited primary productivity in Castle Lake. Follow-up studies showed that Mo additions of 50 nmol L\(^{-1}\) to the epilimnion led to increased NO\(_3^-\) uptake (up to 60%), but only when significant NO\(_3^-\) (>1 µmol L\(^{-1}\)) was present early in the summer (Axler et al., 1980).

When investigating Mo requirements for NO\(_3^-\) assimilation in natural aquatic systems with multiple chemical species of N, it is important to consider the influence of ammonium (NH\(_4^+\)). Above a species-specific threshold, NH\(_4^+\) is either preferred over NO\(_3^-\) or inhibits NO\(_3^-\) uptake (Dortch, 1990) and therefore lowers cellular Mo requirements (Ichioka and Arnon, 1955; Peschek, 1979) because NH\(_4^+\) assimilation does not utilize the Mo-containing nitrate reductase enzyme. In Castle Lake, the epilimnetic plankton community is more susceptible to NH\(_4^+\) suppression of NO\(_3^-\) assimilation than the hypolimnetic community (Axler et al., 1980; Axler and Goldman, 1981; Priscu et al., 1985), likely because regenerated NH\(_4^+\) is the primary N source for epilimnetic phytoplankton for most of the growing season (Axler et al., 1982; Axler et al., 1981). Therefore, it is reasonable to expect that Mo requirements for N assimilation increase with depth in Castle Lake.
The aim of this study was to investigate the role of Mo availability in constraining NO$_3^-$ assimilation by freshwater planktonic microbial communities. This work builds from previous studies that relied on isotopic tracer ($^{15}$N and $^{14}$C) uptake experiments (Axler et al., 1980, 1982; Axler and Goldman, 1981; Axler and Reuter, 1996; Goldman, 1960). To do this, we used a three-pronged approach including geochemical observations, NO$_3^-$ assimilation enzyme activity bioassays, and a chemostat study with the freshwater green alga *Scenedesmus acutus*. Furthermore, we extended previous studies of Castle Lake Mo bioavailability (Bachmann and Goldman, 1964; Goldman, 1966) by obtaining high-resolution dissolved Mo profiles from the middle of Castle Lake, and performed a literature review that revealed that Castle Lake Mo concentrations (2-4 nmol L$^{-1}$) are not unusual for lakes around the world. These findings collectively support the theory that Mo availability can limit NO$_3^-$ assimilation in freshwater ecosystems when NH$_4^+$ is scarce.

**Methods**

*Field sampling and experimental set-up*—The Castle Lake Long-Term Research Program (www.castellake.ucdavis.edu) measured temperature, light, dissolved oxygen (O$_2$), chlorophyll $a$ (Chl $a$), NO$_3^-$, and NH$_4^+$ concentrations from a sampling raft located above the deepest point in the lake (32 m) as described in Huovinen et al. (1999). Samples for dissolved Mo analysis were collected off the raft on 16 July 2008 and 27 June 2009 at 1-m resolution using a 7-L Van Dorn sampler. Samples were filtered through 0.2-µm Supor® membrane
syringe filters (Pall). Concentrated nitric acid was added to a final molarity of 0.32 mol L\(^{-1}\). Metal concentrations were measured using inductively-coupled plasma mass spectrometry (ICP-MS).

For bottle incubation experiments, water samples were collected at 3, 15 and 25 meters from the raft with the Van Dorn sampler. Zooplankton were removed by filtering water through a 75-µm mesh screen into 1-L HCl-washed polycarbonate bottles. Bottles were then spiked with either no nutrients (control), 100 µmol L\(^{-1}\) sodium nitrate (+N), 100 nmol L\(^{-1}\) sodium molybdate (+Mo) or both nutrients (100 µmol L\(^{-1}\) sodium nitrate and 100 nmol L\(^{-1}\) sodium molybdate; +N +Mo). These concentrations were chosen so as to increase Mo and NO\(_3^-\) content by over two orders of magnitude. In 2009, potassium nitrate enriched with \(^{15}\)N (10 atom %, Aldrich) was used instead of sodium nitrate. Four replicate bottles per treatment were incubated at their collection depth (3, 15 or 25 m) for 4 days. In 2008, there was one experiment performed (24-28 July) whereas three experiments were performed in 2009 (24-28 June; 28 June-2 July; 5-9 July). At the end of each experiment, bottles were brought to the surface and transported back to the lakeshore lab (~10 min); during transport, bottles were immersed in water collected from the depth of incubation for each condition in order to maintain \textit{in situ} temperatures. Water was filtered onto 47-mm Pall A/E filters for enzyme activity analyses and protein concentration measurements (see below). Water was filtered onto 47-mm Whatman glass fiber filters (GF/F) for Chl \(a\)
measurements, and onto combusted 25-mm Whatman GF/F filters for C, N and δ¹⁵N analysis.

Laboratory analyses—In 2008, activity assays were used to measure the in vitro activity of the first enzyme, nitrate reductase (NR), and the second-to-last enzyme, glutamine synthetase (GS), involved in NO₃⁻ conversion into amino acids. In 2009, the ¹⁵N-tracer method was used to measure the fraction of labeled ¹⁵NO₃⁻ assimilated into biomass. In addition, protein concentrations (2008), C and N analyses (2009) and Chl a measurements (2009) were taken. Different analyses were performed in 2008 vs. 2009 due to problems with Chl a quantification in 2008 and problems with the lakeshore laboratory spectrophotometer in 2009.

NR activity was assayed following a slightly modified version of the protocol established by Berges & Harrison (1995). Pall A/E filters were inserted into glass homogenizing tubes (Cole-Parmer) and 1 mL of NR extraction buffer was added, consisting of potassium phosphate buffer (200 mmol L⁻¹, pH 7.9), ethylenediaminetetraacetic acid (EDTA, 5 mmol L⁻¹), bovine serum albumin (BSA, 3%), dithiothreitol (DTT, 1 mmol L⁻¹), polyvinylpyrrolidone (PVP, 0.3% wt/vol) and Triton X-100 (0.1%). Soluble proteins were extracted using a Teflon homogenizer (30 s, 4000 rpm). Cell membranes were pelleted by centrifugation (~5000 rpm) for 5 min. The following reagents were added to a new tube: 200 μL of homogenate, 580 μL potassium phosphate buffer (200 mmol L⁻¹, pH 7.9), 100 μL of nicotinamide adenine dinucleotide (NADH, 2 mmol L⁻¹), 20 μL flavin adenine dinucleotide (FAD, 1 mmol L⁻¹), and 100 μL of potassium nitrate (100
mmol L⁻¹). Tubes were vortexed, and blanks were prepared by immediately stopping the reaction. The rest of the samples were incubated for 30 min in a covered water bath filled with water collected from the depth of incubation in the lake. The reaction was stopped by adding 1 mL zinc acetate (0.55 mol L⁻¹). Excess NADH was oxidized by adding 20 µL of phenazine methosulfate (125 mmol L⁻¹). Samples were then centrifuged at maximum speed for 10 min, and then 0.5 mL of sulfanilamide and N-(1-napthyl)-ethylenediamine solutions were added. Samples were transferred to cuvettes and the nitrite (NO₂⁻) formed during the incubation was determined by absorption at a wavelength of 543 nm on a spectrophotometer (Shimadzu UV16OU) using a calibration curve made from sodium nitrite solutions.

GS activity was measured following a slightly modified version of the Slawyk & Rodier (1988) protocol. Pall A/E filters were inserted into glass homogenizing tubes (Cole-Parmer) and 4 mL of protein extraction buffer consisting of cold imidazole (50 mmol L⁻¹, pH 7.0) and DTT (0.6 mmol L⁻¹) was added. Proteins were extracted using a Teflon homogenizer (30 s, 4000 rpm) and cell membranes were pelleted by centrifugation (~5000 rpm) for 10 min. Then, 1 mL of supernatant was transferred to a clean centrifuge tube and 1 mL of assay mixture consisting of glutamate (31 mmol L⁻¹), adenosine triphosphate (6 mmol L⁻¹), magnesium sulfate (80 mmol L⁻¹) and hydroxylamine (8 mmol L⁻¹) in imidazole buffer (50 mmol L⁻¹, pH 7.0) was added. Blanks were prepared by immediately stopping the reaction. The rest of the samples were incubated for 1
hour at the same conditions as the NR assay, and the reaction was stopped by adding 1 mL of freshly-prepared solution containing 3.2 g of ferric chloride hexahydrate and 4.0 g of trichloroacetic acid in 100 ml of 0.5 N HCl. Samples were transferred to cuvettes and the concentration of γ-glutamyl hydroxamate (γ-GH) formed during the incubation by absorption at a wavelength of 540 nm on a spectrophotometer (Shimadzu UV16OU) using a calibration curve made from γ-GH solutions.

Soluble protein concentrations were quantified on imidazole-extracted homogenate from the GS activity assay (see above) dotted onto a membrane and stained with the EZQ® Protein Quantification Kit (Molecular Probes, Invitrogen). The membrane was dried and read on a FLx800 Microplate Fluorescence Reader (Bio-Tek Instruments) ~1 week later.

Combusted Whatman GF/F filters were analyzed for C and N concentrations and δ^{15}N values using a Costech Elemental Combustion System 4010 coupled to Thermo Delta Plus Advantage isotope ratio mass spectrometer. The δ^{15}N values were converted into {^{15}NO_3^-} uptake rates using the equations of Dugdale & Wilkerson (1986).

Samples for Chl a analysis were placed on dry ice and then stored at -80°C until analysis. Chl a was extracted from GF/F filters in methanol at 4°C in the dark for 16-20 hours. Chl a concentration was measured by fluorometric analysis using a Turner BioSystems Model TD-700 fluorometer with internal phaeophytin correction calibrated using a spinach Chl a standard (Sigma Aldrich).
Chemostat experiments—The green alga *S. acutus* was grown axenically in triplicate chemostats at 25°C and ~150 µmol photons m⁻² s⁻¹ photosynthetically active radiance in three variations on the media COMBO (Kilham et al., 1998). Media containing 90 µmol L⁻¹ Mo, with an N:P ratio of 5 (200 mmol L⁻¹ NO₃⁻: 40 mmol L⁻¹ PO₄³⁻) diluted at 0.5 d⁻¹ was abbreviated “MON” (moderately N-limited) media using terminology of Sterner et al. (1993). MON media diluted at 0.1 d⁻¹ was abbreviated “LON” (severely N-limited) (Sterner et al., 1993). MON media containing no added Mo (<1 nmol l⁻¹ Mo) was abbreviated “LOMO”. After two weeks of growth in chemostats, NR activity in 20 mL of algal outflow was measured using the same method as described above. At the same time, 5-20 mL of algal outflow was filtered onto 25 mm GF/F filters, the filter was inserted into a glass homogenizing tube (Cole-Parmer) and 1 mL of 90% acetone was added. Chl *a* was extracted using a Teflon homogenizer (1 min; 4,000 rpm). The acetone extract was then transferred to a 15-ml tube, vortexed again and centrifuged at top speed (15,000 rpm) for 5 min. Absorbance at 664, 647 and 750 nm was measured on a ThermoSpectronic Genesys 20 spectrophotometer and Chl *a* was quantified using the equation: Chl *a* [mg l⁻¹] = (11.93*(Abs₆₆₄-Abs₇₅₀)-1.93*(Abs₆₄₇-Abs₇₅₀)/(volume_acetone added/volume_culture filtered) (P.G. Falkowski unpub).

At the same time as the NR activity and Chl *a* measurements were being taken, 100 mL of algal outflow was pelleted at 7,500 rpm and washed three times with COMBO basal medium containing N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid sodium salt, but lacking added metals, to remove metals
adsorbed to cell membranes. Biomass was then transferred to acid-cleaned preweighed Savillex beakers, dried, weighed and dissolved in concentrated nitric acid. The samples were dried and redissolved in 0.32 M nitric acid for measurement of Mo concentrations by ICP-MS.

Statistical methods—The statistical software package JMP™ (version 5.0.1.2) was used to perform one-way ANOVA statistical tests of the response of bottle experiments to four treatments (Cntrl, N, Mo and N+Mo) at each depth. P values greater than 0.05 were assumed to represent no significant difference between treatments. Student’s T-test was used to determine P values for the S. acutus experiments.

Results

Lake water chemistry—Inorganic N (NO_3^- and NH_4^+; Table 6) and Mo concentrations (Table 6; Fig. 12) reached higher levels during the sampling period in 2009 than 2008. NO_3^- concentrations were 0.02-0.37 µmol L^-1 in 2008 vs. 0.06-1.15 µmol L^-1 in 2009 and NH_4^+ concentrations were 0.01-0.17 µmol L^-1 in 2008 vs. 0.31-0.90 µmol L^-1 in 2009 (Table 6). The dissolved Mo content of the Castle Lake water column ranged from 2-4 nmol L^-1 (Table 6; Fig. 12A). Surface water Mo concentrations were 2.9 and 2.4 nmol L^-1 in 2008 and 2009, respectively. Both years, Mo concentration minima occurred near the thermocline, where Mo was lower by 0.5-0.8 nmol L^-1 than in shallower and deeper waters. The concentration minimum was offset ~5m deeper in 2008 than 2009. The Mo minimum was located at a shallower depth (5-10 m) than the Chl a maximum (15-102
20 m), whereas Mo minima and dissolved O₂ maxima occurred similar depths (Fig. 12A, B). Dissolved Mo was relatively constant down to about 20 m, and then increased towards the lake bottom (Fig. 12A).

**Biological response to Mo and nitrate additions in Castle Lake and chemostats**—Bioassay experiments in Castle Lake from 2008 showed a consistent positive response at the lower two depths (15 and 25 m) only in the +N +Mo treatments (Fig. 13). This hypolimnetic response occurred for all three measurements: nitrate reductase activity (Fig. 13A), glutamine synthetase activity (Fig. 13B) and soluble protein (Fig. 13C). In 2009, only samples from 3 m showed a positive response in NO₃⁻ uptake rates to Mo addition \((R^2 = 0.77, P = 0.02; \text{Fig } 14A)\); rates increased between 20-70% in +N +Mo compared to +N treatments. No significant response to Mo addition \((P > 0.05)\) was observed for NO₃⁻ uptake rates at 15 m or 25 m, nor for C:N ratios and Chl a content at any depth (Fig. 13B, C). C:N ratios and Chl a content were significantly higher \((P < 0.001)\) at 15 m than at 3 m and 25 m (Fig. 14B, C). When the green alga *S. acutus* was grown without added Mo ("LOMO", Mo blank ~1 nmol L⁻¹), it exhibited significantly lower Chl a, nitrate reductase activity and biomass Mo \((P \leq 0.03)\) than the other two treatments with Mo added (Fig. 15). No significant change in the same three parameters was observed between moderately N-limited ("MON") and severely N-limited ("LON") treatments (Fig. 15).
Discussion

The results of this study show that addition of Mo stimulated NO$_3^-$ assimilation in the Castle Lake hypolimnion in 2008 and in the epilimnion in 2009. In accordance with our field experiments, laboratory experiments with S. acutus confirm that this freshwater alga displays symptoms of Mo limitation (decreased nitrate reductase activity, chlorophyll $a$ and cellular Mo) when Mo is 1 nmol L$^{-1}$, well within the typical range in Castle Lake and other lakes (0.03-13 nmol L$^{-1}$; Table 7). Therefore, Mo limitation may be widespread in freshwaters, as suggested by previous studies that showed stimulation of photosynthetic carbon fixation after Mo addition to lakes in New Zealand, Alaska and California (Goldman, 1964, 1972). Three hypotheses were developed to explain the interannual and depth differences in response to Mo and NO$_3^-$ additions in Castle Lake: (1) preference for NH$_4^+$, or NH$_4^+$ inhibition of NO$_3^-$ uptake, leading to decreased Mo requirements; (2) seasonal succession of phytoplankton species with differing Mo requirements; and (3) differential Mo requirements with depth.

Hypothesis 1: Ammonium influence on nitrate uptake—Only the first step of the NO$_3^-$ assimilation pathway (NO$_3^-$ reduction to nitrite, NO$_2^-$, catalyzed by the nitrate reductase enzyme) requires Mo. When NH$_4^+$ is present, it is either preferred over NO$_3^-$ or inhibits NO$_3^-$ uptake (Dortch, 1990). In either case, Mo requirements decrease because NH$_4^+$ assimilation does not utilize Mo (Ichioka and Arnon, 1955; Peschek, 1979). Regeneration of NH$_4^+$ by zooplankton excretion and microbial mineralization are important N sources for the Castle Lake.
Lake phytoplankton community (Axler et al., 1981; Zehr et al., 1985). NH$_4^+$ assimilation rates are significantly higher than those of NO$_3^-$ throughout the summer (Axler et al., 1982; Axler et al., 1981) and mid to late-summer regeneration of NH$_4^+$ can contribute more than 50% of the total N assimilated in the epilimnion (Axler et al., 1981). Small amounts of NH$_4^+$ (~0.3-0.4 µmol L$^{-1}$) can inhibit NO$_3^-$ assimilation by more than 75% in the Castle Lake epilimnion, whereas higher NH$_4^+$ concentrations are required for inhibition at greater depths. At 20 m, ~3 µmol L$^{-1}$ NH$_4^+$ was required to significantly inhibit NO$_3^-$ assimilation, whereas at 25 m, enrichment with 5.4 µmol L$^{-1}$ NH$_4^+$ resulted in minimal inhibition (Priscu et al., 1985).

In this present study’s bioassay experiments, ambient NH$_4^+$ concentrations were consistently 0.1-1 µmol L$^{-1}$, except at 15 m and 25 m in 2008, where they were <0.02 µmol L$^{-1}$ (Table 6). Added Mo strongly stimulated NO$_3^-$ assimilation at these depths in accordance with minimal NH$_4^+$ availability. The NH$_4^+$ preference/inhibition hypothesis predicts suppressed Mo requirements at 3 m both years when NH$_4^+$ was > 0.1 µmol L$^{-1}$. Results from 3 m in 2008 follow this prediction; no evidence of increased NO$_3^-$ assimilation was observed with Mo addition. However, contrary to the prediction, Mo stimulated NO$_3^-$ uptake at 3 m in 2009 where NH$_4^+$ was ~0.5 µmol L$^{-1}$. Furthermore, Mo did not stimulate NO$_3^-$ assimilation in the hypolimnion in 2009, despite NH$_4^+$ concentrations below the hypolimnetic NO$_3^-$ inhibition threshold of ~3 µmol L$^{-1}$ (Priscu et al., 1985).
Hypothesis 2: Seasonal succession of plankton species with differing Mo requirements—Different types of plankton vary in their Mo requirements, depending on factors such as their primary N source and whether or not they possess high-affinity molybdate (MoO$_4^{2-}$) uptake systems. A study by Wallen & Cartier (1975) showed that the freshwater diatom *Navicula pelliculosa* was more susceptible to Mo limitation of photosynthesis and NO$_3^-$ uptake than the freshwater green alga *Chlamydomonas reinhardtii*, likely due to the presence of a high-affinity eukaryotic MoO$_4^{2-}$ uptake system (MOT1) in *C. reinhardtii* that is absent from diatoms (Tejada-Jimenez et al., 2007). The MOT1 uptake system is not present in all green algae since *Scenedesmus* (this study; Arnon et al., 1955) and another green alga, *Chlorella* (Loneragan and Arnon, 1954; Walker, 1953) are susceptible to Mo limitation of NO$_3^-$ assimilation. The spotty distribution of MOT1 in eukaryotes was confirmed by a recent bioinformatic survey (Zhang and Gladyshev, 2010). In contrast, high-affinity MoO$_4^{2-}$ uptake systems (ModABC) are widely distributed in prokaryotes (Self et al., 2001; Zhang and Gladyshev, 2008).

Some of the differences in the response of Castle Lake bioassay experiments to Mo additions between 2008 and 2009 may have been due to successional changes in plankton assemblages through the summer. Diatoms – which have been shown to be more prone to Mo limitation than other algal species (Wallen and Cartier, 1975) – decline in the Castle Lake epilimnion and increase in the hypolimnion through the summer (Huovinen et al., 1999). Such a
seasonal succession could explain why NO$_3^-$ assimilation in the epilimnion was stimulated by Mo earlier in the season in 2009 (late June to early July) whereas the Mo response was found in the hypolimnion later in the season in 2008. It is also possible that chrysophytes (golden algae) contributed to some of the epilimnetic response in 2009; the chrysophyte alga *Dinobryon sertularia* showed the greatest response to experimental Mo additions to Castle Lake in the 1960’s (Goldman, 1972; Jassby and Goldman, 1974), and chrysophytes comprise a significant portion of phytoplankton populations in the epilimnion in the early summer (Huovinen et al., 1999).

In contrast to diatoms and chrysophytes, cyanobacteria – in which ModABC uptake systems are very common (Chapter 3; Thiel et al., 2002; Zahalak et al., 2004) – become more abundant in the Castle Lake epilimnion throughout the summer (e.g., Huovinen et al., 1999). The presence of low-Mo adapted cyanobacteria may help explain the lack of significant changes in glutamine synthetase activity and protein content in response to Mo addition to epilimnetic water in bioassays later in the summer in 2008. It should be noted that the nitrate reductase activity results presented here do not include potential activity arising from cyanobacteria because this particular assay is specific only for organisms that use NADH as an electron donor, namely eukaryotic algae, heterotrophic bacteria and anoxicogenic photoautotrophs (Berges, 1997; Moreno-Vivian et al., 1999), whereas cyanobacteria use ferredoxin as the electron donor to nitrate reductase (Moreno-Vivian et al., 1999).
Hypothesis 3: Differential Mo Requirements with Depth—Our observations of interannual differences in response to Mo addition may be a product of sampling later in the growing season in 2008 than 2009. Mo gradually declines throughout the summer in Lake Donk, Belgium (Dumont, 1972) and in Eshwaite Water, England (Achterberg et al., 1997). Our results suggest a similar decline in Mo throughout the summer in Castle Lake since Mo was significantly lower later in the summer of 2008 than earlier in the summer of 2009 (P = 0.002; Fig. 12A; Table 6). The finding of Mo minima in the Castle Lake epilimnion during both summers suggests that dissolved Mo is being taken up biologically in excess of that supplied by watershed and sediment inputs.

The concurrence of the dissolved Mo minima and dissolved O\textsubscript{2} maxima near the thermocline (Fig. 12A) may provide clues as to which Castle Lake microbial community is responsible for the prominent Mo drawdown between 5-10 m depth. Positive heterograde O\textsubscript{2} profiles, like those of Castle Lake, can arise from high phytoplankton photosynthesis and/or diffusion of O\textsubscript{2} (produced by submerged macrophytes, periphyton, or phytoplankton) from the littoral zone into the metalimnion (Wetzel, 2001). The littoral zone of Castle Lake is an extensive shallow (3-5 m) platform with a well-studied benthic periphyton community that includes an expansive epipelic plain comprised of a mixed community of diatoms, chlorophytes and non-heterocystous cyanobacteria, and a fringing epilithic community dominated by N\textsubscript{2}-fixing heterocystous cyanobacteria (Axler and Reuter, 1996; Loeb and Reuter, 1981; Reuter and Axler, 1988; Reuter and Axler,
1992; Reuter et al., 1985), that have high Mo requirements (Chapter 3). Even when epilimnetic water is severely depleted in NO$_3^-$, the overlying epipelic periphyton can still obtain a significant amount of their N-nutrition by fixing N$_2$ or assimilating interstitial porewater nitrate that diffuses upward from the sediments (Reuter and Axler, 1992; Reuter et al., 1985). Therefore, this population represents a potentially important sink for available Mo. Neither heterocystous cyanobacteria, nor N$_2$-fixation, have been found in the planktonic communities of the lake over the many years of intensive monitoring (Reuter et al. 1985; C.R. Goldman, unpubl.). This fact, together with the extremely low rates of planktonic nitrate assimilation rates previously measured in the epilimnion (Axler et al. 1982), suggests that the Mo minimum at the thermocline likely represents demand by benthic periphyton.

Furthermore, the dissolved O$_2$ gradient likely affects Mo availability by influencing the redox state and chemical speciation of Mo. Whereas MoO$_4^{2-}$ (molybdate, in which Mo is present in the 6+ redox state) is generally thought to be the major chemical species of Mo in fully oxygenated waters, Mo can be converted to thiomolybdate or reduced to Mo$^{5+}$ under low O$_2$ conditions. For instance, Mo$^{5+}$ comprises up to 15% of the total dissolved Mo pool in the bottom waters at the head of the Peconic River Estuary where O$_2$ is 10 µmol L$^{-1}$ (Wang et al., 2009). It is unlikely that Mo is present as thiomolybdate (MoO$_x$S$_{4-x}^{2-}$) in Castle Lake because total sulfide concentrations are low (<2 µmol L$^{-1}$ in the deepest bottom waters; Chapter 5) and thiomolybdates do not form when
hydrogen sulfide (H$_2$S) concentrations are below 11 µmol L$^{-1}$ (Erickson and Helz, 2000). However, Mo$^{5+}$ chemical species (in particular, Mo$_2$O$_4^{2+}$) may be present in the decreased dissolved O$_2$ conditions of Castle Lake’s hypolimnion during the summer growing season. Mo$^{5+}$ may also be more bioavailable to plankton than Mo$^{6+}$ because Mo$^{5+}$ is the redox state of Mo in enzymes (see refs in Wang et al., 2009) and does not compete with sulfate during uptake as may be the case for MoO$_4^{2-}$ (Howarth and Cole, 1985; Howarth et al., 1988). Thus, it is possible that Mo is more bioavailable to organisms in the hypolimnion where lower dissolved O$_2$ may lead to increased abundance of Mo$^{5+}$. However, changes in Mo speciation do little to explain interannual hypolimnetic differences in response to Mo additions because dissolved O$_2$ concentrations were very similar in 2008 and in 2009 (Fig. 12A).

Although we have only measured dissolved Mo concentrations in the summer, it is likely that Mo availability in Castle Lake varies throughout the year in response to hydrologic regimes (e.g. snowmelt runoff, storm runoff, baseflow), microbial demand (seasonal patterns of primary productivity), and Mo speciation (see above). For example, there are pronounced winter depletions of Mo in similarly dimictic Lake Donk (Dumont, 1972) and Linsley Pond, Connecticut (Cowgill, 1977) that may also occur in Castle Lake, which freezes over every winter and does not thaw until late spring. Samples of Castle Lake water taken from under the ice in April and May 2009 had much lower dissolved Mo concentrations (0.1-0.9 nmol L$^{-1}$; Chapter 5) than surface lake water in mid-July
2008 and late June 2009 (2.4-2.9 nmol L\(^{-1}\); Fig. 12A). Thus, it is possible that planktonic communities are more Mo-limited during the winter than in the summer months, in accordance with the finding that lower concentrations of Mo stimulated primary productivity in January than in June or October (Goldman, 1960).

**Conclusions**

Pronounced differences in planktonic microbial responses to Mo and NO\(_3^-\) added singularly or in combination to Castle Lake water were observed between 2008 and 2009. In late July 2008, strong evidence of Mo stimulation of NO\(_3^-\) assimilation was observed in the hypolimnion when both nutrients were added together. Earlier in the 2009 growing season (late-June to early-July), the only evidence of Mo limitation was increased epilimnetic \(^{15}\)NO\(_3^-\) uptake. After consideration of three plausible hypotheses to explain interannual and depth differences in the bioassay Mo and NO\(_3^-\) additions, we conclude that interannual differences were best explained by a combination of microbial plankton preference for NH\(_4^+\) over NO\(_3^-\) and/or NH\(_4^+\) inhibition of NO\(_3^-\) uptake and seasonal succession of plankton species with differing Mo requirements. The observed epilimnetic Mo draw-down was likely a result of Mo demand by littoral periphyton communities that fix N\(_2\) and also assimilate NO\(_3^-\). Chemostat experiments confirmed that a common freshwater green alga experiences Mo-limitation of NO\(_3^-\) assimilation at Mo concentrations typical of lakes around the world.
In sum, this study builds off of previous work on Mo-N interactions in Castle Lake (Axler et al., 1980; Axler and Goldman, 1981; Goldman, 1960) by providing new lines of evidence that low Mo availability can limit NO$_3^-$ assimilation when NH$_4^+$ is scarce. More research is required to determine whether low Mo limits N$_2$ fixation in Castle Lake periphyton as previously suggested by Goldman (1960, 1961, 1972).

This chapter is in preparation for the journal *Freshwater Biology*. The coauthor is Richard Axler and he has provided consent for inclusion of this paper in the dissertation (Appendix A).
Table 6

Physical and chemical properties of the Castle Lake water column during the time periods that the Mo-NO$_3^-$ addition experiments were performed. Mo concentrations are provided for July 16, 2008 and June 27, 2009.

<table>
<thead>
<tr>
<th>Water depth Date (Year)</th>
<th>Temp. (°C)</th>
<th>Light ($\mu$E m$^{-2}$ s$^{-1}$)</th>
<th>O$_2$ (µmol L$^{-1}$)</th>
<th>NO$_3^-$ (µmol L$^{-1}$)</th>
<th>NH$_4^+$ (µmol L$^{-1}$)</th>
<th>Mo (nmol L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3m</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>June 24 (2009)</td>
<td>17</td>
<td>665</td>
<td>247</td>
<td>0.08</td>
<td>0.47</td>
<td>2.1</td>
</tr>
<tr>
<td>July 1 (2009)</td>
<td>21</td>
<td>617</td>
<td>241</td>
<td>0.08</td>
<td>0.42</td>
<td>--</td>
</tr>
<tr>
<td>July 8 (2009)</td>
<td>20</td>
<td>636</td>
<td>222</td>
<td>0.06</td>
<td>0.55</td>
<td>--</td>
</tr>
<tr>
<td>July 23 (2008)</td>
<td>21</td>
<td>522</td>
<td>172</td>
<td>0.02</td>
<td>0.17</td>
<td>2.7</td>
</tr>
<tr>
<td>15m</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>June 24 (2009)</td>
<td>4.5</td>
<td>27.9</td>
<td>244</td>
<td>0.10</td>
<td>0.31</td>
<td>3.0</td>
</tr>
<tr>
<td>July 1 (2009)</td>
<td>4.7</td>
<td>36.9</td>
<td>284</td>
<td>0.11</td>
<td>0.42</td>
<td>--</td>
</tr>
<tr>
<td>July 8 (2009)</td>
<td>4.8</td>
<td>37.6</td>
<td>288</td>
<td>0.08</td>
<td>0.61</td>
<td>--</td>
</tr>
<tr>
<td>July 23 (2008)</td>
<td>5.4</td>
<td>54.5</td>
<td>225</td>
<td>0.03</td>
<td>0.01</td>
<td>2.5</td>
</tr>
<tr>
<td>25m</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>June 24 (2009)</td>
<td>4.1</td>
<td>1.9</td>
<td>109</td>
<td>1.15</td>
<td>0.90</td>
<td>3.2</td>
</tr>
<tr>
<td>July 1 (2009)</td>
<td>4.1</td>
<td>2.5</td>
<td>81</td>
<td>1.14</td>
<td>0.88</td>
<td>--</td>
</tr>
<tr>
<td>July 8 (2009)</td>
<td>4.2</td>
<td>2.6</td>
<td>97</td>
<td>0.78</td>
<td>0.37</td>
<td>--</td>
</tr>
<tr>
<td>July 23 (2008)</td>
<td>4.0</td>
<td>3.4</td>
<td>75</td>
<td>0.37</td>
<td>0.02</td>
<td>2.4</td>
</tr>
</tbody>
</table>
Table 7

Dissolved Mo concentrations for Castle Lake and other lakes around the world.

<table>
<thead>
<tr>
<th>Lake name and location (number of lakes in study)</th>
<th>Dissolved Mo (nmol L⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castle Lake, California, USA</td>
<td>2-4</td>
<td>This study; Bachmann &amp; Goldman (1964)</td>
</tr>
<tr>
<td>Clear Lake, Colorado, USA</td>
<td>1-4</td>
<td>Elser &amp; Glass, unpub. data</td>
</tr>
<tr>
<td>Esthwaite Water, England</td>
<td>0.1-2.6</td>
<td>Achterberg et al. (1997)</td>
</tr>
<tr>
<td>Linsley Pond, Connecticut, USA</td>
<td>0.4-2.7</td>
<td>Cowgill (1977)</td>
</tr>
<tr>
<td>New Zealand lakes (3)</td>
<td>&lt;0.7</td>
<td>Goldman (1964)</td>
</tr>
<tr>
<td>Alaska lakes (3)</td>
<td>≤0.6</td>
<td>Goldman (1964)</td>
</tr>
<tr>
<td>Mirror Lake, New Hampshire, USA</td>
<td>0.1-0.3</td>
<td>Cole et al. (1986)</td>
</tr>
<tr>
<td>Japan lakes (13)</td>
<td>0.5-13</td>
<td>Sugawara et al. (1961)</td>
</tr>
<tr>
<td>Lake Greifen, Switzerland</td>
<td>3-5</td>
<td>Magyar et al. (1993)</td>
</tr>
<tr>
<td>Lake Insjön, Sweden</td>
<td>6.4</td>
<td>Lithner et al. (2000)</td>
</tr>
<tr>
<td>Lake Lundsjön, Sweden</td>
<td>0.8</td>
<td>Lithner et al. (2000)</td>
</tr>
<tr>
<td>Eastern Canadian lakes (4)</td>
<td>0.1-3.4</td>
<td>Chappaz et al. (2008)</td>
</tr>
<tr>
<td>Hall Lake, Washington, USA</td>
<td>1-2</td>
<td>Balisterieri et al. (1994)</td>
</tr>
<tr>
<td>Sierra Nevada lakes (170), California, USA</td>
<td>0.03-10</td>
<td>Bradford et al. (1968)</td>
</tr>
<tr>
<td>Northern Germany lakes (8)</td>
<td>0.5-10</td>
<td>Groth (1971)</td>
</tr>
<tr>
<td>Amazonas, Brazil lakes (3)</td>
<td>4-8</td>
<td>Groth (1971)</td>
</tr>
</tbody>
</table>
Chapter Four Figure Captions

Figure 12. Dissolved oxygen (O\textsubscript{2}; squares), dissolved molybdenum (Mo; circles), average light (squares), temperature (circles) and chlorophyll a concentrations (Chl a; diamonds) with depth in Castle Lake, from water column samples collected on 16 July 2008 (white symbols) and 27 June 2009 (black symbols).

Figure 13. Results of 2008 bioassay experiments at three depths in Castle Lake for control (Cntrl), +nitrate (N), +molybdenum (Mo) and +nitrate +molybdenum (N+Mo) treatments: (A) nitrate reductase activity; (B) glutamine synthetase activity; (C) soluble protein content (“n.d.” on the 15 m nitrate reductase activity sample stands for “no data”). The following samples had undetectable activity: nitrate reductase at 3, 15 and 25 m for the control, nitrate reductase at 25 m for +N and glutamine synthetase at 25 m for the control.

Figure 14. Averaged results of three 2009 bioassay experiments at three depths in Castle Lake for the same four treatments as in Fig. 2: (A) nitrate uptake rates; (B) seston carbon:nitrogen ratio and (C) chlorophyll a content (“n.d.” stands for “no data” for the control and +Mo treatments for (A)). Bars not connected by the same lower-case letter are significantly different.

Figure 15. Average chlorophyll a, nitrate reductase activity and biomass Mo content for the freshwater green alga *Scenedesmus acutus* grown for two weeks in chemostats under three treatments: “LOMO” (without added Mo but with the same nitrate concentration as MON media), “LON” (severely nitrate-limited
media) and “MON” (moderately nitrate-limited media). Bars not connected by the same lower-case letter are significantly different.
Figure 12
Figure 13

A. Nitrate reductase activity (μmol NO₂⁻ min⁻¹ mL⁻¹ x 10^12)

B. Glutamine synthetase activity (μmol GH₃⁻ h⁻¹ L⁻¹)

C. Soluble protein (μg mL⁻¹)

Treatment:
- Cntrl
- N
- Mo
- N+Mo

Legend:
- 3m
- 15m
- 25m
- n.d.
Figure 15
IDENTIFYING MAJOR SOURCES OF MOLYBDENUM TO CASTLE LAKE, CALIFORNIA: ANTHROPOGENIC, HISTORICAL AND NATURAL INPUTS

Abstract

Lakes are important for storage of the essential micronutrient molybdenum (Mo) during its transfer from the continents to the oceans, but little is known about the major sources and sinks for Mo in lacustrine systems. I studied Mo cycling in Castle Lake, a small subalpine lake in the Klamath-Siskiyou Mountains of Northern California underlain primarily by mafic and ultramafic geologic formations. I measured molybdenum (Mo) concentrations in a suite of bulk solids (lake sediments, soils and bedrock) and aqueous samples (sediment porewaters, soil runoff, spring waters, snow and ice) from Castle Lake and its watershed. Lake sediments had elevated Mo (7-36 ppm) compared to soils and bedrock (0.2-2 ppm). Sediment porewaters had higher Mo (4-15 nM) than lake water (2-4 nM), soil runoff (0.1-6.2 nM), snowmelt (≤0.1 nM), lake ice (0.3-2.2 nM) and local spring waters (0.03-2.72 nM). I used the numerical model PROFILE to estimate net reaction rate of Mo in the porewater. Porewater Mo flux (0.3-0.4 nmol cm\(^{-2}\) yr\(^{-1}\)) was roughly equal to Mo fluxes from surface inflow and outflow whereas Mo burial fluxes were significantly higher (11.4 nmol cm\(^{-2}\) yr\(^{-1}\)). Model calculations ruled out diagenesis as a source of Mo to lake sediments; diagenetic Mo always represented ≤ 3% of the total Mo concentration in sediment. Since dissolved Mo fluxes were minimal, and atmospheric Mo
deposition was estimated to be a minor source of Mo (<1 nmol cm\(^{-2}\) yr\(^{-1}\)), the largest source of Mo today is likely particulate matter from the watershed (~10 mol yr\(^{-1}\)). Historical Mo sources include: two whole-lake experimental Mo additions in the 1960s, and atmospheric deposition from extensive copper smelting that occurred south of Castle Lake from 1896 to 1919. Bulk lake sediments had negative \(\delta^{98/95}\)Mo values, ranging from -0.5 to -1.0 (± 0.1) ‰, consistent with a major input of Mo from iron oxyhydroxides and organic matter, that preferentially incorporate the light isotopes of Mo.

**Introduction**

Molybdenum (Mo) is an essential element for all life. Mo concentrations in the oceans have changed significantly over Earth history, likely affecting the evolution of early organisms (Chapter 2; Anbar and Knoll, 2002; Scott et al., 2008b). The riverine supply of Mo to seawater is controlled by the oxygen content of the atmosphere; before the rise of oxygen at ~2.4 billion years ago, there was minimal weathering of continental Mo sulfides and, therefore, Mo in seawater and freshwater was extremely scarce (Anbar et al., 2007; Scott et al., 2008b). Even after the rise of oxygen, Mo remained low in freshwaters (<20 nM; Howarth et al., 1988; Chappaz et al., 2008a; Chapter 4) because it is a minor element in the Earth’s crust (1-2 ppm). Mo is the most abundant transition metal in seawater today (105 nM; Collier, 1985) because it is present as the soluble chemical species molybdate under oxic conditions and biological requirements are small relative to the amount of Mo available.
Lakes are important mid-way reservoirs during the transfer of Mo from the continents to the oceans. While numerous studies have investigated Mo removal to marine sediments (Crusius et al., 1996; McManus et al., 2006; Morford et al., 2007; Zheng et al., 2000), there have been few studies of the mechanisms of Mo sequestration in lake sediments. Lacustrine sediments can be either sources or sinks for Mo depending on redox conditions of the overlying water (Chappaz et al., 2008a; Schaller et al., 1997). A recent study investigated Mo systematics in a permanently stratified lake (Dahl et al., 2010), but such meromictic lakes are rare on a global scale. In temperate regions, lakes that mix twice a year (dimictic lakes) are by far the most common lake type (Wetzel, 2001). The aim of this study was to determine the sources and sinks of Mo in a dimictic lake in an igneous terrain with dissolved Mo concentrations typical of freshwaters.

I chose a lake with very well-characterized hydrogeological and biological parameters to study the Mo budget and isotope systematics. Castle Lake has been used as a model ecosystem to study the importance of Mo in biology since the 1960’s, when it was discovered that primary productivity in the lake was limited by Mo availability (Goldman, 1960; Goldman, 1966). The goals of our study were to estimate the contribution of diagenetic processes to sediment Mo enrichments, and to determine the major sources of Mo to Castle Lake. To accomplish these tasks, I combined high-precision Mo concentration and isotopic measurements from a suite of water, soil and sediment samples. I used the numerical code,
PROFILE (Berg et al., 1998) to estimate net reaction rate in the porewater and, subsequently, determine the relative contributions of diagenetic processes, dissolved and particulate watershed inputs, and anthropogenic deposition to Mo enrichments in Castle Lake sediments.

**Methods**

*Site description*—Castle Lake is a small, subalpine lake situated at an altitude of 1706 m in a glacial cirque basin in the Klamath-Siskiyou Mountains of Northern California (Fig. 16, 17). The lake has a maximum depth of ~34 m and a mean depth of 11.4 m (Fig. 16). The lake’s surface area is 0.2 km². Castle Lake is composed of a deep bowl carved by glacial erosion and a shallow (3-5 m) littoral plain. The basin bedrock includes granodiorite, gabbro and serpentinized peridotite (Throckmorton, 1978; Fig 17). Lake inflow originates from a small pond (Heart Lake) at a higher elevation to the south, and outflow is through Castle Lake Creek (Fig. 16). There is additional inflow from springs arising from faults in the bedrock (Goldman, 1961). The lake’s hydraulic residence time is 2-5 years (Goldman and de Amezaga, 1984). The drainage basin of this lake is uninhabited; human activity in the watershed is essentially limited to summer research and recreational activities. The watershed also has not been affected by wildfires since the 1930’s, or by lumbering since the 1980’s. The sediment contains a surface flocculent layer, underlain by sediment containing 50% fine-to-medium grained sand, and 49% silt and clay (Beatty, 1968). Oxygen penetrates to
a depth of ~3 cm in the shallow water sediments (Neame, 1975). At the deepest part of Castle Lake (~35 m), the hypolimnion becomes anoxic during the summer.

Sampling—Source waters were sampled from numerous locations in the Castle Lake basin throughout 2008 and 2009 (Fig. 16; Table 8). Most source waters were exposed at the surface and collected by hand. Runoff samples from sites B1-B5 were sampled using collectors that trapped water flowing between organic and mineral soil layers. Runoff solution collectors were composed of a partially opened (but screened) polyethylene surface placed at the organic-mineral soil interface, and a buried 2.5 gallon bucket to collect runoff. A sample tube and a vent tube ran up from the collection bucket and were fixed to a stake approximately 3 m aboveground to allow for collection under snowpack (see Miller et al. (2006) for a detailed description). Snowmelt from sites S1 and S2 was collected using snowmelt collectors, constructed from open-top containers above closed, buried containers. The top containers collect snow or rainwater that was directed by a tube to the bottom collection containers. Collection under snowpack was enabled by the same tube system described above (see Susfalk and Johnson (2002) for a detailed description of snowmelt solution collectors). Ice was sampled from four locations on the lake surface from February to May 2009. All samples were filtered through a 0.2-µm Supor® membrane syringe filter (Pall) and acidified with HNO₃ to 0.32 M (calculated concentration based on volume of concentrated acid added to solution) after collection.
Soil samples were collected using a clean trowel and Ziploc® bags. Samples were placed in plastic containers and dried at 100°C for ~24 h the same day as collection. After drying, samples were sieved to <2mm, ground with a ceramic mortar and pestle, and then ground finer with a tungsten ring-and-puck pulverizer. In addition, a sample of granodiorite was collected from an outcrop near Heart Lake (Fig. 16) and ground using the ring-and-puck pulverizer.

A 50-cm sediment core was collected in 1-m acrylic core liner with a 7.6-cm outside diameter (6.7-cm inside diameter) gravity corer deployed by hand in 35 m of water on July 26, 2008 near the center of the deep basin of Castle Lake (Fig. 16). At the time the core was taken, bottom water oxygen was <1 mg L⁻¹. The sediments recovered close to the sediment-water interface (SWI) were flocculent, suggesting minimal loss of surface material during coring. Overlying water was removed by siphoning, and each core was sectioned on-site with a hydraulic extruder and plastic sectioning equipment that was thoroughly washed with lake water between each slice. The core was sectioned at 1-cm intervals throughout its length. Each sediment slice was deposited into a clean, tared, quart-size Ziploc® bag sealed without excess air and kept in the dark at 4°C for shipping and storage prior to drying at 50°C and grinding in a ball mill. A discrete volcanic ash layer was observed at 25-26 cm depth in the core. Tare weights and wet sample weights from each core section were recorded after sectioning at the Castle Lake Limnological Research Laboratory. Water content and porosity were
calculated from the dry weights of sections of an adjacent core taken on the same
day as the core used in our analyses.

Porewater was collected by *in situ* dialysis using polypropylene “peepers”
deployed by SCUBA divers on June 19, 2009. Peepers were prepared and
sampled according to methods described in detail in Alfaro-De La Torre and
Tessier (2002) and Chappaz et al. (2008a). Briefly, peepers were immersed in
10% HNO₃ for 7 d, rinsed thoroughly with ultrapure water (18.2 MΩ·cm), and
stored for 14 d under nitrogen atmosphere (renewed daily) until use, to ensure no
oxygen contamination. Peeper cells were then filled with ultrapure water, covered
with a Gelman HT-200 polysulfone membrane (0.2-µm pore size) and stored for 7
d under a nitrogen atmosphere. Four peepers were deployed at each site for 21 d.
The shallow, oxic site (12 m) had a bottom water dissolved oxygen concentration
of ~10 mg L⁻¹ (Fig. 1a). The deep, suboxic site (35 m) had a bottom water
dissolved oxygen concentration of ~0.4 mg L⁻¹ (Fig. 16). (Oxygen values are
given for the day of collection.) The peepers were designed to collect sediment
porewater at 1-cm intervals from 10 cm above the SWI to 50 cm below. However,
due to poor visibility, the peepers were inserted deeper in the sediment than
intended: 2 cm for the oxic site, and 8 cm for the suboxic site. Hence, I adjusted
the data representation on the porewater figures to reflect deeper installation of
the peepers than anticipated.

*Analyses*—Porewater Mo, iron (Fe) and manganese (Mn) concentrations
were measured using an inductively coupled plasma mass spectrometer (ICP-MS;
Thermo Scientific X series). Certified materials NIST 1643e (water standard) and NIST 1646a (estuarine sediment) were used to determine precision (<2% for Mo, n=6) and accuracy (9% for Mo). Porewater total sulfide ($\sum S^{2-} = H_2S + HS^- + S^{2-}$) concentrations were measured by spectrophotometry (Cline, 1969). Porewater pH was measured using a pH meter (Denver Instruments, Model 220). Sediment samples for metal analysis were ashed at 550°C for ~16 h to remove organic carbon ($C_{org}$) and completely digested in HNO$_3$ and HF. Sediment Mo, Fe, Mn and Al were determined by ICP-MS. Carbon and nitrogen analysis was performed on an Elemental Analyzer (Costech 4010) using the NIST 2710 soil standard for calibration and glycine standards for accuracy (2% for C and 3% for N). Sediments were analyzed for Mo isotopic composition following the Zr element spike method (Barling et al., 2001) and the $^{97-100}$Mo double spike method (Dahl et al., 2010) on a Neptune multi-collector ICP-MS (Thermo Scientific). The measured $^{98}$Mo/$^{95}$Mo ratios were compared to the ASU in-house standard “RochMo 2” (Alfa Aesar Specpure Lot # 802309E). Errors are reported as the 2σ standard deviation (SD) reproducibility for replicate analyses of the same sample.

**Results**

Castle Lake source-water Mo concentrations are shown in Table 8. Sediment porewater concentration profiles of Mo, Fe, Mn, sulfide and pH for the oxic and suboxic bottom water sites are shown in Fig. 18. Solid phase concentration profiles of Mo, Fe, Mn, N and $C_{org}$ are shown in Fig. 19. Hereafter,
[X] and {X} correspond to the concentration of species X in the aqueous and solid phases, respectively.

Lake source waters and outflow—Castle Lake source waters contained less dissolved [Mo] than the lake water itself (2-4 nM; Chapter 4) and were often similar to the field blank (0.07 nM; Table 8). Heart Lake waters contained the lowest [Mo] (≤0.1 nM). Runoff collected directly along the western side of the lake basin (W1-W6) had [Mo] that ranged from 0.1-0.5 nM. Runoff collected between organic and mineral soil layers (B1, B2 and B3) and springs (M1, M2, M3 and M4) displayed a broader range of [Mo], up to 1.2 nM Mo. Spring M5, located within a patch of alder trees on the northeastern lake shore (Fig. 16), contained 2.7 nM [Mo], higher than any of the other springs. Sites in which multiple time points were available generally showed minimal [Mo] fluctuation throughout the year. Exceptions were sites B4 and B5, located within the alder grove and near the Castle Lake Limnological Research Laboratory, respectively (Fig. 16). From May to August 2009, [Mo] at site B4 increased from 0.2 to 1.2 nM, and [Mo] at site B5 increased from 0.1 to 6.2 nM. Lake ice [Mo] sampled throughout the 2008-2009 winter ranged from 0.4-2.2 nM. Lake water sampled from under the ice contained lower [Mo] than the ice (<1 nM). Outflow from Castle Lake ranged from 0.9-2.3 nM over the summer of 2009 (Table 8).

Sediment porewaters—In both the oxic and suboxic sites, [Mo] ranged from ~3-15 nM, however the [Mo] profiles at the two sites were very different (Fig. 18a, b). At the oxic site, [Mo] increased from 3 nM at the SWI to 10 nM at 5
cm depth, and stayed constant at ~10 nM from 5 cm to the bottom of the profile. The other oxic profile increased from 3 nM at the SWI to 13.5 nM at 5 cm depth, declined to a minimum of ~4 nM at 10-15 cm, increased to ~10 nM from 15 to 25 cm, and then stayed constant at ~10 nM from 25 cm to the bottom of the profile (Fig. 18a). Both [Mo] profiles for the suboxic site decreased from the SWI to 5 cm depth; [Mo] concentrations went from ~15 nM at the surface to ~5 nM and remained constant at ~5 nM from the 5 cm depth to the bottom of the profile (Fig. 18b). Maximum values of [Mo] for the oxic site were found within 2.5-3.5 cm below the SWI, and coincided with maximum [Fe] (Fig. 18c). In contrast, maximum [Mo] and [Fe] for the suboxic site were present at the SWI (Fig. 18b, d). These Mo concentrations are in general five times higher than those measured in interstitial water from a suite of Canadian lakes (Chappaz et al., 2008a) and are also elevated above [Mo] in any of the source waters. Sulfide concentrations were undetectable in the overlying water of the oxic site and down to a depth of 3 cm; sulfide became measurable (~1 µM) between 3 cm and 50 cm (Fig. 18e). At the deepest site sulfide was measurable (~1 µM) even above the SWI (Fig. 18f) since dissolved oxygen was low (0.4 mg L⁻¹), consistent with suboxic conditions.

**Solid phases: sediment, soil and bedrock**—Sediment {Mo} contents were high relative to soil and bedrock samples and fairly constant with depth. Sediment {Mo} ranged from 7-36 ppm, consistent with previous measurements of 11.5-28 ppm Mo in a Castle Lake sediment core (Goldman, 1966). Mo/Al molar ratios ranged from 1-5 x 10⁻⁴ (Fig. 19a), nearly two orders of magnitude higher than
average Earth crust (6-19 x 10^{-6}; Taylor and McLennan, 1995) and the Castle Lake granodiorite bedrock (2 x 10^{-6}) that I measured. Throughout the core, \{Fe\} and \{Mn\} ranged from 1.7-3.2% and 367-790 ppm, respectively (Fig. 19b). \{N\} and \{C_{org}\} ranged from 0.5-1.5% and 6-16%, respectively (Fig. 19c). The range of \{C_{org}\} was similar to previous measurements (Carlton, 1984; Sanders et al., 2008) and displayed a sharp decline at ~25 cm where a volcanic ash layer was identified. Soil and granodiorite \{Mo\} was typically <700 ppb, much lower than the lake sediment, with one exception, wetland soil, where Mo was 2.2 ppm (Table 9).

_Sediment Mo isotopes_—The top several centimeters of the core contained significantly lighter Mo isotopic compositions (δ^{98/95}Mo values as light as -0.8 to -0.9 ± 0.1 ‰) than most of the sediments at greater depths in the core, which averaged -0.7 ± 0.1 ‰ (Fig. 19d). The heaviest δ^{98/95}Mo measured was -0.5 ± 0.1 ‰ at 11-12 cm, and corresponded to a \{Mo\} minimum as well as \{Fe\} and \{Mn\} maxima. The lightest δ^{98/95}Mo measured (40-41 cm) was -1.0 ± 0.1 ‰, and roughly corresponded to a local \{Mo\} maximum in the lower half of the core.

**Discussion**

Castle Lake sediments were elevated in Mo. They had Mo/Al ratios that were two orders of magnitude higher than average Earth crust, and Mo concentrations in the same range as those of sediments from British Columbian lakes, where elevated Mo is used as a geochemical indicator of porphyry Mo deposits (Cook, 2000). I considered five possible sources to explain the elevated
sediment Mo: (1) diagenetic processes; (2) dissolved watershed and groundwater sources; (3) two whole lake Mo addition experiments that occurred in 1963 and 1969; (4) smelting activities from the late 1800s-early 1900s; (5) particulate sources from atmospheric deposition and watershed inputs. I eliminated diagenetic processes as an important source of Mo to the sediments by obtaining net reaction rates of Mo in the porewater. I used those rates, along with physical and chemical sediment measurements, to model the amount of sediment Mo that could be accounted for by diagenesis. I ruled out dissolved Mo inputs from groundwater and watershed inflow as important sources of Mo, but calculated that dissolved Mo addition in the 1960s could have contributed a sizeable amount of Mo to the lake sediments. I calculated that the contribution of settling particles to the total flux of Mo into the sediments was significantly greater than the diffusive flux across the SWI. This finding suggests that the largest input of Mo to the sediments today is from deposition of particulate Mo, primarily from watershed sources rather than atmospheric deposition. Finally, I discuss how the Mo isotope data can be used to constrain the relative importance of Mo sources.

*Modeling of porewater Mo profiles*—In order to test whether diagenesis was an important contributor to elevated \( \{\text{Mo}\} \) in the sediments, I first had to obtain net reaction rates of Mo in porewaters. To estimate these rates, I used a reaction-transport model over the 0 to 10 cm depth range because many studies have shown that diagenetic processes are confined to sediments depths <10 cm (e.g. Gallon et al., 2004; Laforte et al., 2005). The following one-dimensional
mass conservation equation (Boudreau, 1997) was used to define the depth intervals (zones) where Mo is produced or consumed from porewater and to estimate the net reaction rate associated with each zone, assuming that the time scale of temporal changes of the [Mo] profiles is long relative to the Mo reaction rates (i.e., assuming steady state) and neglecting advective fluxes due to sediment compaction and groundwater flow:

\[
\left( \frac{\partial \phi \left[ Mo \right]}{\partial t} \right)_x = \frac{\partial}{\partial x} \left( \phi \left(D_s + D_B \right) \frac{\partial \left[ Mo \right]}{\partial x} \right) + \phi \alpha \left( \left[ Mo \right]_{\text{burrow}} - \left[ Mo \right] \right) + R_{\text{net}}^{Mo} = 0 \tag{1}
\]

In this equation: \( x \) is depth (cm; positive downward from the sediment water interface), \( \phi \) is sediment porosity, \( t \) is time (s), \( D_s \) is the effective diffusion coefficient (cm\(^2\) s\(^{-1}\)), \( D_B \) is the biodiffusion coefficient (cm\(^2\) s\(^{-1}\)), \( \left[ Mo \right]_{\text{burrow}} \) is the concentration of dissolved Mo in the burrows of benthic animals (mol cm\(^{-3}\) of porewater; which I assume to be identical to that in the water overlying the sediments), \( \left[ Mo \right] \) is the porewater Mo concentration (mol cm\(^{-3}\)), \( \alpha \) is the bioirrigation coefficient (s\(^{-1}\)), and \( R_{\text{net}}^{Mo} \) is the net rate (mol cm\(^{-3}\) of whole sediment s\(^{-1}\)) for porewater Mo production (\( R_{\text{net}}^{Mo} > 0 \)) or consumption (\( R_{\text{net}}^{Mo} < 0 \)). Although the steady-state assumption may not be strictly valid for an environment subject to variations in dissolved oxygen and temperature, it can be a useful guide to explore the parameters that cause either the sequestration or the mobilization of trace elements (Chappaz et al., 2008b, 2010; Gallon et al., 2004).

\( R_{\text{net}}^{Mo} \) was estimated by solving Eq. (1) using PROFILE (Berg et al., 1998) with average dissolved Mo profiles, and porosity values measured in a core.
adjacent to the suboxic profile, taken in 2008. I also assumed that $D_s = \phi^2 D_w$ (Berner, 1980), where $D_w$ is the tracer diffusion coefficient of MoO$_4^{2-}$ at 4°C ($5.2 \times 10^{-6}$ cm$^2$ s$^{-1}$) corrected for in situ temperature with the Stokes-Einstein equation (Li and Gregory, 1974). Thermodynamic calculations using MINEQL+ (Schecher and McAvoy, 1998) and the database from Chappaz et al. (2008a) predict that MoO$_4^{2-}$ is the dominant Mo species in porewater for both sites. For the oxic site, I used a biodiffusion coefficient $D_b$ of $2.6 \times 10^{-9}$ cm$^2$ s$^{-1}$. This value was based on the work of Brownstein et al. (unpublished data) who determined the most abundant benthic animal at Castle Lake at 10 m depth in mid-June 2008 was *Diptera chironomidae chironomidae* (1298 individuals m$^{-2}$), and on laboratory measurements of biodiffusion coefficients per individual for chironomids (Matisoff and Wang, 2000). The bioirrigation coefficient $\alpha$ for the oxic site was assumed to decrease linearly from $\alpha_0$ at the SWI to zero at 10 cm depth, since chironomids are generally not found below this depth (Matisoff and Wang, 1998). Eq. (2) was used to estimate $\alpha_0$ (Boudreau, 1984):

$$\alpha_0 = \frac{D_b r_i}{(r_2^2 - r_1^2)(r_a - r_i)}$$  \hspace{1cm} (2)

where $r_i$ is the radius of a chironomid’s tube (0.1 cm), $r_2$ is half the distance between adjacent tubes (1.5 cm), and $r_a$ is equal to $r_2/2$. Because seasonal anoxia in the bottom waters of the deepest site prevents the development of benthic communities (there were only 43 oligochaetes m$^{-2}$ at 30 m depth in mid-June
2008; Brownstein et al. unpublished data), I assumed negligible bioturbation and bioirrigation (i.e., $D_B = \alpha = 0$ in Eq. (1)) for the 35-m suboxic site.

Fitting of the porewater data by PROFILE (Fig. 20) is excellent ($r^2 = 0.99$). For the oxic site, I eliminated the data from the profile that displayed an anomalous Mo minimum at ~15 cm depth (Fig. 18a). The Mo minimum was likely the result of a chironomid burrow since [Mo] in that zone was the same as that of the overlying water (~4 nM). For the other oxic profile, PROFILE defined three distinct reaction zones (in terms of depth intervals): a zone just below the SWI where [Mo] was consumed, and two lower zones where [Mo] was produced, with the faster production zone overlying the slower one. The net reaction rate values $R_{\text{net}}^{\text{Mo}}$ are given in Table 10. Our results are similar to those of Chappaz et al. (2008a), and consistent with Mo adsorption onto Fe oxyhydroxides at the SWI (consumption zone) and release 1 cm below the SWI where Fe oxyhydroxides are dissolved (production zone). For the suboxic site, the model defined a production zone between 0 and ~1 cm depth overlying a slower consumption zone. These particular conditions would promote the dissolution of Fe oxyhydroxides (absence of dissolved oxygen) but prevent the formation of Fe sulfide (not enough sulfide), leading to a net export of [Mo] from the sediments.

Influence of diagenesis on sedimentary Mo—Diagenetic modeling of the porewater Mo profiles can provide information on Mo cycling and improve our understanding of Mo diagenesis. Assuming that sediment mixing is negligible in Castle Lake, the measured sediment Mo concentrations represent the sum of the
Mo concentration in the settling particles deposited at the SWI and that of the Mo added or removed from the solid phase by diagenetic reactions during sediment burial. To estimate the latter concentration, I used the following equation (Chappaz et al., 2008a) that relates the net removal or production rate of dissolved Mo to the net rate of Mo fixation to or release from the solid phase:

\[
R_{\text{net}}^{\text{Mo}} = \phi \left( \frac{d[\text{Mo}]}{dt} \right)_{\text{reaction}} = -m \left( \frac{d\{\text{Mo}\}}{dt} \right)_{\text{reaction}}
\]  

In this equation, \( m \) is the average dry bulk density (g cm\(^{-3} \)) of whole sediment) measured for an adjacent core taken immediately after our sediment core was sampled in 2008, and \( \{\text{Mo}\} \) is the solid phase Mo concentration (mol g\(^{-1} \)). The subscript “reaction” indicates reaction rates in solution and solid phase. From Eq. (3), it follows that:

\[
d\{\text{Mo}\} = -\frac{R_{\text{net}}^{\text{Mo}}}{m} \ dt = -\frac{R_{\text{net}}^{\text{Mo}}}{mv_S} \ dx
\]  

and

\[
\{\text{Mo}\}_{\text{diagenetic}} = -\int_{x=0}^{x=x_i} \frac{R_{\text{net}}^{\text{Mo}}}{mv_S} \ dx = -\sum_{x=0}^{x=x_i} \frac{R_{\text{net}}^{\text{Mo}}}{mv_S} \Delta x
\]  

where \( x_i \) is the depth of a sediment layer and \( v_s \) is the average sedimentation rate (0.23 cm yr\(^{-1} \)) given by Sanders et al. (2008). \( \{\text{Mo}\}_{\text{diagenetic}} \) can be viewed as the solid Mo concentration gained or lost by a layer of sediment during its burial. To obtain the Mo concentration at the time of sediment deposition (\( \{\text{Mo}\}_{\text{deposited}} \)), the values of \( \{\text{Mo}\}_{\text{diagenetic}} \) are subtracted from those of measured Mo (\( \{\text{Mo}\}_{\text{measured}} \)). Using an average of the \( R_{\text{net}}^{\text{Mo}} \) values (obtained with
PROFILE) for both sites to constrain our model with geochemical conditions occurring during a year for Castle Lake (seasonally anoxic), our calculations show that diagenesis results in a removal of a small amount of Mo (at most 3% of \(\{\text{Mo}\}_{\text{measured}}\)). This result indicates that the influence of diagenetic processes on the sedimentary record of Mo in Castle Lake was negligible and, if anything, resulted in a removal of Mo from the sediment instead of an addition to it, ruling out diagenetic sources of Mo.

*Groundwater and watershed dissolved Mo inputs*—The measured fluxes of dissolved Mo to and from Castle Lake were small. Surface inflow has an average [Mo] of 0.5 nM (Table 1) and an inflow rate of 1386 m\(^3\) d\(^{-1}\) (Goldman, 1961), yielding a Mo source of 0.25 mol Mo yr\(^{-1}\) \(\approx\) 0.25 nmol Mo cm\(^{-2}\) yr\(^{-1}\). Outflow has average [Mo] of 1.5 nM (Table 1) and an outflow rate of 800 m\(^3\) d\(^{-1}\) (Goldman, 1961), yielding an outflow loss term of 0.4 mol Mo yr\(^{-1}\) \(\approx\) 0.4 nmol Mo cm\(^{-2}\) yr\(^{-1}\). These fluxes are shown in box model for the Castle Lake Mo budget, given in Fig. 21. Assuming that our sediment porewater profiles are representative for the entire lake, groundwater contributions of [Mo] were negligible because the porewater profiles do not show any increase in concentration at depth (Fig. 18a,b), although I acknowledge there could be groundwater inputs to the lake at depths greater than those sampled by the peeper, or at other sites where samples were not taken. In contrast to other lakes where snow (Sugawara et al., 1961) and ice (Cowgill, 1977) contribute a significant source of Mo, snow and ice [Mo] concentrations were lower than lake water in Castle Lake, suggesting that about
half the ice pack is from snowfall and that snow is a negligible source of Mo to Castle Lake.

While modern dissolved fluxes of Mo are small, two significant historical inputs occurred in the 1960s. In July 1963 and again in June 1969, ~6 kg of Mo was added as sodium molybdate to the epilimnion of Castle Lake to stimulate primary production in the lake (Goldman, 1966; Goldman, 1972). These Mo additions increased dissolved Mo in the epilimnion from ~2-5 nM to 50-80 nM. From measurements of \{Mo\} concentrations in surface sediments made before and after the 1963 Mo addition, Goldman (1966) calculated that at least 10% of the added Mo or as much as 3.14 μg (32.7 nmol) Mo cm\(^{-2}\) of sediment was lost to sedimentation. Since net loss of Mo from the lake water stopped in 1965 (Goldman, 1966), the maximum Mo sedimentation rate in 1963 and 1964 would have been ~16 nmol Mo cm\(^{-2}\) yr\(^{-1}\). Although no published values exist, the Mo sedimentation rate after the 1969 addition was presumably similar to that after the 1963 addition. Assuming from geochronology presented in Sanders et al. (2008) that the volcanic ash layer at 25 cm marks the 1914-1917 volcanic eruptions of Mt. Lassen, and that sedimentation has been constant over the past ~100 years, 1963-1965 would correspond to 11-12 cm depth and 1969-1971 to 9.5-10.5 cm depth. It is possible that the steady increase in sediment \{Mo\} concentrations between 11.5 and 7.5 cm represents sedimentation of Mo added in the 1960s; in fact, the \{Mo\} concentration at 7.5 cm is the highest in the whole core. However,
high concentrations of \( \text{Mo} \) (>30 ppm) also occur at greater depths, suggesting there were other historical sources of Mo to Castle Lake.

**Fluxes of Mo**—The present-day accumulation of Mo in the sediments of Castle Lake for the oxic site \( J_{\text{acc}}^{\text{Mo}} \) is the sum of the fluxes of Mo deposited at the SWI with settling particles \( J_{\text{dep}}^{\text{Mo}} \) and those of dissolved Mo transported by molecular diffusion \( J_{D}^{\text{Mo}} \), bioirrigation \( J_{I}^{\text{Mo}} \) and bioturbation \( J_{B}^{\text{Mo}} \):

\[
J_{\text{acc}}^{\text{Mo}} = J_{\text{dep}}^{\text{Mo}} + J_{D}^{\text{Mo}} + J_{I}^{\text{Mo}} + J_{B}^{\text{Mo}} \quad (6)
\]

Due to the seasonal anoxia at the suboxic site, Eq. (6) can be reduced to:

\[
J_{\text{acc}}^{\text{Mo}} = J_{\text{dep}}^{\text{Mo}} + J_{D}^{\text{Mo}} \quad (7)
\]

An estimated value for \( J_{\text{dep}}^{\text{Mo}} \) \((3.8 \times 10^{-16} \text{ mol cm}^{-2} \text{ s}^{-1})\) was obtained by multiplying the average sediment accumulation rate \((0.04 \text{ g cm}^{-2} \text{ yr}^{-1} = 1.3 \times 10^{-9} \text{ g cm}^{-2} \text{ s}^{-1})\) determined by Sanders et al. (2008) for Castle Lake by the value of \( \text{Mo} \) measured at the topmost sediment layer \((3 \times 10^{-7} \text{ mol g}^{-1})\) since \( \text{Mo} \) diagenetic was negligible. I also calculated \( J_{\text{dep}}^{\text{Mo}} \) using the ash layer at 25 cm and the following equation:

\[
J_{\text{dep}}^{\text{Mo}} = \sum_{x=0}^{x=L_{i}} \phi(1 - \beta) \{\text{Mo}\} \Delta x \quad (8)
\]

where \( \beta \) is the water content of the sediment. This method produced a similar value \((3.5 \times 10^{-16} \text{ mol cm}^{-2} \text{ s}^{-1})\) as the value of \( J_{\text{dep}}^{\text{Mo}} \) produced by multiplying the sediment accumulation rate by \( \{\text{Mo}\}_{\text{measured}} \). These two \( J_{\text{dep}}^{\text{Mo}} \) values were averaged to give \( 3.7 (\pm 0.2) \times 10^{-16} \text{ mol Mo cm}^{-2} \text{ s}^{-1} \) and converted
into appropriate units for the box model, providing a sediment burial flux of 11.5 ± 0.7 nmol Mo cm\(^{-2}\) yr\(^{-1}\), far greater than the dissolved surface inflow flux of ~0.25 nmol Mo cm\(^{-2}\) yr\(^{-1}\) (Fig. 21) and comparable, but slightly lower, than the sediment burial rate for Mo in meromictic Lake Cadagno (16 ± 4 nmol Mo cm\(^{-2}\) yr\(^{-1}\); Dahl et al., 2010).

The values for \(J_D^{Mo}\) and \(J_I^{Mo}\) were provided as output of the PROFILE code (Table 10). The value for \(J_B^{Mo}\) was calculated using the following equation:

\[
J_B^{Mo} = \frac{D_S J_D^{Mo}}{D_W} \quad (9)
\]

The negative sign for \(J_D^{Mo}\) for both the oxic (-0.54 x 10\(^{-18}\) mol Mo cm\(^{-2}\) s\(^{-1}\)) and suboxic (-8.62 x 10\(^{-18}\) mol Mo cm\(^{-2}\) s\(^{-1}\)) sites indicates that sediments are a source of Mo to the lake (Table 10), and that they are a greater source when bottom waters are suboxic than oxic. This finding is consistent with observations made in seasonally anoxic lacustrine basins (Davison, 1981; Davison, 1982).

Converting \(J_D^{Mo}\) to the proper units for the box model’s sediment loss term, I obtain a value of 0.3 nmol Mo cm\(^{-2}\) yr\(^{-1}\), a small flux of the same magnitude as that coming in from dissolved surface inflow (Fig. 21). In sum, the contribution of \(J_D^{Mo}\), \(J_I^{Mo}\) and \(J_B^{Mo}\) to \(J_{acc}^{Mo}\) is minor. This result supports our previous finding that the contribution of \{Mo\}_\text{diagenetic} is negligible and suggests that the source of Mo to Castle Lake sediments today is largely derived from particulate Mo, either from atmospheric deposition or transport from the watershed.
Atmospheric and watershed particulate Mo inputs—Particulate Mo can be carried to lakes by deposition from the atmosphere or through transport of suspended material from the watershed. Atmospheric deposition of anthropogenic Mo can be significant when lakes are located in the wind path of fuel (coal and oil) combustion and/or base metal smelting (Chappaz et al., 2008a; Pacyna and Pacyna, 2001; Telmer et al., 2004). The atmospheric source has been found to be a major contributor of Mo to sediments in lakes in eastern Canada (Chappaz et al., 2008a), similar to the findings for Hg deposition in Castle Lake (Sanders et al., 2008). A historical source of atmospheric Mo deposition may have been extensive copper smelting that occurred south of Castle Lake from 1896 to 1919 around the Iron Mountain Richard Mine. This smelting resulted in vegetation kills due to the high metal content of the smelter smoke (Smith, 2010). The smelting likely contributed ~1 nmol Mo cm\(^{-2}\) yr\(^{-1}\) (Fig. 21), the maximum flux of atmospheric Mo in eastern Canadian lakes heavily affected by anthropogenically-sourced Mo from smelting over the 20\(^{th}\) century (Chappaz et al., 2008a). Integrated over the 23 years of intense smelting activity, it is possible that significant Mo was delivered to the Castle Lake system from smelter smoke. Assuming a total flux of ~23 nmol Mo cm\(^{-2}\) over a watershed area of 2 km\(^{2}\), smelter smoke could have deposited ~45 kg of Mo into the Castle Lake basin. If this Mo has been gradually weathered into the lake, it could explain why Mo has remained elevated in the sediment core throughout the last ~100 years and would account for the vast majority (~70%) of the sediment Mo. Alternatively, there could have been another source of Mo after
the closure of the last of the copper smelters in 1919. It is likely that population growth in Northern California over the remainder of the 20th and into the 21st century contributed to the high Mo concentrations in Castle Lake sediments due to Mo pollution as a result of coal and oil combustion (Pacyna and Pacyna, 2001). While atmospheric flux of Mo is unlikely to be a major source of particulate Mo to Castle Lake today, its accumulation in the early 20th centuries may have had long-lasting impacts on the Mo cycle in Castle Lake.

The unaccounted source of ~10 mol Mo yr⁻¹ is likely brought in largely by particulate Mo inputs in source waters (Fig. 21). Particles from the watershed can be associated with detrital (pre-existing weathered mineral grains) or non-detrital (smelting dust, organic matter and Fe-Mn oxyhydroxides) material. The Castle Lake watershed is underlain predominantly by ultramafic (peridotite) and mafic (gabbro) rocks, with some felsic (granodiorite) outcrops (Fig. 17). The sample of granodiorite I sampled had very low Mo (~200 ppb; Table 9), similar to average ultramafic rocks (Kuroda and Sandell, 1954; Turekian and Wedepohl, 1961). Average basaltic rocks typically contain the highest Mo concentrations of igneous rocks (~1.5 ppm; Kuroda and Sandell, 1954; Turekian and Wedepohl, 1961). Non-detrital Mo is likely present in concentrations of ~1-10 ppm Mo, which encompasses the Mo abundances in wetland organic soils (2.2 ppm; Table 9) and riverine particulate matter (0.5-7 ppm; Yigiterhan et al., 2008). It is possible that smelting dust elevated in Mo deposited in the early 20th century is also contributing to non-detrital particulate Mo sources in the watershed since Mo in
smelter smoke becomes less soluble with increasing distance from the smelter (Telmer et al., 2004). Assuming that particulate matter \(\{\text{Mo}\}\) concentrations are \(\sim1-10\) ppm, a suspended sediment load of \(\sim0.1-1 \times 10^9\) g yr\(^{-1}\) would be required to bring in \(\sim10\) mol Mo yr\(^{-1}\). This sediment load is within the range reported for similar subalpine watersheds, for example those draining the western slope of Lake Tahoe (Stubblefield et al., 2009). Suspended sediment loading to Castle Lake was not been measured due to logistical constraints associated with evaluating seasonal snowmelt characteristics. However, it is likely that spring snowmelt events provide the largest annual pulse of suspended sediment to Castle Lake, with some additional high concentration pulses in the summer, especially after thunderstorms when mineral soil is hydrophobic and water rushes through the organic layer.

*Mo isotopic constraints on Mo sources*—Mo isotopes may be useful for elucidating the sources of Mo to lake sediments. All of the Castle Lake sediment samples I measured had negative \(\delta^{98}\text{Mo}\) values, ranging from \(-0.5\) to \(-0.9\)‰. Since detrital Mo (as the mineral molybdenite) tends to be close to 0‰ or slightly positive (Hannah et al., 2007; Malinovsky et al., 2007), the isotope data suggest that detrital Mo is a minor contributor of Mo. Dissolved Mo in rivers also generally has positive \(\delta^{98}\text{Mo}\) values, ranging from \(~0\) to \(+2.4\)‰ (Archer and Vance, 2008; Pearce et al., 2010), so dissolved Mo is unlikely to be the dominant source of Mo to the Castle Lake sediments. Instead, the negative \(\delta^{98}\text{Mo}\) bulk sediment isotopic compositions likely reflect the major modern source of Mo to
Castle Lake, non-detrital particulate matter from watershed inputs. It is possible that the light Mo isotopic composition could be a result of the input of Mo particles from smelter smoke, if the molybdenites in the copper ores that were smelted in the early 1990’s were isotopically fractionated. Goldberg et al. (2009) reported preferential adsorption of light Mo isotopes to Fe oxyhydroxides, with fractionation factors ($\Delta^{98}\text{Mo}$) ranging from -0.8 to -2.2‰ depending on mineralogy. Mn oxyhydroxides are likely less abundant than Fe oxyhydroxides in Castle Lake, but those present will have an even stronger preference for light Mo isotopes, with $\Delta^{98}\text{Mo} \approx 3/2 \Delta^{97}\text{Mo} = -2.4$ to -2.9‰ between Mo absorbed to Mn oxyhydroxides and dissolved Mo (Barling and Anbar, 2004; Wasylenki et al., 2008). It is also likely that there is a fractionation of Mo isotopes during incorporation into lake organic matter, although it is probably of a smaller magnitude; Wasylenki et al. (2007) report a fractionation factor of $\Delta^{98}\text{Mo} \approx 3/2 \Delta^{97}\text{Mo} = -0.45$‰ and Zerkle et al. (2011) report fractionation factors of -0.2 to -1.0‰ (± 0.2‰) between microbial cells and growth medium. The trend towards lighter $\delta^{98}\text{Mo}$ values at the top of the core (Fig. 19d) likely reflects abundant Fe (and some Mn) oxyhydroxide particles, that are reduced upon burial. The sediment profile’s consistently negative $\delta^{98}\text{Mo}$ values suggest that most of the light Mo brought in on particles (Fe oxyhydroxides or smelter material) stays in the sediment in other host phases, such as organic matter, upon burial. In accordance with this hypothesis, Malinovsky et al. (2007) found that Mo isotopic ratios in sediment cores from a lake with high concentrations of suspended Fe and
Mn oxyhydroxide particles were also negative (bulk sediment $\delta^{98}\text{Mo} \approx 3/2 \delta^{97}\text{Mo} = -0.44$ to $-1.37\%$) and surmised that efficient removal of dissolved Mo from the water column by Fe-Mn oxyhydroxides was the primary fractionation mechanism of Mo. Finally, I did not observe an obvious isotopic excursion coinciding with the increased Mo sedimentation in the 1960s-1970s, although two of three lightest isotopic compositions I measured were contained in the core section between 8 and 12 cm. This suggests that the isotopic composition of the added sodium molybdate was similar to natural sources.

**Conclusions**

I found that the major modern source of Mo to Castle Lake was likely non-detrital particulate sources from the watershed. Minor sources of Mo to the lake included dissolved source inflow, particulate atmospheric deposition and release of Mo from the sediment. Groundwater and snow inputs are likely negligible. Historical sources of Mo include experimental additions of large quantities of dissolved sodium molybdate in the 1960s and atmospheric deposition of Mo due to metal smelting activities from 1896 to 1919. Mo sources are balanced by the major sink of Mo: sediment burial. Sediments are highly enriched in Mo compared to the watershed bedrock and average crustal Mo abundances. Porewater profiling indicates that diagenetic processes are not an important source of Mo to Castle Lake sediments. Instead, the sediment obtains most of its Mo from settling particles. The light Mo isotopic composition of the
sediment suggests that adsorption of Mo onto Fe–Mn oxyhydroxide particles and organic matter are important processes in the sequestration of Mo in the sediment.

This chapter is in preparation for the journal *Geochimica Cosmochimica Acta*. Coauthors include Anthony Chappaz, Brooke Eustis, Alan C. Heyvaert, David Waetjen, Hilairy E. Hartnett and Ariel D. Anbar. All co-authors have provided consent for inclusion of this paper in the dissertation (Appendix A).
Table 8

Mo concentrations of source waters to and outflow from Castle Lake.

<table>
<thead>
<tr>
<th>Sample description</th>
<th>Sample location</th>
<th>Date collected</th>
<th>Mo [nM] (σ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake ice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>28 Feb. 2009</td>
<td>1.01 (0.11)*</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>28 Feb. 2009</td>
<td>1.97 (0.35)*</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>4 Apr. 2009</td>
<td>0.44 (0.04)</td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td>28 Feb. 2009</td>
<td>2.01 (0.49)*</td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td>2 May 2009</td>
<td>0.48 (0.02)</td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td>3 May 2009</td>
<td>2.24 (0.05)</td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>4 Apr. 2009</td>
<td>0.56 (0.22)</td>
<td></td>
</tr>
<tr>
<td>Lake water under ice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>16 May 2009</td>
<td>0.07 (0.02)</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>4 Apr. 2009</td>
<td>0.72 (0.07)</td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>4 Apr. 2009</td>
<td>0.94 (0.14)</td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td>3 May 2009</td>
<td>0.35 (0.01)</td>
<td></td>
</tr>
<tr>
<td>Heart Lake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>16 July 2008</td>
<td>B.D.*</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>5 July 2009</td>
<td>0.02 (0.02)</td>
<td></td>
</tr>
<tr>
<td>Heart Lake outflow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HO</td>
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<td></td>
<td>11 August 2009</td>
<td>2.27 (0.07)</td>
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*aLocations are plotted on map in Fig. 16; *bCollected using a runoff collector (see text for description); *cCollected using a snowmelt collector; *below detection; *average and 1σ standard deviation of two duplicate samples run separately.
Table 9

Mo concentrations of bulk soils and bedrock in the Castle Lake watershed.

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<th>Sample description</th>
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<th>Mo [ppb] (σ)</th>
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<tr>
<td>Granodiorite</td>
<td>G</td>
<td>209 (9)</td>
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<tr>
<td>Heart Lake granodiorite mineral soil, beneath pines</td>
<td>HGM</td>
<td>462 (6)</td>
</tr>
<tr>
<td>Heart Lake granodiorite organic soil, beneath pines</td>
<td>HGO</td>
<td>192 (1)</td>
</tr>
<tr>
<td>Manzanita organic soil</td>
<td>MZO</td>
<td>338 (2)</td>
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<td>East forest organic soil</td>
<td>B1O</td>
<td>241 (8)</td>
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<td>Northwest forest organic soil</td>
<td>B2O</td>
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<td>Northwest forest organic soil</td>
<td>B3O</td>
<td>368 (7)</td>
</tr>
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<td>Alder area organic soil</td>
<td>B4O</td>
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</tr>
<tr>
<td>Organic soil, beneath huckleberry oak</td>
<td>B5O</td>
<td>640 (4)</td>
</tr>
<tr>
<td>East spring organic soil</td>
<td>M2O</td>
<td>594 (3)</td>
</tr>
<tr>
<td>Wetland organic soil</td>
<td>WO</td>
<td>2210 (9)</td>
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</table>

1Locations are plotted in Fig. 16
Table 10

Modeling results for Castle Lake: net rates of porewater Mo production or consumption as a function of depth in the sediments ($R_{net}^{Mo}$), as well as fluxes of Mo deposited with settling particles ($J_{dep}^{Mo}$) and fluxes of dissolved Mo across the SWI due to diffusion ($J_{D}^{Mo}$), bioirrigation ($J_{I}^{Mo}$) and biodiffusion ($J_{B}^{Mo}$).

<table>
<thead>
<tr>
<th>Sites</th>
<th>Zone</th>
<th>Depth interval</th>
<th>$R_{net}^{Mo}$</th>
<th>$J_{dep}^{Mo}$</th>
<th>$J_{D}^{Mo}$</th>
<th>$J_{I}^{Mo}$</th>
<th>$J_{B}^{Mo}$</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>cm</td>
<td>$10^{-15}$ mol cm$^{-2}$ s$^{-1}$</td>
<td>$10^{-16}$ mol cm$^{-2}$ s$^{-1}$</td>
<td>$10^{-18}$ mol cm$^{-2}$ s$^{-1}$</td>
<td></td>
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<tr>
<td>Oxic</td>
<td>(-) consumption</td>
<td>0 to 1.06</td>
<td>-1.66</td>
<td>N.D.</td>
<td>-0.54</td>
<td>-7.6</td>
<td>-2.71 x 10$^{-4}$</td>
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<td></td>
<td>(+) production</td>
<td>1.06 to 4.22</td>
<td>0.72</td>
<td>N.D.</td>
<td>-8.62</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>(+) production</td>
<td>4.22 to 9.50</td>
<td>0.06</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Suboxic</td>
<td>(+) production</td>
<td>0 to 1.06</td>
<td>1.77</td>
<td>3.6 ± 0.2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>(-) consumption</td>
<td>1.06 to 9.50</td>
<td>-0.11</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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</table>
Chapter Five Figure Captions

Figure 16. Map showing the location of Castle Lake in Northern California (inset), and the Castle Lake watershed with locations of source water collection sites, sediment porewater and solid phase (soil, bedrock and sediment core) sampling sites indicated.

Figure 17. Map showing the geologic units underlying the Castle Lake watershed. Oig: Ordovician heterogeneous intrusive gabbro; Olg: Ordovician layered gabbro; Ou: Ordovician cumulus ultramafic rock; Pcc: Permian diorite Castle Crags plug; Ph: Permian diorite dikes and plugs; Qu: Quaternary deposits; pOu: pre-Ordovician peridotite. Geology was mapped by Brooke Eustis.

Figure 18. Duplicate porewater profiles of dissolved Mo (a,b), Fe and Mn (c,d), sulfide and pH (e,f) for Castle Lake in July 2009 from oxic (shallow water) and suboxic (deep lake locations). The horizontal broken line indicates the sediment-water interface (SWI). Sulfide above the SWI in panel e was below detection.

Figure 19. Depth bulk solid phase concentration profiles of Mo and Mo:Al ratios (a), Fe and Mn (b), organic C and N (c) and stable Mo isotopic composition (d) in a sediment core collected in July 2008. The N and C<sub>org</sub> minima at 25cm depth correspond to a volcanic ash layer. Black circles were <sup>97-100</sup>Mo double-spiked. White circles were spiked with Zr.

Figure 20. Depth profiles of measured (open circles) and modeled (dotted gray line) porewater Mo concentrations; the black line represents the Mo net reaction rate (\( R_{net}^{Mo} \)) profile. Two porewater Mo profiles were averaged to model the
suboxic profile; only one porewater Mo profile was used for the oxic profile because the other profile showed evidence of significant bioturbation.

**Figure 21.** Generalized box model for modern Mo sources and sinks in the Castle Lake system in units of mol Mo yr\(^{-1}\) ≈ nmol Mo cm\(^{-2}\) yr\(^{-1}\). Explanation of each of the fluxes is given in the text. In 1963-1965 and 1969-1971(?), there was an additional Mo source from two sodium molybdate addition experiments (≤ 16 nmol cm\(^{-2}\) yr\(^{-1}\)).
Figure 17

Legend
- Geomorphological Events
- Cirque Basin Dikes
- Lake Inferred Fault
- Cirque Face Inferred Fault
- Inferred Fault

Geology Legend
- Olg
- Olg
- Ou
- Pcc
- Ph
- Qu
- pOu

Castle Lake, California

Limnology Lab

Castle Lake

Heart Lake

Meters

0 250 500 1,000

N S W E
Figure 18
Figure 21

Castle Lake water column [Mo] = 2-4 nM

- Atmospheric deposition <1
- Surface outflow 0.4
- Sediment escape 0.3
- Sediment burial 11.5 ± 0.7
- Average sediment (Mo) 23 ppm
- Surface source waters 0.3
- Particulates ~10 (Fe ox, org. matter, other?)
- Groundwater ~0

Diagram showing the flow of molybdenum in Castle Lake with various input and output pathways and their respective values.
Abstract

Molybdenum (Mo) is an essential trace element for all life, and is particularly important for the enzyme that catalyzes N\textsubscript{2} fixation, nitrogenase (Nif). Despite high Mo requirements for N\textsubscript{2} fixers, Mo concentrations are low (<5 nM) in most freshwaters. Low Mo may limit N\textsubscript{2} fixation by freshwater N\textsubscript{2} fixers, such as heterocystous cyanobacteria. In previous work, I found evidence that the heterocystous cyanobacterium Nostoc sp. PCC 7120 stores Mo when concentrations are >1000 nM in the growth medium. I hypothesized that this excess cellular Mo is bound to the small molybdate-binding protein Mop and expected that mop transcripts and protein levels would increase with increasing Mo concentration, in accordance with Mop’s role as a Mo storage protein. Here, I test this hypothesis by examining Mop transcription and protein levels in cultures of Nostoc sp. PCC 7120 grown at three medium Mo concentrations (1, 150 and 3000 nM) for 16 days. Transfers into fresh growth medium were performed every 3-5 days. On the fourth transfer, 3000 nM Mo medium was added to all bottles. Contrary to my prediction, I found that mop transcription was up-regulated after two transfers into 1 nM Mo medium, remained elevated for the third transfer, and then decreased upon addition of 3000 nM Mo on the fourth transfer. There was excellent correlation of mop and nif\textsubscript{D} gene expression at all three Mo concentrations.
concentrations, suggesting that these two genes are co-regulated. The mop gene is located on the distal end of the nif operon (but on the opposite strand as the nif genes) so its transcription could be under the same control as nif genes. Up-regulation of the nif operon under low-Mo may be an adaptation to scavenge cellular Mo by overproducing the NifD protein to which the Fe-Mo-cofactor binds. Mop protein levels also did not follow predicted the pattern: Mop was present after multiple transfers into 1 nM Mo medium, but occurred as a smaller band than in protein samples from 150 and 3000 nM Mo treatments, suggesting that Mop subunit composition changes as a function of medium Mo concentration. It is possible that when cellular Mo is scarce, the smaller Mop protein functions as a Mo transfer protein and donates Mo to enzymes such as NifD. When Mo is abundant in cells, the larger multimeric Mop protein likely functions as a storage protein. In sum, this study suggests that cellular regulation of Mo homeostasis in heterocystous cyanobacteria is more complex than previously expected due to transcriptional co-regulation of mop and nif genes and post-translational changes in Mop protein subunit composition as a function of cellular Mo content.

**Introduction**

Molybdenum (Mo) is an essential trace element for all life, and is particularly important for microbial acquisition of nitrogen in the forms of dinitrogen (N₂) gas and nitrate (NO₃⁻) due to its presence as a co-factor in the enzymes nitrogenase and nitrate reductase, respectively. While Mo is relatively
abundant (~105 nM) in the open ocean today (Collier, 1985), it is present in trace quantities (<5 nM) in most freshwaters and soils (Howarth et al., 1988). Low availability of Mo limits N₂ fixation in tropical forest soils (Barron et al., 2008) and NO₃⁻ assimilation in subalpine lakes (Chapter 4; Axler et al., 1980). Therefore, terrestrial and freshwater microorganisms that rely on Mo for N acquisition possess a variety of mechanisms to combat Mo limitation. Most bacteria encode high-affinity Mo uptake systems in order to selectively access Mo at low-nanomolar concentrations (Pau, 2004; Self et al., 2001; Zhang and Gladyshev, 2008). Some soil bacteria have been found to excrete Mo-chelating ligands (“molybdophores”) that solubilize Mo from minerals (Bellenger et al., 2008; Liermann et al., 2005). In addition, two types of prokaryotic Mo storage proteins have been discovered (Fenske et al., 2005; Pau, 2004). The first, “MoSto,” stores up to 90 atoms of Mo as an oxide mineral but is present in only a few strains of heterotrophic soil bacteria and purple non-sulfur bacteria (Fenske et al., 2005; Schemberg et al., 2008). The second Mo storage protein, Mop, binds only 8 atoms of Mo per hexamer (Schüttelkopf et al., 2002), but it is much more widespread in prokaryotes than MoSto. Therefore, Mop is likely the main microbial Mo storage mechanism for combating Mo limitation in freshwaters and soils.

Mop was first discovered in the anaerobic N₂-fixing bacterium *Clostridium pasteurianum* (Elliott and Mortenson, 1975, 1977). After purification and characterization, Mop was found to be a small protein of only 69 amino acids
Subsequently, crystal structures of Mop from diverse N₂-fixing anaerobic bacteria were solved and provided further support for the role of Mop as a Mo storage protein (Grunden and Shanmugam, 1997; Pau, 2004). However, there remains minimal information about Mo storage and regulation of Mop in the dominant N₂ fixers in aquatic ecosystems: heterocystous cyanobacteria. These bacteria perform N₂ fixation in specialized cells called “heterocysts,” that protect the extremely oxygen-sensitive nitrogenase enzyme complex from photosynthetically-produced O₂. Heterocystous cyanobacteria can survive for extended periods of time after Mo reserves are depleted in growth media, likely due to expression of both high-affinity molybdate uptake systems and Mop proteins (Chapter 3; Thiel et al., 2002; Zahalak et al., 2004). Previously, we observed extremely high intracellular Mo accumulation in the heterocystous cyanobacterium *Nostoc* sp. PCC 7120 grown on high Mo (1500 nM), but we do not know for certain if this accumulation was a consequence of Mo storage by Mop (Chapter 3). Proteomic analyses have shown that Mop is one of the ~100 most abundant proteins in *Nostoc* sp. PCC 7120 (Sazuka, 2003), lending support to an important role for this protein, likely in Mo storage.

In this study, I examined the effect of media and cellular Mo concentration on Mop expression at both transcriptional and translational levels in *Nostoc* sp. PCC 7120. I predicted that:
• Mop transcript and protein levels would be highly correlated since bacterial mRNA is translated into protein as soon as it is transcribed;
• Mop transcription and translation would be minimal at low Mo if *Nostoc* was using Mo for enzymatic activity, not storage;
• Mop transcription and translation would increase with Mo concentrations in the growth media, particularly above 1500 nM where I have previously observed luxury Mo storage (Chapter 3).

Instead, the results showed a different pattern: *mop* transcription was up-regulated at 1 nM Mo compared to higher concentrations, but the Mop protein present at 1 nM Mo folded into dimers instead of larger multimers at higher concentrations (150 and 3000 nM). These results reveal a more complex regulation of Mop in *Nostoc* than previously predicted.

**Methods**

Culture experiments with N$_2$-fixing *Nostoc* sp. PCC 7120 were performed at three Mo concentrations: low (<1 nM), medium (~150 nM) and high (~3000 nM). Cultures were transferred into fresh media with the same concentration of Mo as the starting media every 3-5 days for a total of four transfers (16 days). Three transfers were made in order to acclimate the cyanobacteria to each of the three Mo concentrations and to force cyanobacteria in the low-Mo treatments to deplete their cellular Mo reserves. On the fourth transfer, high Mo media (~3000 nM) was added to all bottles to measure how low- and medium-Mo treatments responded to high-Mo addition. Samples were taken for chlorophyll *a* (Chl *a*),
cellular Mo content, RNA (for mop and nifD transcript measurements) and protein (for immunoblotting with Mop antibodies).

*Culture conditions and experimental set-up—Nostoc sp. PCC 7120 was grown axenically in HCl-washed sterile 500-mL polycarbonate bottles under constant light (150 μmol m⁻² s⁻¹ irradiance) at 25°C for 16 days. Cultures were grown in modified BG-11 media (Allen and Arnon, 1955), lacking NH₄⁺ but with added Ni (170 nM), V (200 nM) and Mo at three concentrations (1, 150, and 3000 nM). The experiments were run in triplicate. Inductively coupled plasma mass spectrometry (ICP-MS) measurements of fresh media and supernatant sampled at the end of each transfer period confirmed that the Mo concentrations were within range of expected values (<1 nM = 0.6-0.9 nM, ~150 nM = 117-161 nM, and ~3000 nM = 2328-3833 nM). At the beginning of the experiment (transfer 0), a consistent volume of stock culture grown in modified BG-11 media with 3000 nM Mo was pelleted at 7,400 rpm for 20 mins, washed three times with media of the proper Mo concentration for the experiment (<1, ~150 or ~3000 nM) and transferred into bottles filled with 300 mL of each type of media. Cultures were continuously bubbled with room air sterilized by filtration through 0.2-μm Supor® membrane syringe filters (Pall). Cultures were diluted to optical clarity every 3-5 days (transfers 1, 2 and 3) with media from the same batch used to initiate the experiment. On the 4th transfer, all bottles were filled with media of the highest Mo concentration (~3000 nM). Samples were taken daily to quantify Chl a, while all other types of samples were taken just before dilution (i.e.,
transfer-1 samples were taken on day 3, transfer-2 samples were taken on day 7, transfer-3 samples were taken on day 11 and transfer-4 samples were taken on day 16).

**Growth rates**—Growth rates were calculated from Chl a concentrations. Cells were pelleted from 2 mL culture samples and methanol was used to extract pigments. Chl a was measured at a wavelength of 665 nm on a Beckman Coulter DU 730 UV/Vis spectrophotometer, using the equation: Chl a [µg mL⁻¹] = 13.42 x Abs⁶⁶⁵ nm (Porra, 2002). The instantaneous growth rate (µ, d⁻¹) was calculated at the end of each transfer period using the equation µ = (lnNᵣ - lnN₀) t⁻¹, where N₀ is the original Chl a concentration at the zero time point and Nᵣ is the Chl a concentration after a given amount of time after transferring (t, in days).

**Cellular Mo**—Between 50 to 100-mL were sampled from each culture at the end of each transfer period and centrifuged at 7,400 RPM for 20 min in 50-mL centrifuge tubes. Washing and digestion protocols were the same as in a previous study (Chapter 3).

**RNA extraction**—At the end of each transfer period, 25 mL of culture were centrifuged in 50-mL centrifuge tubes at 7,400 RPM for 20 min. The supernatant was poured off and RNA was extracted from the cell pellet using the FastRNA Pro Blue kit according to the protocol of the manufacturer (Qbiogene, Irvine, CA). RNA pellets were resuspended in 100 µL of nuclease-free water (Ambion, Austin, TX). Resuspended RNA was checked for integrity by visualization of ribosomal bands on an ethidium bromide-stained agarose gel and quantified by
the ratio of absorbance at 260 vs. 280 nm on a Nanodrop (Thermo Fischer, Wilmington, DE). RNA samples were treated with RQ1 RNase-free DNase according to the protocol of the manufacturer (Promega, Madison, WI). Samples were then ethanol precipitated, resuspended in 38 µL of nuclease-water and reexamined for degradation as described above. A portion of the RNA was used in cDNA synthesis. The remainder was stored at -20°C and used later as the non-reverse-transcribed control for quantitative PCR (qPCR).

**cDNA synthesis**—RNA was converted to single-strand cDNA using 2 pmol of either *mop* or *nifD* forward and reverse primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocol at an extension temperature of 55°C for 60 min. Commercially manufactured primers (Integrated DNA Technologies, Coralville, IA) were resuspended to 5µM and tested on *Nostoc* sp. PCC 7120 genomic DNA (gDNA) for specificity and efficiency before use with cDNA. The *mop* primer sequences were: 5’-ATGGAAGTTAGCGACGTAATTTTC-3’ (forward) and 5’-ATCAACAGCAACTATCATC ATCTGAGGA-3’ (reverse). The *nif* primer sequences were: 5’–CGGTTACTGTGCTTGGTCTGGTC–3’ (forward) and 5’–GCGTCGTTAGCGATGTGGTGTC–3’ (reverse). The 20 µL reaction was diluted 1:5 with sterile H₂O and stored at −20°C for later analysis by qPCR.

**Quantitative PCR**—Quantitative PCR of cDNA was performed in 25 µL reactions according to the manufacturer's protocol, using 2x Brilliant® SYBR Green (Stratagene, La Jolla, CA, USA) master mix and the same primers as listed
above. Quantitative PCR was also performed on non-reverse-transcribed RNA to check for carryover of gDNA. A calibration curve was prepared over a concentration range of $10^{-6}$ to 1 ng using a plasmid (pBAD-TOPO; Invitrogen, Carlsbad, CA) containing the same DNA sequence amplified by the mop or nifD primers. The fluorescence of the accumulated product was measured at each extension step on a Mx3005P thermal cycler (Stratagene, La Jolla, CA), using the following PCR parameters: initial denaturation at 95°C for 10 min; 40 cycles consisting of 95°C for 30 s; 53°C for 1 min; and 72°C for 1 min. A melting curve was generated over the temperature range 55-95°C following each qPCR run. The melting curves consistently displayed only one peak, suggesting that only the segment of the gene of interest was amplified during the qPCR reaction. After log linearization of fluorescence versus cycle number curves and background subtraction of fluorescence, $C_t$ values were obtained at a specific fluorescence level. These $C_t$ values were compared to the calibration curve to quantify expression of nifD and mop. Data analysis was performed with MxPro software (Stratagene, La Jolla, CA).

*Mop antibody production and immunoblotting*—Polyclonal IgY antibodies against the Mop protein from Nostoc sp. PCC 7120 were raised in chicken and purified by antigen-affinity chromatography by GenWay Biotech Inc. (San Diego, CA). At the end of each transfer period in the experiment, samples for protein analysis were sampled, stored, extracted and quantified using the same method described in Chapter 3. Immunoblotting was performed on extracted sample
proteins separated by gel electrophoresis on 16% Tricine gels (NuSep iGels). The gel was transferred to a polyvinylidene fluoride membrane and blotted with the antibodies against Mop conjugated to IgY HRP (GAYFC-HRP, Genway, San Diego, CA). Western visualization was performed with SuperSignal chemiluminescent substrate (Pierce) on a bioimager (UVP, San Diego, CA).

**Results**

*Identification of Mop storage protein*—A search of the previously published genome of *Nostoc* sp. PCC 7120 (Kaneko et al., 2001) revealed a single copy of a gene encoding a 69 amino acid protein sequence homologous to known Mop proteins of the same size from other bacteria (Fig. 22). The protein from *Nostoc* sp. PCC 7120 exhibited 38-44% amino acid sequence identity and 75-79% amino acid sequence similarity to other characterized Mop proteins from *Clostridium pasteurianum, Hemophilus influenzae* and *Sporomusa ovata*. In its crystallized state, Mop is a hexamer that binds 8 molecules of molybdate (MoO$_4^{2-}$) (Duhme et al., 1999). There are two different types of molybdate-binding sites in each Mop hexamer: two higher-affinity Type 1 sites that each have 12 hydrogen bonds between molybdate and the protein, and six lower-affinity Type 2 sites that each have 8 hydrogen bonds between molybdate and the protein (Schüttelkopf et al., 2002; Wagner et al., 2000). Mop relinquishes Mo from its binding sites when it is required for incorporation into Mo-requiring proteins like nitrogenase (Elliott and Mortenson, 1975; Hinton and Mortenson, 1985a, b).
Alignment of the protein sequence of Mop from *Nostoc* sp. PCC 7120 with sequences derived from solved crystal structures of Mop from *Clostridium pasteurianum* (Schüttelkopf et al., 2002) and *Sporomusa ovata* (Wagner et al., 2000) revealed that amino acid residues that act as ligands to molybdate ions in Type 2 sites are highly conserved (Schüttelkopf et al., 2002), particularly the residues Ser\(^4\), Arg\(^6\), Ser\(^{43}\) and Lys\(^{60}\) (Fig. 22). There was slightly more variability in the other Type 2 binding residues 40 and 61. These are both serines in *C. pasteurianum* but are threonines in *S. ovata* and *Nostoc* sp. PCC 7120 for residue 40 and are alanines for *S. ovata* and *H. influenzae* for residue 61. Amino acids that serve as ligands for Type 1 binding sites (Val\(^{20}\), Val\(^{21}\) and Thr\(^{22}\) using the residue notation from *C. pasteurianum*) are much less conserved than Type 2 binding sites, particularly for residues 20 and 22 (Fig. 22).

*Location of mop in genome*—Searches of *mop* genes in sequenced microbial genomes using the Integrated Microbial Genomes website (Markowitz et al., 2010; http://img.jgi.doe.gov) reveal in most microbes that contain the *mop* gene, it is located near high-affinity (ModABC) molybdate uptake systems. However, in freshwater diazotrophic cyanobacteria (including the heterocystous strains *Nostoc* sp. PCC 7120, *Anabaena variabilis* sp. ATCC 29413, *Nostoc punctiforme* sp. PCC 73012, and the non-heterocystous strains of *Synechococcus* sp. OSA and OSB isolated from Octopus Spring, Yellowstone National Park), *mop* is located at the distal end of the *nif* operon in the opposite orientation to the rest of the *nif* genes (Böhme, 1998). This is also the case for other diazotrophs.
with sequenced genomes, such as *Bradyrhizobium* spp., *Clostridium kluyveri*, *Methanosarcina acetivorans*, *Methanosarcina barkeri fusaro*, *Methylobacterium* sp. 4-46, *Rhodopseudomonas palustris* and *Zymomonas mobilis* subsp. *mobilis*.

**Response of growth rates and cellular Mo to medium Mo concentration**—
In the experiment with *Nostoc* sp. PCC 7120 grown at 1, 150 and 3000 nM Mo, growth rates and cellular Mo concentrations varied based on Mo levels in the growth media. Both parameters declined in the 1 nM Mo treatments after each transfer into fresh growth media (transfers 1-3; Fig 23a,b). Growth rates stayed fairly constant for the 150 and 3000 nM Mo treatments from transfer 1 to 3 (0.5-0.8 d⁻¹; Fig. 23a). Cellular Mo dropped slightly (19±6 to 9±1 ppm) from transfer 1 to 2 for the 150 nM Mo treatments, but remained constant from transfer 2 to 3. The opposite trend occurred for the 3000 nM treatments: cellular Mo increased from transfer 1 to 2 (26±15 to 67±27 ppm) and then remained constant from transfer 2 to 3 (Fig. 23b). After transfer 4 into 3000 nM Mo media, growth rates for the 1 nM Mo treatments increased significantly (<0.2 to 0.5-0.8 d⁻¹), as did cellular Mo (2±1 to 44±7 ppm; Fig 23a, b). The 150 nM Mo treatments showed less dramatic increases, whereas the 3000 nM Mo treatments exhibited a slight decrease in growth rate and cellular Mo after transfer 4 (Fig. 23a, b).

**Gene expression**—Expression of *mop* and *nifD* genes was up-regulated into the low-Mo (1 nM) treatment compared the 150 and 3000 nM treatments. Expression of *mop* was not statistically different in any of three Mo treatments after transfer 1 (P = 0.1). After transfer 2, *mop* gene expression became
significantly elevated in the 1 nM treatment compared to the two higher Mo treatments (150 and 3000 nM; P < 0.001) and stayed elevated through transfer 3 (P < 0.001). Gene expression of *mop* dropped back to basal levels after transfer 4 (Fig 24a). Expression of *mop* did not differ significantly between the 150 and 3000 nM Mo treatments, nor was there significant variation of gene expression for these two treatments throughout the experiment (Fig. 24a). The results for *nifD* gene expression were more variable between bottles for each treatment (Fig. 24b), however the same general trend held: elevated *nifD* expression in the 1 nM Mo treatment, particularly by transfer 3 (P < 0.001), with a return to basal levels after 3000 nM was added in transfer 4 (Fig 24b). Gene expression of *mop* and *nifD* showed a near 1:1 correlation (Fig. 25).

*Mop protein expression*—Mop was not observed as a single subunit of 7 kDa in any of the experimental samples despite the use of denaturing conditions (sodium dodecyl sulfate addition and boiling) during protein extraction from cells pelleted after each transfer and during gel electrophoresis. Instead, Mop was most often present as 1-2 bands at a higher molecular weight (~37 and 50 kDa), likely as multimers of Mop subunits (Fig. 26). I verified that these multiple banding patterns were not caused by unspecific binding of the anti-Mop antibodies to proteins other than Mop by testing negative controls of protein extracts from other cyanobacteria that lack the *mop* gene (*Synechocystis* PCC 6803 and *Thermosynechococcus elongatus*). These tests showed minimal binding to the antibody (Fig. 27). I also verified that the Mop antibody was not binding to the
transcriptional regulator ModE, the only other protein in *Nostoc* sp. PCC 7120 with significant sequence similarity to Mop, by demonstrating that the banding patterns of protein extracts of a mutant –*modE* strain of *Anabaena variabilis* ATCC 29413 were very similar to those of *Nostoc* sp. PCC 7120 (Fig. 27). Although these negative controls provide good evidence of the binding specificity of the Mop antibody, protein sequencing of the bands is the definitive final step to prove that they are all in fact 100% Mop protein.

Marked shifts in the relative intensity and size distribution of immunoblot bands occurred between different treatments and transfers, reflecting changes in the Mop protein expression level and subunit composition, respectively. For example, minimal Mop protein (i.e. faint bands) was present in the 1 nM Mo treatment after transfer 1, but at transfer 2, there were clearly-defined protein bands at ~50, ~37 and 10-15 kDa (Fig. 26). Since Mop is 69 amino acids (7 kDa), these bands are likely actually 7 kDa x 7 (49 kDa), 7 kDa x 5 (35 kDa) and 7 kDa x 2 (14 kDa). Although Mop has been crystallized in hexameric form (Schüttelkopf et al., 2002; Wagner et al., 2000), a 7 kDa x 6 (42 kDa) band was not observed these immunoblots. The ~50 and ~37 kDa bands disappeared after transfer 3, but the smaller band at 10-15 kDa remained. In 3000 nM Mo treatments, Mop was present as a single band at ~37 kDa after transfer 1, whereas minimal protein was present in transfers 2 and 3. Protein expression levels and subunit composition did not change significantly for the 150 nM Mo treatments, although there was more variability between triplicate bottles in the intermediate
Mo concentration treatment than the other treatments. After 3000 nM Mo was added to all of the bottles in transfer 4, the banding pattern became consistent in all bottles with two clearly-defined bands: one at ~37 kDa and the other at ~50 kDa (Fig. 26).

**Discussion**

Regulation of Mo homeostasis in heterocystous cyanobacteria is more complex than expected, likely due to transcriptional co-regulation of mop and nif genes and to post-translational changes in subunit composition of Mop protein. Below I discuss how my findings lead to this conclusion and the major implications of this study, starting with the physiological response of heterocystous cyanobacteria to Mo limitation and excess, and then moving to a new model for regulation of mop and nif genes in heterocystous cyanobacteria.

_Mo requirements for nitrogen fixation: influence on growth rate and cellular Mo_—In Chapter 3, I showed that at cellular Mo concentrations <2 µg Mo g⁻¹ dry biomass (equivalent to 1 µmol Mo mol⁻¹ C), heterocystous cyanobacteria did not measurably fix nitrogen. Therefore, I attribute the drop in growth rate after transfers 2 and 3 in the 1 nM Mo treatments to limitation of nitrogen fixation by scarcity of cellular Mo. This interpretation is supported by the observation that growth rates and cellular Mo concentration both returned to maximal levels after 3000 nM Mo media was added to these low-Mo cultures before transfer 4 (Fig. 23a,b). Of particular interest is the extremely high Mo accumulation (215 µg Mo g⁻¹ biomass, over 2x any of the other samples) after transfer 4, when 3000 nM Mo
media was added, in one of the bottles previously grown at 1 nM Mo. This particular culture was the only one that fell below the threshold cellular Mo concentration of 2 µg Mo g^{-1} biomass established in Chapter 3 after transfer 3, so its nitrogen fixation activity was likely impaired by insufficient Mo although nitrogen fixation rates were not measured. A hypothesis resulting from this result is that severely Mo-limited cultures (< 2 µg Mo g^{-1} biomass) have higher capacity than moderately Mo-limited cultures (2-5 µg Mo g^{-1} biomass) for luxury Mo accumulation after high concentrations of Mo are added back, but this remains to be thoroughly tested in future experiments.

Luxury Mo accumulation in the 3000 nM Mo treatments in this study was not as elevated as the 1500 nM Mo treatments in Chapter 3. The highest cellular Mo in the 3000 nM Mo treatments was 100 µg Mo g^{-1} biomass, whereas the highest Mo concentration in the 1500 nM Mo treatments was 485 µg Mo g^{-1} biomass. Cellular Mo in the 3000 nM Mo treatments actually declined after the 4^{th} transfer, suggesting that long-term exposure to 3000 nM Mo may be detrimental to growth of the organism, since growth rates were lower after the 4^{th} transfer into 3000 nM Mo media than in previous transfers. This theory is supported by the previous study that showed a decline in nitrogenase expression and activity between media Mo concentrations of 100 and 1500 nM (Chapter 3), but the threshold above which Mo becomes toxic to cyanobacteria remains unknown.

Influence of Mo concentration on transcriptional regulation of mop—
Comparison of our findings to previous studies on the influence of Mo
concentration on mop transcriptional expression revealed key differences between species. In both C. pasteurianum and E. acidaminophilum, low Mo concentration does not suppress transcription of the mop gene (Hinton and Mortenson, 1985b; Makdessi et al., 2004). In C. pasteurianum, this finding was attributed to production of Mop in apoprotein form (lacking Mo) when Mo was low in the growth medium, followed by Mo binding to the protein when Mo concentrations rose above 1000 nM Mo (Hinton and Mortenson, 1985b). However, there is no data available to compare transcription at low vs. high Mo concentrations in C. pasteurianum and E. acidaminophilum. Such a study has been performed for the purple non-sulfur anoxygenic photosynthetic bacterium Rhodobacter capsulatus. In this species, mop transcription was over ten-fold higher when Mo concentrations were 10 µM vs. when no Mo was added (Wiethaus et al., 2006).

Our study of Nostoc sp. PCC 7120 showed the exact opposite response: we found that mop transcription was >2-fold higher when Mo was low in the growth media (<1 nM) compared to higher Mo concentrations (~150 and ~3000 nM).

This inverse response may be due to co-regulation of mop and nifD transcription in Nostoc sp. PCC 7120, as implied by the close correlation between mop and nifD transcript levels (Fig. 25). Up-regulation of mop transcription at the onset of Mo limitation may be primarily driven by the cellular response to elevate expression of the entire nif operon to compensate for declining nitrogenase activity, as supported by previous observations of elevated NifDK and NifH protein expression after short-term Mo-limitation in Nostoc sp. PCC 7120 (Glass
et al., 2007). Co-regulation likely occurs because mop is located at the distal end of the nif operon in this species and may be under the control of the same transcriptional regulators, such as the global nitrogen regulator NtcA, which binds to a promoter sequence GTA (N8) TAC upstream of the nif operon (Herrero et al., 2001). I identified the NtcA promoter sequence upstream of mop gene, lending additional support to the theory that mop and nifD are both regulated by nitrogen availability in this species.

Species-specific differences in metal concentration regulation of storage protein expression have also been observed for other systems. For example, like our findings for Mo limitation and mop transcripts, low Fe concentration results in an increase of the number of transcripts of the Fe-storage protein ferritin (Fer2) in the green alga Chlamydomonas reinhardtii (Long et al., 2008), whereas transcription of ferritins in marine pennate diatoms are down-regulated at low Fe (Marchetti et al., 2009). Since there is so much variation in the transcriptional regulation of even the same metal-storage protein between species, it is not possible to assume a priori that all species regulate metal storage in the same manner.

*Influence of Mo concentration on Mop protein expression*—The finding that Mop protein was present as numerous bands in denaturing conditions and never as a single 7 kDa subunit suggests that Mop is a very stable protein that refolds in SDS buffer after boiling (although autoclaving results in complete disintegration of the protein; data not shown). Since denaturing conditions were used in gel
electrophoresis, the distribution of observed protein bands in immunoblots is almost surely different than in native conditions within cell. Nonetheless, differences in protein banding distribution can be compared between transfers and treatments by assuming that the way in which the Mop protein refolds after boiling reflects its folding preferences within the cell.

Changes in the intensity of immunoblot protein bands at the lowest Mo concentration (<1 nM) scale with copies of mop transcripts, whereas changes in the size distribution of protein bands likely reflect post-translational changes to Mop folding. After transfer 1, Mop protein expression conformed with the expectation that minimal protein would be present in cells grown without added Mo (<1 nM Mo blank). However, after transfer 2, Mop protein was observed in three distinct bands in the <1 nM Mo treatment. This increase in Mop protein expression from transfer 1 to 2 reflects a similar up-regulation in copies of mop transcripts. After transfer 3, Mop protein was still strongly expressed, but only present as a single band between 10-15 kDa. The differences in Mop protein sizes between transfers 2 and 3 may reflect post-translational changes to Mop’s folding structure. It is likely that the dimeric structure has diminished ability to store molybdate, and instead functions as a transfer protein to Mo-enzymes like NifD.

After transfer 4, protein was again present as two larger bands (~37 and ~50 kDa), suggesting that add-back of 3000 nM to the media resulted in restoration of Mop’s Mo storage capacity.
Conclusions and Future Directions

This study revealed a more complex regulatory scheme for Mo homeostasis in heterocystous cyanobacterium than predicted. Instead of being down-regulated at low Mo, mop gene expression was >2-fold higher when Mo was low in the growth media (<1 nM) compared to higher Mo concentrations (~150 and ~3000 nM). The same was true for expression of the nifD gene, the gene that encodes the nitrogenase subunit that binds the Mo-Fe-cofactor. It is likely that mop and nifD transcription are co-regulated in Nostoc sp. PCC 7120. Smaller subunit composition of Mop at low Mo may indicate a different function for this protein; when it is not needed for a storage role, perhaps Mop is used as a Mo-transfer protein. Future studies will be needed to test whether the smaller Mop protein that is present in low-Mo cells interacts with Mo-containing enzymes like NifD. Furthermore, the percentage of Mo in the cell that is bound to Mop remains to be quantified.

Beyond the scope of this study, but an important area for future investigation, is the influence of nitrogen source on Mo storage. The experiments reported here were all performed under N₂-fixing conditions, but it is likely that Mop expression is regulated by the chemical form of N that is available due to the higher Mo requirements for N₂ fixation vs. growth on other N sources such as NH₄⁺ and NO₃⁻ (Chapter 2; Chapter 3; Raven, 1988). In support of this prediction, mop (alr1428) gene expression is up-regulated in Nostoc sp. PCC 7120 when grown under N₂-fixing conditions compared to growth on NH₄⁺ and NO₃⁻ (Ehira
et al., 2003). Furthermore, \textit{mop} mutants (strain BMB92) of \textit{Nostoc} sp. PCC 7120 grown on NO$_3^-$ have similar growth rates to wild-type cells, whereas the same mutants grown diazotrophically have decreased growth rates and nitrogenase activity (Masepohl et al., 1997). Similarly, when NH$_4^+$ was added to the original $^{99}$Mo-labeling experiments in \textit{C. pasteurianum}, $^{99}$Mo became completely exchangeable, suggesting that Mop was not produced by the cells grown on NH$_4^+$ (Elliott and Mortenson, 1976). This finding is consistent with the recent studies of \textit{R. capsulatus}, for which transcription of \textit{mop} is almost twice as high in N$_2$-fixing vs. NH$_4^+$-grown cultures (Wiethaus et al., 2006). In contrast, MoSto is constitutively expressed regardless of whether \textit{Azotobacter vinelandii} is grown on NH$_4^+$ or fixing N$_2$ (Fenske et al., 2005). Future studies will be required to resolve how Mop protein expression is affected by changes in N source in heterocystous cyanobacteria.

This study has elucidated mechanisms behind the maintenance of Mo homeostasis in a model cyanobacterium isolated from a freshwater environment where Mo is likely scarce. Since marine cyanobacteria lack \textit{mop} genes (Chapter 3), it is possible that they possess a different pathway for maintaining Mo homeostasis or have lost/never acquired such a pathway because they live in seawater where Mo is not likely a limiting micronutrient.

This chapter is in preparation for the journal \textit{Applied and Environmental Microbiology}. Coauthors include Felisa Wolfe-Simon, Amisha Poret-Peterson,
and Ariel D. Anbar. All co-authors have provided consent for inclusion of this paper in the dissertation (Appendix A).
Chapter Six Figure Captions

**Figure 22.** Alignment of bacterial Mop protein sequences from organisms with solved protein structures (*Clostridium pasteurianum*, *Hemophilus influenzae*, *Sporomusa ovata*) and *Nostoc* sp. PCC 7120 aligned with Multalin (http://prodes.toulouse.inra.fr/multalin/multalin.html). Figure adapted from (Schüttelkopf et al., 2002). Amino acids highlighted in grey are identical between *Nostoc* and two of the other Mop protein sequences. The number “1” indicates amino acids involved in Type-1 molybdate binding and the number “2” indicates amino acids involved in Type-2 molybdate binding (see text for details).

**Figure 23.** *Nostoc* sp. PCC 7120 growth rate (A) and cellular Mo (B) plotted for four transfers and three Mo concentrations. Vertical arrows indicate the addition of 3000 nM to all treatment bottles.

**Figure 24.** *Nostoc* sp. PCC 7120 mop (A) and nifD (B) gene expression (gene copies normalized to total RNA) plotted for four transfers and three Mo concentrations. Vertical arrows indicate the addition of 3000 nM to all treatment bottles.

**Figure 25.** Transcript levels of mop vs. nifD display high correlation ($R^2 = 0.62$), suggesting that transcription of these two genes is co-regulated. Line is best fit to the data.

**Figure 26.** Mop immunoblots of protein extracts for three Mo concentration treatments in triplicate and four transfers. Between transfers 3 and 4, 3000 nM Mo
was added to all bottles. All lanes loaded with 10 µg protein (see text for protein quantification methods).

**Figure 27.** Negative control (*Synechocystis* PCC 6803 and *Thermosynechococcus elongatus*) and positive control (Mop purified protein supplied by GenWay, wild-type *Nostoc* sp. PCC 7120 and –modE *Anabaena variabilis* ATCC 29413) Mop immunoblots used to check for antibody binding specificity.
Figure 23

A

Growth rate (d^{-1})

- 1 nM Mo
- 150 nM Mo
- 3000 nM Mo

B

Cellular Mo

(µg g^{-1} dry biomass)

1  2  3  4

Transfer #
Figure 24

(A) mop gene expression (copies mop ng⁻¹ total RNA)

(B) nifD gene expression (copies nifD ng⁻¹ total RNA)

Legend:
- ☀ 1 nM Mo
- ● 150 nM Mo
- ● 3000 nM Mo
Figure 25

The figure shows a scatter plot correlating nifD gene expression (copies nifD ng\(^{-1}\) total RNA) with mop gene expression (copies mop ng\(^{-1}\) total RNA). The linear regression equation is given by:

\[ y = 0.88x - 4.4 \times 10^4 \]

The R\(^2\) value is 0.62, indicating a moderate correlation between the two gene expressions.
Figure 26

Mo: 1 nM  150 nM  3000 nM

Transfer 1

Transfer 2

Transfer 3

Transfer 4
Figure 27

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mop protein purified (1 ng)</td>
<td></td>
</tr>
<tr>
<td>Synechocystis PCC 6803</td>
<td></td>
</tr>
<tr>
<td>Thermo-&lt;sup&gt;+&lt;/sup&gt;synechococcus elongatus</td>
<td></td>
</tr>
<tr>
<td>Nostoc sp. PCC 7120</td>
<td></td>
</tr>
<tr>
<td>modE mutant Anabaena variabilis</td>
<td></td>
</tr>
</tbody>
</table>

Western Blot with Anti-Mop Antibodies

- Run on 16% Tris-Tricine Gel at 150 V for 1 hour
- Transferred to PDVF membrane at 40 V for 4 hours
- Primary antibody: 50 uL: 50 mL 1% milk PBS-T
- Secondary antibody: 5 uL: 50 mL 1% milk PBS-T
- SuperSignal West Pico Chemilumiscent Substrate

0- Blank
1- 2 ng Mop protein
2- 10 ug protein extracted from Synechocystis sp PCC 6803 (does not have mop gene)
3- 10 ug protein extracted from Thermo-<sup>+</sup>synechococcus elongatus (does not have mop gene)
4- 10 ug protein extracted from Nostoc sp. PCC 7120 (does have mop gene)
Chapter 7

CONCLUSIONS

The research presented in this dissertation provides a foundation for understanding Mo requirements for microbial nitrogen assimilation and freshwater Mo biogeochemical cycling, but there remain numerous avenues for future research on these subject. In this chapter, I summarize the major findings of each previous chapter and suggest directions for the next steps for future research.

In Chapter 2, we related the evolution of metalloenzymes involved in N-assimilation to changing marine metal availability. We presented a scheme whereby Fe-based NH$_4^+$ and NO$_2^-$ enzymes evolved in the Fe-rich Archean, followed by Fe-Fe, Fe-V and Fe-Mo nitrogenases once the N demands of the expanding microbial community exceeded the fixed N available. Mo-containing nitrate reductases likely evolved as nitrate and Mo concentrations rose during the Great Oxygenation Event or perhaps slightly prior to it. We suggested that differential Mo requirements in nitrate reductases of cyanobacteria vs. eukaryotic algae may have contributed to the long lag before algal radiation in the Neoproterozoic.

This chapter laid a framework for future work, including the rest of this dissertation. In order to determine how biological pathways coevolved with changing Earth enviroments, we proposed an integrated geobiological approach: coupling high-resolution studies of metal concentrations through time with laboratory experiments to explore the physiological changes that organisms
experience at representative metal concentrations. We suggested that future work be focused on variations in seawater abundance of bioessential metals that were understudied compared to Fe and Mo (i.e. Zn, Ni, Cu, Co, V). Since Chapter 2 was published in 2009, great strides have been made in this direction by measurement of metals in banded iron formations and black shales. This research has yielded a clearer picture of Ni (Konhauser et al., 2009) and Zn (Planavsky et al., 2010) bioavailability in seawater through time. However, there are still many metals that are understudied: Cu, V, Co, W, Mn and others. Great strides in reconstructing nitrogen geochemistry have been made in recent years through N-isotopic characterization of ancient sediments (Garvin et al., 2009) and the newly pioneered use of compound-specific nitrogen isotopes. However, this work has focused on inorganic nitrogen bioavailability; little remains known about changes in dissolved organic nitrogen (i.e. urea and amino acids) through time.

On the biological side, Chapter 2 recommends further study of microbial metal requirements, strategies for metal uptake and intracellular allocation of metals, particularly for bioessential metals that underwent drastic seawater concentration changes over Earth history. Chapters 3 and 6 took this approach to study Mo in heterocystous cyanobacteria, but much work remains for analogous studies of Cu, Ni, Zn, V, Co, etc. Further future work includes (1) characterization of the range of variation in microbial metal quotas, (2) studies of secondary metal requirements for biosynthesis of metal-cofactors and for accessory proteins in environmentally-relevant microbes and (3) more precise phylogenetic dating of
the evolution of enzymes that catalyze reactions at the heart of biogeochemical cycles.

In Chapter 3, we compared Mo requirements for N₂ fixation in two species of heterocystous cyanobacteria: one freshwater strain and one coastal strain. We found that <1 nM Mo induced N-limitation in both species, but the N₂ fixation rates dropped earlier in the coastal strain than the freshwater strain. When Mo was high (>1 µM), the freshwater strain hyperaccumulated Mo whereas the coastal strain did not. We hypothesized that the high Mo content and extended time required for N₂-fixation to decrease in the freshwater strain was due to expression of the Mo-storage gene mop. These predictions were tested in Chapter 6, in which the regulation of mop at both transcriptional and translational levels was studied at three Mo concentrations. The regulation was found to be more complex than originally expected: instead of finding no mop transcripts and protein at low Mo, I found that mop transcription was actually upregulated and Mop protein was present. However, evidence of a Mo storage role for Mop was supported by immunoblots, which showed a change in subunit composition between samples grown at low Mo (1 nM) for multiple transfers vs. higher Mo (150 and 3000 nM). Mop protein in low-Mo treatments was present as a small protein (likely a dimer) whereas Mop protein in the higher-Mo treatments was present as a larger multimeric protein. This difference in subunit composition suggests that Mop may have different functions at low vs. high Mo; it might
function as a multimeric Mo storage protein at high Mo and as a dimeric Mo transfer protein at low Mo.

Future work is required to tie up several loose ends in Chapter 6. Although negative controls showed a lack of unspecific binding of the antibody, protein sequencing of chemiluminescent bands in the immunoblots is needed to confirm that all of the bands shown are in fact 100% Mop protein. It would also be very informative to study a mop deletion strain of Nostoc sp. PCC 7120 to test if it does indeed lack the ability to accumulate Mo intracellularly. A –mop strain would also be the most appropriate negative control for immunoblots. More experiments are needed to resolve how mop transcriptional and translational regulation is influenced by changes in N source (NH₄, NO₃, or N₂). Future studies are also needed to test whether the smaller Mop protein that is present in low-Mo cells interacts with Mo-containing enzymes like NifD and also to quantify the percentage of Mo in the cell that is bound to Mop.

**Chapter 4** and **Chapter 5** were studies conducted at Castle Lake of the Mo requirements for biological nitrate assimilation and Mo biogeochemical cycling, respectively. In Chapter 4, I found that addition of Mo stimulated NO₃⁻ assimilation in the Castle Lake hypolimnion in 2008 and in the epilimnion in 2009. These interannual and depth response differences were explained by preference for NH₄⁺ over NO₃⁻ when NH₄⁺ was available and seasonal succession of plankton species with differing Mo requirements. A particularly intriguing finding of Chapter 4 was a dissolved Mo minimum in the Castle Lake epilimnion
that occurred at the same depth as N₂-fixing periphyton communities in the littoral zone. More research is required to determine whether low Mo limits N₂ fixation in Castle Lake periphyton. Furthermore, the alder endosymbiont N₂-fixing communities growing along the east shore of the lake would also be an ideal population to study Mo limitation of soil bacteria and their methods for coping with low Mo (molybdophore excretion, storage proteins, etc).

Lastly, Chapter 5 presented a study of sources and sinks of Mo in Castle Lake and suggests that the largest source of Mo today is likely particulate matter from the watershed. Future work is needed to measure Mo concentrations in the particulate matter in source waters feeding into the lake to confirm that they could supply the amount of Mo needed to balance the box model. It would also be very informative to measure the Mo isotopic composition of other samples from the watershed, most importantly lake water and atmospheric sources of Mo, in order to obtain a more complete model of the Mo isotopic balance of a typical freshwater system and to quantify what fraction of the isotopically-light Mo in the lake sediments is coming from atmospheric sources.
REFERENCES


Bortels, H., 1940. On the importance of molybdenum for nitrogen fixation by Nostoc. Archives of Microbiology 11, 155-186 (written in German).


diazotrophic cyanobacteria *Trichodesmium* spp. Applied and Environmental Microbiology 58, 3122-3129.


Glass, J., Krieg, M., Wolfe-Simon, F., Anbar, A., 2007. Trace metal controls on the efficiency of nitrogen fixation: assessing microbial metal requirements in ancient ocean, American Society of Limnology and Oceanography Aquatic Sciences Meeting; Santa Fe, NM; Abstract 1037


fixation rates and iron: carbon ratios of field populations. Limnology and Oceanography 48, 1869-1884.


Lu, G., Lindqvist, Y., Schneider, G., Dwivedi, U., Campbell, W., 1995. Structural studies on corn nitrate reductase: refined structure of the cytochrome b reductase fragment at 2.5 Å, its ADP complex and an active-site mutant and modeling of the cytochrome b domain. Journal of Molecular Biology 248, 931-948.


Marchetti, A., Maldonado, M.T., Lane, E.S., Harrison, P.J., 2006. Iron requirements of the pennate diatom Pseudo-nitzschia: Comparison of oceanic (high-nitrate, low-chlorophyll waters) and coastal species. Limnology and Oceanography 51, 2092-2101.


Planavsky, N., Scott, C., Gill, B.C., Bekker, A., Lyons, T.W., 2010. Tracking Zn bioavailability through time: New insights from sulfidic black shales, American Geophysical Union, Fall Meeting 2010, abstract #OS33E-1510


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APPENDIX A

APPROVAL LETTERS FROM COAUTHORS
"I approve that Jennifer Glass include the following papers, which I co-authored, as chapters in her PhD dissertation:"

Glass JB, F Wolfe-Simon, AT Poret-Peterson, AD Anbar. Regulation of Mop, a molybdenum storage protein, in the cyanobacterium Nostoc PCC 7120. In prep. for Applied and Environmental Microbiology


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Approval for inclusion of papers in PhD dissertation.

1 message

Felisa Wolfe-Simon <felisawolfesimon@gmail.com>  
Tue, Mar 8, 2011 at 9:50 PM

To: Jennifer Glass <jbglass1@asu.edu>

Dear members of the Ph.D. dissertation committee,

I approve that Jennifer Glass include the following papers, which I co-authored or am co-authoring, as chapters in her PhD dissertation:


Wishing her the best of luck in her career.

All the best,

Felisa Wolfe-Simon, Ph.D.
approval
2 messages

James Elser <j.elser@asu.edu>  Tue, Mar 8, 2011 at 10:11 PM
To: Jennifer Glass <jbglass1@asu.edu>

I approve that Jennifer Glass include the following paper, which I co-authored, as a chapter in her PhD dissertation: "


--
James Elser
Adjunct Professor & Parents Association Professor
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Associate Dean
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Arizona State University
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Lab web site: [http://www.eliserlab.asu.edu/index.html](http://www.eliserlab.asu.edu/index.html)
Dept web site: [http://soils.asu.edu/faculty/elser.php](http://soils.asu.edu/faculty/elser.php)
Sustainable P initiative: [http://sustainablep.asu.edu/](http://sustainablep.asu.edu/)
P is for? [http://www.youtube.com/watch?v=RPPawofv3kY](http://www.youtube.com/watch?v=RPPawofv3kY)
I approve that Jennifer Glass include the following paper in review, which I co-authored, as a chapter in her PhD dissertation:


Alan C. Heyvaert, Ph.D.
Associate Director
Center for Watershed Environmental Sustainability
Assistant Research Professor
Division of Hydrologic Sciences
Desert Research Institute
2215 Raggio Parkway
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I approve that Jennifer Glass include the following paper in review, which I co-authored, as a chapter in her PhD dissertation:


Sincerely,

Anthony Chappaz
Brooke Eustis <bneustis@gmail.com>
To: Jennifer Glass <jbglass1@asu.edu>

Dear Jennifer,

"I approve that Jennifer Glass include the following paper in review, which I co-authored, as a chapter in her PhD dissertation:"


Thank you,

Brooke Eustis
Environmental Sciences Graduate Program
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1664 N. Virginia St. / MS 186
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http://www.castelake.ucdavis.edu/
I approve that Jennifer Glass include the following paper in review, which I co-authored, as a chapter in her PhD dissertation:


Sincerely,
Dave

---
David P. Waetjen
Information Center for the Environment
University of California, Davis
http://ice.ucdavis.edu/people/dwaetjen
Request for approval statement to include paper in dissertation

Hilairy Hartnett <h.hartnett@asu.edu>                              Thu, Apr 21, 2011 at 2:08 PM
To: Jennifer Glass <jbglass1@asu.edu>

To whom it may concern:

I approve that Jennifer Glass include the following paper in review, which I co-authored, as a chapter in her PhD dissertation:


Sincerely,

Hilairy Ellen Hartnett

Hilairy Ellen Hartnett, Associate Professor
School of Earth and Space Exploration
Department of Chemistry and Biochemistry
Arizona State University
Hi Jen,

Good luck on your interview!

I approve that Jennifer Glass include the following paper, which I am co-authoring, as a chapter in her Ph.D. dissertation.

Glass J.B., F. Wolfe-Simon, A.T. Pore-Peterson, A.D. Anbar. Regulation of Mop, a molybdenum storage protein, in the cyanobacterium *Nostoc PCC 7120*. In prep. for *Applied and Environmental Microbiology*

Amisha Pore-Peterson, March 8, 2011

Amisha

[Quoted text hidden]

--

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Request for approval statement to include paper in dissertation

raxler@nrri.umn.edu <raxler@nrri.umn.edu>  
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Cc: raxler@nrri.umn.edu

Mon, Apr 25, 2011 at 6:52 PM

Dear Jennifer & ASU Thesis Committee Members -

I approve that Jennifer Glass include the following paper in review, which I co-authored, as a chapter in her PhD dissertation:


Richard Axler

“it all comes down to your water”
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