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Coevolution of metal availability and nitrogen assimilation in cyanobacteria and algae

J. B. GLASS,[1] F. WOLFE-SIMON[2,3] AND A. D. ANBAR[1,3]

[1]*School of Earth and Space Exploration, Arizona State University, Tempe AZ 85287, USA*
[2]*Department of Earth and Planetary Sciences, Harvard University, Cambridge, MA 02467, USA, and*
[3]*Department of Chemistry and Biochemistry, Arizona State University, Tempe, AZ 85287, USA*

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' *c.* 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.

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Corresponding author: Jennifer Glass. Tel.: 480-244-3603; fax: 262-244-3605; e-mail: jennifer.b.glass@asu.edu

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 (μM) (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, the dominant form of N in the oceans, other than particulate N bound up in living and dead organisms. However, it is also highly nonreactive because of the strength of the N-N bond. Breaking this triple bond is energetically expensive, and so the organisms that can assimilate N_2 only do so when there are no other options.
- Dissolved ammonium (NH_4^+), 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_4^+ is exceedingly low in

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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_3^-), are more abundant than NH_4^+. Collectively, these dissolved inorganic forms of N are known as DIN. Although the concentration of NO_3^- is low in surface waters (0.2 μM) as a result of biological removal, it can reach up to ~40 μM in deep waters (Sharp, 1983). Thus, upwelling events contribute major influxes of NO_3^- to the photic zone. A nitrite (NO_2^-) maximum exists at the base of the euphotic zone in stratified water columns, where the NO_2^- concentration can reach 4.5 μM (Lomas & 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 & Anderson, 2003).

Assimilation of these various forms of N requires a number of complex enzymes (Falkowski, 1983; Berges & Mulholland 2008). 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 cofactors 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; Morel, 2008). 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, because of their low solubilities and rapid removal on particles (Morel & 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 (Moffett *et al.*, 1997; Achterberg *et al.*, 1999), 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 in seawater (Xue *et al.*, 1995; Achterberg *et al.*, 1997), whereas Ni concentrations show little salinity dependence (Xue *et al.*, 2001). Mo exhibits the opposite trend, with very low concentrations (<1–20 nM) in freshwater (Sugawara *et al.*, 1961; Bachmann & Goldman, 1964; Howarth & Cole, 1985; Cole *et al.*, 1993).

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 (Martin & Fitzwater, 1988; Price *et al.*, 1991; Coale *et al.*, 1996). Marine cyanobacteria have been shown to fix N_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 as a result of 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, because of its long residence time and high stability in oxic seawater, but Mo scarcity may limit N uptake, and hence primary productivity, in freshwater (Goldman, 1960; Axler *et al.*, 1980).

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)) as a result of 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.*, 2008). 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 (Falkowski, 1997; Anbar & Knoll, 2002; Anbar, 2008).

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_2, whereas eukaryotes cannot; eukaryotes assimilate fixed forms of N from the environment or (in the case of heterotrophs) from their food.

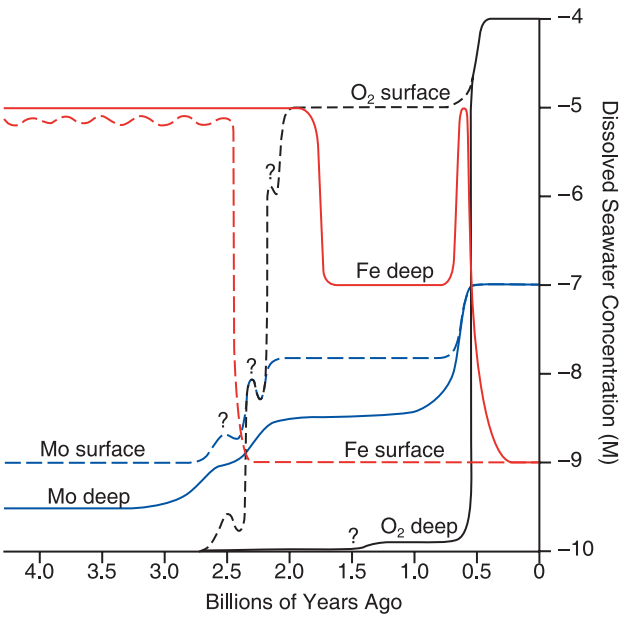

Fig. 1 Notional estimated surface (dotted lines) and deep (solid lines) concentrations of Fe (red), Mo (blue) and O_2 (black) in seawater through time, modeled after Zerkle *et al*. (2005) with data updated for Mo (Anbar *et al*., 2007; Scott *et al*., 2008), Fe (Canfield *et al*., 2008) and surface ocean O_2 (Holland, 2006).

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 & 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 N 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 ('diazotrophs'), on the other hand, can perform this reaction at ambient pressures and temperatures. Heterotrophic and photosynthetic diazotrophs 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 diazotrophs are cyanobacteria (Capone & Carpenter, 1982; Capone *et al*., 1997; Stal & Zehr, 2008). Conversion of N_2 gas into NH_3 requires electrons, ATP and the metal-rich enzyme nitrogenase (abbreviated Nif), following the reaction:

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 16ADP + 16P_i + 2NH_3 + H_2 \quad \text{(eqn 1)}$$

The eight electrons required for each catalytic cycle are supplied by ferredoxin, a small [2Fe-2S]-cluster protein that accepts electrons from the photosynthetic electron transport chain (Schrautemeier & Bohme, 1985). The product NH_3 gas is rapidly converted into the soluble NH_4^+ and directed into glutamine and glutamate biosynthesis (Wolk *et al*., 1976; Meeks *et al*., 1978; Carpenter *et al*., 1992) (see Ammonium Assimilation section).

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 N_2 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 & Ludden, 2005, 2008).

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 & Rees, 1996; Kustka *et al*., 2003a), although all Fe-S cluster-binding

Table 1 Characteristics of the key enzymes involved in N assimilation in phytoplankton

Enzyme	Metal co-factors	Substrates	Product	Distribution	Oxygenic photoautotrophs from which protein has been purified or overexpressed
Nitrogenase (Nif, 2-3 (?) subunits per enzyme)	4Fe-4S (NifH), 8Fe-8S, Mo(V/Fe)-7Fe-9S (NifDK)	**N_2**, $8H^+$, $8e^-$, 16ATP	**$2NH_3$**, H_2, 16ADP, $16P_i$	Cyanobacteria	*Anabaena cylindrica* (Hallenbeck *et al*., 1979)
Ferrodoxin-nitrate reductase (NarB)	4Fe-4S, molybdopterin guanine dinucleotide (Mo)	**NO_3^-**, $2e^-$, $2H^+$	**NO_2^-**	Cyanobacteria	*Plectonema boryanum* (Ida & Mikami, 1983; Mikami & Ida, 1984); *Synechococcus* sp. PCC 7942 (Rubio *et al*., 2002); *Aphanothece halophytica* (Thaivanich & Incharoensakdi, 2007)
NAD(P)H-nitrate reductase (NR, 4 subunits per enzyme)	Cytochrome b_{557} (Fe), sulfite-oxidase type molybdopterin (Mo)	**NO_3^-**, $2e^-$, $2H^+$	**NO_2^-**	Algae	*Chlorella vulgaris* (Solomonson, 1975); *Ankistrodesmus braunii* (de la Rosa *et al*., 1980, 1981)
Ferrodoxin-nitrite reductase (NiR)	4Fe-4S, siroheme (Fe)	**NO_2^-**, $6e^-$, $8H^+$	**NH_3**	All	*Anabaena* sp. *7119* (Mendez & Vega, 1981); *Chlamydomonas reinhardtii* (Romero *et al*., 1987); *Phormidium laminosum* (Arizmendi & Serra, 1990)
Glutamine synthetase (GS)	Mn (?)	**NH_4^+**, Glutamate, ATP	**Glutamine** ($C_2H_{10}N_2O_3$)	All	*Anabaena* sp. CA (Stacey *et al*., 1977); *Nostoc* sp. PCC 7120 (Orr *et al*., 1981); *Chlorella kessleri* (Sumar *et al*., 1984); *Anacystis nidulans* (Florencio & Ramos, 1985); *Chlorella sorokiniana* (Beudeker & Tabita, 1985); *Phormidium laminosum* (Blanco *et al*., 1989); *Calothrix* sp. PCC 7601 (Merida *et al*., 1990); *Synechocystis* sp. PCC 6803 (Merida *et al*., 1990; Garcia-Dominguez *et al*., 1997) *Prochlorococcus* spp. (El Alaoui *et al*., 2003)
Ferredoxin-glutamate synthase (GltS)	3Fe-4S	**Glutamine** ($C_2H_{10}N_2O_3$), 2-oxo-glutarate, $2e^-$, $2H^+$	**2Glutamate** ($C_5H_9NO_4$)	All (mainly used by cyanobacteria)	*Chlamydomonas reinhardtii* (Gotor *et al*., 1990); *Synechococcus* sp. PCC 6301 (Marques *et al*., 1992); *Synechocystis* PCC 6803 (Ravasio *et al*., 2002)
NADH-glutamate synthase (GltBD)	4Fe-4S (GltD), 4Fe-4S, 3Fe-4S (GltB)	**Glutamine** ($C_2H_{10}N_2O_3$), 2-oxoglutarate, $2e^-$, $2H^+$	**2Glutamate** ($C_5H_9NO_4$)	Some cyanobacteria, mainly used by eukaryotes	*Chlamydomonas reinhardtii* (Marquez *et al*., 1984)
Urease (Ure, usually 3 subunits per enzyme)	2Ni (di-nuclear)	**Urea**	**NH_3**, H_2CO_3	Cyanobacteria/red algae	*Spirulina maxima* (Carvajal *et al*., 1982); *Anabaena doliolum* (Rai, 1989); *Anabaena cylindrica* (Argall *et al*., 1992); *Leptolyngbya boryana* (Jahns *et al*., 1995); *Anabaena* sp. PCC 7120 (Jahns *et al*., 1995); *Synechococcus* sp. WH 7805 (Collier *et al*., 1999)
Urea amidolyase	–	**Urea**	**Allo-phanate**	Green algae	No purification has been published.
Allophanate hydrolyase	–	**Allophanate**	**NH_3**	Green algae	*Chlamydomonas reinhardtii* (Maitz *et al*., 1982)
Cu-amino acid oxidase (CuAO, 2 subunits per enzyme)	Cu (mononuclear)	**Amines** (RCH_2NH_2), O_2	**NH_3**, H_2O_2, RCHO	Dinoflagellates/ coccolithophores/ cyanobacteria/green algae	No purification has been published.

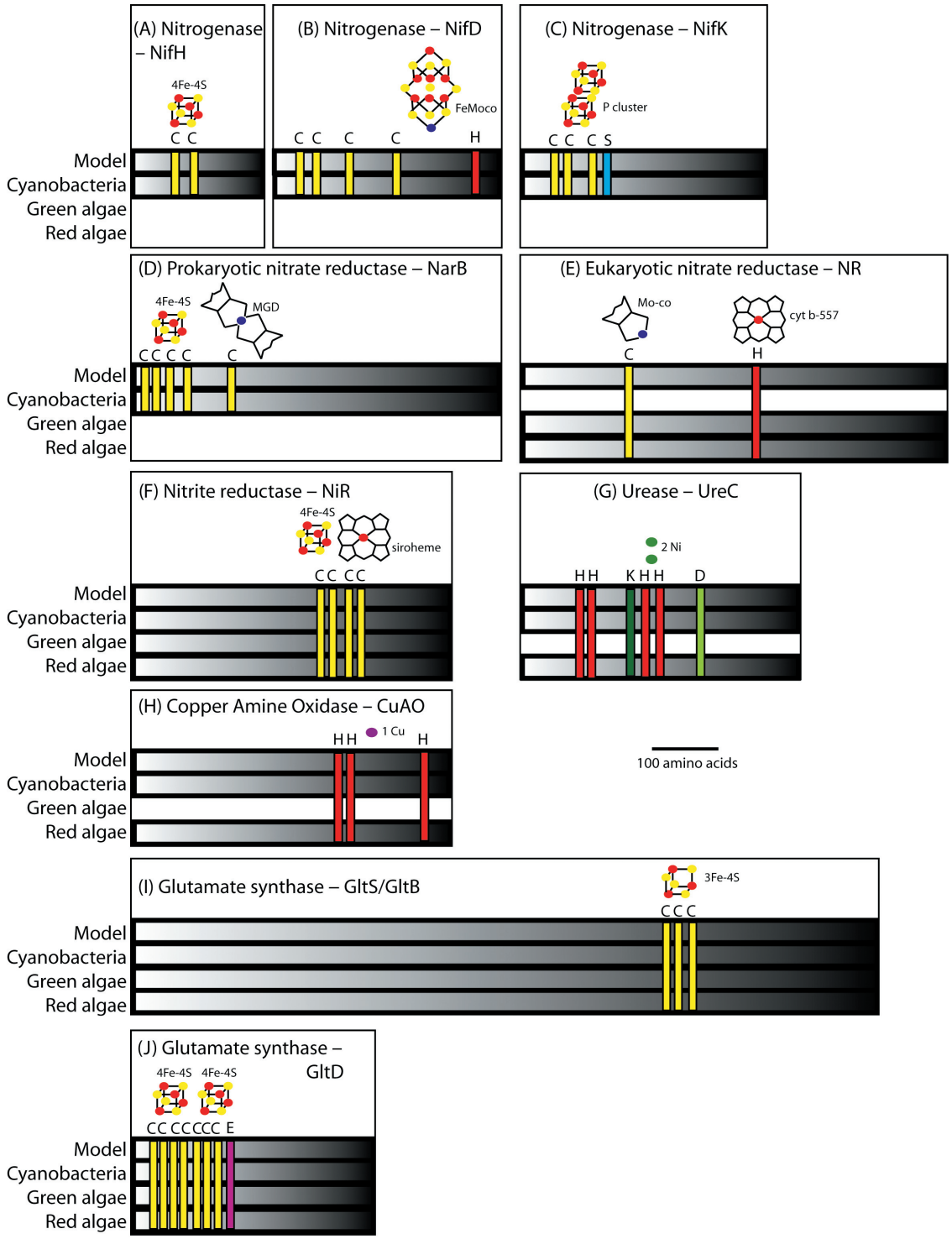

Fig. 2 Schematic of N assimilatory protein alignments. Each horizontal shaded gray square represents a sequence of amino acid that folds into a protein. Each vertical colored bar represents the position of a conserved (present in the same position in the same proteins from different organisms) metal-binding 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. See Supporting Information (Supplementary text for Fig. 2) for details of the construction of the figure and for model organism species names.

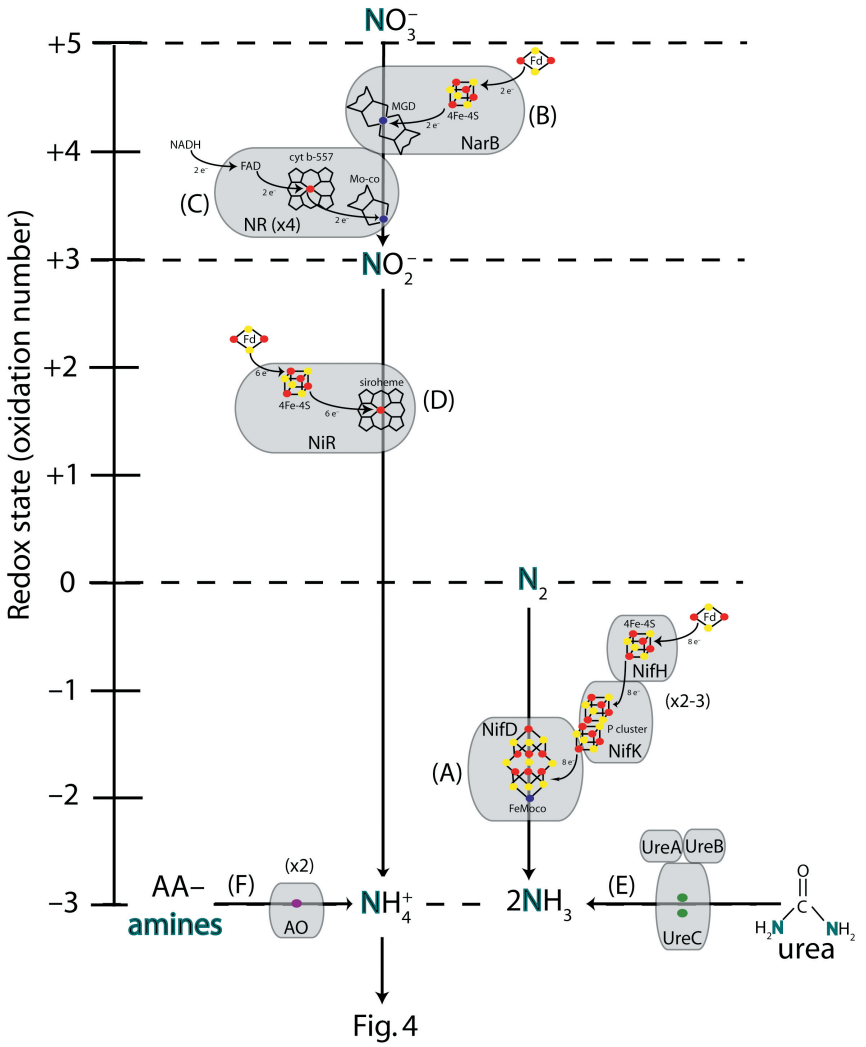

Fig. 3 N assimilation pathways plotted versus redox state of N with metal cofactors colored (Fe: red, Mo: blue, Ni: green, Cu: purple, yellow atoms are S atoms), including N_2 fixation (a), prokaryotic NO_3^- reduction (b), eukaryotic NO_3^- reduction (c), NO_2^- reduction (d), urea hydrolysis (e) and amine oxidation (f). All assimilation pathways lead to 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.

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 N_2 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 characterizing the metal quotas of photosynthetic N_2 fixation in the sea.

The importance of Mo to N_2 fixation has been well-established since the 1930s (Bortels, 1930; Bortels, 1940). Nitrogenases that utilize alternatives to Mo were discovered more recently (Burns *et al.*, 1971; Bishop *et al.*, 1980). These alternative nitrogenases are distinct proteins encoded by separate genes (Robson *et al.*, 1986; Chisnell *et al.*, 1988; Pau *et al.*, 1989), which are expressed when Mo concentrations fall below 100 nM (Joerger & 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 *Anabaena* (Kentemich *et al.*, 1988; Thiel, 1993; Boison *et al.*, 2006). 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 *Anabaena variabilis* ATCC 29413 VnfD protein (587 amino acids) returns marine peptides with only 34% identity at most. These are likely NifD proteins hits, as Nif and Vnf proteins share ~30% sequence similarity (Raymond *et al.*, 2004). No cyanobacteria have been found to contain *anf* genes.

Nitrate reduction

Most, though not all, phytoplankton can assimilate NO_3^-. Prokaryotes and eukaryotes express evolutionarily-distinct proteins (Stolz & Basu, 2002) for this purpose, but regardless both kinds of nitrate reductases require Mo. NO_3^- assimilation

has been extensively reviewed for cyanobacteria, algae and plants (Guerrero *et al.*, 1981; Berges, 1997; Campbell, 1999; Moreno-Vivian *et al.*, 1999). In all cases, NO_3^- assimilation begins with the two-electron reduction to NO_2^-:

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \qquad \text{(eqn 2)}$$

The protein responsible for the reduction of NO_3^- to 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 & Myers, 1967; Manzano *et al.*, 1976; Mikami & Ida, 1984; Hirasawa *et al.*, 2004). Biochemical studies of NarB have shown that this protein binds one [4Fe-4S] cluster and one molybdopterin cofactor with cysteines (Rubio *et al.*, 1999; Rubio *et al.*, 2002) (Figs. 2D,3B, Table 1). Two electrons travel from ferredoxin through the [4Fe-4S] 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. NO_3^- then binds to the reduced Mo and is reduced to NO_2^-, returning Mo to the 6+ state (Jepson *et al.*, 2004).

Eukaryotic nitrate reductase arose through convergent evolution to serve the same role as NarB. This enzyme, abbreviated as NR, utilizes nicotinamide adenine dinucleotide (phosphate) ((NAD(P)H)), an organic cofactor that 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 dinucleutide (FAD) (Zumft *et al.*, 1970), cytochrome b_{557} (Ahmed & Spiller, 1976), and molybdopterin of a slightly different structure than in NarB (Solomonson *et al.*, 1975; Solomonson *et al.*, 1986) (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 green and red algae with primary plastid symbionts derived from cyanobacteria (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 NO_3^- is reduced to 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 algal NRs (de la Rosa *et al.*, 1981; de la Rosa, 1983; Solomonson *et al.*, 1986). Although the physiological requirement of Mo for nitrate reduction has been established for fungi (Steinberg, 1937; Nicholas & Nason, 1954; Nicholas *et al.*, 1954) and green algae (Arnon *et al.*, 1955; Aparicio *et al.*, 1971; 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 from 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 *et al.*, 1981; de la Rosa, 1983). Further study is necessary to determine whether Mo content is in fact variable in eukaryotic NR.

NO_3^- reduction also occurs in anaerobic bacteria, which utilize NO_3^- as a terminal electron acceptor during dissimilatory 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 (Antipova *et al.*, 1998; Antipov *et al.*, 2003, 2005).

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 NH_4^+ (Kuznetsova *et al.*, 2004), following the overall reaction:

$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O \qquad \text{(eqn 3)}$$

Biochemical and phylogenetic evidence suggests that NiR in 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 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 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 algae and plants, catalyzes the hydrolysis of urea, producing two molecules of ammonia and one molecule of carbonic acid (Fig. 2E, Table 1):

$$H_2N\text{-}CO\text{-}NH_2 + 2H_2O \rightarrow 2NH_3 + H_2CO_3 \qquad \text{(eqn 4)}$$

Urease isolated from cyanobacteria and diatoms contains three subunits, usually two small and one large (Fig. 3E) (Carvajal *et al.*, 1982; Rai, 1989; Argall *et al.*, 1992; Jahns *et al.*, 1995; Collier *et al.*, 1999; Palinska *et al.*, 2000). 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 (Oliveira & Antia, 1984, 1986a,b; Egleston & Morel, 2008) and marine cyanobacteria *Synechococcus* (Dupont *et al.*, 2007) on urea as the sole N source. Urease activity has been shown to increase with rising Ni concentration (Mackerras & Smith, 1986; Price & 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 does not require Ni for urea assimilation (Rees & 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 & 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 & 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.

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_2O_2), α-keto acids and extracellular ammonia, following the reaction:

$$RCH_2NH_2 + O_2 \rightarrow H_2O_2 + RCHO + NH_3 \qquad \text{(eqn 5)}$$

The NH_3 is subsequently taken up through specific transporters and assimilated by the organism (see Ammonium Assimilation section). Amine oxidase activity has been observed for phytoplankton with secondary plastid symbionts derived from red algae including coccolithophorids and dinoflagellates (Palenik & Morel, 1990a,b, 1991). Although the Cu content of algal CuAO has not been confirmed, it is likely 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 & 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):

$$\text{Glutamate} + \text{ATP} + NH_4^+ \rightarrow \text{Glutamine} + \text{ADP} + P_i \qquad \text{(eqn 6)}$$

GS is most often a large, multi-subunit protein (Stacey *et al.*, 1977; Sampaio *et al.*, 1979; Orr *et al.*, 1981; Sumar *et al.*, 1984; Beudeker & Tabita, 1985; Florencio & Ramos, 1985; Merida *et al.*, 1990; Yuan *et al.*, 2001; El Alaoui *et al.*, 2003) 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 archeal domains (Raymond, 2005).

The second enzyme in the GS-GltS pathway is glutamate synthase (GltS, also known as GlsF or GOGAT) (Suzuki & 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:

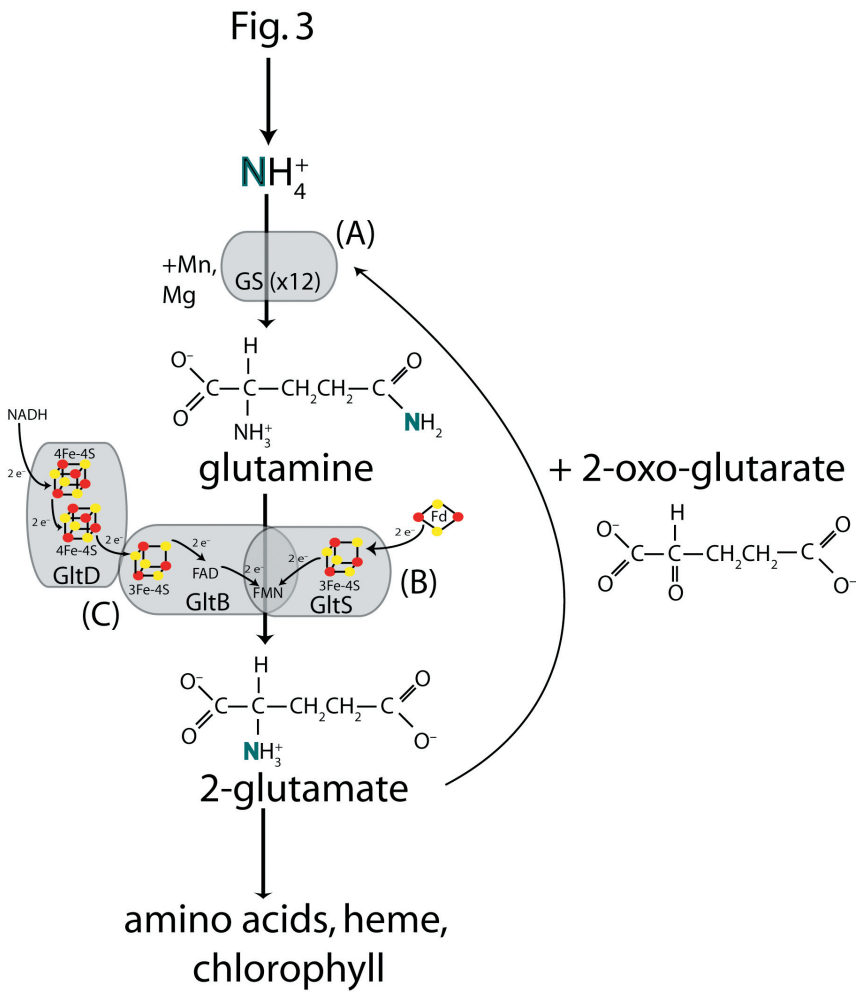

Fig. 4 The NH_4^+ assimilation pathway in primary producers with Fe colored in red and S colored in yellow. NH_4^+ taken up from the environment or reduced via the pathways in Fig. 3 is converted to glutamine (A) and subsequently to two molecules of glutamate with either ferredoxin (B) or NADH (C) supplying 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.

$$\text{Glutamine} + \text{2-oxoglutarate} + 2e^- + 2H^+ \rightarrow 2\ \text{Glutamate} \quad \text{(eqn 7)}$$

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 (Merida *et al.*, 1990; Marques *et al.*, 1992; Navarro *et al.*, 2000; Ravasio *et al.*, 2002) (Fig. 4B). 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, 2003), which are conserved in GltS sequences from other cyanobacterial and algae (Fig. 2I), supporting the essential role for Fe in phytoplankton glutamate synthesis. However, although algae possesses genes encoding GltS-like cyanobacteria, many express a larger two-protein complex, abbreviated GltBD, for glutamine assimilation (Cullimore & Sims, 1981; Marquez *et al.*, 1984) (Fig. 4C, Table 1). Like NR (see Nitrate Reduction section), GltBD accepts electrons from NADH instead of ferredoxin (Clayton & 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 & 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 11 Fe atoms per enzyme complex, compared to only 3 in GltS. Why would utilizing NADH instead of ferredoxin as an electron donor for glutamate synthesis require a nearly fourfold 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 a nonendosymbiotic 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 & Ahmed, 1986).

N 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 (Rouxel *et al.*, 2005; Anbar & Rouxel, 2007; Anbar *et al.*, 2007; Scott *et al.*, 2008), two 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 (Saito *et al.*, 2003; Morel, 2008).

We consider this possibility in the succeeding discussion, 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 (Fig. 1). 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 N 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 & 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 O_2 began to rise, new enzymes utilizing more oxidized N substrates and metals made more bioavailable by oxygenation evolved.

The anoxic Archean

Molecular O_2 was absent or scarce in the Archean atmosphere and oceans (Holland, 2006). Under these conditions, the most abundant form of bioavailable N was probably NH_4^+, rather than NO_3^- as today (Boyd & Philippot, 1998; Beaumont & Robert, 1999; Papineau *et al.*, 2005). Some N may also have been present as NO_2^-. However, then, as now, the vast majority of N in the oceans and atmosphere was present as N_2 (Kasting & Catling, 2003), which is not readily bioavailable.

Before the evolution of biological N_2 fixation, the supply of fixed N to the oceans would have been limited by the rate of abiotic N_2 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 & 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 (Yung & McElroy, 1979; Mancinelli & McKay, 1988; Navarro-González *et al.*, 1998; Navarro-Gonzalez *et al.*, 2001). Bolide impacts and subaerial volcanoes could have supplied additional fixed N as N-oxides (Mancinelli & McKay, 1988; Mather *et al.*, 2004). In the ocean, NO_2^- could have been abiotically reduced to NH_4^+ by abundant ferrous Fe (Summers & 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; Valentin *et al.*, 1993; Raymond, 2005), 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 (Kump, 2005; Rouxel *et al.*, 2005; Johnson *et al.*, 2008). Fe content may have fluctuated in response to relative input from hydrothermal vents versus export as Fe oxides in banded iron formations (Rouxel *et al.*, 2005). The question of whether N assimilation first evolved at seafloor 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 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.*, 2008).

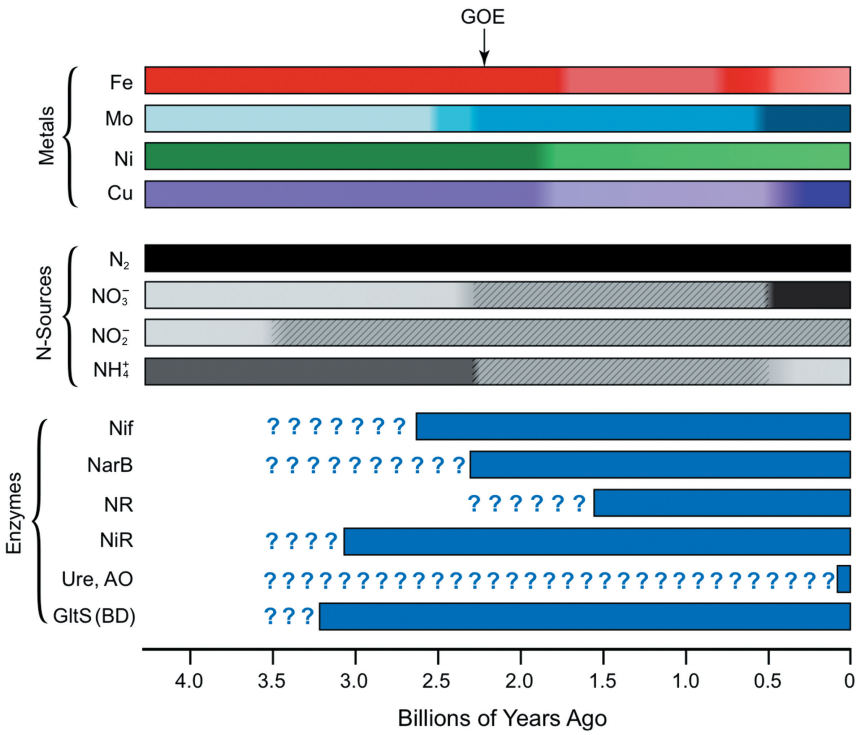

Fig. 5 Trends in the relative abundance of metals involved in N assimilation (Fe, Mo, Ni and Cu) and N species (N_2, NO_3^-, NO_2^-, NH_4^+ urea and amino acids) through time. Color darkness represents relative average seawater concentration, with darker colors representing higher concentrations, and lighter colors representing lower concentrations. Note that very little data exist 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.

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 N_2 fixation evolved much earlier. The high similarity between *nifD*, *nifK*, and *nifE* and *nifN* 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 O_2-sensitivity of all forms of nitrogenase, which implies that they evolved before 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 & Williams, 1991) (Fig. 1). Intriguingly, if Anf is ancestral it is conceivable that this enzyme complex developed for reasons other than N_2 fixation. Anf is the least effective nitrogenase of the three, but is the most effective at H_2 production (Schneider *et al.*, 1997). Thus, Anf may have initially evolved in Fe-rich oceans for H_2 production and subsequently been adopted for nitrogenase function (Silver & Postgate, 1973; Normand & Bousquet, 1989; Fani *et al.*, 2000). 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.

The oxygen transition

Oxygenic photosynthesis may have evolved by 2.7 Ga (Brocks *et al.*, 1999, 2003a,b; Eigenbrode & 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 & Kopp, 2008; Rasmussen *et al.*, 2008). However, few dispute that by 2.3 Ga, O_2 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, whereas the deep sea remained euxinic (Fig. 1) (Poulton *et al.*, 2004). This rise of O_2 would have stabilized NO_2^- and then NO_3^- in the ocean (Fig. 5) (Beaumont & Robert, 1999; Papineau *et al.*, 2005).

The reason for the delay of at least 300 Myr 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-O_2 condition (Holland, 2002; Catling & Claire, 2005; Fennel *et al.*, 2005; Kump & 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 (MoO_4^{2-}), 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 O_2 was commonly produced but not yet pervasive.

During this period, the residence times of both O_2 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 p.p.m. 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 & 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 as a result of the rise of marine Mo concentrations (Figs 1, 5) (Anbar *et al.*, 2007; Scott *et al.*, 2008). This hypothesis is consistent with the N isotopic data of Garvin *et al.* (2009). The key advantage of Mo is that Nif is ~15 times more efficient at *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 (Garvin *et al.*, 2009). 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 & 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 as early as 2.5 Ga (Garvin *et al.*, 2009), providing a minimum age for the origin of this metabolism. These signatures are also seen in ~2.1 Ga sediments (Boyd & Philippot, 1998; Beaumont & Robert, 1999; Papineau *et al.*, 2005).

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 & Imhoff, 2000; Philippot, 2002) between 3 and 4 Ga (Feng *et al.*, 1997), with subsequent passage of the gene among prokaryotes through horizontal gene transfer (Stolz & 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 (see Nitrate Reduction section). 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 toward competition between marine organisms for dominance. Cyanobacteria prevailed during the first three quarters of Earth history, whereas algae rose to prevalence in the Phanerozoic (Falkowski *et al.*, 2004; Knoll, 2007). 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 the 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_2S in deep ocean waters (Canfield, 1998). This counterintuitive consequence arises from the enhanced influx of sulfate as a result of 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_2S generation could have exceeded the rate of O_2 supply to deep waters in many places, resulting in an 'H_2S 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 (Shen *et al.*, 2003; Arnold *et al.*, 2004; Poulton *et al.*, 2004; Brocks *et al.*, 2005; Scott *et al.*, 2008).

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

Table 2 Comparison of the specific activities of prokaryotic (P) to different eukaryotic (E) nitrate reductase enzymes

Specific activity (μmoles NO_2 formed min^{-1} mg $enzyme^{-1}$)	Species	Reference
20–40	*Synechococcus* PCC 7942 (cyanobacteria, P)	Rubio *et al*. (2002)
72–80	*Ankistrodesmus braunii* (green alga, E)	de la Rosa *et al*. (1981)
83–86	*Chorella vulgaris* (green alga, E)	Solomonson *et al*. (1975)
2050	*Thalassiosira pseudonana* (diatom, E)	Amy & Garrett (1974)

precipitated a crash in Fe concentrations compared to those common in the Archean (Fig. 1).

Under these conditions, 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 & Knoll, 2002). Anf and Vnf do not exist in marine cyanobacteria today, so it is possible that Mo was absolutely necessary for cyanobacterial N_2 fixation throughout Earth's history. Experiments testing this hypothesis have shown that the rates of growth and N_2 fixation by certain cyanobacteria drop when the concentration of Mo is <5 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.*, 2008). Combined, these experiments and observations suggest that Mo availability in mid-Proterozoic oceans was close to the levels that are problematic for N_2 fixation by extant cyanobacteria, and at or below that level in the Archean.

Would Proterozoic oceans have contained sufficient Mo for NO_3^- assimilation? We sought to determine the cyanobacterial Mo requirements for NO_3^- assimilation by growing the filamentous, heterocystous cyanobacterium *Nostoc* sp. PCC 7120 with 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 1 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 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 (see Nitrate Reduction section). Thus, cyanobacteria could have used NO_3^- as an alternative N source if 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 resulting from their ability to assimilate NO_3^- even at low Mo concentrations.

Phylogenetic evidence suggests that NR arose soon after the origin of eukaryotes (Stolz & Basu, 2002) in the late Archean (Brocks *et al.*, 1999; Hedges *et al.*, 2001; Brocks *et al.*, 2003a,b). The Mo requirement for eukaryotic nitrate reductase (NR)

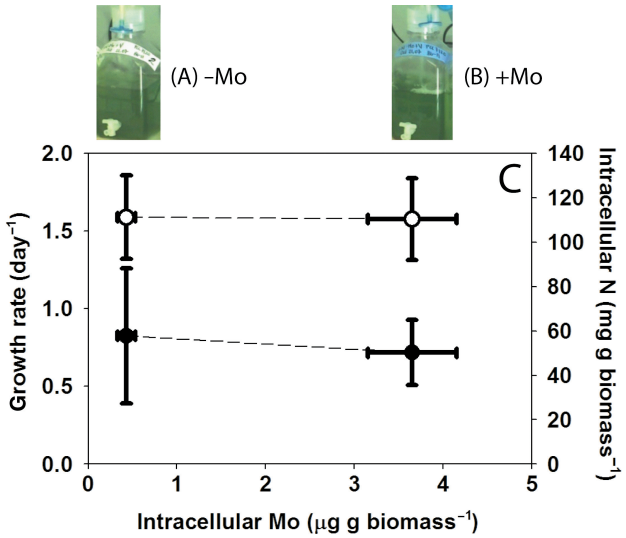

Fig. 6 Results of an experiment in which the heterocystous cyanobacterium *Nostoc* sp. PCC 7120 was grown for 1 month and diluted every 3–5 days with new media containing NO_3^- (15 mM) and either no Mo ('–Mo', blank was 0.5 nM) or 1500 nM Mo as sodium molybdate. (A) and (B) Photographs of one replicate culture bottle from each condition with Mo (A) and without Mo (B) on the final day of the experiment. (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). Chlorophyll concentrations were 1.0 mg L^{-1} in both conditions on the last day of the experiment. These results show that cyanobacteria are adapted to cope with very low environmental Mo levels when they are grown with a source of nitrate. See Supporting Information (Supplementary text for Fig. 6) for details about the growth conditions of this experiment.

may be considerably higher than that of the cyanobacterial NarB enzyme. NR requires four times as much Mo per enzyme as NarB per enzyme complex, as a result of NR's tetrameric structure. However, NR is up to 100× 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, benthic bacteria actively assimilate and denitrify NO_3^- whereas phytoplankton (dominated by algae) show little NO_3^- uptake (Axler & 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., 2008; Wille *et al.*, 2008) especially because eukaryotes lack the ability to directly utilize N_2. The scenario of Mo limitation of eukaryote proliferation fits well with the fossil record of algae which displays a radiation of green algae in the Cambrian (Revill *et al.*, 1994; Knoll, 2007). 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.*, 2008; 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.*, 2008). 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 & 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. 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 (Kuypers *et al.*, 2004; Ohkouchi *et al.*, 2006; Junium & Arthur, 2007; Kashiyama *et al.*, 2008), 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 & Price, 1996; Maldonado & Price, 2000; Marchetti *et al.*, 2006). As shown in Fig. 7, the intracellular Fe content is lower in algae than cyanobacteria even when the expected Fe demand based on the N assimilation pathway being used is similar. This suggests that modern algae can subsist on lower Fe quotas than cyanobacteria, and may partially explain the success of algae (especially diatoms) in the modern ocean. Metal-N limitation likely affected not only competition between eukaryotes versus prokaryotes but also competition between algal divisions (e.g. red versus 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 (see DON Assimilation section). 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 versus eukaryotes. For example, cyanobacteria, which fared better than 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 were low in Mo compared to today (Scott *et al.*, 2008). 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 cyanobacteria to 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

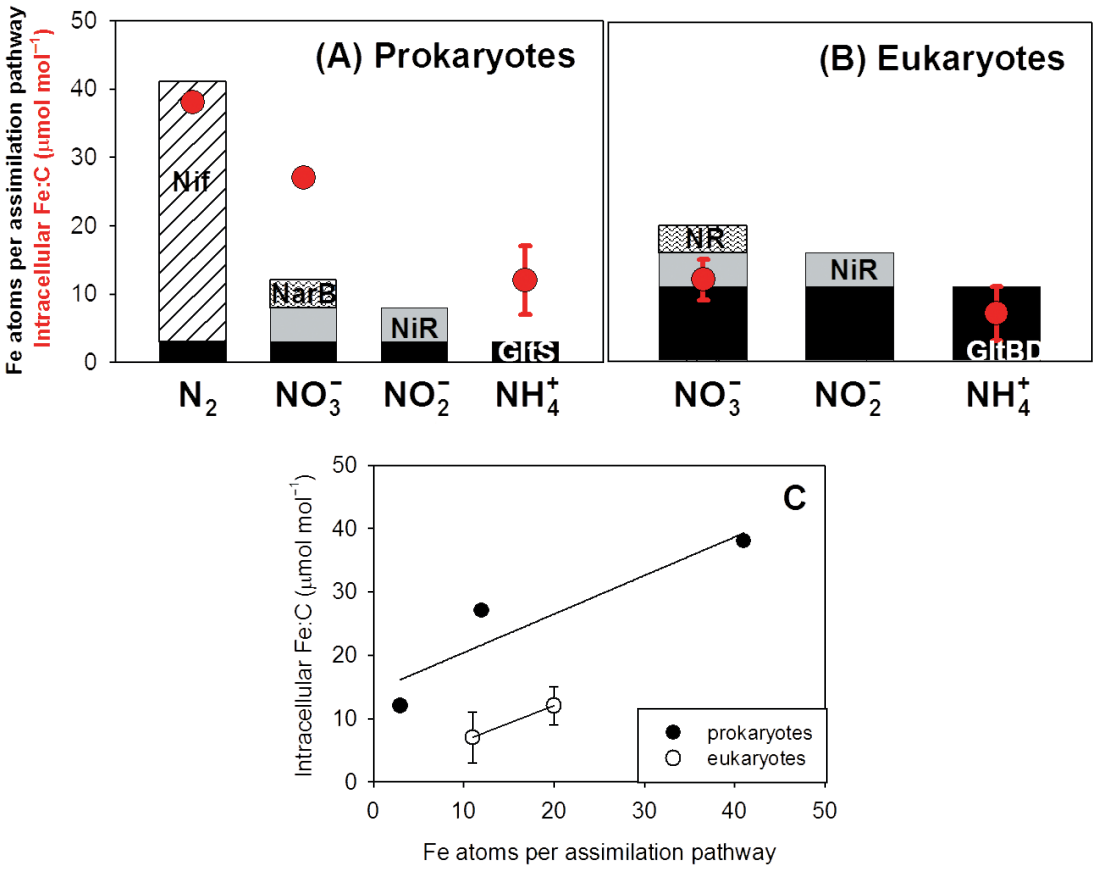

Fig. 7 Comparison of Fe requirements for assimilation of different N sources for photosynthetic (A) prokaryotes (cyanobacteria) and (B) eukaryotes (secondary red algae). Bar graphs show the distribution of Fe atoms present in enzymes used to assimilate N_2 (prokaryotes only; Nif and GltS), NO_3^- (NarB 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) because of 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 *Synechoccocus* sp. (Kudo & Harrison, 1997); NH_4^+-supported growth of species cyanobacterium *Trichodesmium* IMS 101 (Kustka *et al*., 2003b) and *Synechoccocus* sp. (Kudo & Harrison, 1997). (b) NO_3^- and NH_4^+-supported growth of marine diatoms *Thalassiosira pseudonana* and *T. weissfloggi* (Maldonado & Price, 1996). (C) Intracellular Fe versus 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 number 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 cyanobacterium adaptation to the Precambrian ocean, where Fe levels were higher.

support N_2 fixation and eukaryotic NO_3^- assimilation. Better understanding of metal concentrations through time, coupled with laboratory experiments to explore the physiological changes that organisms experience at representative metal concentrations, is 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; Shen *et al*., 2001; Habicht *et al*., 2002; Farquhar *et al*., 2003; Johnston *et al*., 2005). That precedent should serve as a model for further study of the evolutionary consequences of changing metal abundances.

In the succeeding discussion, 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 & Knoll, 2002; Rouxel *et al*., 2005; Anbar *et al*., 2007; Scott *et al*., 2008), 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 (Konhauser *et al*., in press). Theoretical arguments suggest that Cu was lowest during the Proterozoic (Saito *et al*., 2003; Zerkle *et al*., 2005; Buick, 2007). Future geochemical studies and data compilations are needed to explore these and other such possibilities.

Further work is also required to gain greater knowledge of 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 & Robert, 1999); more efforts are needed as analytical abilities advance. Previous studies have explored how marine inorganic N concentrations varied through ocean history (Beaumont & 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 expressing high-affinity uptake systems to acquire Fe at minute quantities from seawater (Hutchins *et al.*, 1999; Maldonado & Price, 2001; Kraemer *et al.*, 2005). Marine diazotrophic cyanobacteria cycle Fe between nitrogenase and photosynthetic metalloproteins on a diurnal cycle, thus minimizing overall Fe requirements (Berman-Frank *et al.*, 2001; Tuit *et al.*, 2004; Saito *et al.*, 2008). Fe and N are also linked through regulatory networks that coordinate Fe uptake in accordance with requirements for N assimilatory enzymes (Maldonado & Price, 2000; López-Gomollón *et al.*, 2007). Additionally, some cyanobacteria store Fe in ferritin as a hedge against Fe scarcity (Castruita *et al.*, 2006, 2007). Other adaptations involve enzyme substitutions. For example, flavodoxin, an alternative to ferredoxin (see Nitrogen Fixation section) 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; Schemberg *et al.*, 2007). 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 biosysnthesis also requires Cu. This represents an important future research direction, as an additional Cu requirement for NO_3^- 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 versus eukaryotes. Studies of intracellular Mo, Ni and Cu demand and partitioning are just beginning. Intracellular Mo has been shown to be higher in N_2-fixing versus NO_3^--grown cyanobacteria (Tuit *et al.*, 2004). Early studies showed that several species of algae had an absolute requirement for Mo when grown on NO_3^- as the sole N source (Walker, 1953; Loneragan & Arnon, 1954; Arnon *et al.*, 1955; Wallen & Cartier, 1975). Future studies could investigate the Mo requirements for NO_3^- assimilation for eukaryotes compared with 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 algae could be measured in order to ascertain whether such low Cu concentrations could have limited N assimilation from amines 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 (Raymond & Segre, 2006). 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.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Supplementary Text Figure 2. Detail of the construction of Figure 2 and of model organism species names.

Supplementary Text Figure 6. Details of the growth conditions of the experiment shown in Figure 6.

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