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The evolution of Earth's biogeochemical nitrogen cycle

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Abstract

Nitrogen is an essential nutrient for all life on Earth and it acts as a major control on biological productivity in the modern ocean. Accurate reconstructions of the evolution of life over the course of the last four billion years therefore demand a better understanding of nitrogen bioavailability and speciation through time. The biogeochemical nitrogen cycle has evidently been closely tied to the redox state of the ocean and atmosphere. Multiple lines of evidence indicate that the Earth's surface has passed in a non-linear fashion from an anoxic state in the Hadean to an oxic state in the later Phanerozoic. It is therefore likely that the nitrogen cycle has changed markedly over time, with potentially severe implications for the productivity and evolution of the biosphere. Here we compile nitrogen isotope data from the literature and review our current understanding of the evolution of the nitrogen cycle, with particular emphasis on the Precambrian. Combined with recent work on redox conditions, trace metal availability, sulfur and iron cycling on the early Earth, we then use the nitrogen isotope record as a platform to test existing and new hypotheses about biogeochemical pathways that may have controlled nitrogen availability through time. Among other things, we conclude that (a) abiotic nitrogen sources were likely insufficient to sustain a large biosphere, thus favoring an early origin of biological N₂ fixation, (b) evidence of nitrate in the Neoproterozoic and Paleoproterozoic confirm current views of increasing surface oxygen levels at those times, (c) abundant ferrous iron and sulfide in the mid-Precambrian ocean may have affected the speciation and size of the fixed nitrogen reservoir, and (d) nitrate availability alone was not a major driver of eukaryotic evolution.

Keywords

nitrogen cycle; nitrogen isotopes; Precambrian; evolution

1. Introduction

Nitrogen is a major nutrient that is essential to all life on Earth and it is likely that this is no accident of chemical evolution, for at least three reasons. First, it is an abundant element in the cosmos and in Earth's atmosphere (Henry et al., 2000; Marty, 2012); second, it forms versatile covalent bonds with carbon that are integral to the functioning of organic biomolecules; and third, nitrogen is redox-active in the stability field of liquid water and is thus a potent source

of electrochemical energy for biological metabolism (Schoepp-Cothenet et al., 2012). A better understanding of what constrained the evolution of life on Earth therefore demands a reconstruction of the biogeochemical nitrogen cycle over time; in particular its role as a limiting nutrient affecting biological evolution and ecology (Anbar and Knoll, 2002).

Evolution of the nitrogen cycle may also have had significant effects on the continued habitability of the Earth. The partial pressure of nitrogen gas (N_2) in the atmosphere controls the degree of pressure-broadening of greenhouse gas adsorption and thus surface temperature (Goldblatt et al., 2009). Moreover, some nitrogenous gaseous species are powerful greenhouse gases themselves, such as nitrous oxide (N_2O) and nitrogen dioxide (NO_2) (Byrne and Goldblatt, 2014). These gases can also affect climate indirectly by their role in the formation of ozone (O_3), which is a powerful greenhouse agent in the troposphere. Ammonia (NH_3) is also a potent greenhouse gas but is often neglected due to its short atmospheric residence time resulting from rapid photolytic destruction. However, if NH_3 flux rates to the atmosphere are high, then this photolysis barrier can be overcome, with a resulting effect on surface temperatures (Kuhn and Atreya, 1979). Lastly, nitrogenous gas species can react with carbonaceous compounds to form aerosol hazes such as photochemical smog or organic particulates like the tholins of Titan. These have an anti-greenhouse effect and cool temperatures by physically blocking solar insolation.

The redox state of Earth's surface plays an important role in the biogeochemical nitrogen cycle because nitrogen is highly redox-active. Nitrate (NO_3^-), the most oxidized nitrogen species (N^{+V}), is stable under oxic conditions. Denitrification, *i.e.* reduction of nitrate to more reduced forms including N_2 (N^0), N_2O (N^{+I}) or NH_4^+ (N^{+III}), usually occurs at a higher Eh than reduction of ferric iron and sulfite (Fig. 1). The onset of widespread nitrification – the oxidation of NH_4^+ to NO_3^- – is thus plausibly linked to the appearance of oxygenic photosynthesis and the accumulation of O_2 in atmosphere and oceans. The strong redox activity further suggests that the nitrogen cycle may have been linked to iron and sulfur cycles. In their reduced form, both Fe and S could have acted as electron donors during denitrification, whereas the oxidized species may have played a role in nitrogen remineralization, but the linkages are so far unclear. Furthermore, it has been hypothesized that extensive euxinia (anoxia with dissolved HS^-) may have lowered the concentration of transition metals such as Mo or Cu in the Precambrian ocean (Anbar and Knoll, 2002; Buick, 2007a). Many of these elements are essential cofactors in the nitrogen cycle and their absence could have limited nitrogen bioavailability.

Over recent years, it has become apparent that the nitrogen biogeochemical cycle has changed markedly over geological time, especially during the Archean and Proterozoic (e.g. Ader et al., 2014; Garvin et al., 2009; Godfrey and Falkowski, 2009; Koehler et al., in review; Stüeken, 2013; Stüeken et al., 2015b; Stüeken et al., 2015c). These changes apparently occurred partly in response to biological evolution and partly as a result of varying environmental conditions, particularly the redox state of the ocean and atmosphere. In this paper, we will review these key events in nitrogen-cycle evolution, concentrating on the Precambrian when the most radical changes occurred. Based on inferred trends in the evolution of the nitrogen cycle, we will revisit key questions about abiotic and biotic nitrogen fixation, nitrogen speciation in seawater and the relationship between nitrogen availability and the evolution of eukaryotes.

2. Nitrogen and redox

2.1. Current working model of Earth's redox evolution

Nitrogen is redox-sensitive in the stability field of liquid water, and therefore the oxygenation of Earth's atmosphere and oceans has played a major role in the evolution of the global biogeochemical nitrogen cycle. Constraining the history of oxygen production and accumulation in surface environments is thus required to arrive at accurate reconstructions of nitrogen speciation and bioavailability through time. The oldest possible signs of oxygenic photosynthesis date back to 3.8 Gyr (reviewed in Buick, 2008). Recent work proposes a minimum age of 3.0 Gyr (Crowe et al., 2013; Planavsky et al., 2014a). However, prior to ~2.3 Gyr, atmospheric O₂ did likely not accumulate to more than 10⁻⁵ times present atmospheric levels (PAL), *i.e.* the threshold for mass-independent fractionation (MIF) in sulfur isotopes (Pavlov and Kasting, 2002), and it may even have been significantly lower (Kurzweil et al., 2013). Before 2.75 Ga, oxidative weathering is thought to have been trivial on a global scale (Stüeken et al., 2012) and, if present, restricted to local areas where cyanobacteria may have been thriving (Crowe et al., 2013; Lalonde and Konhauser, 2015). There are no documented occurrences of euxinia in the Paleo- and Meso-Archean, suggesting that marine sulfate concentrations were very low, possibly <0.2 mM (Habicht et al., 2002). This may have changed around 2.75 Gyr when multiple lines of evidence, including elevated Mo, sulfide and organic carbon concentrations, and isotopic fractionations suggest that terrestrial and shallow marine environments became increasingly oxygenated (Eigenbrode and Freeman, 2006; Gregory et al., 2015; Kendall et al., 2010; Kurzweil et al., 2013; Kurzweil et al., 2015; Siebert et al., 2005; Wille et al., 2007; Zerkle et al., 2012). The deep ocean probably continued to be anoxic in the late Archean, but euxinia developed along some continental margins, at least temporarily (e.g. Reinhard et al., 2009; Scott et al., 2011). Oxidative weathering of the continents may have become more important (Stüeken et al., 2012), particularly during temporary and possibly local increases of free O₂ in surface environments as indicated by geochemical evidence for pulses of enhanced oxidative weathering (Anbar et al., 2007; Kendall et al., 2015a; Stüeken et al., 2015a). Such oxidative weathering pulses can be reconciled with the persistence of sulfur MIF throughout this time, because oxygen production and weathering may have been restricted to microbial mats on land while the atmosphere stayed anoxic (Lalonde and Konhauser, 2015). This model is also consistent with the record of redox-sensitive minerals that rule out oxygen accumulation in river waters (England et al., 2002; Johnson et al., 2014; Rasmussen and Buick, 1999). Furthermore, the MIF signature can be reworked from older sediments, which may thus overprint intervals where MIF production was subdued (Reinhard et al., 2013a).

The disappearance of MIF in the early Paleoproterozoic rock record, around 2.4-2.3 Gyr, combined with photochemical models, suggests that atmospheric *p*O₂ rose permanently above 10⁻⁵ PAL, quenching sulfur MIF production and causing the "Great Oxidation Event" (GOE) (Bekker et al., 2004; Luo et al., 2016; Pavlov and Kasting, 2002). It has been proposed that oxygen levels may even have approached modern levels during a brief "O₂ overshoot" between 2.3 Gyr and 2.05 Gyr and then declined again afterwards (Bekker and Holland, 2012; Partin et al., 2013). According to models based on Cr isotopes, oxygen may have been as low as 10⁻³ PAL during the Mesoproterozoic (Planavsky et al., 2014b). Sulfur isotopes and evaporites indicate that marine sulfate concentrations probably tracked *p*O₂ with a peak during the Paleoproterozoic (Planavsky et al., 2012; Schröder et al., 2008), followed by a minimum between ~1.7 Ga and ~1.0 Ga (Kah et al., 2004; Luo et al., 2014b; Scott et al., 2014). The Mesoproterozoic ocean was likely ferruginous at depth, but euxinia apparently prevailed along continental margins (Arnold et al., 2004; Planavsky et al., 2011; Sperling et al., 2015). Atmospheric *p*O₂ probably reached near-modern levels during the "Neoproterozoic Oxygenation Event" (NOE) between 0.8 Gyr and

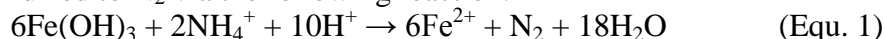
0.65 Gyr (Kah and Bartley, 2011; Och and Shields-Zhou, 2012; Planavsky et al., 2014b), but the deep ocean may not have become permanently oxygenated until the rise of land plants in the Devonian (Dahl et al., 2010; Sperling et al., 2015; but see Chen et al., 2015, for an alternative view).

2.2. Nitrogen as a redox proxy

Nitrogen undergoes redox transformations at moderately high Eh levels, which makes it a useful proxy for oxygen abundances. Nitrate has a higher redox potential than ferric iron and sulfate, *i.e.* it is stable at higher Eh except under acidic conditions and extremely low Fe^{2+} concentration. (**Fig. 1**). Hence sulfate and Fe^{3+} are usually not strong enough oxidizers to produce nitrate or nitrite from organic-bound NH_4^+ . Iron oxides can only oxidize NH_4^+ to NO_2^- or NO_3^- at pH 6-8 if NH_4^+ concentrations are at least in the 100 μM range at vanishingly low NO_2^- (<nM) while Fe^{2+} levels are less than a few nM (**Fig. 2a**), which is unrealistic for the iron-rich Precambrian ocean (**Section 2.3**). Photosynthetic oxidation of NH_4^+ is unknown; the only photosynthetic pathway in the nitrogen cycle that has so far been detected oxidizes NO_2^- to NO_3^- (Griffin et al., 2007). Hence free O_2 appears to be a prerequisite for the production of significant amounts of nitrate under neutral to alkaline conditions. MnO_2 may be the only alternative oxidizer that can take NH_4^+ to NO_3^- (as well as to NO_2^- and N_2) over a wide pH range (Hulth et al., 1999; Luther et al., 1997), but unlike ferric iron, which can be produced by anoxygenic photosynthesizers, MnO_2 requires O_2 itself, and it is not clear if Mn-driven ammonium oxidation actually occurs in marine settings (Thamdrup and Dalsgaard, 2000). The inference of nitrification at any time in Earth's history therefore implies the presence of at least some appreciable amount of O_2 in surface waters. A quantitative estimate is difficult, but it needs to be high enough that the amount of nitrate that is produced can become a major nitrogen source. Otherwise it would not be detectable in the $\delta^{15}\text{N}$ record (**Section 3.1**). For illustration, the maximum nitrate concentration in the Black Sea, where nitrate is too scarce to leave an isotopic signature (Fulton et al., 2012), is roughly 3.5 μM (Fuchsman and Murray, 2008), whereas the open ocean has concentrations up to around 35 μM with nitrate being the major nitrogen source (Sverdrup et al., 1942). The threshold may lie somewhere between these two bounds, but it is probably variable, depending on productivity and the relative abundances of prokaryotes and eukaryotes.

2.3. Effects of ferruginous conditions on the nitrogen cycle

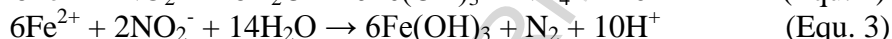
A characteristic feature of most of the Precambrian ocean is the abundance of dissolved ferrous iron (Fe^{2+}) in the deep sea (Poulton and Canfield, 2011). Canfield (2005) estimated concentrations of 40-120 μM in the Archean compared to <1nM today (Moore and Braucher, 2007), which raises the question about potential effects on the nitrogen cycle. Possible routes include (a) oxidation of NH_4^+ by Fe^{3+} , and (b) reduction of NO_2^- or NO_3^- by Fe^{2+} . As noted above, iron-driven NH_4^+ oxidation to NO_2^- or NO_3^- is thermodynamically infeasible under realistic marine conditions (pH ~ 6-8, Grotzinger and Kasting, 1993) with μM amounts of Fe^{2+} . However, NH_4^+ can be oxidized to N_2 via the following reaction:



Between pH 6 and 8, ΔG_r is negative, *i.e.* the reaction is favored, if $[\text{Fe}^{2+}]$ is 100 μM or less and $[\text{NH}_4^+]$ is 1 μM or higher (**Fig. 2b,c**). For comparison, the Black Sea has up to 100 μM NH_4^+ at

depth (Brewer and Murray, 1973). Given the abundance of iron-oxide-bearing banded iron formation in the Archean and Paleoproterozoic (Isley and Abbott, 1999), this mechanism could have constituted a significant sink of nitrogen from the ocean and provided a nitrogen flux back to the atmosphere prior to the widespread appearance of denitrification in the Paleoproterozoic (**Section 4.4**).

The reduction of NO_2^- or NO_3^- by Fe^{2+} could also have been important, following either one of the two following reactions:



Between pH 6-8 and with 1-100 μM [Fe^{2+}] and 0.8 bar N_2 , both reactions are thermodynamically feasible ($\Delta G_r < -150$ kJ/mol), even with an unrealistic amount of 1 M [NH_4^+] and as little as 1 nM [NO_2^-]. The overall conclusion does not change if NO_3^- is used instead of NO_2^- . Importantly, ΔG_r is always significantly more negative if N_2 rather than NH_4^+ is the end-product. Hence reaction 3 would usually be favored. Both reactions can be catalyzed by chemolithoautotrophic microbes over a wide range of temperatures, but the reduction to NH_4^+ seems to be less common (Weber et al., 2006). Under ambient temperatures, microbial catalysis greatly accelerates nitrite and nitrate reduction (Weber et al., 2001), but it could occur abiotically under hydrothermal conditions (Brandes et al., 1998; Summers and Chang, 1993). As discussed in **Section 5.1**, this pathway could have lessened the supply of lightning-derived NO_x to the earliest biosphere or prebiotic reaction networks, because a significant fraction of NO_x would likely have been returned to the atmosphere as N_2 gas instead of being converted into the more bioavailable form NH_4^+ .

Another implication of denitrification with ferrous iron is that nitrate and nitrite concentrations must have been negligible in the Precambrian deep ocean. Euxinic zones would have also excluded nitrate from the water column, because like Fe^{2+} , HS^- and other reduced sulfur compounds are powerful nitrate reductants (Shao et al., 2010). The same is true for Mn^{2+} (Luther et al., 1997), H_2 (Karanasios et al., 2010) and CH_4 (Raghoebarsing et al., 2006).

2.4. Euxinia and nitrogen

The observations of sulfide-driven denitrification (Section 2.1, Shao et al., 2010), combined with the significantly higher metabolic energy gain from nitrate reduction compared to sulfate reduction (Froelich et al., 1979) has led to the hypothesis that euxinia and nitrate are mutually exclusive (Boyle et al., 2013). In other words, one should never see evidence of both in the same sample. Where euxinia is observed, N_2 -fixation should have been the dominant mode of nitrogen acquisition. At first glance, this hypothesis is not born out by existing data, because there are numerous high $\delta^{15}\text{N}$ values reflecting nitrate uptake (**Section 3.1**) in euxinic black shales from Archean and Proterozoic basins; nitrogen isotopes are not correlated with pyritization of reactive iron, which usually increases with intensification of euxinia (**Fig. 3**). This observation can perhaps be reconciled with thermodynamics if the water column in these basins was strongly stratified. Iron speciation, which is the most commonly used proxy for detecting euxinia (Poulton and Canfield, 2005; Raiswell et al., 1988), primarily reflects bottom water conditions close to the sediment-water interface; it is not directly sensitive to processes in the surface ocean. The dominant mode of nitrogen metabolism, on the other hand, is controlled by regions of highest productivity, which is usually the photic zone. Biomass produced in the photic zone will eventually sink and carry its nitrogen isotopic signature to the sediments. Hence the

combination of the two proxies – $\delta^{15}\text{N}$ and iron speciation – may in fact be a powerful tool to reconstruct gradients from oxic surface waters to euxinic or ferruginous bottom waters. If both nitrate and euxinia are reflected in sediments, then it may imply that the chemocline did not extend up into the photic zone. Biomarker evidence may be used to test this hypothesis (e.g. Brocks et al., 2005).

3. The nitrogen isotope proxy in deep time

Stable nitrogen isotopes (99.6337% ^{14}N and 0.3663% ^{15}N) are the most widely used tool for reconstructing nitrogen cycling in ancient environments, because they are fractionated by several permil during many biogeochemical reactions (**Fig. 4**) (Casciotti, 2009; Robinson, 2001). Atmospheric N_2 , the largest surface reservoir of nitrogen, is used as a reference standard and therefore has a $\delta^{15}\text{N}$ value of 0‰, where $\delta^{15}\text{N} = [({}^{15}\text{N}/{}^{14}\text{N})_{\text{sample}}/({}^{15}\text{N}/{}^{14}\text{N})_{\text{air}} - 1] \cdot 1000$. In the following, fractionations will be defined as $\varepsilon \approx \delta^{15}\text{N}_{\text{product}} - \delta^{15}\text{N}_{\text{reactant}}$, *i.e.* positive values indicate that the product is isotopically heavier.

3.1. A primer on nitrogen isotopes in geological samples

Biological nitrogen fixation (diazotrophy; reduction of N_2 to NH_4^+ , **Table 1**) with the most common Mo-based nitrogenase enzyme imparts a small fractionation of -1‰ on average with a range from -2‰ to +1‰, except under Fe^{2+} rich conditions or in thermophilic cultures where it can be as large as -4‰ (Nishizawa et al., 2014; Zerkle et al., 2008; Zhang et al., 2014). Alternative nitrogenases containing V or Fe instead of Mo are less efficient and impart fractionations of -6‰ to -8‰ (Zhang et al., 2014); there is so far no evidence of biological N_2 fixation by alternative nitrogenases in the Precambrian $\delta^{15}\text{N}$ record (**Section 4**). Diagenetic remineralization of organic-bound ammonium to dissolved NH_4^+ renders residual biomass isotopically heavier by 1.4-2.3‰ under oxic conditions (Freudenthal et al., 2001; Lehman et al., 2002; Moebius, 2013), but under anoxic conditions the effect is probably much smaller and maybe even reversed if light nitrogen is added by anaerobic diazotrophs (Lehman et al., 2002; Robinson et al., 2012) (**Section 3.2**). In an anoxic environment where fixed nitrogen is scarce and Mo-based nitrogen fixation is the major mode of nitrogen acquisition, one would thus expect $\delta^{15}\text{N}$ values between -2‰ and +1‰ in carbonaceous sediments. The Black Sea is a modern example (Fulton et al., 2012); anoxic events in the Cretaceous oceans with values down to -7‰ may represent cases where alternative nitrogenases were dominant (Zhang et al., 2014).

The NH_4^+ that is released during organic matter degradation can accumulate in anoxic waters such as the modern Black Sea (Fuchsman and Murray, 2008) and serve as a nitrogen source. Non-quantitative NH_4^+ assimilation imparts a fractionation of up to -27‰ when $[\text{NH}_4^+] > 20 \mu\text{M}$, but the fractionation decreases to -4‰ at lower concentrations (Hoch et al., 1992; Pennock et al., 1996; Waser et al., 1998). Under NH_4^+ replete conditions one may thus expect light biomass in underlying sediments (*i.e.* organisms that assimilated a small proportion of the available NH_4^+ pool) and a complementary heavy reservoir in sediments elsewhere, representing uptake of the residual heavy dissolved NH_4^+ . There is no known modern example of this scenario, because in the case of the Black Sea, the dissolved NH_4^+ does not reach far into the photic zone where most productivity occurs (Fuchsman and Murray, 2008). However, this mechanism has

been invoked for Cretaceous anoxic events (Higgins et al., 2012) and the late Paleoproterozoic (Papineau et al., 2009).

In the presence of O₂, NH₄⁺ is rapidly re-oxidized ('nitrified') to NO₂⁻ ($\epsilon = +14\text{‰}$ to $+38\text{‰}$, Casciotti et al., 2003) and subsequently to NO₃⁻ ($\epsilon = -13\text{‰}$, Casciotti, 2009). In sum, the resulting NO₃⁻ is thus isotopically lighter than the original biomass. Nitrification is rapid and has been observed at nM O₂ levels in marine oxygen minimum zones (Füssel et al., 2012; Thamdrup et al., 2012). One would thus expect it to go to completion in most cases without leaving an isotopic trace in the geologic record. However, incomplete nitrification has been reported from sediments in the Bering Sea where biomass becomes increasingly heavy from assimilation of the residual NH₄⁺ (Granger et al., 2011; Morales et al., 2014). The light NO₂⁻ was lost by subsequent denitrification. It is unlikely that this mechanism is important on a global scale, because it lacks an output channel of heavy nitrogen from seawater and would thus throw the marine nitrogen cycle out of balance, counter to what is observed (Sigman et al., 2009). But it could presumably occur in individual basins, as proposed for late Archean lakes (Thomazo et al., 2011). It may be recognizable by a large scatter in sedimentary $\delta^{15}\text{N}$ data as in the modern Bering Sea where organic N ranges from $+2\text{‰}$ to $+18\text{‰}$ (Morales et al., 2014). This scatter may result from fluctuations between complete and incomplete nitrification over short timescales.

When nitrification goes to completion, the resulting NO₃⁻ becomes a major source of nitrogen for aerobic organisms. Fractionations associated with NO₃⁻ assimilation are often not expressed because assimilation typically goes to completion (Altabet and Francois, 1994; Thunell et al., 2004), except in modern Arctic settings (Somes et al., 2010). Under oxic conditions, marine sediments therefore approach the isotopic composition of NO₃⁻ in the overlying water column (Altabet and Francois, 1994; Thunell et al., 2004). This value is on average $+5\text{‰}$ in the modern ocean (range mostly between $+3\text{‰}$ and $+14\text{‰}$), which is largely a result of isotopic fractionations imparted by denitrification. According to the canonical view, roughly 25% of all NO₃⁻ is denitrified to N₂ in oxygen minimum zones with an average fractionation of -25‰ (range -5‰ to -30‰) and $\sim 75\%$ is denitrified in sediments with net fractionations of 0‰ (Devol, 2015; Sigman et al., 2009). Water-column denitrification thus returns isotopically light N₂ to the atmosphere ($\delta^{15}\text{N}_{\text{N}_2} \sim -20\text{‰}$), pushing residual NO₃⁻ up to $+5\text{‰}$ (**Fig. 5**). Sedimentary denitrification acting on this residual heavy NO₃⁻ pool then returns heavy N₂ to the atmosphere ($\delta^{15}\text{N}_{\text{N}_2} \sim +5\text{‰}$) because it does not impart any additional fractionation. More recent work suggests that sedimentary denitrification imparts a net fractionation of -3‰ (Kessler et al., 2014; Rooze and Meile, 2016), *i.e.* producing N₂ gas with $\delta^{15}\text{N}_{\text{N}_2} = +2\text{‰}$, while water-column denitrification only has a net fractionation of -12.5‰ ($\delta^{15}\text{N}_{\text{N}_2} = -7.5\text{‰}$) (Kritee et al., 2012; Rooze and Meile, 2016). In that case, the mass balance of the two processes would be 68% to 32%. Some denitrification may also be taken up by anammox (**Section 3.2.1**). In both models, the two return pathways lead to an isotopically balanced system. Deposition of biomass constitutes another heavy sink ($\delta^{15}\text{N}_{\text{org}} \sim +5\text{‰}$), but it is comparatively minor under oxic conditions and has probably been balanced by volcanism and/or weathering of sedimentary rocks over geologic timescales (Berner, 2006). Unaltered ancient marine sediments that contain organic matter and fall within the modern marine range of $+3$ to $+14\text{‰}$ are usually interpreted as reflecting an aerobic nitrogen cycle as in today's ocean (see Algeo *et al.*, 2014, and Stüeken *et al.*, 2015, for compilations of references). The absolute value will depend on the ratio of water-column to sedimentary denitrification, the net fractionation associated with water-column denitrification, contributions of anammox and DNRA (**Section 3.2.2**), and possible admixtures of diazotrophic biomass with a composition around -1‰ .

Independent constraints on those parameters are usually lacking, and so elevated $\delta^{15}\text{N}$ values are strictly speaking a qualitative rather than a quantitative proxy for aerobic nitrogen cycling.

3.2. The role of minor nitrogen pathways

The following pathways are less well explored, but may have been important in some environments or certain time periods.

3.2.1. Anaerobic ammonium oxidation (anammox)

Anaerobic ammonium oxidation coupled to nitrite reduction (abbreviated anammox) was only recognized relatively recently (Mulder et al., 1995; Van de Graaf et al., 1995), but it appears to play a significant role in nitrogen cycling in modern oxygen minimum zones (Kalvelage et al., 2013; Lam et al., 2009). This metabolism converts nitrite and ammonium to N_2 via a hydrazine (N_2H_4) intermediate. At present, it is unclear whether the essential nitrogen transforming enzymes utilize iron or copper cofactors. Phylogenetic analysis has identified both iron and copper binding domains in the genome of anammox bacteria (Ali et al., 2015), and copper limitation of anammox activity has been observed in the field (Jacquot et al., 2013). The evolutionary history of anammox is also poorly known. Since it has only been documented from a single bacterial phylum (Planctomycetes), it may be a recent evolutionary invention (Strous et al., 1999; Strous et al., 2006) (but see Brochier and Philippe, 2002, for an alternative view). However, the ingredients for anammox – ammonium and nitrite – may have existed in appreciable abundances in parts of the ocean since the late Archean, especially at times when the ocean was stratified (**Sections 2.1, 2.4, 4.3**). Unfortunately, the nitrogen isotopic fractionations imparted by anammox bacteria are too similar to those of canonical denitrifiers to distinguishing between the two metabolisms in the geologic record (Brunner et al., 2013).

3.2.2. Dissimilatory nitrate reduction to ammonium (DNRA)

There are additional biotic nitrogen transformation pathways that may play important biogeochemical roles, but at the moment are poorly understood. One is the dissimilatory nitrate reduction to ammonium (DNRA). All denitrifiers are capable of reducing nitrate as the first step of canonical denitrification, but denitrification continues toward an end product of N_2 gas while DNRA produces ammonium. This process has long been known to occur in sediments (Sørensen, 1978), and it has recently been observed in the water column of the Namibian oxygen minimum zone (Kartal et al., 2007). In the water column, DNRA may provide up to 100% of the ammonium utilized by anammox bacteria (Kartal et al., 2007; Lam et al., 2009), while in some coastal settings, DNRA is thought to be responsible for ~30% of all nitrate reduction (Giblin et al., 2013). It is therefore an important biogeochemical process.

The isotopic fractionation associated with DNRA has not yet been studied systematically, but may be on the order of -30‰ with the residual nitrate becoming heavier (McCready et al., 1983). If so, then DNRA may be difficult to distinguish from denitrification, unless evidence of both the light ammonium and the heavy nitrate are preserved separately in different samples from the same basin. So far, no dataset exists where this is the case. Establishing the importance of DNRA in the Precambrian is important, because unlike denitrification to N_2 , DNRA retains all

nitrogen in the marine ecosystem, provided that the resulting NH_4^+ is not subsequently consumed by anammox.

3.2.3. NH_3 volatilization

Large positive fractionations can also occur under anoxic conditions if the pH of the water column is highly alkaline. At standard conditions, dissolved NH_4^+ dissociates to NH_3 with a pKa of 9.25 (*i.e.* at pH 9.25, both species exist at equal abundances), and NH_3 can be lost by volatilization with a fractionation of -45‰ (Li et al., 2012). The residual heavy NH_4^+ can be preserved in biomass. This mechanism has been reported from modern and ancient alkaline lakes (Stüeken et al., 2015c; Talbot and Johannessen, 1992).

3.3. Preservation of nitrogen isotopes in the rock record

A major challenge in nitrogen isotope studies of ancient rocks is the potential for diagenetic and metamorphic alteration (reviewed by Ader et al., 2016; Robinson et al., 2012). Diagenetic effects differ markedly between anoxic and oxic depositional environments. Under oxic diagenetic conditions in both natural and laboratory settings, $\delta^{15}\text{N}$ values of bulk sediments can increase by up to ~4‰, especially when sedimentation rates are low and the exposure to oxygen is relatively long (e.g. Altabet et al., 1999; Freudenthal et al., 2001; Lehman et al., 2002). This increase is likely due to isotopic fractionations during NH_4^+ release and partial oxidation in pore waters (Moebius, 2013; Prokopenko et al., 2006). In contrast, diagenesis under anoxic conditions, or under oxic conditions with high sedimentation rates, only imparts small isotopic fractionations of <1‰, making bulk sedimentary nitrogen slightly lighter (Altabet et al., 1999; Lehman et al., 2002; Thunell et al., 2004). Anoxic diagenesis therefore has minimal effect on the utility of the $\delta^{15}\text{N}$ proxy in deep time. Importantly, this is despite the fact more than half of the organic nitrogen can be converted to clay-bound NH_4^+ within the sediments (Müller, 1977; Rosenfeld, 1979; Schroeder and McLain, 1998). The conclusion that the effects of anoxic alteration are small is further supported by the preservation of consistent $\delta^{15}\text{N}$ offsets between biomarkers and bulk sediments from redox-stratified basins (Higgins et al., 2010; Higgins et al., 2012; Sachs and Repeta, 1999). Overall, light $\delta^{15}\text{N}$ values (<+2‰) consistent with anaerobic nitrogen cycling are likely close to primary compositions, whereas positive $\delta^{15}\text{N}$ values (>+2‰), which are commonly interpreted as evidence of aerobic nitrogen cycling, may have been altered and elevated during oxic diagenesis. This caveat prohibits quantitative interpretations of positive $\delta^{15}\text{N}$ values from ancient oxic settings. Nevertheless, these trends still imply that positive $\delta^{15}\text{N}$ values can be interpreted as evidence of oxic conditions, favoring nitrification of NH_4^+ to NO_3^- , either in pore waters or in the water column. Even though it is not possible to use absolute $\delta^{15}\text{N}$ values from sedimentary rocks to calculate, for example, the degree of nitrate uptake in the photic zone (e.g. Fig. 5b), it remains valid to use them as a qualitative indicator for the stability of NO_3^- in the environment during the time of sediment deposition.

Metamorphism can increase sedimentary nitrogen isotope ratios by preferential volatilization of isotopically light NH_3 or N_2 (Bauersachs et al., 2009; Bebout et al., 1999; Bebout and Fogel, 1992; Boyd and Phillippot, 1998; Haendel et al., 1986; Jia, 2006; Mingram and Bräuer, 2001; Palya et al., 2011). However, the overall effect is variable and not always expressed (Busigny et al., 2003; Plessen et al., 2010; Yui et al., 2009), which prohibits precise corrections. However, the effect appears to be insignificant below greenschist facies (<1‰) and

minor within the greenschist facies (1-2‰) (reviewed by Ader et al., 2016; Thomazo and Papineau, 2013). Rocks from upper greenschist to amphibolite facies or above may be metamorphically altered by several permil and are therefore generally avoided in studies aiming to reconstruct biogeochemical nitrogen cycling in deep time.

There is less consensus regarding which nitrogen phase best preserves primary isotopic ratios. As noted above, more than half of the organic nitrogen can migrate into clay minerals during diagenesis. The residual organic-bound nitrogen phase can be analyzed separately by kerogen extraction, *i.e.* removal of silicate-bound ammonium with hydrofluoric acid (e.g. Godfrey and Falkowski, 2009; Kump et al., 2011; Stüeken et al., 2015b; Stüeken et al., 2015c), and this kerogen-phase is sometimes considered to be a more accurate recorder of primary nitrogen isotope ratios (Godfrey and Falkowski, 2009; Kump et al., 2011). However, experimental work suggests that kerogen can equilibrate isotopically with ammonium from exogenous fluids (Schimmelmann and Lis, 2010). Such fluids may be derived from other proximal organic-rich sediments (Svensen et al., 2008). Indeed, Paleoproterozoic sediments with evidence of metasomatic alteration show large offsets of up to 13‰ between kerogen-bound and silicate-bound nitrogen (Godfrey et al., 2013). Bulk rock $\delta^{15}\text{N}$ is enriched by +4‰ compared to unperturbed rocks from the same basin, while kerogen-bound $\delta^{15}\text{N}$ is depleted by -7‰. Hence metasomatic alteration is overall more pronounced in the kerogen fraction, meaning that kerogen extracts may not necessarily be a high-fidelity proxy for primary compositions. Additional problems can arise from contamination during the extraction process. In some studies, bulk rock C/N ratios of some samples are higher than the C/N ratios of the corresponding kerogen extracts (Godfrey and Falkowski, 2009; Kump et al., 2011), which is physically impossible. In other studies, the reproducibility of $\delta^{15}\text{N}$ analyses in kerogen extracts is unusually poor with sometimes more than 10‰ difference between replicates (Beaumont and Robert, 1999). These issues highlight that additional care must be taken when analyzing and interpreting kerogen-bound nitrogen. Bulk rock $\delta^{15}\text{N}$ values may therefore be the more accurate proxy if metasomatic alteration can be ruled out. Through uptake into clay minerals (Schroeder and McLain, 1998), bulk rocks can retain some of that nitrogen that was lost from organic matter during diagenesis and metamorphism and may therefore provide a more complete record of the primary biomass. Potential contributions of detrital ammonium need to be taken into account. Detrital ammonium addition may be significant in organic-poor siliciclastic sediments and needs to be addressed on a case-by-case basis (e.g. Ader et al., 2014; Busigny et al., 2013; Stüeken et al., 2015c). It is worth noting that there does not appear to be a significant difference in bulk $\delta^{15}\text{N}$ between lithologies (**Appendix 2**), which to first order may further suggest that variable ammonium retention or addition as a function of sample mineralogy does not lead to vast isotopic alteration of bulk rocks. In the following, we will focus on bulk rock $\delta^{15}\text{N}$, noting that more work is needed to settle this debate.

4. The geologic record of Earth's nitrogen cycle

There is a growing body of literature about nitrogen isotopes in ancient sedimentary rocks (**Fig. 6**) that allow us to reconstruct a plausible evolutionary pathway for Earth's biogeochemical nitrogen cycle. This reconstruction gains additional importance since Johnson & Goldblatt (2015) calculated that the continental crust and its sedimentary cover contain roughly half as much nitrogen as the atmosphere with 1-2 orders of magnitude higher concentrations than the mantle. This large nitrogen reservoir was most likely created by biologically-mediated draw-

down of atmospheric N₂. Interest in this question has surged with the development of paleobarometric proxies (Kavanagh and Goldblatt, 2015; Kite et al., 2014; Marty et al., 2013; Som et al., 2016; Som et al., 2012) and evidence for a decrease in N₂ pressure from >0.5 bar at 3.5 Gyr to <0.5 bar at 2.7 Gyr (Goldblatt et al., 2009; Marty et al., 2013; Som et al., 2016). A better understanding of biological nitrogen utilization in the past is therefore necessary to more accurately reconstruct atmospheric evolution.

4.1. Hadean and Paleoarchean (~4.6 – 3.5 Gyr)

There are so far no reported measurements of nitrogen isotope ratios older than ~3.8 Gyr. In rocks between 3.8 Gyr and 3.5 Gyr, negative values down to -4.4‰ (Fig. 6, 7) have been interpreted as representing hydrothermal NH₄⁺ assimilation by thermophilic microbes (Pinti et al., 2001; Pinti et al., 2009), metasomatic alteration (Li et al., 2014; van Zuilen et al., 2005), or biological N₂ fixation with alternative V or Fe nitrogenases (Zhang et al., 2014). Positive values up to +12.2‰ from rocks of similar age are most commonly interpreted as reflecting metamorphic overprinting, which is known to increase δ¹⁵N (Papineau et al., 2005; Thomazo and Papineau, 2013). The entire range of values could in theory also result from biological processes (Table 1). However, many of these samples are metamorphosed to amphibolite facies and/or were collected from hydrothermal veins (Beaumont and Robert, 1999; Pinti et al., 2001; Pinti et al., 2009; Ueno et al., 2004). Secondary alteration can therefore not be ruled out at this stage, prohibiting any firm conclusions about the Paleoarchean nitrogen cycle. Ammonium concentrations up to several hundred ppm in metasedimentary biotite grains at ~3.75 Gyr suggest that biological nitrogen consumption had already evolved at this time (Stüeken, in press), but so far no particular metabolism can be inferred from these data. Even less is known about the Hadean where no data exist.

4.2. Mesoarchean (3.5-2.8 Gyr)

While δ¹⁵N data from the Paleoarchean are difficult to interpret due to the wide scatter in individual geological units, the Mesoarchean rock record shows more much more tightly clustered datasets (Fig. 7). The global mean from four different marine sections from 3.3-2.8 Ga is +1.1 ± 1.9‰ (n = 46) (Stüeken et al., 2015b; Yamaguchi, 2002). Most of these data points fall well within the range of Mo-based biological N₂ fixation (-2‰ to +1‰, Zhang et al., 2014), especially if possible metamorphic alteration of rocks at greenschist facies (+2.2 ± 1.9‰, n = 26) is taken into account. If correct, this conclusion would imply that alternative nitrogenases, which use Fe (Anf) or V (Vnf) instead of Mo and which cause larger isotopic fractionations up to -8‰ (Zhang et al., 2014), were either insignificant or had not yet evolved. Preferential Mo utilization would contradict the canonical view that Mesoarchean seawater was rich in ferrous iron but low in molybdenum (Anbar, 2008; Poulton and Canfield, 2011) (Section 5.3). However, molecular phylogenies suggest that Anf and Vnf are in fact more recent inventions, because they are only found in very few organisms that also possess and preferentially produce Nif (Boyd and Peters, 2013; McGlynn et al., 2012). Furthermore, Mo isotope data from 3.0 Gyr iron formations suggest appreciable levels of mobile molybdate ions in seawater (Planavsky et al., 2014a). Sufficient molybdenum concentrations in seawater could have been sustained by either localized oxidative weathering (Crowe et al., 2013; Guy et al., 2012; Planavsky et al., 2014a) or by low-

temperature hydrothermal fluids which may contribute ~10% of the Mo flux into the modern ocean (McManus et al., 2002).

As is true for most of Earth's history, only a few well-preserved rocks are available from the Mesoarchean. It is therefore impossible to know if biological Mo-based N₂ fixation occurred globally, or if other reactions were more important in settings that are not represented in current sample sets. However, the data do at least suggest that the nitrogenase enzyme evolved prior to 3.2 Gyr (Stüeken et al., 2015b). Furthermore, the inference of an anaerobic nitrogen cycle at this time is consistent with current views of Archean redox states (**Section 2.1**).

4.3. Neoarchean (2.8-2.5 Gyr)

The Neoarchean rock record contains the first $\delta^{15}\text{N}_{\text{bulk}}$ values that are elevated above the N₂-fixation range and are likely primary biologic signals rather than metamorphic or diagenetic overprints (**Fig. 6, 7**). Around 2.7 Gyr, marine $\delta^{15}\text{N}_{\text{bulk}}$ values from continental shelf settings rise from a Mesoarchean mean of $+1.1\text{‰} \pm 1.9\text{‰}$ (Stüeken et al., 2015b; Yamaguchi, 2002) to a Neoarchean mean of $+4.2\text{‰} \pm 2.0\text{‰}$ ($n = 160$, Busigny et al., 2013; Garvin et al., 2009; Godfrey and Falkowski, 2009; Stüeken et al., 2015c; Yamaguchi, 2002). Enriched $\delta^{15}\text{N}_{\text{bulk}}$ values up to $+7.5\text{‰}$ from the Hamersley Group at 2.5 Gyr are notably transient, *i.e.* restricted to a proposed temporary increase in surface oxygen levels (Anbar et al., 2007; Garvin et al., 2009), suggesting that nitrogen cycling was dynamic in space and time (**Fig. 8b**). While values near 0‰ probably reflect anaerobic nitrogen cycling dominated by Mo-based N₂-fixation, the moderately high values can be interpreted as evidence of an episodic or localized aerobic nitrogen cycle reminiscent of the modern ocean, featuring nitrification, denitrification, and perhaps anammox (Busigny et al., 2013; Garvin et al., 2009; Godfrey and Falkowski, 2009). Models suggest that dissolved oxygen concentrations in marine oxygen oases may have been on the order of 1-10 μM at this time (Olson et al., 2013). This would have been sufficient for aerobic nitrogen metabolisms, which have been observed at $< 1 \mu\text{M}$ O₂ levels in modern oxygen minimum zones and cultures (Füssel et al., 2012; Martens-Habbenha et al., 2009; Thamdrup et al., 2012). The onset of oxidative weathering of the continents would have raised the flux of Mo and other nutrients to the ocean (Lalonde and Konhauser, 2015; Reinhard et al., 2009; Stüeken et al., 2012) and perhaps facilitated nitrogen fixation as well as denitrification because, like nitrogenase, nitrate reductase is a Mo-containing enzyme (Godfrey and Glass, 2011). On the other hand, the expansion of euxinia in the Neoarchean (Reinhard et al., 2009; Scott et al., 2011) could have drawn down marine Mo levels and limited its bioavailability. The balance of these effects is unknown, and may have fluctuated, perhaps on similar timescales to fluctuations in atmospheric redox states (Izon et al., 2015; Zerkle et al., 2012). As noted above, sample limitation prohibits inferences about global nitrogen cycling, and so it is possible that occurrences of aerobic activity were local phenomena. But regardless of the spatial extent, repeated occurrences of positive $\delta^{15}\text{N}$ values coinciding with other indications of temporary oxygenation provide compelling evidence that biological nitrification and denitrification had evolved by ~2.7 Gyr.

The Neoarchean may have marked the first time in Earth's history when the biosphere promoted a significant flux of N₂ back into the atmosphere through coupled nitrification and denitrification. Until the Neoarchean, only minor amounts of nitrogen may have been returned to the atmosphere by dissociation of dissolved NH₄⁺ to volatile NH₃, but this flux would have been small if NH₄⁺ concentrations were low (**Section 5.4**). It has been hypothesized that the onset of nitrification/denitrification could thus have created a deficit of fixed nitrogen in water

column by converting dissolved and organic-bound NH_4^+ to N_2 (Fennel et al., 2005). This deficit may in turn have enhanced biological N_2 -fixation rates. However, given the possible patchiness of these processes in the Neoproterozoic, the overall effect may have been relatively minor until the Paleoproterozoic.

4.4. Paleoproterozoic (2.5-1.6 Gyr)

Compared to the relatively abundant nitrogen data from the preceding Neoproterozoic and the following Mesoproterozoic, data from the Paleoproterozoic are rather scarce. This is unfortunate, because this time interval potentially has the most interesting nitrogen isotope record of all as major changes in the Earth's redox state apparently occurred then. Firstly, the "Great Oxidation Event" when the atmosphere became permanently oxygenated at levels above 10^{-5} times present atmospheric levels occurred early in the era around 2.4-2.3 Gyr (Bekker et al., 2004). This presumably meant that the surface ocean also became permanently oxygenated, potentially allowing an aerobic nitrogen cycle to prevail globally (**Fig. 8c**). This may have been further exacerbated during the proposed "oxygen overshoot" at 2.3-2.05 Gyr (Bekker and Holland, 2012). Furthermore, the expansion of euxinia and the initiation of the "Boring Billion" years of purported biogeochemical stasis around 1.85 Gyr, when atmospheric oxygen levels evidently dropped to levels insufficient to maintain more than a few mM of sulfate in the ocean (Luo et al., 2014b), may have resulted in a range of biogeochemical feedbacks between metal supply and nitrogen availability and speciation. However, the available data are only sufficient for providing hints as to whether these predicted effects on nitrogen cycling actually occurred.

The only Paleoproterozoic nitrogen isotopic data prior to the ~2.35 Gyr Great Oxidation Event come from the 2.48-2.46 Gyr Brockman banded iron formation of Australia (Busigny et al., 2013). These range from +0.4 to +13.4‰, though almost all are between +4 and +10‰. These consistently heavy values were explained as representing either i) a fully anoxic nitrogen cycle with partial assimilation of ammonium, ii) largely anoxic with partial nitrification in oxygen oases or by photoferrothrophic Fe^{3+} , followed by complete denitrification, or iii) an aerobic nitrogen cycle with fractionation controlled by partial denitrification, as today (Busigny et al., 2013). The first alternative requires a complementary reservoir of isotopically light nitrogen, which has so far not been found. Although this option cannot be fully ruled out with the limited number of data points, there are so far no signs in its favor. The second pathway occurs now in non-marine settings but could plausibly have operated in a stratified pre-oxygenation ocean. However, it produces a wide range of heavy fractionations unlike the consistently moderate values observed in the Brockman, and it creates an isotopic imbalance in the ocean if it occurs on a global scale (**Section 3.1**). Thus the third option of aerobic nitrogen cycling, already known to operate in the Neoproterozoic, seems most likely – noting that sample limitation from a wider range of environments precludes greater certainty. If correct, then persistent aerobic cycling commenced shortly before the Great Oxygenation Event.

After atmospheric oxygenation, the hypothesized "oxygen overshoot" during the Lomagundi-Jatuli carbon isotope excursions between 2.22-2.06 Gyr (Bekker and Holland, 2012) should have greatly increased the availability of nitrate and could have enhanced denitrification rates leading to heavier $\delta^{15}\text{N}$ in sediments. Data from the 2.2-2.1 Gyr Ashanti Belt of Ghana (Jia and Kerrich, 2004) support these conjectures, as all samples fall between the notably heavy values of +9 to +13‰. Corroborating $\delta^{15}\text{N}_{\text{bulk}}$ data come from the 2.09-1.98 Gyr upper Zaonaga Formation in the Fennoscandian Shield (Kump et al., 2011) which also reach +7 to +10‰. These

values are as heavy as or heavier than most parts of the modern ocean and are thus also supportive of oxidizing conditions with abundant nitrate in surface waters and vigorous denitrification below in a strong chemocline. This conclusion is further supported by elevated iodate abundances in carbonates of similar age (Hardisty et al., 2014). Iodate has a similar redox potential as nitrate, and so if iodate was thermodynamically stable in surface waters, it is plausible that nitrate was stable as well.

Following the “oxygen overshoot”, phosphoritic shales in the ~2.0 Gyr Aravalli Group, India show a very wide range of $\delta^{15}\text{N}_{\text{bulk}}$ from -5 to +27‰ whereas non-phosphatic shales have a narrower range from +5 to +12‰ (Papineau et al., 2013; Papineau et al., 2009). These generally positive values have been interpreted as reflecting vigorous denitrification under conditions of high productivity nourished by abundant but variable phosphorus levels but with a chemocline above anoxic bottom waters at differing depths. However, the negative values under phosphoritic conditions may result from nitrogen becoming limiting under phosphorus excess, prompting nitrogen fixation to dominate the nitrogen cycle. The extremely heavy values in some phosphoritic samples have been attributed to anaerobic decomposition of organic matter already isotopically heavy and then subsequent assimilation of the liberated ammonium, imparting further enrichment in the heavy isotope in the incorporating biomass (Papineau et al., 2009). If correct, this would be the so far only known site in the Precambrian where dissolved NH_4^+ levels were high enough to result in an isotopic fractionation during assimilation. It is unknown if this would have been a local or global feature at this time.

The onset of widespread euxinic (sulfidic) conditions in the late Paleoproterozoic was marked by further changes in nitrogen cycling. In the 1.88-1.85 Gyr Gunflint and Biwabik Iron Formations of North America, where bottom waters were ferruginous and deposition was exclusively on the outer shelf, variable $\delta^{15}\text{N}$ values range from +2 to +9‰ (Godfrey et al., 2013). The overlying 1.85 – 1.83 Gyr Rove and Virginia Formations, where nearer-shore bottom water was euxinic, have heavier $\delta^{15}\text{N}_{\text{org}}$ values (+5 to +9‰) by an average of +1.5‰ than deeper ferruginous facies (Godfrey et al., 2013). These spatial patterns have been interpreted as representing denitrification of abundant nitrate onshore and a relatively higher contribution of N_2 fixation offshore (Godfrey et al., 2013). Although such a trend has so far only been reported from one basin of late Paleoproterozoic age, it may foreshadow the stronger cross-basinal isotopic gradients evident in the Mesoproterozoic.

4.5. Mesoproterozoic (1.6-0.8 Gyr)

Throughout the Mesoproterozoic, a return to ocean stratification and widespread euxinia may have controlled the nitrogen cycle (Anbar and Knoll, 2002). Canfield (1998) first proposed that from ~1.8 Gyr onwards, mildly oxygenated surface waters overlay a euxinic deep ocean, and that widespread microbial sulfate reduction to sulfide curtailed the deposition of iron oxides on marine shelves. Over the past decade, numerous isotopic and trace metal geochemical data have refined this hypothesis. The newly emerging view is one of a ferruginous deep ocean, euxinic continental margins and oxic surface waters (Planavsky et al., 2011; Shen et al., 2003) with significant spatial and temporal heterogeneity (Gilleaudeau and Kah, 2015; Sperling et al., 2014). Carbon isotopes further suggest that near-shore waters were dominated by aerobic metabolisms, while anaerobic processes became more important with depth (Luo et al., 2014a), especially in restricted epicontinental basins (Gilleaudeau and Kah, 2013a). Existing records of nitrogen isotopes suggest that nitrogen speciation and bioavailability may have mirrored these patterns.

The Mesoproterozoic Belt basin (~1.5 Gyr) shows a facies-dependent trend in $\delta^{15}\text{N}_{\text{bulk}}$ from ~0‰ on average in deep water facies to +3.7‰ in peritidal facies (Stüeken, 2013). This distinct onshore-offshore trend was interpreted to reflect a gradient from aerobic conditions with bioavailable nitrate along oxic basin margins to anaerobic, N_2 -fixation dominated regimes in anoxic waters offshore where nitrate was limiting (**Fig. 8d**). Any nitrate that was produced offshore was likely re-reduced rapidly and quantitatively such that it did not leave a geochemical signature. Transects of the Roper and Bangemall basins of similar age show comparable gradients on the order of ~1.5‰ in the same direction (Koehler et al., in review). An important difference between these basins is that while offshore samples from the Belt basin have $\delta^{15}\text{N}_{\text{bulk}}$ values that consistently fall within the range of nitrogen fixation, some offshore samples from the Roper and Bangemall basins are slightly enriched relative to fractionations solely caused by fixation, suggesting a small degree of available nitrate and partial denitrification. This difference may be linked to basin geometry and the degree of restriction (Koehler et al., in review). However, all three basins show a peak in $\delta^{15}\text{N}_{\text{bulk}}$ values (and hence evidence of nitrate availability) in near-shore depositional facies and a minimum in offshore facies. Furthermore, there is no evidence of Fe- or V-based biological N_2 fixation in the form of light $\delta^{15}\text{N}$ values (-6‰ to -8‰, **Table 1**), suggesting that Mo was bioavailable despite low concentrations in mid-Proterozoic seawater (**Section 5.3**). Of course, three basins cannot capture all heterogeneity in the global ocean at this time, but based on existing data it appears that the Mesoproterozoic ocean may have been much more nitrate-depleted than its Paleo- and Neoproterozoic counterparts. Both atmospheric O_2 and dissolved SO_4^{2-} may have dropped after the Paleoproterozoic (Luo et al., 2014b; Planavsky et al., 2014b), and so a concurrent decline in NO_3^- levels makes intuitive sense.

4.6. Neoproterozoic (1.0-0.5 Gyr)

Investigations of nitrogen isotopes in the Neoproterozoic are relatively scarce, but existing records suggest that in the late Neoproterozoic (~750-550 Myr) nitrogen cycling looked much like it does today (Ader et al., 2014) (**Fig. 8e**). The distributions of nitrogen isotopes in these sedimentary successions analyzed to date converge on a mode of ~+4‰, similar to the modern mode of ~+5‰ (Tesdal et al., 2013), and basinal gradients have so far not been documented (Ader et al., 2014). A negative $\delta^{15}\text{N}$ excursion during the Shuram carbon isotope excursion is exception (Kikumoto et al., 2014). The most parsimonious explanation for this similarity to the modern ocean is that – as today – NO_3^- persisted throughout large parts of the water column and was reduced to N_2 via water column denitrification in local oxygen minimum zones, leaving a positive isotopic signature in residual NO_3^- and resulting biomass. This interpretation implies expansion of oxygenated water masses in the Neoproterozoic. Although various paleoredox proxies indeed point to an increase in atmospheric and oceanic oxygen between ~800 and ~550 Ma (Canfield et al., 2007; Fike et al., 2006; Johnston et al., 2012; Kendall et al., 2015b; Planavsky et al., 2014b; Sahoo et al., 2012), others suggest anoxic conditions in roughly coeval strata (Canfield et al., 2008; Johnston et al., 2013; Schröder and Grotzinger, 2007; Sperling et al., 2015). Hence like the Neoproterozoic, the Neoproterozoic may have been a time of dynamic spatial and temporal redox fluctuations where NO_3^- was not abundant globally at all times. Future studies may reveal more heterogeneity in nitrogen isotopic data than is currently known.

The temporal correlation between oxygenation, a succession of low-latitude glaciations (Hoffman et al., 1998), and the rise of architecturally complex life (Marshall, 2006; Narbonne, 2005) invites many hypotheses proposing cause-and-effect relationships between these events (Butterfield, 2009; Lenton et al., 2014; Sperling et al., 2013), but so far no single compelling story exists. A high-resolution record of nitrogen isotope ratios across Neoproterozoic glaciations is lacking, and so possible linkages between these events and the evolution of the nitrogen cycle are unknown. But if oxic conditions generally expanded during the Neoproterozoic, and if the chemocline receded to deeper waters, then this may mean that the marine nitrogen cycle became balanced (*i.e.* the major fluxes – nitrogen fixation and denitrification – were of roughly the same magnitude). Nitrate or trace metal limitation of denitrification would have been lifted or weakened by the increase in atmospheric and oceanic oxygen.

4.7. Phanerozoic (since ~0.5 Gyr)

A recent compilation of $\delta^{15}\text{N}$ data through the Phanerozoic revealed a surprisingly dynamic picture with values near 0‰ and evidence of basinal stratification (Cremonese et al., 2014; Wang et al., 2013) in the early Paleozoic, a rise to values $> +2\%$ with a maximum of $+8\%$ from the early Carboniferous to the end of the Permian, a return to low values around 0‰ during most of the Mesozoic, and finally positive values around $+4\%$ from the late Cretaceous onwards (Algeo et al., 2014). These long-term Phanerozoic nitrogen isotope trends are usually ascribed to climatic rather than redox fluctuations (Algeo et al., 2014; Altabet, 2007). According to this model, lower isotopic values (-2% to $+2\%$) occurred in greenhouse climates when sea-level was higher, such that the dominant locus of denitrification was ocean sediments rather than the water column. The net fractionation of denitrification would thus have been negligible and seawater nitrate would have had a composition around 0‰ (Algeo *et al.* 2014) (**Fig. 5b**). This hypothesis is supported by the canonical view that the final step in the oxygenation of Earth's surface occurred in the Neoproterozoic (**Section 2.1**) and that therefore the Phanerozoic ocean was fully oxygenated with only brief interruptions during anoxic events that favored a temporary return to anaerobic nitrogen cycling (e.g. Junium and Arthur, 2007; Ohkouchi et al., 2006; Rau et al., 1987; Schoepfer et al., 2012; Sephton et al., 2002). However, it is possible that the redox state of the ocean was not stable in the early Phanerozoic, and this may have significantly affected nitrogen cycling. A recent statistical investigation of redox proxy data suggests that oxygenation may have been protracted across the Neoproterozoic-Phanerozoic boundary, and atmospheric/oceanic oxygen levels may have remained significantly lower than modern values until as late as the Devonian (Sperling et al., 2015). If this were the case, then heterogeneous ocean redox states could still have dictated nitrogen speciation and cycling during the Paleozoic. With this possibility remaining open, more detailed basinal analyses of Paleozoic nitrogen isotopes (e.g. LaPorte et al., 2009; Luo et al., 2015) are required to parse out the differential influence of sea-level- and redox-driven variations in Phanerozoic nitrogen cycling.

Over the last 70 million years nitrogen isotope values in marine sediments have been relatively stable ($+3.8 \pm 1.9\%$, Algeo et al., 2014), apart from short-term perturbations after the last glacial maximum in the Pleistocene (Altabet, 2007). These may have been caused by a large input of nitrogen from land during the continental glaciation (McElroy, 1983) and the oceans may still be recovering from this process (Christensen et al., 1987).

5. Revisiting major themes in Precambrian nitrogen cycling

The emerging picture of the evolution of Earth's biogeochemical nitrogen cycle allows us to revisit key questions about biological innovations and ocean chemistry.

5.1. Was there a significant source of abiotically fixed nitrogen?

While biological N_2 fixation is by far the major source of nitrogen to the biosphere today (e.g. Ward, 2012), it is conceivable that abiotic sources were significant earlier in Earth's history, especially prior to the origin of the nitrogenase enzyme.

As shown in **Table 2**, the highest proposed abiotic flux of fixed nitrogen is N_2 reduction on TiO_2 . However, this flux may be grossly overestimated, because (a) it assumes widespread deserts with abundant TiO_2 as today, and (b) most NH_3 that was produced was probably rapidly photolyzed back to N_2 (cf. Kuhn and Atreya, 1979). In the absence of this TiO_2 catalysis mechanism, N_2 oxidation to NO_x species in post-impact plumes becomes the major source ($\sim 10^{12}$ mol/yr) (Kasting, 1990), which, however, could have been insignificant over stretches of many millions of years when no impacts occurred and it would have declined rapidly after ~ 3.8 Gyr (Kasting, 1990). Hydrothermal reduction of N_2 to NH_4^+ could have generated 10^9 - 10^{12} mol/yr of fixed nitrogen, according to some scaled laboratory estimates (Smirnov et al., 2008); however, those calculations assume an arbitrary reaction yield and an unlimited supply of the FeNi alloy that acts as a reductant or catalyst. Furthermore, modern hydrothermal fluids that do not assimilate nitrogen from organic-rich sediments are noticeably NH_4^+ -poor (< 0.01 mM, Lilley et al., 1993; von Damm, 1990). Assuming a modern hydrothermal water flux of 0.5 - $6 \cdot 10^{13}$ kg/yr (Elderfield and Schultz, 1996; Emerson and Hedges, 2008), these low concentrations indicate an NH_4^+ flux of $\sim 10^8$ - 10^9 mol/yr or less, despite the fact that the modern ocean is rich in nitrate. Nitrate can also be reduced to NH_4^+ (Brandes et al., 1998) and should thus raise the total NH_4^+ flux. Hence even if hydrothermal activity was ten times more vigorous in the earlier Precambrian, estimates of more than 10^9 mol/yr seem implausible.

Thus the major continuous sources (*i.e.* excluding impacts) of fixed nitrogen would have been lightning and volcanic eruptions, and a realistic steady-state abiotic production flux would probably have been on the order of 10^{10} - 10^{11} mol N/yr. Rates of up to 10^{12} mol N/yr may have been achieved under high pCH_4 (1000 ppmv) and $< 1\%$ pCO_2 favoring the production of HCN later in the Archean (Tian et al., 2011; Zahnle, 1986), but pCH_4 would probably have been low in the Hadean under prebiotic conditions prior to the origin of methanogenic microbes, lowering this flux to 10^9 mol N/yr. Lightning and volcanism both generate NO_x species which could have been reduced abiotically into the more bioavailable NH_4^+ in hydrothermal vents, catalyzed by sulfide minerals or native metals (Brandes et al., 1998; Singireddy et al., 2012; Smirnov et al., 2008; Summers and Chang, 1993), provided that the reduction did not stop at N_2 (**Section 2.3**). Reduction of NO_x to N_2 may have greatly diminished the abiotic supply of fixed nitrogen to prebiotic reactions and the earliest biosphere.

Today the marine biosphere fixes around 10^{13} mol N/yr (e.g. Ward, 2012). Whether or not abiotic nitrogen sources were sufficient in the Precambrian would have depended on relative rates of primary productivity. Prior to the evolution of oxygenic photosynthesis, productivity may have been more than 10 times smaller than today, but once oxygenic photosynthesis evolved, it may have only been 5-10 times lower than modern levels throughout the Precambrian (Canfield et al., 2010; Canfield et al., 2006). The presumed constancy of organic carbon burial since at least 3.5 Gyr (Krissansen-Totton et al., 2015) perhaps further supports a continuously

high nitrogen demand similar to the modern, exceeding plausible abiotic fluxes. A significant abiotic nitrogen source is further inconsistent with the isotopic record. Although isotopic fractionations imparted by abiotic N_2 fixation mechanisms are not well known, laboratory and field measurements suggest that they span over 5-25‰ and perhaps more (Kuga et al., 2014; Moore, 1977). In particular HCN – the potentially largest source under high pCH_4 and low pCO_2 – may have been fractionated by tens to hundreds of permil relative to N_2 (Kuga et al., 2014; Liang et al., 2007). Where NO_x was produced, additional fractionations would likely have occurred during biotic or abiotic NO_x reduction to N_2 or NH_4^+ . The Archean isotope record from 3.5 Gyr onwards does not show evidence of such large fractionations and is therefore unresponsive of a significant abiotic source of fixed nitrogen. This inference may be a further indication of an ancient origin of biological N_2 fixation.

5.2. Was there an Archean ‘nitrogen crisis’?

It has previously been proposed that a diminishing supply of abiotically fixed nitrogen resulted in a ‘nitrogen crisis’ in the Neoproterozoic and triggered the invention of the nitrogenase enzyme (Navarro-Gonzalez et al., 2001). It has further been suggested (Boyd et al., 2011b) that the first nitrogenase enzymes responsible for nitrogen fixation in the early biosphere were “proto-nitrogenases” ancestral to the current forms and thus not as specific or efficient at reducing unreactive N_2 to bioavailable NH_3 . Based on inferred phylogenetic relationships between nitrogenase and bacteriochlorophylls, the timing of the takeover from proto-nitrogenase by modern Mo-nitrogenase has been estimated as occurring around 1.5-2.2 Gyr (Boyd et al., 2011a).

However, without effective biological nitrogen fixation, the potential volume of biomass should have been restricted, thus restraining opportunities for evolutionary experimentation and diversification due to the fewer number of evolving lineages in a small biota. This may be at odds with evidence for high metabolic diversity by 3.5 Gyr (Buick, 2007b). Furthermore, based on the consistent pattern and tight distribution of nitrogen isotope fractionation around a $\delta^{15}N_{bulk}$ value of 0‰ \pm 1.5‰ from 3.2 Gyr to 2.5 Gyr (except during temporary oxygenation events) in rocks with high post-metamorphic organic carbon levels of ~5% (Garvin et al., 2009; Stüeken et al., 2015b), it appears that a highly efficient and isotopically specific nitrogen-fixing metabolic pathway was already operative. This implies that an enzyme with the capabilities of and very similar to modern Mo-nitrogenase with an iron-molybdenum cofactor at its active site was involved as early as 3.2 Gyr. This proposition is not incompatible with the idea of an ancestral nitrogenase lacking FeMo-co preceding Mo-nitrogenase; it is just incompatible with the timing of the transition. We note that the time constraints used to calculate when this transition occurred (Boyd et al., 2011a) are extremely conservative, in particular the placement of oxygenic photosynthesis at 2.5 Gyr. As there is now abundant evidence for the evolution of oxygenic photosynthesis well before this late date, perhaps even as early as 3.8 Gyr (Buick, 2008; Rosing and Frei, 2004), a revised calculation should yield molecular clock ages more compatible with the isotopic data. Secondly, increasing evidence for an origin of the nitrogenase enzyme in thermophilic methanogenic Archaea – perhaps the most ancient phylum on Earth – (Boyd et al., 2011a; Mehta and Baross, 2006; Nishizawa et al., 2014; Raymond et al., 2004) may suggest that nitrogenase is ancient and predates the radiation of cyanobacteria. If so, then abiotic nitrogen sources alone may already have been bio-limiting long before the possible evidence of cyanobacterial activity at 3.8 Gyr (Kasting and Siefert, 2001).

5.3. Was molybdenum a limiting constituent in nitrogen metabolisms?

Molybdenum is an important constituent of nitrogenase and nitrate reductase (Fig. 1, Godfrey and Glass, 2011). Even though Mo isotopes indicate that some dissolved Mo was present in seawater back to 3.0 Gyr, possibly sourced from mild oxidative weathering or hydrothermal inputs (McManus et al., 2002; Planavsky et al., 2014a), dissolved Mo concentrations in the Precambrian ocean may have been as low as 1-10 nM compared to 100 nM today (Anbar, 2008; Reinhard et al., 2013b; Scott et al., 2008). This raises the question of whether one or both of these enzymes were Mo-limited during early evolution (Anbar and Knoll, 2002).

Regarding assimilatory nitrate reductase, culturing experiments suggest that the cyanobacterial version of this enzyme functions normally with as little as 0.5 nM Mo (Glass et al., 2009). Hence this metabolism may not have been affected by Mo shortage. However, eukaryotic nitrate reductases appear to be more sensitive, as shown in one experiment where their activity could be stimulated by raising Mo concentrations from < 2nM to 50nM (Axler et al., 1980). It is thus conceivable that eukaryotes were outcompeted for nitrate in Mo-depleted oceans in the Precambrian (Anbar and Knoll, 2002). Hence, even though there is evidence of nitrate availability from the Neoproterozoic onwards (**Section 4.3**), this does not necessarily imply that eukaryotes would have been ecologically significant. The minimum Mo requirements of dissimilatory nitrate reductases have to our knowledge not been studied.

With regards to nitrogen fixation, the situation is much more ambiguous. Under experimental conditions with cultures of modern cyanobacteria, N₂-fixation rates decline significantly if Mo drops below 5-10nM, depending on the organism used (Glass et al., 2010; Zerkle et al., 2006). Fixation rates are still measurable but 80% suppressed with 1-5nM Mo (Zerkle et al., 2006). These thresholds coincide with the 1-10nM range of Mo that has most recently been proposed for the mid-Proterozoic ocean (Reinhard et al., 2013b), making Mo-limitation conceivable. This interpretation has even stimulated the hypothesis that planktonic cyanobacteria, *i.e.* the major diazotrophs in the modern ocean, did not evolve until the Neoproterozoic rise of Mo to near modern levels (Sánchez-Baracaldo et al., 2014) (**Fig. 6**). If so, then the earlier marine biosphere would have been severely nitrogen-limited. On the other hand, we see nitrogen isotopic evidence for Mo-based nitrogen fixation in marine shales dating back to the Neoproterozoic (Stüeken et al., 2015b). From 3.2 Gyr onwards, $\delta^{15}\text{N}$ values around 0‰ are common in sampled anoxic environments throughout the rest of the Archean and much of the Proterozoic (**Section 4.2, 4.3, 4.5**). There is so far no isotopic evidence of Fe- or V-based nitrogenase ($\delta^{15}\text{N} < -6\text{‰}$, **Table 1**) at any time in the Precambrian. So, it is possible that Mo scarcity affected the overall rate of N₂ fixation, but it did evidently not inhibit the expression of the nitrogenase enzyme.

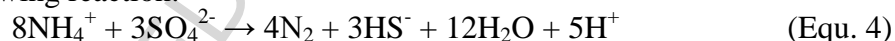
We see two possible solutions to this conundrum. First, the cultures of modern cyanobacteria used in experiments may not have been representative of their Precambrian counterparts. Ancient microbes, in particular anaerobes, may have been better adapted to low Mo concentrations than modern ones. Second, Mo could have been made more bioavailable in Precambrian seawater if it was organically complexed by molybdophores or molybdate-binding siderophores, which are known to be excreted by some diazotrophs (Bellenger et al., 2008; Duhme et al., 1996; Liermann et al., 2005). The evolutionary history of molybdophores is unknown, but they could have facilitated Mo weathering (Liermann et al., 2005) as well as

uptake from the water column (Duhme et al., 1996). In addition, organisms may have expressed enzymatic adaptations to facilitate Mo uptake and storage in their cells (Glass et al., 2013).

5.4. Was the Precambrian ocean rich in NH_4^+ ?

It is often assumed that anoxic Precambrian oceans would have been rich in NH_4^+ (Beaumont and Robert, 1999; Falkowski and Godfrey, 2008), perhaps similar to the modern Black Sea (100 μM , Brewer and Murray, 1973), but this hypothesis has so far not been verified. In a completely anoxic world with negligible amounts of sulfate and oxygen, as presumably in the earlier Archean or in the ferruginous deep ocean of the Mesoproterozoic (Planavsky et al., 2011; Poulton and Canfield, 2011), the major mode of nitrogen acquisition would likely have been biological N_2 fixation, with minor contributions of fixed nitrogen from lightning or volcanism (see above). NH_4^+ would have been released from degrading biomass by passive ammonification and during heterotrophic methanogenesis or fermentation, but these fluxes may have been relatively low, especially if degradation occurred in sediments where NH_4^+ could be trapped by clay minerals. Hence in ferruginous basins the dissolved marine NH_4^+ reservoir may have been fairly small.

With the influx of sulfate, substantially from the Neoproterozoic onwards (Stüeken et al., 2012), some organic matter would have been respired by microbial sulfate reduction. As noted above, sulfate is not a strong enough oxidizer to convert NH_4^+ into NO_2^- or NO_3^- , but it can generate N_2 via the following reaction:



This reaction is thermodynamically feasible if NH_4^+ concentrations and $\text{SO}_4^{2-}/\text{HS}^-$ ratios are high, and it is favored with increasing pH (**Fig. 9**). It is conceivable that close to the chemocline these conditions were met, releasing N_2 back into the atmosphere. However, as SO_4^{2-} became depleted with depth, as in modern euxinic basins (reviewed by Algeo and Lyons, 2006), the reaction would probably have become unfavorable. While some organic carbon may still have been converted to CO_2 under those conditions, the organic-bound ammonium would likely have accumulated in the water column, as in the Black Sea. In other words, an ecosystem dominated by sulfate reduction, for instance along continental margins in the late Archean and mid-Proterozoic when euxinia was more widespread (Arnold et al., 2004; Kendall et al., 2009; Kendall et al., 2011; Partin et al., 2015; Poulton et al., 2004; Reinhard et al., 2009; Scott et al., 2011; Sperling et al., 2015), may have favored high NH_4^+ concentrations in deep water. Large quantities of dissolved NH_4^+ may thus have been a rather localized phenomenon, restricted to euxinic environments.

There are additional arguments in support of relatively low NH_4^+ concentrations in most of the Precambrian deep ocean. First, upwelling of a large NH_4^+ reservoir should have led to non-quantitative NH_4^+ assimilation and associated large isotopic fractionations (**Table 1**). However, apart from one Paleoproterozoic $\delta^{15}\text{N}$ dataset that has been explained by that mechanism (Papineau et al., 2009), there is so far no evidence of partial NH_4^+ assimilation and hence for a large deep marine NH_4^+ reservoir. This observation may suggest that NH_4^+ concentrations were similar to or lower than modern marine NO_3^- levels ($\sim 30\mu\text{M}$), which are assimilated quantitatively in most environments except at high latitudes (Altabet and Francois, 1994; Somes et al., 2010). The Black Sea with its 100 μM of NH_4^+ is an imperfect analogue, because upwelling is weak and so this NH_4^+ is not readily bioavailable to organisms at the surface (Özsoy and Ünlüata, 1997). Second, nitrogen fixation is an energetically costly

metabolism, requiring 16 ATP molecules per N_2 molecule (e.g. Hoffman et al., 2014). If NH_4^+ had built up to high concentrations and was transported to the photic zone, then biological N_2 fixation would presumably have slowed down until the NH_4^+ reservoir had returned to lower levels, especially if low concentrations of Mo limited the rate of biological N_2 fixation (**Section 5.3**). Thus there is so far no evidence and no a priori reason to expect a large NH_4^+ reservoir exceeding modern NO_3^- levels in most of the Precambrian deep ocean.

5.5. Could nitrous oxide have been a significant greenhouse gas in the Proterozoic?

Nitrous oxide (N_2O) is a potent greenhouse gas that can have a significant warming effect, depending on partial pressures of carbon dioxide (CO_2) or methane (CH_4) (Byrne and Goldblatt, 2014). N_2O primarily enters the atmosphere via “leakage” in the biological nitrification and denitrification pathways as a metabolic byproduct that escapes reduction to N_2 . On the modern Earth, this comprises a small portion of the overall denitrification flux, but there is reason to believe that the biological N_2O flux has varied along with the evolution of the nitrogen cycle through the Precambrian. The final step in the denitrification pathway – reduction of N_2O to N_2 – is only known to be catalyzed by the N2OR enzyme, which is a multi-copper protein containing 12 Cu atoms per dimer (Glass and Orphan, 2012; Haltia et al., 2003). Similar to Mo and many other transition metals, Cu is rapidly scavenged from the water column in the presence of hydrogen sulfide. Modeling of mid-Proterozoic ocean euxinia predicts that Cu should be the most strongly sequestered trace metal (Saito et al., 2003), which would render Cu essentially unavailable for microbial nitrogen transformations. Prior to the radiation of nitrification and denitrification in the Neoproterozoic (**Section 4.3**), there would probably not have been any significant N_2O flux from the ocean to the atmosphere. However, from the Paleoproterozoic onwards, when we see persistent evidence of aerobic nitrogen cycling (**Section 4.4**), there may have been significant production of N_2O in suboxic waters, if Cu-limitation inhibited efficient N_2O reduction to N_2 .

This has led to the hypothesis that especially in the Mesoproterozoic, when both aerobic nitrogen cycling and euxinia have been documented (**Section 4.5**), N_2O may have been a significant greenhouse gas (Buick, 2007a). This idea is supported by experimental evidence showing that cultures of denitrifying bacteria indeed accumulate N_2O at Cu concentrations $< \sim 3nM$ (Granger and Ward, 2003). According to some models, the resulting Mesoproterozoic N_2O flux to the atmosphere could have been as high as 20 times the modern flux (Buick, 2007a; Roberson et al., 2011).

However, given new atmospheric oxygen constraints, this flux may have been insufficient to achieve N_2O mixing ratios greater than a few ppbv. The accumulation of N_2O in the atmosphere depends on the rate of photolytic destruction in the stratosphere, which on the early Earth would have been much higher than in today’s ozone-shielded atmosphere. Therefore the ability of N_2O to act as a significant greenhouse gas depends critically on the level of atmospheric oxygen (in addition to CO_2 and CH_4 , Byrne and Goldblatt, 2014). At pO_2 levels higher than 0.1 PAL, nitrous oxide can become an important greenhouse gas if the flux rates were somewhat elevated above modern levels (Roberson et al., 2011). However, an upper limit of 0.001 PAL for mid-Proterozoic pO_2 has been proposed by Planavsky et al. (2014b) which is much lower than the threshold at which N_2O mixing ratios decline markedly below the modern value of ~ 330 ppbv regardless of the flux rate (Roberson et al., 2011). High CO_2 and CH_4 levels may further reduce the greenhouse effect of N_2O (Byrne and Goldblatt, 2014). Thus, the

accuracy of the mid-Proterozoic pO_2 , pCO_2 and pCH_4 estimates is critical to the assessment of the N_2O -greenhouse hypothesis and further work needs to be done to assess the plausibility of this scenario.

When atmospheric and oceanic oxygen levels began to approach modern values in the Neoproterozoic, euxinia receded (Sperling et al., 2015) and the Cu inhibition of denitrification would have disappeared as well. However, there may have been periods of time in the Paleoproterozoic and early Paleozoic when euxinia was prevalent while pO_2 was significantly higher than in the Mesoproterozoic (Sperling et al., 2015). If this was the case, then those two intervals may be promising candidates for an N_2O -greenhouse.

5.6. Did the evolution of the nitrogen cycle play a role in eukaryogenesis?

The advent of aerobic nitrogen cycling in the late Neoproterozoic, even if transient and localized, could have permitted the evolution of eukaryotes, as these organisms cannot fix nitrogen and preferentially assimilate nitrate whereas prokaryotes can fix nitrogen or preferentially assimilate ammonium (Fawcett et al., 2011). However, there is no strong evidence that eukaryotes existed at this time (French et al., 2015). Also the apparent expansion of nitrate availability in the early Paleoproterozoic evidently did not trigger the rise of eukaryotes to ecological dominance, as microfossil evidence of their existence does not appear until the late Paleoproterozoic (Knoll et al., 2006) and hydrocarbon biomarker evidence is not certain until the late Neoproterozoic (Summons et al., 1988). Although nitrate was perhaps necessary, it was likely not sufficient for eukaryogenesis.

Once eukaryotes had evolved in the late Paleoproterozoic, nitrate segregation into shallow coastal regions during the late Paleoproterozoic and Mesoproterozoic would have geographically restricted the distribution of eukaryotes, which may have retarded their evolutionary diversification and hindered their ecological dominance outside these regions (Stüeken, 2013). Perhaps not until nitrate became widely available throughout the ocean upon deep ocean oxygenation in the late Neoproterozoic (or perhaps even later) did eukaryotes take over the dominant ecological role and develop the diverse evolutionary aspect that we see today.

6. Conclusions

The evolution of the biogeochemical nitrogen cycle has been driven by biological innovations and the progressive, non-linear oxygenation of Earth's atmosphere and ocean. In particular, nitrogen isotopes can inform us about which metabolisms were active in ancient environments. N_2 fixation may have been a dominant nitrogen uptake pathway in the Mesoarchean, consistent with widespread anoxia; nitrate became locally and temporarily more abundant in the Neoproterozoic, consistent with brief and possibly restricted increases in surface O_2 ; and the Mesoproterozoic ocean was stratified with nitrate restricted to shallow waters, similar to stratification in sulfur at that time. From the Neoproterozoic onwards, nitrate appears to have been more common, though not permanently present. The inferred evolution of the nitrogen cycle thus strengthens current views of the progressive oxygenation of Earth's atmosphere and oceans.

Additional work is needed to fill major gaps in the database and to provide more accurate answers to some of the key questions about the Precambrian nitrogen cycle. Based on current evidence outlined above we draw the following conclusions:

1. Biological N₂ fixation appears to be a very ancient metabolism going back to at least 3.2 Gyr (Mehta and Baross, 2006; Raymond et al., 2004; Stüeken et al., 2015b). Abiotic nitrogen sources from lightning, volcanism and photochemistry were likely insufficient to sustain a significant biosphere (**Section 5.1, 5.2**), especially after the evolution of oxygenic photosynthesis and a concurrent increase in bioproductivity (Canfield et al., 2010).
2. Molybdenum appears to have been sufficiently available to express nitrogenase enzymes since at least 3.2 Gyr, and it was evidently preferred over Fe and V nitrogenases throughout the entire Precambrian (Garvin et al., 2009; Godfrey and Falkowski, 2009; Koehler et al., in review; Stüeken, 2013; Stüeken et al., 2015b; Yamaguchi, 2002). Low Mo concentrations in seawater may have been compensated by biological adaptations (**Section 5.5**, e.g. Glass et al., 2013).
3. Following the first evidence for an oceanic nitrate reservoir at ~2.7 Ga, dissolved nitrate levels were probably low and spatially heterogeneous throughout most of the Precambrian (**Fig. 6, Section 4.3, 4.4, 4.5**). This may have restricted the radiation of eukaryotes through much of the Proterozoic. On the other hand, nitrate availability alone was likely not the main driver of eukaryote evolution (**Section 5.6**).
4. Biologically-mediated nitrogen burial goes back to at least 3.8 Gyr, potentially affecting the atmospheric N₂ reservoir (Goldblatt et al., 2009; Johnson and Goldblatt, 2015; Papineau et al., 2005; Som et al., 2016; Stüeken, in press; Stüeken et al., in review). Furthermore, biological N₂O production may have been pronounced in the Proterozoic and possible in the early Paleozoic (**Section 5.5**, Buick, 2007a; Roberson et al., 2011). Through both of these processes, the nitrogen cycle could plausibly have played a role in global climate evolution, but missing constraints on fluxes and feedbacks prohibit firm quantitative assessments at this stage.

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Tables

Table 1: Metabolic nitrogen pathways and associated isotopic fractionations.

Ammonification can be abiotic and is just included for completeness. Metal catalysts and isotopic fractionations are taken from the literature (Brunner et al., 2013; Buick, 2007a; Casciotti, 2009; Godfrey and Glass, 2011; Hoch et al., 1992; McCready et al., 1983; Nishizawa et al., 2014; Pennock et al., 1996; Waser et al., 1998; Zerkle et al., 2008; Zhang et al., 2014).

Fractionations are defined as $\epsilon \approx \delta^{15}\text{N}_{\text{product}} - \delta^{15}\text{N}_{\text{reactant}}$. Int. = intermediate reaction steps.

pathway		reaction	metals	fractionation [%]
N ₂ fixation		$\text{N}_2 + 6\text{e}^- + 8\text{H}^+ \rightarrow 2\text{NH}_4^+ (\rightarrow \text{org-NH}_2)$	Fe, Mo, (V)	Fe, V: -8 to -6. Mo: -2 to +1 (-4 with Mo in high Fe)
ammonification		$\text{org-NH}_2 + 2\text{H}^+ \rightarrow \text{NH}_4^+$		+1
ammonium assimilation		$\text{NH}_4^+ \rightarrow \text{org-NH}_2 + \text{H}^+$	Fe	-4 to -27
nitrification	<i>net:</i>	$\text{NH}_4^+ + 3\text{H}_2\text{O} \rightarrow \text{NO}_3^- + 8\text{e}^- + 10\text{H}^+$	Fe, Mo, Cu	-1 to -25
	<i>int:</i>	$\text{NH}_4^+ + \text{H}_2\text{O} \rightarrow \text{NH}_2\text{OH} + 2\text{e}^- + 5\text{H}^+$	Fe, Cu	
		$\text{NH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + 4\text{e}^- + 5\text{H}^+$	Fe	
		$\text{NO}_2^- + \text{H}_2\text{O} \rightarrow \text{NO}_3^- + 2\text{e}^- + 2\text{H}^+$	Fe, Mo	
nitrate assimilation		$\text{NO}_3^- + 8\text{e}^- + 10\text{H}^+ \rightarrow \text{NH}_4^+ + 3\text{H}_2\text{O} (\rightarrow \text{org-NH}_2)$	Fe, Mo	-5 to -10
denitrification	<i>net:</i>	$2\text{NO}_3^- + 10\text{e}^- + 12\text{H}^+ \rightarrow \text{N}_2 + 6\text{H}_2\text{O}$	Fe, Mo, Cu	-5 to -30
	<i>int:</i>	$\text{NO}_3^- + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$	Fe, Mo	
		$\text{NO}_2^- + \text{e}^- + 2\text{H}^+ \rightarrow \text{NO} + \text{H}_2\text{O}$	Fe, (Cu)	
		$2\text{NO} + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$	Fe, (Cu)	
		$\text{N}_2\text{O} + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{N}_2 + \text{H}_2\text{O}$	Cu	
DNRA		$\text{NO}_3^- + 8\text{e}^- + 10\text{H}^+ \rightarrow \text{NH}_4^+ + 3\text{H}_2\text{O}$	Fe, Mo	-30 (?)
ANAMMOX	<i>net:</i>	$\text{NO}_2^- + \text{NH}_4^+ \rightarrow \text{N}_2 + 2\text{H}_2\text{O}$	Fe, Cu?	-26 on NH ₄ ⁺ , -16 on NO ₂ ⁻
	<i>int:</i>	$\text{NO}_2^- + 2\text{H}^+ + \text{e}^- \rightarrow \text{NO} + \text{H}_2\text{O}$	Fe, Cu?	
		$\text{NO} + \text{NH}_4^+ + 2\text{H}^+ + 3\text{e}^- \rightarrow \text{N}_2\text{H}_4 + \text{H}_2\text{O}$	Fe	
		$\text{N}_2\text{H}_4 \rightarrow \text{N}_2 + 4\text{H}^+ + 4\text{e}^-$	Fe	

Table 2: Estimates of abiotic N₂ fixation rates compiled from the literature. Values are given in mol N per year. NO_x species can undergo further reduction to NH₄⁺ under hydrothermal conditions (e.g. Brandes *et al.* 1998) and are therefore also relevant as a source of organic ammonium. Cat = solid mineral catalyst. References: 1. Smirnov *et al.* (2008), 2. Chyba and Sagan (1992), 3. Kasting (1990), 4. Mather *et al.* (2004), 5. Nna-Mvondo *et al.* (2005), 6. Navarro-Gonzalez *et al.* (1998), 7. Navarro-Gonzalez *et al.* (2001), 8. Kasting and Walker (1981) 9. Tian *et al.* (2011) and Zahnle (1986), 10. Schoonen & Xu (2001), 11. Brandes *et al.* (1998), 12. Henderson-Sellers & Schwartz (1980). 13. Canfield *et al.* (2010). Notes: a. production rate increases with CO₂ but low CO₂ levels may be more realistic (e.g. Kasting and Siefert, 2001); b. the minimum rate applies at low atmospheric CH₄ levels and before the onset of widespread biological methanogenesis; c. assumes regeneration of FeNi alloy catalysts at high rates equivalent to water flux through ocean crust, but tectonic regeneration is likely rate-limiting; d. produced NH₃ may be rapidly destroyed by photolysis (e.g. Kuhn and Atreya, 1979). Furthermore, assumes extensive deserts, which may not be realistic on the early Earth.

mechanism	reactants	product	min.	avg	max.	ref.
impact delivery		reduced N	1·10 ³		1·10 ⁵	1
interplanetary dust		reduced N		7.1·10 ⁸		2
post-impact plume	N ₂ + CO ₂	NO _x		1.6·10 ¹²		3
volcanic eruptions	N ₂ + CO ₂	HNO ₃	10 ⁹		10 ¹¹	4
coronal discharge	N ₂ + CO ₂	NO _x		7.1·10 ⁷		5
volcanic lightning	N ₂ + CO ₂	NO _x		1.7·10 ¹⁰		6
thunder-storm lightning ^a	N ₂ + CO ₂	NO _x	1.9·10 ⁸		2.1·10 ¹⁰	7
lightning	N ₂ + CO ₂	NO _x		2.9·10 ¹⁰		8
UV photolysis ^b	N + CH ₃	HCN	2.7·10 ⁹		2.7·10 ¹²	9
hydrothermal reduction	N ₂ + HS ⁻ (FeS _x cat.)	NH ₄ ⁺			8.5·10 ⁷	10
hydrothermal reduction	N ₂ + Fe ⁰ , Fe ₃ O ₄ (cat.)	NH ₄ ⁺	1·10 ⁹		1·10 ¹¹	11
hydrothermal reduction ^c	N ₂ + Fe ⁰ Ni ⁰ (cat.)	NH ₄ ⁺	1·10 ⁹		1·10 ¹²	1
photochemical reduction ^d	N ₂ + H ₂ O (TiO ₂ cat.)	NH ₃	4.9·10 ¹¹		4.9·10 ¹²	12
modern N ₂ fixation	N ₂ + nitrogenase	org. NH ₄ ⁺		10 ¹³		13

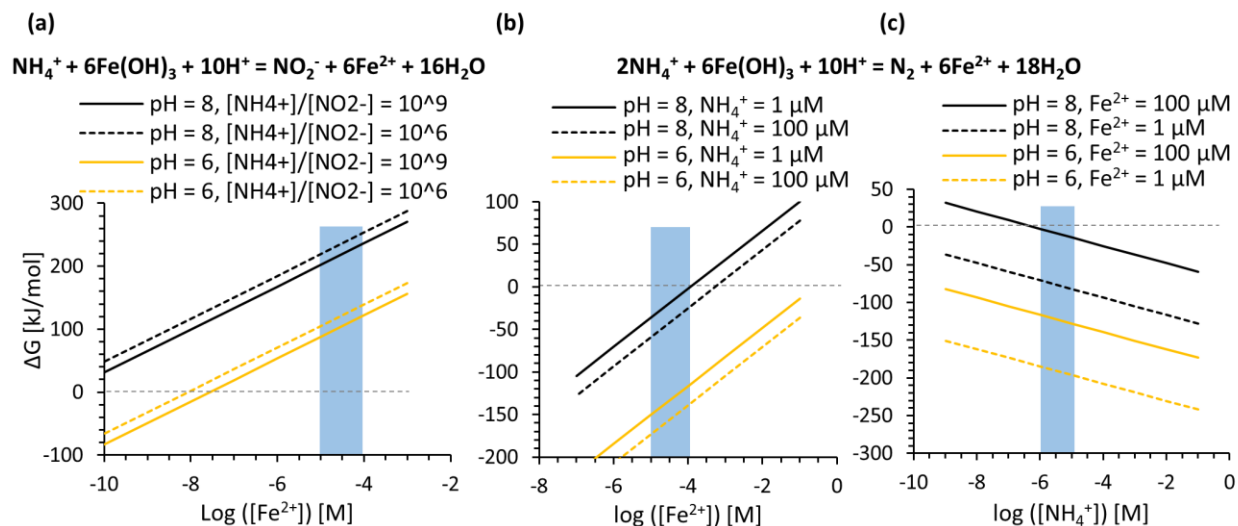


Figure 2: Thermodynamic feasibility of iron-driven ammonium oxidation to NO_2^- and N_2 . Calculations were made with thermodynamic data from Stumm & Morgan (1996). (a) ΔG_r for oxidation to NO_2^- as a function of Fe^{2+} ratios for pH 6 (yellow) and pH 8 (black) at $[\text{NH}_4^+]/[\text{NO}_2^-]$ ratios of 10^9 (solid) and 10^6 (dashed), blue bar = range of Fe^{2+} levels proposed for the Archean (Canfield, 2005). (b) ΔG_r for oxidation to N_2 as a function of dissolved Fe^{2+} for pH 6 (yellow) and pH 8 (black) with NH_4^+ concentrations of 10^{-6} M (solid) and 10^{-4} M (dashed). (c) ΔG_r for oxidation to N_2 as a function of dissolved NH_4^+ for pH 6 (yellow) and pH 8 (black) with Fe^{2+} concentrations of 10^{-6} M (dashed) and 10^{-4} M (solid), blue bar = range of total N (ammonium or nitrate) levels in the modern ocean. Reactions are thermodynamically feasible if $\Delta G_r < 0$ (grey dashed line).

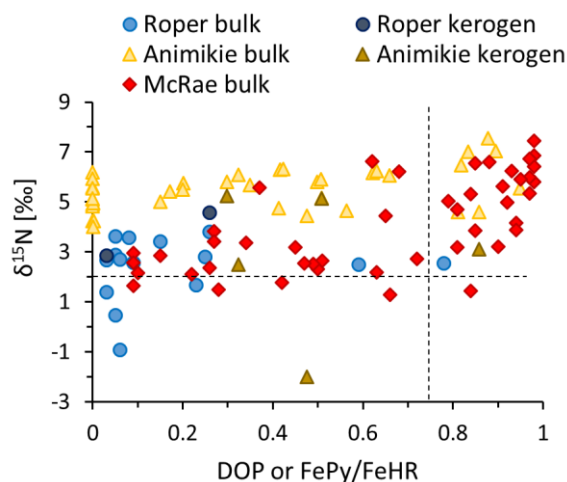


Figure 3: $\delta^{15}\text{N}$ versus iron pyritization. DOP = degree of pyritization, defined as $\text{Fe}_{\text{Py}}/(\text{Fe}_{\text{Py}}+\text{Fe}_{\text{HCl}})$, where Fe_{Py} = pyrite-bound iron and Fe_{HCl} = HCl-soluble iron; euxinia is inferred above ~ 0.75 (vertical dashed line) (Raiswell et al., 1988). $\text{Fe}_{\text{Py}}/\text{Fe}_{\text{HR}}$ stands for Fe_{Py} over highly reactive iron, which comprises iron bound in carbonate, iron oxides and pyrite; euxinia is inferred above 0.7-0.8, if the ratio of Fe_{HR} over total iron exceeds 0.38 (Poulton and Canfield, 2005), which is fulfilled in these samples. Therefore, both methods provide comparable results. DOP applies to the Roper Gp (1.4 Gyr, Koehler et al., in review; Shen et al., 2003), $\text{Fe}_{\text{Py}}/\text{Fe}_{\text{HR}}$ applies to data from the Mt. McRae Fm (2.5 Gyr, Garvin et al., 2009; Reinhard et al., 2009) and from the Animikie Gp (1.86 Gyr, Godfrey and Falkowski, 2009; Poulton et al., 2004). Aerobic nitrogen cycling is commonly inferred where $\delta^{15}\text{N}$ is greater than $\sim +2\text{‰}$ (horizontal dashed line). Data points in the top right quadrant violate the model of Boyle *et al.* (2013)

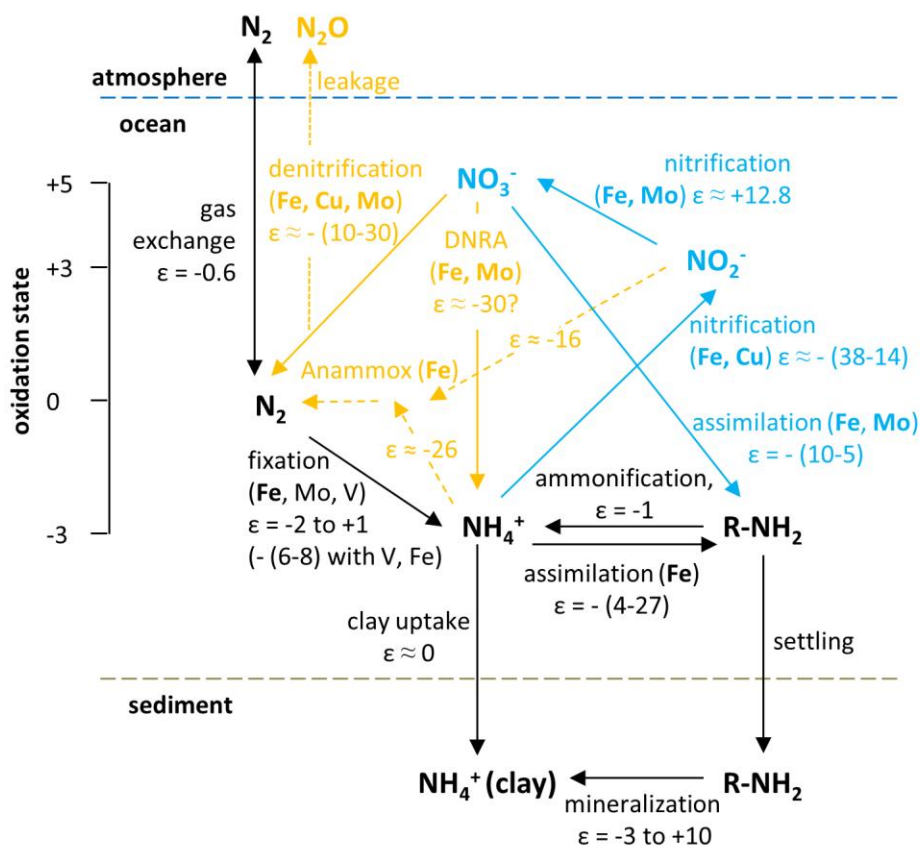


Figure 4: Illustration of the marine nitrogen cycle. Essential metal cofactors are in bold. Black pathways show an anaerobic nitrogen cycle as envisioned for the earlier Archean, blue pathways mark oxic processes, and orange mark suboxic processes. Oxic and suboxic pathways did probably not radiate until the Neoproterozoic. See Table 1 and text for references. Adapted from Stüeken (2013) with additional information from McCready (1983), Zhang *et al.* (2014) and Brunner *et al.* (2013).

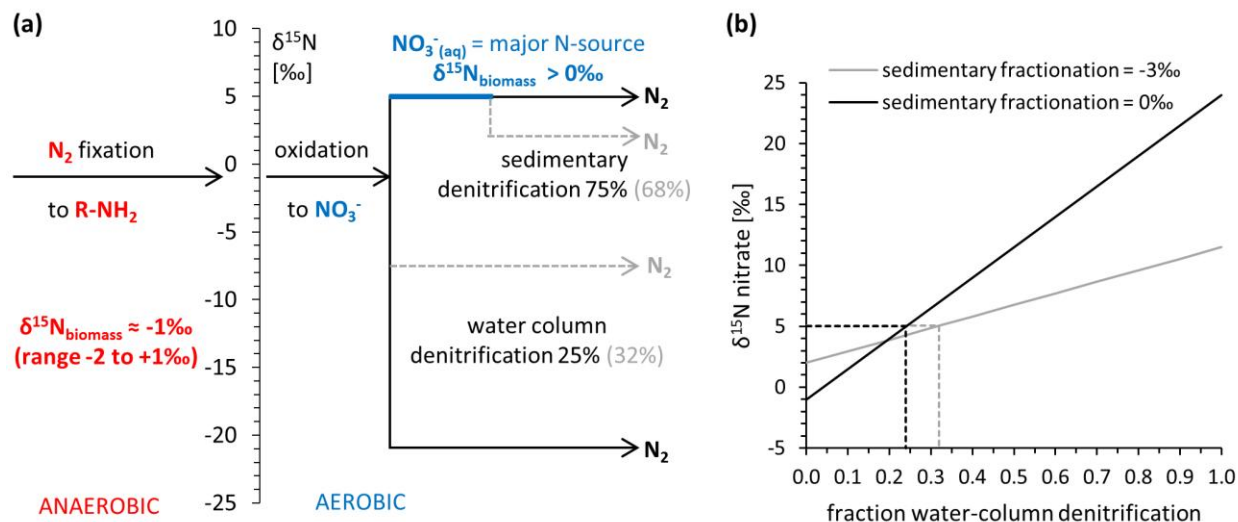


Figure 5: Nitrogen isotope mass balance in the modern ocean. (a) Mass balance, model, following the general formulation: $\delta^{15}\text{N}_{\text{N}_2\text{atmosphere}} + \epsilon_{\text{N}_2\text{fixation}} = \delta^{15}\text{N}_{\text{NO}_3^-} + \epsilon_{\text{denitrification}}$, where $\epsilon_{\text{denitrification}} = f_{\text{sedimentary}} \cdot \epsilon_{\text{sedimentary}} + f_{\text{watercolumn}} \cdot \epsilon_{\text{watercolumn}}$. Nitrogen enters the system via N_2 fixation with an average fractionation of -1‰ ($\epsilon_{\text{N}_2\text{fixation}}$). In anoxic settings, this constitutes the major nitrogen source. In oxic settings, aerobic nitrogen cycling quantitatively converts NH_4^+ released from biomass into NO_3^- without net fractionation. The resulting NO_3^- with $\delta^{15}\text{N}_{\text{NO}_3^-}$ is reduced back to N_2 in locally suboxic regions in the water column and in sediments. Black solid arrows = traditional model, where water-column fractionation imparts a large fractionation of -25‰ ($\epsilon_{\text{watercolumn}}$), but only makes up $\sim 25\%$ of all denitrification ($f_{\text{watercolumn}}$). Sedimentary denitrification ($f_{\text{sedimentary}}$) imparts no significant net fractionation ($\epsilon_{\text{sedimentary}}$). Grey dashed arrows = new emerging model, where sedimentary denitrification imparts a small fractionation of -3‰ and water-column denitrification imparts a smaller net fractionation of -12.5‰ on average. Adapted from Sigman et al. (2009). (b) The effect of the degree of water-column denitrification on the composition of residual NO_3^- . Black = traditional model without fractionation in sediments; grey = new model with fractionation of -3‰ in sediments. Dashed lines = mass balance in the modern ocean where $\delta^{15}\text{N}_{\text{NO}_3^-} \approx +5\text{‰}$. The composition of NO_3^- is transferred to biomass (and hence sediments) via quantitative NO_3^- assimilation in the photic zone. A higher degree of water-column denitrification can result in heavier $\delta^{15}\text{N}$ values.

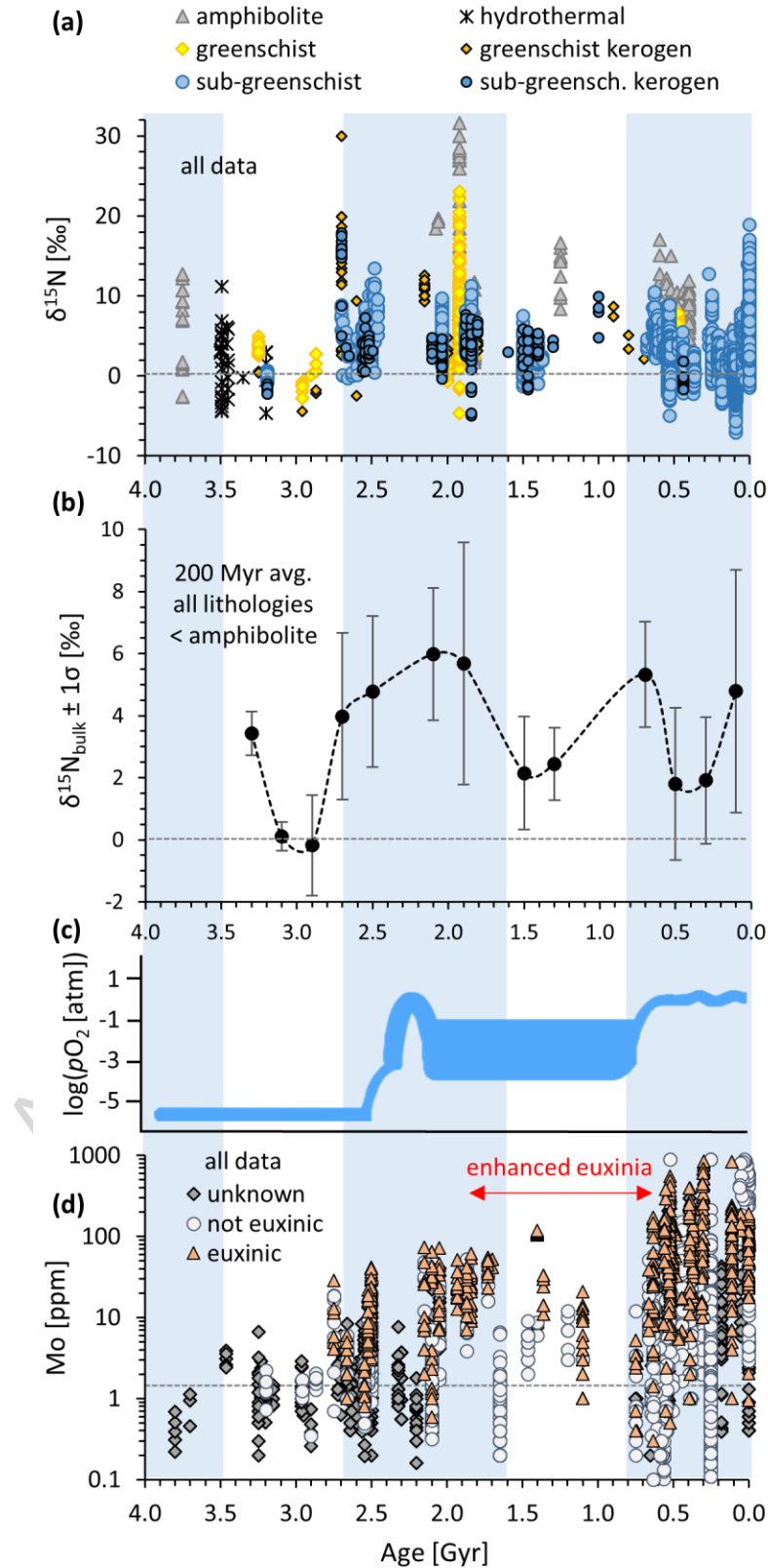


Figure 6: Secular trends in nitrogen, oxygen and molybdenum. (a) Published nitrogen isotope data (Appendix), separated by metamorphic grade as specified in the original papers. (b)

200 Myr average of all sub-greenschist and greenschist nitrogen isotope data, $\pm 1\sigma$ for each bin. (c) Current view of atmospheric pO_2 adapted from Lyons (2014). (d) Mo concentrations in marine shales compiled from the literature (Chen et al., 2015; Dahl et al., 2010; Gilleaudeau and Kah, 2013b; Little et al., 2015; Reinhard et al., 2013b, and references therein), sorted by redox state of the overlying water column as specified by the authors. Dashed horizontal line = average upper continental crust for Mo (Rudnick and Gao, 2014), and atmospheric $\delta^{15}N$ for nitrogen isotopes. Vertical blue shading marks boundaries between time periods as discussed in the text.

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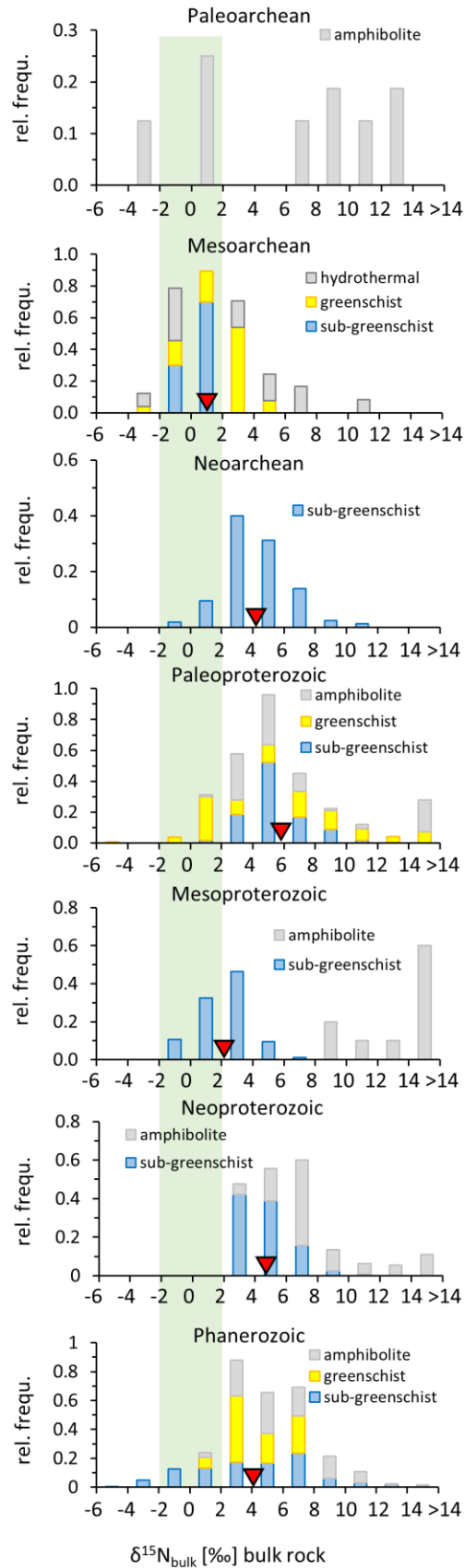


Figure 7: Histograms of bulk nitrogen isotope data for different time periods. Data include all lithologies and are subdivided by metamorphic grade. Vertical green shaded bar = most common range of Mo-based biological N₂ fixation (**Table 1**). In the presence of high Fe concentrations, or with alternative nitrogenases, fractionations can be more negative. Red triangle = average of sub-greenschist and greenschist samples in each interval.

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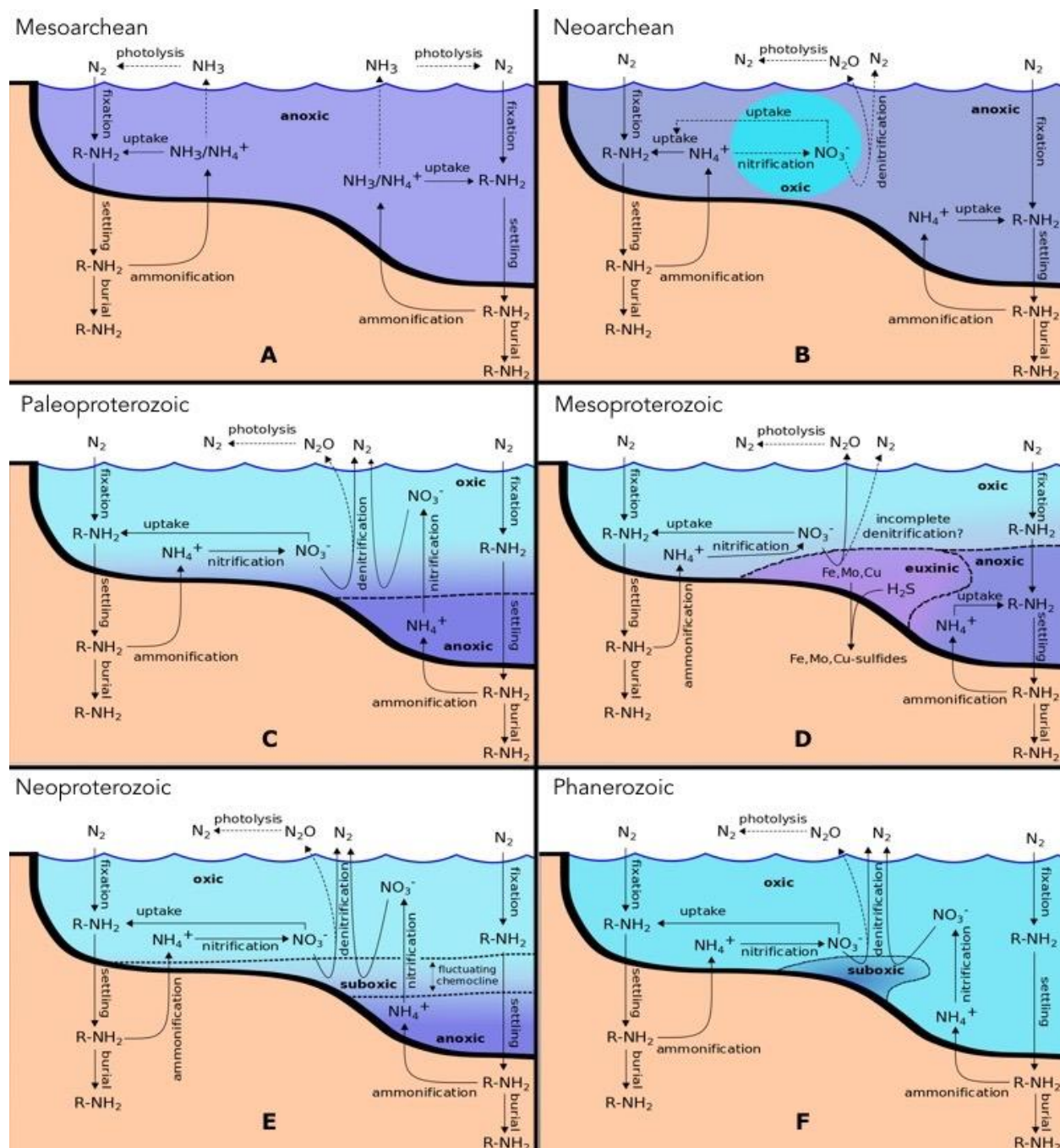


Figure 8: Sketch of the nitrogen cycle for different stages in Earth's history. Near-shore environments are depicted on the left, with a progression to outer shelf and deep ocean settings. Organic (reduced) nitrogen is depicted as “R-NH₂” and minor/intermittent processes are depicted with dotted arrows. (a) Mesoarchean, 3.2-2.8Gyr. (b) Neoproterozoic, 2.8-2.5Gyr. (c) Paleoproterozoic, 2.5-1.8Gyr. (d) Mesoproterozoic, 1.8-0.8Gyr. (e) Neoproterozoic, 0.8-0.5Gyr. (f) Phanerozoic, 0.5 to modern. To avoid clutter, sedimentary denitrification has been omitted. Today, this process accounts for ~70% of the total denitrification flux as calculated by isotopic mass balance (Sigman et al., 2009), and it is implied here wherever water-column denitrification

is depicted. The relative contributions of water-column and sedimentary denitrification may have varied in the past.

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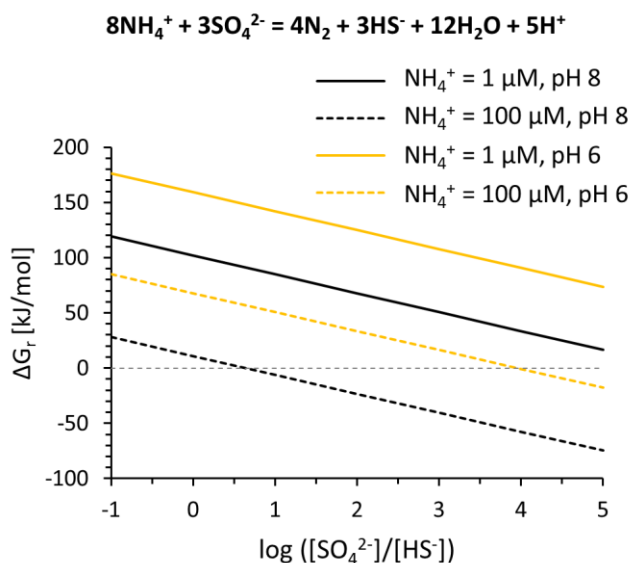


Figure 9: Thermodynamic feasibility of sulfate-driven ammonium oxidation to N_2 .

Calculations were made with thermodynamic data from Stumm & Morgan (1996). The free energy of the reaction (ΔG_r) is a function of NH_4^+ concentrations (10^{-4} M in dashed, 10^{-6} M solid), pH (6 orange, 8 black), and the ratio of SO_4^{2-} to HS^- on the x-axis. Reactions are thermodynamically feasible if $\Delta G_r < 0$.

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