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A REDUCED, ABIOTIC NITROGEN CYCLE BEFORE THE RISE OF OXYGEN

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Abstract:

Nitrogen is a critical element for all known life, where it is used in essential biomolecules such as amino and nucleic acids. However, most nitrogen on Earth is found as relatively inert N_2 in the atmosphere, and must be "fixed" to more bioavailable forms before it can be incorporated into biomass. Fixed nitrogen can further be interconverted between a range of oxidized and reduced forms in a complex web of biogeochemical reactions driven by diverse microorganisms in order to conserve energy in addition to using nitrogen for biosynthesis. This redox cycling is a function of the oxidation state of Earth surface environments, and therefore cannot be assumed to have been present on the early Earth, particularly before the evolution of oxygenic photosynthesis and oxygenation of the atmosphere. Here, we consider which steps in the modern nitrogen cycle may have been present on the early Earth before the introduction of molecular oxygen into biology and geochemistry. This includes incorporation of biochemical and phylogenetic evidence for the antiquity of microbial nitrogen metabolisms, as well as geochemical- and photochemical- model based estimates for abiotic processes. We conclude that before the evolution of oxygenic photosynthesis, the nitrogen cycle was largely abiotic, consisting of atmospheric and geological transformations of reduced nitrogen species. Depending on the productivity of the early biosphere, the nitrogen demand of the biosphere may have

been met by abiotic nitrogen fixation processes, consistent with a late origin of biological nitrogen fixation via the nitrogenase enzyme, or an early evolution of nitrogenase for cyanide uptake followed by later cooption for N_2 fixation.

Introduction

The evolution of oxygenic photosynthesis led to the Great Oxygenation Event (GOE) ~2.3 billion years ago (Gya), when O_2 first accumulated to significant concentrations in the atmosphere (Fischer et al. 2016). At this critical juncture, O_2 was introduced into a primarily anoxic global biosphere and geochemical landscape, which in turn revolutionized the nature of the ecology and Earth surface environments as a whole (Ward et al. 2016, Fischer et al. 2016). Before the GOE, oxygenic photosynthesis (and the ability to use water as an electron donor for C fixation) was unavailable to drive high rates of primary productivity that we see today, and O_2 was unavailable for the efficient and complex aerobic heterotrophy and biogeochemical cycles that dominate modern environments (Ward et al. 2017). The size of the biosphere and the structure of biogeochemical cycles before the introduction of O_2 is not well understood, but must have been very different than the modern biosphere fueled by oxygenic photosynthesis and aerobic respiration.

In particular, the pre-GOE, Archean nitrogen cycle must have worked very differently than it does today. Nitrogen is a critical element for all known life, where it is used in essential biomolecules such as amino and nucleic acids. However, most nitrogen on Earth is found as relatively inert N_2 in the atmosphere, and must be “fixed” to more

bioavailable forms before it can be incorporated into biomass (Canfield et al. 2010). Fixed nitrogen can further be interconverted between a range of oxidized and reduced forms in a complex web of biogeochemical reactions driven by diverse microbes that utilize these transformations to conserve energy (Figure 1) in addition to using nitrogen for biosynthesis (Canfield et al. 2010). These reactions include processes that return fixed nitrogen to the atmospheric N_2 pool (denitrification and anammox) making this a closed cycle, maintaining a balance of atmospheric N_2 and fixed N over long timescales (Canfield et al. 2010). This redox cycling, which is a function of the oxidation state of Earth surface environments, cannot be assumed to have been present in the Archean (Ward et al. 2017, Zerkle et al. 2017). For instance, a critical step in the nitrogen cycle—nitrification—has an absolute requirement for O_2 (Klotz and Stein 2008). Nitrification is the only known biological process to oxidize NH_3 to high-valent oxidized N species such as NO_2^- and NO_3^-, which can serve as the substrates for denitrification and anammox to drive the return of fixed nitrogen to N_2 (Mancinelli and McKay 1988). As a result, without O_2 the N cycle is not a closed loop, but instead a vector of nitrogen toward reduced forms.

Furthermore, it is unclear what processes supplied fixed nitrogen to the early biosphere. During recent Earth history, the vast majority of nitrogen fixation has been through biological nitrogen fixation using the nitrogenase enzyme (Canfield et al. 2010); however, it is unclear when nitrogenase evolved. Hypotheses for the origin of nitrogenase range from its presence in the Last Universal Common Ancestor (Fani et al. 2000, Weiss et al. 2016), to evolving much later, close to the GOE (Raymond et al. 2004, Boyd et al.

2011). Without a good understanding of the evolutionary history of biological nitrogen fixation, it is unclear when this process became available to supply fixed nitrogen to the biosphere. Before the evolution of nitrogenase, nitrogen must have been sourced through abiotic mechanisms of converting N_2 into more bioavailable forms, including fixation of N_2 into NO by lightning (Navarro-Gonzalez et al. 1998) or HCN by photochemistry (Tian et al. 2011). The products and fluxes of nitrogen fixation by these abiotic processes is not well constrained, as it is dependent on poorly understood aspects of atmospheric chemistry (such as the ratio of CO_2 to CH_4) (Navarro-Gonzalez et al. 2001). Moreover, because these abiotic fluxes could not be tuned to keep pace with primary productivity as biological nitrogen fixation can (Tyrrell 1999), fixed nitrogen may have eventually become limiting to productivity (Navarro-Gonzalez et al. 2001) or may have proceeded in excess of biological demands and led to depletion of the atmospheric N_2 reservoir (Som et al. 2016) or accumulation of reduced nitrogen in the oceans (Ward et al. 2017). In order to determine when the modern, aerobically driven, biologically closed nitrogen cycle evolved, and what the nature of the earlier nitrogen cycle may have been, we must better constrain the fluxes of nitrogen through the early Earth and the evolutionary history of individual steps in the nitrogen cycle that exist today.

Here, we integrate what is known about the evolution and early history of the nitrogen cycle, and attempt to constrain the drivers and fluxes included in the nitrogen cycle before the introduction of O_2 to biogeochemical cycles following the evolution of oxygenic photosynthesis. We conclude that the modern, aerobic, biologically driven nitrogen cycle is a relatively recent innovation, likely postdating the evolution of

oxygenic photosynthesis and not arising sooner than the Great Oxygenation Event ~2.3 Gya. Before this time, the nitrogen cycle was dominated by reduced species and abiotic processes, primarily the fixation of N_2 to reduced forms by lightning and photochemistry. Reduced nitrogen likely accumulated in the oceans, providing a nutrient source in excess of demands by early electron donor-limited primary productivity. The nitrogen cycle was likely closed by photolysis of a tenuous atmospheric ammonia pool in equilibrium with the ammonia-rich ocean.

Evolution of the biological nitrogen cycle

The modern nitrogen cycle has an absolute requirement for molecular oxygen. Once N_2 is fixed to reduced forms like ammonia, the only known biological pathways for returning nitrogen to the atmosphere are denitrification and anammox, both of which utilize oxidized nitrogen species—NO, nitrite, or nitrate—as substrates. Aerobic nitrification is the only known biological pathway to convert reduced nitrogen into these forms, and so in the absence of molecular oxygen the closed nitrogen cycle collapses to a vector converting N_2 to ammonia.

Nitrification, the sequential oxidation of ammonia to nitrite and nitrate using O_2, is a thermodynamically favorable reaction catalyzed by various groups of microbes. This includes ammonia oxidation by various Proteobacteria and Thaumarchaea, nitrite oxidation by Nitrospirae, Nitrospinae, various Proteobacteria, and one strain of Chloroflexi (Stein and Klotz 2016, Bock and Wagner 2006, Sorokin et al. 2012), and “comammox”, or complete nitrification of ammonia to nitrate, by some Nitrospirae (Daims et al. 2015, van Kessel et

al. 2015). The phylogenetic distribution of these organisms is sparse, and restricted to fairly derived clades, consistent with a relatively late origin and distribution via horizontal gene transfer. Interpretations of a late origin for nitrification are supported by its oxygen requirements, which suggest an origin after the evolution of oxygenic photosynthesis by Cyanobacteria and oxygenation of the atmosphere ~2.3 Gya. Although nitrification has been shown to proceed at oxygen concentrations down to ~10 nM, it exhibits strong oxygen concentration dependence at these ranges, with a half saturation constant of 0.5-1 μM O_2 (Bristow et al. 2016). As a result, nitrification is almost certainly unviable under the trace oxygen concentrations permissible by Archean pO2 proxies (e.g. Johnson et al. 2014). Nitrification, and a complete aerobic nitrogen cycle, may have evolved hand-in-hand with the GOE (e.g. Zerkle et al. 2017), but not before.

In the absence of nitrification, what then would be the topology of the early biological nitrogen cycle? Were alternative mechanisms in place to produce oxidized nitrogen, or was the nitrogen cycle restricted to reduced forms? These questions can be addressed by combining insights from the rock record about abiotic transformations with comparative biology analyses of the relative age of nitrogen metabolisms. A first order question that must first be answered is whether in the absence of aerobic nitrification as we see it today other sources, either abiotic or biological, could supply oxidized nitrogen species to the early biosphere.

It has been proposed that atmospheric processes, particularly lightning, could fix N_2 into bioavailable forms on the early Earth (Nacarro-Gonzalez et al. 1998). Depending on atmospheric chemistry, the products of this abiotic fixation could range from oxidized

forms like NO (under high CO_2:CH_4 ratios) to reduced phases like HCN (under relatively high methane atmospheres) (Navarro-Gonzalez et al. 2001). The fluxes of nitrogen fixed by these processes could be significant for much of Earth history (Ward et al. 2017), and oxidized nitrogen produced through these processes has been proposed as the source of the first high potential electron acceptors to the biosphere (Ducluzeau et al. 2009). The high pCO_2 necessary for these sources to produce significant oxidized nitrogen, however, are inconsistent with several proxies (e.g. Rye et al. 1995, Blättler et al. 2016), while numerous models suggest that methane was a more significant greenhouse gas in counteracting the Faint Young Sun (e.g. Pavlov et al. 2000, Kasting et al. 2001, Kasting 2005); as a result, abiotic supplies of oxidized nitrogen at this time cannot be assumed to be significant. Even if abiotic fluxes of oxidized nitrogen existed on the early Earth, it is possible that the compounds produced would not have been stable over long enough timescales to be useful to the early biosphere. The Archean ocean is thought to have been relatively rich in dissolved ferrous iron (Holland 1984), and many oxidized nitrogen species, including nitrite and NO, react rapidly with ferrous iron (Klueglein and Kappler 2012, Kopf et al. 2013). As a result, any oxidized nitrogen reaching the Archean ocean from atmospheric processes may have been reduced on timescales of minutes, preventing it from playing a role in the biological nitrogen cycle until after the Rise of Oxygen titrated reduced iron from the oceans.

An alternative route to oxidized nitrogen species in the Archean ocean are uncharacterized anaerobic nitrification pathways. These include the oxidation of ammonia coupled to the reduction of iron oxides (feammox) or to phototrophy (photoammox). Many

novel nitrogen metabolisms have been uncovered in recent years, including aerobic methane oxidation coupled to denitrification (Kits et al. 2015, Skennerton et al. 2015), intraaerobic methane oxidation driven by nitric oxide dismutation (Ettwig et al. 2012), and the combined oxidation of ammonia to nitrite and then nitrate (comammox, van Kessel et al. 2015, Daims et al 2015). It is therefore reasonable that additional nitrogen metabolisms exist in the environment and simply have not yet been discovered. In 1977, Broda predicted the existence of anammox on the basis of thermodynamic calculcations, proving to be one of the most prescient hypotheses in environmental microbiology. In the same paper, he also proposed the existence of photoammox, yet this metabolism has still not been identified in an organism. In order to oxidize ammonia to nitrite or N_2, a total of 6 electrons would need to be transferred without the release of toxic intermediates like hydroxylamine; this is unheard of in the distribution of characterized phototrophs, and so may not be possible. While the phototrophic oxidation of nitrite to nitrate has been described in a small number of Proteobacteria (Griffin et al. 2007, Schott et al. 2010), this metabolism has been shown to have evolved relatively recently (Hemp et al. 2016) and therefore does not reflect an early history of phototrophic nitrification. Further investigation may uncover photoammox organisms in modern or ancient environments, but as of now there is no evidence to suggest that this metabolism has ever evolved.

Similarly, the oxidation of ammonia coupled to iron oxide reduction (feammox) has been proposed but never demonstrated in an isolated organism. This metabolism is thermodynamically favorable under a range of conditions, and would be a logical component of an Archean ecosystem driven by the phototrophic oxidation of iron (Fischer

and Knoll 2009). Evidence for the occurrence of feammox has been described in environments such as wetlands (Clement et al. 2005, Yang et al. 2012), but no conclusive evidence has been presented, nor has a microbe capable of driving this process yet been described. This metabolism, while thermodynamically favorable, is significantly kinetically inhibited. In ammonia oxidizing organisms, ammonia monooxygenase activates ammonia to more accessible forms in an O_2-dependent reaction (Bock and Wagner 2006). The process of methane oxidation is evolutionarily related to ammonia oxidation, using homologous methane monooxygenase enzymes. While some methane oxidizing bacteria are capable of using alternative electron acceptors during methanotrophy (e.g. Kits et al. 2015, Skennerton et al. 2015), these strains retain an absolute requirement for trace O_2 for the initial activation of methane, suggesting that this step is challenging if not impossible to achieve in a fully anaerobic world. The most likely scenario for an anaerobic nitrifier is one analogous to the anaerobic oxidation of methane, achieved by specialized archaea running a process of reverse methanogenesis that is still not fully understood (McGlynn 2017). It is conceivable that a pathway of reverse Dissimilatory Nitrate Reduction to Ammonia could exist, though it has never been demonstrated. Any mechanism of ammonia oxidation without O_2 or oxidized nitrogen as a substrate would therefore require exotic biochemistry, acting in an unknown organism.

It is thermodynamically favorable for feammox to proceed without biological catalysis; however, iron oxides may not have been abundant in the Archean ocean (Ward et al. 2017), and the reaction of iron oxides with ammonia is kinetically inhibited and does not

proceed spontaneously under reasonable timescales under ocean-relevant conditions (Supplemental Information).

Hypotheses about potential sources of oxidized nitrogen on the early Earth can be tested by querying the biological record of metabolisms that make use of these compounds. If a source of oxidized nitrogen was present before the Rise of Oxygen, then metabolisms that make use of these compounds, such as denitrification, should appear to be evolutionarily ancient, predating the evolution of aerobic respiration. If, however, nitrogen respiration appears to have evolved post-O_2, this will increase our confidence that no significant oxidized nitrogen was available to the early biosphere.

In order for the nitrogen cycle to be maintained at steady state over geological timescales, fixation of nitrogen must be balanced by return of nitrogen to the atmospheric N_2 pool. Today, this return is accomplished via denitrification and anammox. These metabolisms make use of oxidized nitrogen species (e.g. nitrite and nitrate) as respiratory electron acceptors coupled to the oxidation of organic carbon or ammonia.

Denitrification is the process of respiratory reduction of nitrate to N_2 via nitrite, nitric oxide, and nitrous oxide. This is a multistep process that can be found as either complete or partial pathways in a range of microbial taxa (Klotz and Stein 2016), utilizing a variety of enzymes for donating electrons from the electron transport chain onto nitrogen. The distribution of denitrification genes appears to be the result of extensive horizontal gene transfer, of entire pathways and individual genes (Jones et al. 2008). Despite the thermodynamic favorability of oxidized nitrogen species as electron acceptors, not all steps in denitrification are coupled to energy conservation, making it a relatively inefficient

pathway in terms of protons translocated per electron (Chen and Strous 2012). Some of the steps in the denitrification pathway are catalyzed by only a single enzyme or by a small group of evolutionarily related proteins. This includes nitric oxide reduction, which is catalyzed by several families of closely related Heme Copper Oxidoreductase enzymes (Hemp et al., in prep). The HCO superfamily is primarily made up of O_2 reductases, but several independent lineages of these enzymes have convergently evolved the ability to catalyze nitrogen chemistry, including putative nitric oxide dismutation, nitrous oxide reduction, and most often nitric oxide reduction (Figure 2)(Hemp et al. in prep). Evolutionary analysis of the origins of NOR enzymes can help reveal the antiquity of denitrification, and, by extension, the oxidized half of the nitrogen cycle.

Structural and phylogenetic analysis of the HCO superfamily has revealed that the A family O_2 reductases, used for aerobic respiration at relatively high oxygen concentrations (>1 micromolar), with nitric oxide reductases evolving from the derived B and C family family O_2 reductases which appear to be secondarily adapted to low oxygen concentrations, having exchanged a conserved proton channel for an oxygen channel, increasing the diffusion of O_2 to the active site at the cost of protons pumped (Hemp et al., in prep).

Nitrogen fixation on the early Earth

The foundational step in the nitrogen cycle is the fixation of atmospheric N_2 to reduced forms that can be incorporated into biomass. On the modern Earth, this is accomplished biologically via the nitrogenase enzyme (Canfield et al. 2010). It is unclear, however,

exactly when biological nitrogen fixation evolved, and therefore whether the biosphere relied on abiotically fixed nitrogen for some period of its early history. The evolution of biological nitrogen fixation via nitrogenase is contested, with estimates for its origin spanning nearly 2 billion years (Fani et al. 2000, Raymond et al. 2004, Boyd et al. 2011, Suteken et al. 2015, Boyd et al. 2015, Weiss et al. 2016). On the early Earth, before the invention of oxygenic photosynthesis, global rates of primary productivity were likely limited by geological fluxes of electron donor compounds like H_2 (Ward et al. 2017). As a result, the efficiency of consumption of these electron donors would be a critical constraint on the overall rates of productivity that result. Nitrogen fixation is an energetically costly process, and for the energy- and electron-limited metabolisms that fueled the Archean biosphere, this process would have likely been a major drain on productivity. It is therefore a reasonable expectation that nitrogenase might not evolve without strong selective pressure for biological nitrogen fixation, likely as a result of the nitrogen demand of biological primary productivity outpacing nitrogen supply through abiotic processes. To constrain when this is likely to occur, we must integrate expectations of abiotic nitrogen fixation processes compared against estimates of primary productivity through time. Abiotic nitrogen fixation on the early Earth likely occurred through a variety of processes, including lightning, photochemistry, and hydrothermal reactions (Ward et al. 2017). While the fluxes through each of these process is not well constrained, and relies on poorly understood aspects of the early Earth system like the CO_2:CH_4 ratio of the atmosphere (Navarro-Gonzalez et al. 2001), their sum can be estimated to within about an order of

magnitude, which is sufficient accuracy for comparison with expectations of early biological productivity.

It has been hypothesized that nitrogenase was present in the last universal common ancestor of life on Earth (Raymond et al 2004, Weiss et al. 2016), but this interpretation is widely disputed (Boyd et al 2011). For much of the Archean Era, abiotic nitrogen fixation through lightning and other processes could have supplied a sufficient nitrogen flux to support the Earth's much less productive biosphere (Vlaeminck et al 2011, Kharecha et al 2005, Ward et al. 2017), but increases in primary productivity and potentially declines in abiotic nitrogen fixation fluxes in the latest Archean and early Paleoproterozoic may have led to a decrease in abiotic nitrogen fixation, possibly driving the evolution of nitrogenase (Navaro-Gonzalez et al 2001, Ward et al. 2017). This nitrogen crisis may have been exacerbated by the Great Oxidation Event, as the rise in atmospheric O_2 would have allowed the nitrification of a large marine ammonium pool that may have developed in the Archean, followed by denitrification and massive loss of accumulated fixed nitrogen (Fennel et al 2005, Ward et al. 2017). Nitrogenase may then have evolved around the GOE (Boyd et al 2011). An alternative scenario for the evolution of nitrogenase is that it initially evolved for the uptake of HCN, produced via atmospheric processes under a high CH_4 atmosphere (Ward et al. 2017). Nitrogenase can be used for the uptake of HCN (Materassi and Balloni 1977, Li et al. 1982, Dekas et al. 2009), and so large fluxes of HCN to the oceans may have led to the evolution of nitrogenase first as a way to detoxify and take up HCN, and was only later coopted to N_2 fixation (Silver and Postgate 1973, Raymond 2005, Ward et al. 2017).

HCN produced via atmospheric processes (e.g. lightning and photochemistry) is expected to be delivered to the oceans by rain on timescales of ~10 years (Zahnle 1986). HCN could then be converted to ammonia via hydrolysis over timescales of decades (Abelson 1966) or directly taken up by biology via nitrogenase (Raymond 2005). Depending on pH, dissolved HCN could even polymerize and subsequently hydrolyze to produce glycine or other amino acids (Abelson 1966). Availability of atmospherically fixed nitrogen to the biosphere would depend partially on the precipitation of cyanide as ferrocyanide ($Fe(CN)_6)^{4-}$) upon reaction with dissolved iron; the production of this compound would depend on the concentration of dissolved iron in the surface ocean, which may have been low relative to the average ocean at this time (Ward et al. 2017) and relative timescales of mixing and cyanide hydrolysis. It is not known if nitrogen in ferrocyanide is accessibly to biology, and so the reaction dynamics and uptake of this phase via nitrogenase could be an important constraint for understanding the supply of nitrogen to the early biosphere. If hydrolysis is relatively slow, this would potentially encourage the early evolution of nitrogenase for the uptake of nitrogen from cyanide or ferrocyanide. However, if conversion of cyanide to ammonia, and subsequent ammonia recycling, is efficient, then the nitrogen demands of the biosphere may have been met or exceeded without nitrogenase.

An abiotic nitrogen cycle before oxygenic photosynthesis

Based on the above discussion, it appears that most steps in the biological nitrogen cycle were not viable before the evolution of oxygenic photosynthesis. Nitrification,

denitrification, and anammox were not present, and biological nitrogen fixation may or may not have evolved. However, the biosphere still needed fixed nitrogen, and so for the biosphere and an N_2-rich atmosphere to coexist a dynamic steady state must have existed between reduced nitrogen species accessible to biology and return of N_2 to the atmosphere. This could have taken the form of a largely abiotic, reduced nitrogen cycle before the evolution of oxygenic photosynthesis. In this model (Figure 3), atmospheric N_2 is fixed to more reduced forms by abiotic processes including lightning, photochemistry, and reactions at hydrothermal vents; organic nitrogen is interconverted with biomass; small fluxes of organic nitrogen are buried and eventually returned to the atmosphere as N_2 following subduction and degassing, but most nitrogen is returned to the atmosphere via equilibration of marine ammonia with the atmosphere and subsequent photolysis. The primary pools of nitrogen would therefore be atmospheric N_2, marine NH_3, organic nitrogen, and atmospheric NH_3. Assuming previous estimates of abiotic nitrogen fixation rates (Ward et al. 2017), burial of organic nitrogen estimated by a 40 C:1 N ratio (Berner 2006) scaled to expected organic carbon burial (Ward et al. 2017), volcanic outgassing rates of nitrogen (Sano et al. 2001), equilibration between dissolved and gaseous ammonia, and estimates of photolysis of atmospheric ammonia (Kasting 1982), a simple steady state model of the Archean nitrogen cycle can be developed. Under these steady state conditions, fixation of N_2 to reduced forms results in accumulation of NH_3 in the oceans, likely more than meeting the nitrogen demands of the electron donor-limited early biosphere, even without recycling or biological nitrogen fixation (Ward et al. 2017). This dissolved ammonia will be in equilibrium with a tenuous atmospheric ammonia phase, which would

be rapidly photolysed back to N_2. Depending on atmospheric composition, temperature, and other variables, dissolved ammonia concentrations in the ocean would range over the order of 1-100 μM, with atmospheric ammonia mixing ratios on the order of 10^{-8}—too low for ammonia to play a significant role in maintaining climate under the faint young sun (Kasting 1982). If significant haze developed in the Archean atmosphere, NH_3 may have been shielded from UV photolysis, increasing the proportion of nitrogen that could be locked into reduced species (Pavlov et al. 2001).

Conclusions

Under the scenario described above, the early, pre-oxygen nitrogen cycle was largely abiotic, consisting of transformations between N_2 and reduced nitrogen species primarily via atmospheric processes. Life's role would have been minor, and consisted largely of interconversion of reduced nitrogen with biomass, and delivery of a portion of that organic nitrogen to sediments. Given the expected low productivity of the early biosphere, the relatively high expected abiotic nitrogen fixation rates, and the likelihood of high nitrogen recycling, biological nitrogen fixation may not have been necessary until relatively late in Earth history, potentially evolving around the time of the Great Oxygenation Event. Alternatively, nitrogenase may have evolved earlier, potentially in a niche nitrogen-limited environment or for the uptake of cyanide into biomass followed later by cooption for N_2 fixation, a scenario that has been hypothesized previously (e.g. Silver and Postgate 1973, Raymond 2005, Ward et al. 2017). Whether nitrogenase predated the GOE or not, it appears that the remainder of the nitrogen cycle did not evolve until this

time. Tests of this hypothesis will hinge on development of an appropriate isotope mass balance framework for interpreting the Archean nitrogen cycle in the context of the fluxes described here.

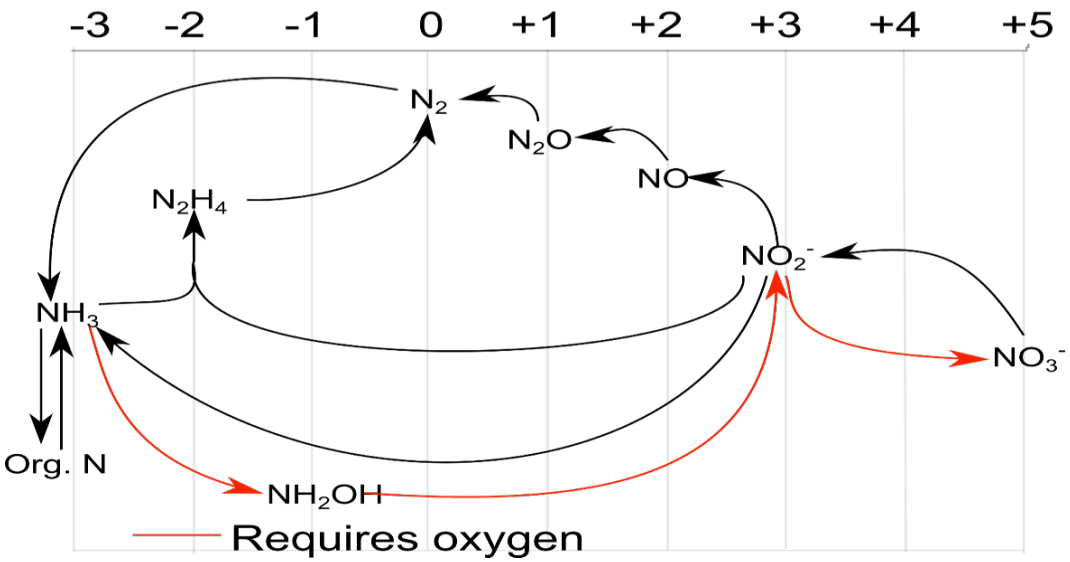

Figure 1: Topology of the nitrogen cycle plotted against the oxidation state of nitrogen. Atmospheric N2 can be fixed into reduced forms like ammonia that can be interconverted with organic nitrogen. Subsequent cycling requires molecular oxygen to drive nitrification to nitrite and nitrate. These compounds can then serve as substrates for denitrification and anammox, returning nitrogen to the atmosphere. Without O2, however, nitrogen would become trapped in reduced forms.

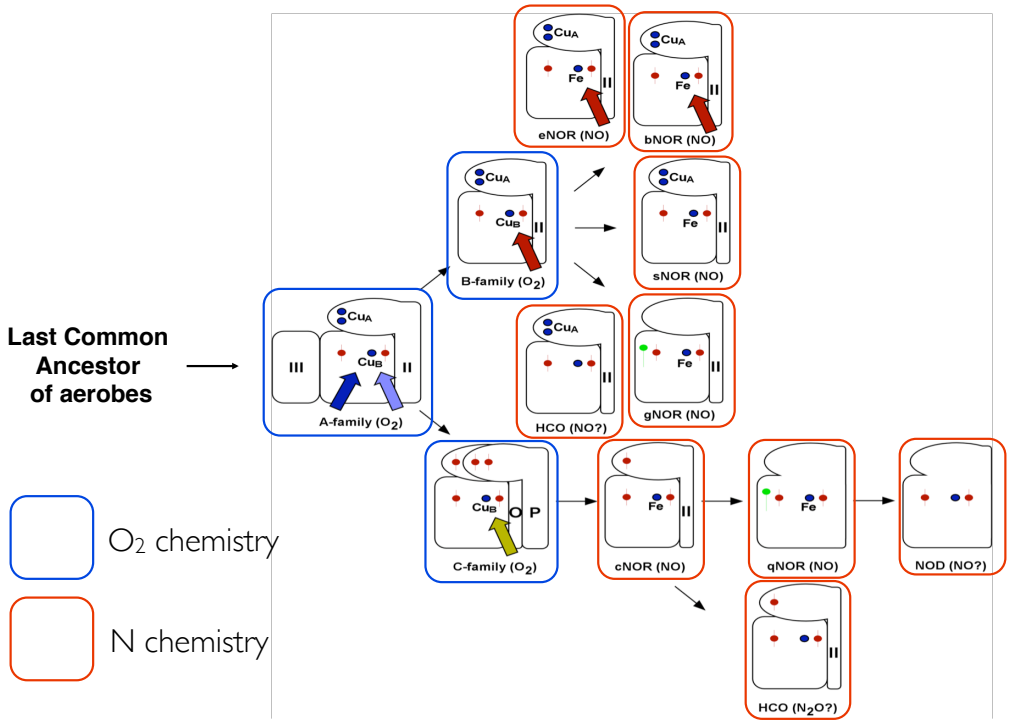

Figure 2: Cartoon of the evolution of the Heme Copper Oxidoreductase superfamily, showing derivation of nitrogen reductases from the A-family O2 reductases. Modified from Hemp et al.

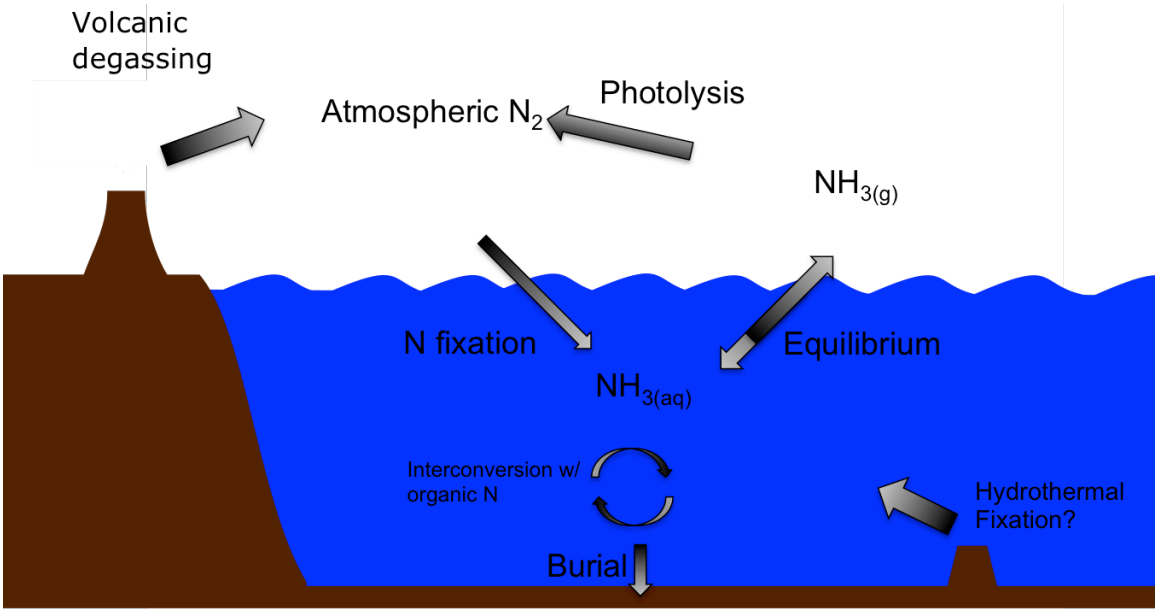

Figure 3: Topology of the pre-oxygen, Archean nitrogen cycle described above. Nitrogen existed in only reduced forms, and was primarily cycled between NH_3 and N_2 via abiotic atmospheric processes.

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Supplemental Information:

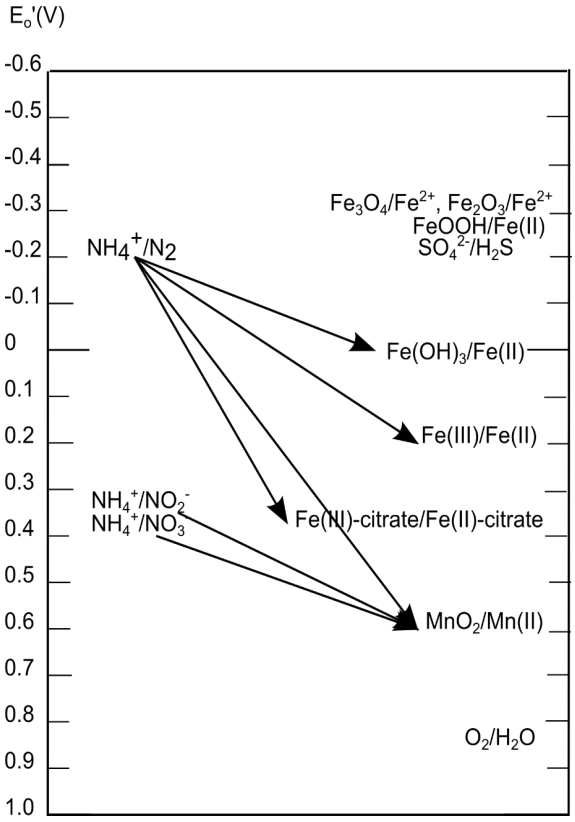
Anaerobic oxidation of ammonium coupled to Fe(III) reduction— "feammox"—could have provided a source of oxidized nitrogen before the evolution of oxygenic photosynthesis, and therefore promoted the closure of the nitrogen cycle without oxygen. Production of N_2 or nitrite by this process is thermodynamically favorable at environmentally reasonable substrate (Supplemental Figure 1, Supplemental Figure 2). While no microorganism has ever been characterized as driving this process, it is conceivable that it could proceed abiotically. To characterize the kinetics of the abiotic reactions, we incubated sterile solutions of ammonium with ferrihydrite (a reactive, environmentally common, poorly-ordered ferric iron phase) and measured the production of reduced iron over time. Results suggest that feammox does not proceed spontaneously

under environmentally relevant conditions, so this process is unlikely to play a role in modern or ancient environments in the absence of biological catalysis. In contrast, similar abiotic incubations containing manganese oxides demonstrate spontaneous reaction with ammonia over timescales of several months. This process ("manoxammox") may be important in some modern environments (Hulth et al. 1999, Luther et al. 1997) but was unlikely to be a factor in the nitrogen cycle before the evolution of oxygenic photosynthesis, as manganese oxides were not present on Earth's surface until around the GOE (Johnson et al. 2013).

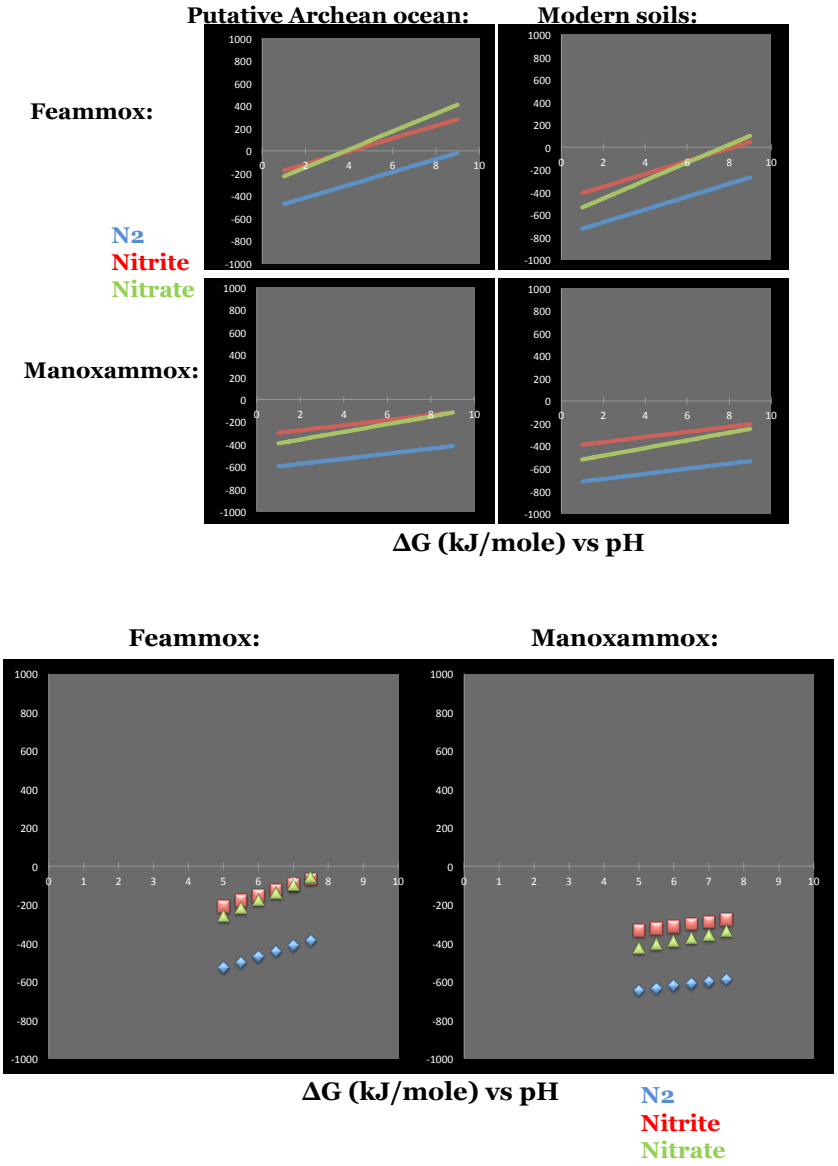
Experiments consisted of serum vials prepared anaerobically with milliQ H_20, buffered with 10mM PIPES and adjusted to pH 5 or 7, to which were added 5-100 mM NH_4Cl and 1 g/L of ferrihydrite (feammox), or either birnessite or colloidal MnO_2 (manoxammox). Vials were then sparged with 100% N_2 and overpressured to 20 psi. Vials were left in the dark at room temperature for the duration of the experiment except during sampling, which was performed in an anaerobic chamber. Measurements were taken via ion chromatography on a Dionex DX500 equipped parallel CS16 cation and AS19 anion columns to measure Mn(II), NO_3^-, NO_2^-, and NH_4^+. Fe(II) was measured using the ferrozine assay (Carter 1971). Sensitivity was linear down to analyte concentrations of ~50μM, and consistent to within ~3%.

No change was detected in the concentrations of any reactants during weekly sampling for 70 days (Supplemental Figure 3). However, following several additional months additional incubation in the dark at room temperature, dissolution of colloidal manganese was determined by visual assessment, likely via reduction by ammonium (Supplemental

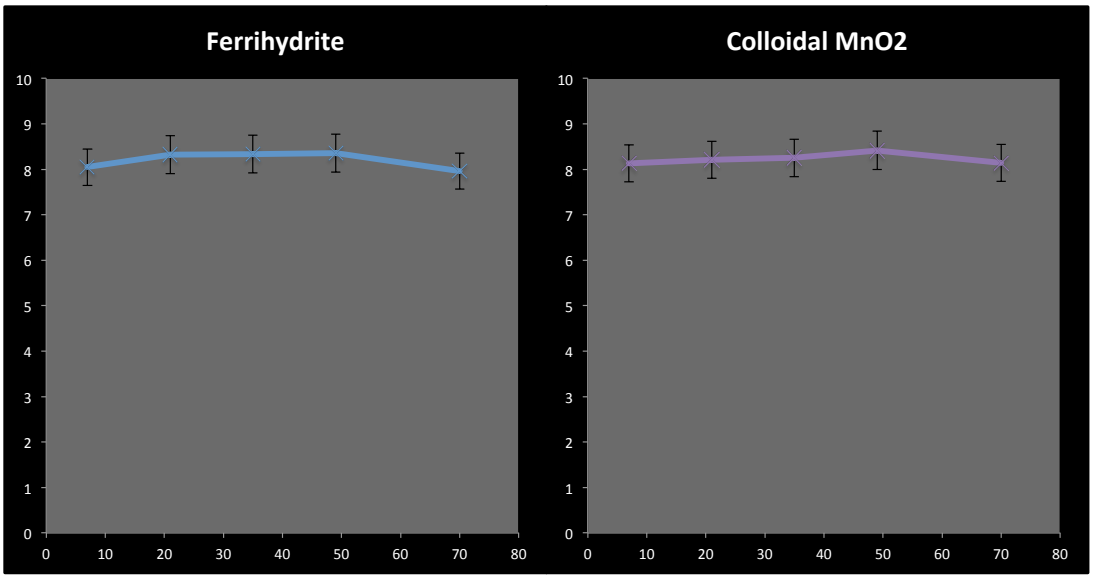
Figure 4). This suggests that manoxammox can proceed spontaneously over timescales of several months, despite no measurable reaction in the first several weeks. No visible change in ferrihydrite or birnessite was observed even up to three years later, indicating that while some poorly ordered manganese oxide phases are capable of spontaneous reaction with ammonia, this is not true of iron oxides or more highly ordered manganese.



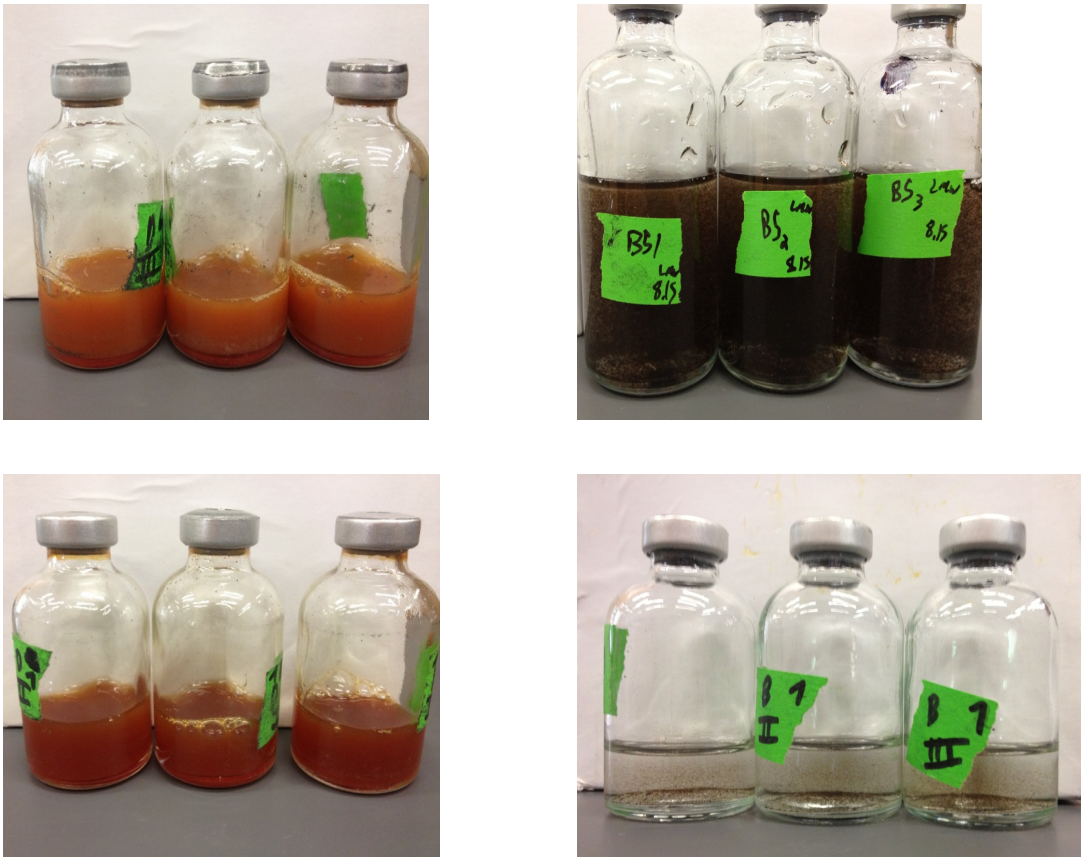
Supplemental Figure 1: Redox ladder with reduction potential of feammox/manoxammox reactions (arrows). Values are calculated at standard state and pH 7.



Supplemental Figure 2: Thermodynamic favorability (ΔG/kJ/mole) against pH for feammox under putative Archean conditions (top left), feammox in modern iron-rich anoxic soils (top right), manoxammox under putative late Archean/early Proterozoic conditions (middle left), and manoxammox in modern manganese-rich anoxic soils (middle right). Below, favorability of feammox and manoxammox, respecitively, under experimental conditions using ferrhydrite and birnessite.



Supplemental Figure 3: Measurements of dissolved ammonium concentrations over 70 days of incubation. No significant change in ammonium was detected in this time.



Supplemental Figure 4: Photographs of incubations. Top, vials as initially prepared, containing dense suspended metal oxides (ferrihydrite at left, colloidal manganese at right). Below, vials after several months of incubation. Ferrihydrite solutions have not noticeably reacted, while colloidal manganese has largely dissolved.

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