



# Cyanobacterial Diazotrophy and Earth's Delayed Oxygenation

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The redox landscape of Earth's ocean-atmosphere system has changed dramatically throughout Earth history. Although Earth's protracted oxygenation is undoubtedly the consequence of cyanobacterial oxygenic photosynthesis, the relationship between biological O<sub>2</sub> production and Earth's redox evolution remains poorly understood. Existing models for Earth's oxygenation cannot adequately explain the nearly 2.5 billion years delay between the origin of oxygenic photosynthesis and the oxygenation of the deep ocean, in large part owing to major deficiencies in our understanding of the coevolution of O<sub>2</sub> and Earth's key biogeochemical cycles (e.g., the N cycle). For example, although possible links between O<sub>2</sub> and N scarcity have been previously explored, the consequences of N<sub>2</sub> limitation for net biological O<sub>2</sub> production have not been examined thoroughly. Here, we revisit the prevailing view that N<sub>2</sub> fixation has always been able to keep pace with P supply and discuss the possibility that bioavailable N, rather than P, limited export production for extended periods of Earth's history. Based on the observation that diazotrophy occurs at the expense of oxygenesis in the modern ocean, we suggest that an N-limited biosphere may be inherently less oxygenic than a P-limited biosphere—and that cyanobacterial diazotrophy was a primary control on the timing and tempo of Earth's oxygenation by modulating net biogenic O<sub>2</sub> fluxes. We further hypothesize that negative feedbacks inhibit the transition between N and P limitation, with the implication that the pervasive accumulation of O<sub>2</sub> in Earth's ocean-atmosphere system may not have been an inevitable consequence of oxygenic photosynthesis by marine cyanobacteria.

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## INTRODUCTION

Cyanobacteria are responsible for two major metabolic innovations: (1) oxygenic photosynthesis and (2) aerobic N<sub>2</sub> fixation. The former initiated the protracted oxygenation of Earth's ocean-atmosphere system and eventually set the stage for the evolution of complex animal life (reviewed by Lyons et al., 2014). The latter is also a remarkable achievement considering that nitrogenase, the enzyme responsible for cleaving the dinitrogen triple bond, is irreversibly inactivated by O<sub>2</sub> (reviewed by Berman-Frank et al., 2003). Despite the apparent incompatibility of oxygenesis and diazotrophy, cyanobacteria dominate N<sub>2</sub> fixation in the modern well-oxygenated ocean (Capone et al., 1997). This uniquely cyanobacterial ability to reconcile both environmental (exogenous) and photosynthetic (endogenous) O<sub>2</sub> with N<sub>2</sub> fixation (diazotrophy) acts to minimize the potential for biospheric N limitation and favors primary production that is broadly limited by the bioavailability

of P in the modern ocean (Redfield, 1958; Tyrrell, 1999). Nevertheless, the details of the co-evolution of cyanobacterial innovation, the O<sub>2</sub> content of the ocean-atmosphere system, and Earth's nutrient cycles during the Precambrian remain unclear. In particular, the origin of oxygenic photosynthesis and the oxygenation of Earth's atmosphere appear to be temporally decoupled, and there are major uncertainties regarding the primary controls on the timing and tempo of Earth's oxygenation (Lyons et al., 2014). The dynamics of the transition from a broadly anaerobic biosphere supported by NH<sub>4</sub><sup>+</sup> and anaerobic diazotrophy to an aerobic biosphere dominated by NO<sub>3</sub><sup>-</sup> assimilation and O<sub>2</sub>-tolerant cyanobacterial diazotrophy are also poorly known (Fennel et al., 2005), muddling our understanding of the causal relationships at play between O<sub>2</sub> production, oxygenation, and marine nutrient cycling.

Herein, we examine the metabolic capabilities of extant diazotrophic cyanobacteria in the context of recent constraints on the oceanographic conditions of the mid-Proterozoic (~1.8 to 0.8 billion years ago or Ga). We discuss the possibility that the spatiotemporal redox landscape of the mid-Proterozoic ocean would have been less than ideal for cyanobacterial N<sub>2</sub> fixation, favoring widespread N scarcity rather than P limitation. We also explore the implications of N limitation for biogenic O<sub>2</sub> fluxes from the marine biosphere, hypothesizing that cyanobacterial production would have been less oxygenic under the O<sub>2</sub> and nutrient conditions that typified the mid-Proterozoic ocean—potentially providing a biological mechanism for stabilizing pO<sub>2</sub> at values much lower than those of the modern Earth.

## EARTH'S REDOX EVOLUTION

The origin of cyanobacterial oxygenic photosynthesis significantly predates the permanent and pervasive oxygenation of Earth's ocean-atmosphere system (Lyons et al., 2014). A growing collection of trace metal and isotopic records collectively indicates the accumulation of dissolved O<sub>2</sub> in highly productive regions of the shallow ocean up to 50–500 million years before the initial oxygenation of the atmosphere ~2.4 Ga (e.g., Anbar et al., 2007; Kendall et al., 2010; Planavsky et al., 2014a)—a conclusion that is supported by recent biogeochemical models (Olson et al., 2013; Reinhard et al., 2013a). Evidence for dissolved O<sub>2</sub> prior to the initial oxygenation of Earth's atmosphere in the early Paleoproterozoic includes N isotope records that capture the onset of coupled nitrification and denitrification, implying metabolic utilization of O<sub>2</sub> in the oxidation of NH<sub>4</sub><sup>+</sup> to N oxyanions in the surface ocean during the late Archean (Garvin et al., 2009; Godfrey and Falkowski, 2009). It is likely that dissolved O<sub>2</sub> also accumulated, at least locally, in freshwater environments and within microbial mats, in isolation from prevailing oceanic and atmospheric conditions (Herman and Kump, 2005; Lalonde and Konhauser, 2015; Sumner et al., 2015). As such, inhibition of nitrogenase by O<sub>2</sub> may have been an issue in both terrestrial and marine oxygen oases well before the first rise of atmospheric oxygen, and an initial inability to fix N<sub>2</sub> aerobically to replenish bioavailable

N lost through denitrification may have limited cyanobacterial proliferation in the late Archean (Scott et al., 2011).

It is also clear that Earth's protracted oxygenation was not unidirectional and featured dramatic variability on a variety of timescales in addition to spatial heterogeneity (Lyons et al., 2014). Although relatively high atmospheric pO<sub>2</sub> has been suggested during the Paleoproterozoic Lomagundi Event ~2.3–2.1 Ga (Bekker and Holland, 2012; Rybacki et al., 2016), several geochemical records collectively suggest that environmental O<sub>2</sub> levels subsequently fell precipitously at the onset of the ensuing Boring Billion (~1.8–0.8 Ga) and remained relatively low until the late Neoproterozoic (Planavsky et al., 2012; Partin et al., 2013). Recent O<sub>2</sub> constraints suggest mid-Proterozoic pO<sub>2</sub> of less than 10<sup>-3</sup> times the present atmospheric level as a long-term average (Planavsky et al., 2014b), with the implication that the dissolved O<sub>2</sub> landscape of the mid-Proterozoic ocean was not markedly different than that of the late Archean (Reinhard et al., 2016). The emerging view is that the surface ocean was likely characterized by low-micromolar O<sub>2</sub> that was highly variable in space and time (Reinhard et al., 2016), while the deep ocean remained anoxic (Poulton and Canfield, 2011)—for the vast majority of Earth history. Indeed, despite evidence for oxygenation in the late Neoproterozoic (Sahoo et al., 2012; Planavsky et al., 2014b), clear evidence for pervasive and permanent oxygenation within the ocean is lacking until the Phanerozoic (Kendall et al., 2015; Sperling et al., 2015). In this view, the so-called Great Oxidation Event of the early Paleoproterozoic has relatively minor ecological implications compared to subsequent oxygenation in the late Neoproterozoic and early Phanerozoic. Meanwhile, it remains unclear why biospheric O<sub>2</sub> production failed to result in stably oxygenated surface environments and deep ocean oxygenation for ~2.5 billion years after cyanobacterial emergence.

## OXYGENATION AND DENITRIFICATION

The persistence of O<sub>2</sub> stratification, that is, the widely held view that O<sub>2</sub> was mostly restricted to the shallow ocean until the Paleozoic, has profound implications for the operation of the N cycle during Precambrian time. Denitrification, the reduction of bioavailable NO<sub>3</sub><sup>-</sup> to N<sub>2</sub> via anaerobic respiration of organic matter, results in a substantial loss of bioavailable N in O<sub>2</sub>-deficient environments. Anaerobic oxidation of NH<sub>4</sub><sup>+</sup> coupled to NO<sub>2</sub><sup>-</sup> reduction (anammox) also represents a leak for bioavailable N as inert N<sub>2</sub> (Kuypers et al., 2005). In the modern well-oxygenated ocean, denitrification and anammox are limited to organic-rich sediments and oxygen minimum zones (OMZs) underlying high-nutrient waters where the subsurface demand for respiratory electron acceptors locally exceeds O<sub>2</sub> supply (Gruber and Sarmiento, 1997). Denitrification and anammox would also have been limited prior to the origin of oxygenic photosynthesis as the result of limited NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> synthesis in an anoxic ocean. The redox-stratified water column of the Proterozoic Earth, however, would have been particularly conducive to widespread loss of bioavailable N, because surface oxygenation would have allowed oxidation of NH<sub>4</sub><sup>+</sup> to N

oxyanions by  $O_2$  in surface environments to support continued subsurface  $N_2$  synthesis via denitrification and anammox (Fennel et al., 2005; Garvin et al., 2009; Godfrey and Falkowski, 2009).

## OXYGENATION AND $N_2$ FIXATION

The likelihood of extensive loss of fixed N through denitrification requires a correspondingly large influx of bioavailable N via diazotrophy at steady-state; if  $N_2$  fixation cannot keep pace with denitrification, export production must decline until denitrification and  $N_2$  fixation are balanced (e.g., Deutsch et al., 2007). Thus, widespread denitrification may potentially modulate the long-term oxidation dynamics of Earth's ocean-atmosphere system by limiting the burial of reduced carbon and sulfur if  $N_2$  fixation was less efficient than today. Indeed, there are several reasons that the Proterozoic surface ocean may have been less than ideal for cyanobacterial diazotrophy. Based on the metabolic capabilities of extant cyanobacteria, we suggest that the low-level, spatially and temporally variable  $O_2$  concentrations that likely characterized the Proterozoic surface ocean would have posed a worst-case scenario for  $N_2$  fixation by cyanobacteria—thus allowing for the possibility of an N-limited biosphere for much of Earth history.

Extant diazotrophic cyanobacteria differ dramatically in their ability to cope with environmental  $O_2$ , and many  $N_2$  fixing cyanobacteria are actually obligate aerobes despite nitrogenase sensitivity to  $O_2$  (reviewed by Fay, 1992; Gallon, 1992). For these cyanobacteria, aerobic respiration provides: (1) a strategy for protecting nitrogenase by contributing to the maintenance of low intracellular  $O_2$  and (2) an energy source to support the demanding task of cleaving the dinitrogen triple bond while simultaneously maintaining  $O_2$  defense mechanisms. For example, despite the need for anoxic conditions *within* heterocysts, these differentiated cells are metabolically costly, and the rate at which  $O_2$  can diffuse into heterocysts can be an important control on the rate of  $N_2$  fixation in low  $O_2$  environments (Stal, 2009). Among modern heterocystous cyanobacteria, the metabolic machinery for  $N_2$  fixation appears to be optimized for present day air-saturated or even modestly elevated  $O_2$  concentrations (e.g.,  $\sim 300 \mu\text{M}$  for *Anabaena cylindrica*; see Tomitani et al., 2006). Ambient  $O_2$  concentrations also appear to control both the rate and frequency of heterocyst growth (Kangatharalingam et al., 1992), with heterocysts grown under  $O_2$ -replete conditions demonstrating lower sensitivity to environmental instability (e.g., diurnal fluctuations in  $O_2$  availability; Rippka and Stanier, 1978). In sum, the ability to cope with inhibitory levels of  $O_2$  via heterocystic cellular differentiation seems, rather ironically, to be favored by persistently elevated  $O_2$ . These observations highlight the possibility that heterocystous diazotrophy may have been challenging for marine cyanobacteria for the vast majority of Earth history. Indeed, unambiguous marine heterocyst fossils are absent until the Phanerozoic (Golubic et al., 1995), and heterocystous cyanobacteria are relatively uncommon in well-oxygenated environments of the modern pelagic ocean (Zehr, 2011).

The non-heterocystous cyanobacteria that are capable of aerobic diazotrophy are generally tolerant of lower  $O_2$  concentrations than the heterocystous cyanobacteria. Nonetheless, some non-heterocystous cyanobacteria are also obligate aerobes, and low  $O_2$  availability may also reduce the rate of non-heterocystous  $N_2$  fixation (reviewed by Fay, 1992). Most aerobic non-heterocystous diazotrophic cyanobacteria optimally fix  $N_2$  at dissolved  $O_2$  concentrations on the order of  $\sim 10$ – $50\%$  of present marine levels (see Tomitani et al., 2006). Thus, it is likely that Proterozoic shallow marine dissolved  $O_2$  concentrations ( $\sim 1\%$  of modern; Reinhard et al., 2016) would have been suboptimal for diazotrophic cyanobacteria that were obligate aerobes. Meanwhile, even  $\sim 1\%$  of modern dissolved  $O_2$  is sufficient to completely inhibit anaerobic  $N_2$  fixation among the majority of microaerobic non-heterocystous cyanobacteria (Bergman et al., 1997) and the obligate anaerobes capable of diazotrophy (e.g., some methanogens; Murray and Zinder, 1984).

## N SCARCITY AND NET $O_2$ PRODUCTION

Not all  $O_2$ -tolerant,  $N_2$ -fixing cyanobacteria meet the energetic demands of  $N_2$  fixation through aerobic respiration. Although  $O_2$  is not typically limiting in the open ocean today, one of the most successful marine diazotrophs, *Trichodesmium*, does not depend on exogenous  $O_2$  to respire stored carbohydrates. Instead, *Trichodesmium* is able to use its photosynthetic enzymes to power  $N_2$  fixation (reviewed by Bergman et al., 2013). However, because *Trichodesmium* performs diazotrophy at the expense of photosynthesis during daylight, midday  $O_2$  production is markedly reduced and potentially reversed when bioavailable N is limiting (Berman-Frank et al., 2001). Several other widespread unicellular marine cyanobacteria also fix  $N_2$  during the day using electron transfer from photosystem I (PSI)—and these cyanobacteria completely lack the ability to perform oxygenic photosynthesis (Zehr et al., 2008; Bothe et al., 2010; Tripp et al., 2010). These alternative strategies for coping with N scarcity would have been particularly advantageous if the ancient marine  $O_2$  landscape was not persistently favorable for either obligately aerobic or anaerobic  $N_2$  fixation. If PSI-dependent  $N_2$  fixation by  $O_2$  tolerant diazotrophic cyanobacteria was more common during Proterozoic time, cyanobacterial production may have been widely anoxygenic for much of Earth history. It follows that even if previous claims that the C isotope record precludes major changes in the burial of reduced organic carbon are correct (e.g., Holland, 2002), the C isotope record does not rule out major changes in net  $O_2$  fluxes from the marine biosphere through time.

This potential for reduced net oxygenation in a low- $O_2$  ocean is potentially exacerbated by the enhanced ecological significance of anoxygenic phototrophy coupled to the oxidation of  $H_2S$  (Johnston et al., 2009; Hamilton et al., 2016). Indeed, biomarkers and several inorganic proxy records point to local subsurface  $H_2S$  accumulation (euxinia) during the Proterozoic (Brocks et al., 2005; Scott et al., 2008; Lyons et al., 2009), despite the apparent persistence of iron-rich (ferruginous)

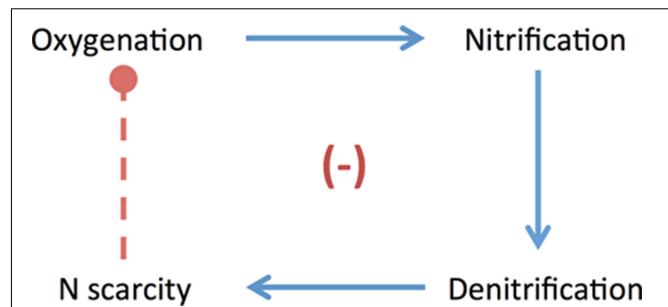
conditions at depth (Planavsky et al., 2011; Poulton and Canfield, 2011). In addition to supporting greater anoxygenic production, euxinia may also increase the likelihood of nitrogenase metal cofactor limitation due to the burial of Mo in euxinic sediments (e.g., Anbar and Knoll, 2002)—even if euxinia was limited to only ~1–10% of the seafloor (Reinhard et al., 2013b). Regardless, because anaerobic respiration using  $\text{SO}_4^{2-}$  as an electron acceptor (sulfate reduction) yields less energy than denitrification, evidence for euxinia is synonymous with evidence for  $\text{NO}_3^-$  depletion (Boyle et al., 2013). The observation that euxinia was relatively common in both space and time during the Proterozoic is therefore fully consistent with the conclusion that N stress may have been problematic for the Proterozoic biosphere, as well as the assertion that the Proterozoic biosphere may have been less oxygenic.

## OXYGEN-NUTRIENT FEEDBACKS

Whereas diazotrophy provides a pathway to alleviate N limitation, P is exclusively sourced to the ocean through crustal weathering. This inability of the marine biosphere to replenish P gives rise to the paradigm of a P-limited biosphere throughout Earth history (Redfield, 1958; Tyrrell, 1999). However, the assumption that cyanobacterial  $\text{N}_2$  fixation could keep pace with P supply during the Precambrian requires revisiting for reasons discussed above. In contrast with the prevailing view that P has limited marine productivity throughout Earth history, we hypothesize that prolonged N deficiency may have been a primary control on biospheric  $\text{O}_2$  fluxes and the tempo of Earth's oxygenation.

As long as the ocean remained stratified with respect to  $\text{O}_2$ , subsurface anoxia would have allowed a positive coupling between surface oxygenation and denitrification: nitrification is enhanced as surface oxygen oases expand, allowing greater loss of fixed N through subsequent subsurface denitrification—promoting N scarcity and providing a stabilizing negative feedback on biogenic  $\text{O}_2$  accumulation (Figure 1; also see Fennel et al., 2005). Complete alleviation of N scarcity via  $\text{N}_2$  fixation would be challenging for the reasons outlined above, which may result in reduced export production and organic C burial in response to oxygenation. Meanwhile, we argue that enhanced biospheric dependence on diazotrophy may also result in a reduction of net  $\text{O}_2$  production per mol organic C burial. Thus, minor oxygenation of a redox-stratified biosphere may strongly disfavor further oxygenation through the reduction of net biological  $\text{O}_2$  production.

The critical coupling between oxygenation and denitrification is reversed under  $\text{O}_2$ -replete conditions. Following the oxygenation of the deep ocean, increases in  $\text{O}_2$  would tend to limit the areal extent of denitrifying conditions in the ocean, minimizing the likelihood of N stress and favoring P limitation (Fennel et al., 2005). Meanwhile, net  $\text{O}_2$  production would be expected to increase as the biospheric dependence on  $\text{N}_2$  fixation and the contribution of anoxygenic phototrophy to export production decreased.



**FIGURE 1 | Nutrient- $\text{O}_2$  feedbacks.** Positive couplings are represented by solid blue arrows; negative couplings are represented by dashed red arrows. In the scenario shown here, minor surface oxygenation encourages nitrification, which allows greater subsurface denitrification (positive couplings). Loss of bioavailable N through denitrification in turn prompts greater  $\text{N}_2$  fixation—and we suggest that enhanced biospheric dependence on cyanobacterial diazotrophy results in reduced net  $\text{O}_2$  production (a negative coupling). The consequence is a stabilizing negative feedback: minor oxygenation results in reduced  $\text{O}_2$  production, strongly disfavoring  $\text{O}_2$  accumulation.

Thus, there may be two stable oxygen-nutrient states for the Earth system following the origin of oxygenic photosynthesis: an  $\text{O}_2$ -stratified, N-limited biosphere and an  $\text{O}_2$ -replete, P limited biosphere (Anbar and Knoll, 2002; Fennel et al., 2005). Indeed, updated C isotope models suggest a permanent increase in organic burial at the end of the Proterozoic (Krissansen-Totton et al., 2015), possibly consistent with the alleviation of N stress and the transition to a P-limited biosphere as the consequence of oxygenation in the late Neoproterozoic.

## CONCLUDING REMARKS

Aerobic diazotrophy was a critical cyanobacterial innovation on par with oxygenic photosynthesis in terms of ecological impact, but it is likely that its environmental context and its contribution to global N cycling has varied dramatically through time. An important implication is that net biogenic  $\text{O}_2$  fluxes may have also varied through time. We hypothesize that low  $\text{O}_2$  availability may perpetuate low  $\text{O}_2$  conditions in the surface ocean, and anoxia at depth, by promoting extensive denitrification and the maintenance of conditions that are unfavorable for diazotrophy, both aerobic and anaerobic. Based on the success of anoxygenic diazotrophic cyanobacteria in the modern ocean, we further suggest that the most favorable cyanobacterial strategies for coping with N deficiency may have resulted in markedly reduced  $\text{O}_2$  production relative to today.

Enhanced biospheric dependence on cyanobacterial diazotrophy during the mid-Proterozoic, therefore, may explain the ~2.5 billion years delay between the Archean origin of oxygenic photosynthesis and the pervasive oxygenation of the ocean-atmosphere system in the Phanerozoic. Considering the likelihood of negative feedbacks that would tend to inhibit mitigation of global N-limitation, the relatively recent transition



away from a world in which O<sub>2</sub> accumulation was spatially limited to oases within the surface ocean toward a more pervasively oxygenated ocean may not be readily understood as the inevitable consequence of continued photosynthetic O<sub>2</sub> production in surface environments. Instead, the transition from an O<sub>2</sub>-stratified, N-deficient marine biosphere to the broadly P-limited and well-oxygenated biosphere may require a yet unknown external perturbation to Earth's O<sub>2</sub> cycle. Although uncertainties remain, it is clear that cyanobacteria have played a highly dynamic role in the protracted oxygenation of the Earth system throughout the Precambrian—extending well beyond their invention of oxygenic photosynthesis roughly three or more billion years ago.

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## AUTHOR CONTRIBUTIONS

SO wrote the paper with insight from all authors.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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