Nitrogenase inhibition limited oxygenation of the Proterozoic atmosphere

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Cyanobacteria produced the atmospheric O₂ that began accumulating 2.4 billion years ago¹, leading to Earth's Great Oxidation Event (GOE)². For nearly 2 billion years following the GOE, O₂ production was restricted and atmospheric oxygen remained low²⁻⁵. Oxygen rose again sharply with the advent of land plants roughly 450 million years ago, which increased atmospheric O₂ through carbon burial⁴⁻⁵. Why did the O₂ content of the atmosphere remain constant and low for more than a billion years despite the existence of O₂-producing cyanobacteria? While geological limitations have been explored²⁻⁷, the limiting factor may have been biological, and enzymatic. Here we propose that O₂ was kept low by oxygen inhibition of nitrogenase activity. Nitrogenase is the sole N₂-fixing enzyme on Earth, and is inactive in air containing 2% or more O₂ by volume⁸. No O₂-resistant nitrogenase enzyme is known⁹⁻¹². We further propose that nitrogenase inhibition by O₂ kept atmospheric O₂ low until upright terrestrial plants physically separated O₂ production in aerial photosynthetic tissues from N₂ fixation in soil, liberating nitrogenase from inhibition by atmospheric O₂.

Current views of oxygen in Earth history (Fig. 1) depict the first traces of O₂ appearing in the atmosphere starting about 2.7 to 2.5 Gy ago¹⁻⁵. During the Great Oxidation Event, or GOE, roughly 2.4 billion years ago², O₂ rose to about 10% of its present atmospheric level (PAL), corresponding to an atmosphere of roughly 2% O₂ by volume², or even less³. Isotopic studies indicate that for roughly 1.5 billion years following the comparatively sudden GOE, further net O₂ accumulation ceased, with atmospheric levels remaining stable and below 10% PAL²⁻⁴.

Fig. 1 | Schematic summary of O₂ accumulation in Earth history. Modified after refs. 1-5. For most of the Proterozoic eon, free O₂ was much less abundant than it is today. Lyons et al. (2014) estimate Proterozoic O₂ in the atmosphere as low as 0.001 PAL while Holland² estimates atmospheric O₂ at around 0.1 to 0.2 PAL. Stolper and Keller⁴ estimate mid-Proterozoic deep ocean dissolved O₂ concentrations at about 11 μM or roughly 0.06 of the present value of 178 μM. “Whiffs” refers to isotope signatures for evidence of transient, local O₂ before the GOE^{1,3}. The Lomagundi excursion is represented as a dotted line because it is included in the summary of ref.³ but not in that of refs.^{1,2,4,5}. Heterocysts are differentiated cells of some cyanobacteria, and protect nitrogenase from inactivation by O₂. Their relevance is that cyanobacteria have an ancient fossil record, but the oldest fossil heterocysts²⁶ are younger than land plants, suggesting that cyanobacteria evolved this mechanism of O₂ protection in response to Phanerozoic O₂ accumulation. PAL; Present Atmospheric Level. GOE; Great Oxidation Event.

With atmospheric O₂ low, marine O₂ stayed low as well. Geochemical evidence suggests that the oceans remained largely anoxic throughout the Proterozoic¹⁻⁵, with a rapid rise to roughly

modern oxygen levels starting around 580 My ago, perhaps as recently as only 430 My ago^{4,5} (Fig. 1). Late increases in atmospheric O₂ implicate the emergence of land plants and terrestrial carbon burial as a causal factor. Today, land plants comprise roughly 97% of Earth's surface-exposed biomass⁷. Their ecological success has been linked to the rise in O₂ because terrestrial sequestration of organic carbon as fibrous biomass protects it from reoxidation to CO₂, curbing O₂ consumption⁴⁻⁵.

What limited oxygen accumulation? A major puzzle of O₂ history is why O₂ rose so late, that is, why atmospheric and marine O₂ levels stayed low for almost 2 billion years despite the existence of cyanobacteria, which were capable of continuous light-driven O₂ production. What held cyanobacteria back, why did O₂ stop accumulating after the GOE and why did it remain low during the Proterozoic, or the “Boring Billion” as it is sometimes called³.

Geochemists have long recognized that Proterozoic O₂ stasis presents a problem and have proposed a number of explanations to account for the delayed oxygen rise. Some proposals posit a steady supply of geochemical reductants from within the Earth, such as Fe²⁺ or S²⁻, reductants that continuously consumed the O₂ produced by cyanobacteria, keeping O₂ low⁸. Other proposals invoke biotically induced changes affecting in the degree of mixing between nutrient rich reservoirs and the photic zone, for example through animal burrowing activity⁹. Germane to many proposals is the concept that crucial nutrients such as molybdenum, which is required for nitrogenase activity, were limited in supply by geochemical factors and that nitrogenase limited primary production for this reason⁶. These proposals and others²⁻⁵ might apply to some areas of the ocean or some phases of Earth's history. But how and why any set of factors should act in concert to keep O₂ low for almost 2 billion years is yet unresolved¹⁰.

Nitrogenase regulates oxygen levels. We propose that O₂-dependent feedback inhibition of a single enzymatic activity limited O₂ accumulation during the boring billion: inhibition of nitrogenase by O₂ gas. Carbon and nitrogen enter the biosphere in distinct chemical reactions catalysed by specific enzymes. For carbon there are six pathways of CO₂ assimilation that differ in age, oxygen tolerance, and key CO₂ reducing enzymes¹¹. For N₂ there is only one entry point into metabolism: nitrogenase¹²⁻¹⁴. Nitrogenase is widespread among cyanobacteria¹². There are Mo, Fe and V containing isoforms of the enzyme that all share a common ancestor and homologous active sites¹³⁻¹⁵.

Fig. 2 | Model of the oxygen-sensitive active site of nitrogenase. Redrawn from ref.¹⁵ with the proposed binding site for N₂.

The nitrogenase active site is replete with metal cofactors (Fig. 2) and harbours a metal coordinated carbide carbon atom, unique among all enzymes known so far¹⁴. Like a blacksmith, nitrogenase uses ancient but robust technology. Nitrogenase has an obligatory H₂ producing side reaction, and it requires 8 electrons and 16 ATP per N₂ fixed, the ATP being consumed at steps that alter the redox potential of FeS clusters via conformational change¹³. Nitrogenase requires numerous assembly factors¹⁴, and has been neither replaced nor improved during evolution, which reveals that the solution that life found to fix N₂ is the only one readily attainable in 4 billion years of physiological engineering by microorganisms. Nitrogenase is a limiting factor. It is inhibited by O₂ in a feedback loop (Fig. 3), and this simple property alone could limit O₂ accumulation over geological time.

Fig. 3 | Inhibitory feedback at nitrogenase. O₂ inhibits nitrogenase, which is required for O₂ production in photosynthesis. A steady state is reached at environmental O₂ levels not exceeding 10% PAL.

Nitrogenase feedback inhibition operates as follows. By dry weight, cells are about 50% carbon and about 10% nitrogen. Cyanobacteria had water as an unlimited reductant for CO₂

fixation, but, for net growth to occur, N₂ incorporation had to keep pace. Nitrogenase is inhibited by oxygen, the product of water oxidation — but there is a threshold of oxygen concentration below which nitrogenase remains active and above which nitrogen fixation ceases completely. If diazotrophic cyanobacteria are grown under conditions where they have sufficient CO₂ and light, and with N₂ as the sole N source, then they grow and accumulate no more than 2% oxygen in their culture atmosphere¹⁶. The 2% O₂ remains constant during prolonged culture growth because this is the O₂ partial pressure beyond which nitrogenase activity becomes inhibited. With greater O₂, nitrogenase is inactivated and there is no fixed N to support further biomass accumulation. With less O₂, nitrogenase outpaces CO₂ fixation until the latter catches up, returning O₂ to 2% in the culture. In cyanobacteria, CO₂ fixation means O₂ production.

In microbial mats, oxygen inhibits nitrogenase activity and nitrogenase gene transcription following the onset of illumination during the natural diel cycle¹⁷. The initial effect of illumination, however, is to increase nitrogenase activity to its maximum value by means of increased ATP and reductant from photosynthetic electron transport. After a lag of a few hours, O₂ concentration becomes inhibitory to nitrogen fixation¹⁷. Because there is no biochemical alternative to nitrogenase for fixing N₂, because there are no O₂ tolerant nitrogenases known, and because reductant for CO₂ fixation was not limiting for cyanobacteria, this feedback loop would have operated, on a planetary scale, for two billion years or more. While primary production using H₂S instead of H₂O as in *Oscillatoria limnetica*¹⁸ is also subject to O₂-feedback inhibition, it would have been limited via the availability of reductant at O₂ levels far below those created by of oxygenic photosynthesis¹⁹, and would not have impacted O₂ accumulation. Nitrogenase is an O₂ inhibited sensor that kept environmental O₂ low throughout the Proterozoic.

Cyanobacteria have evolved mechanisms to avoid nitrogenase inhibition by oxygen¹², including N₂ fixation in the dark²⁰, heterocysts²¹ or filament bundles as in *Trichodesmium*²². Critics might counter that any one of those mechanisms could have bypassed O₂ feedback inhibition. There are three problems with this objection. First, evolution operates without foresight. Second, the mechanisms that cyanobacteria use to deal with modern O₂ levels appear to have arisen independently in diverse phylogenetic lineages, not at the base of cyanobacterial evolution when water oxidation had just been discovered^{23,24}. Third, the oldest uncontroversial fossil heterocysts trace to land ecosystems of the Rhynie chert and are merely

Devonian in age²⁵ (Fig. 1), suggesting that heterocysts arose late in evolution, probably in response to levels of O₂ exceeding 2% by volume. Fossil akinetes — cyanobacterial resting spores — have been found in older sediments^{26,27}, yet there is no direct evidence for heterocysts older than the first land plants.

The concept of limiting metal availability (Mo, V, or Fe) for nitrogenase activity⁶ is an element of many proposals to account for low Proterozoic O₂. Our proposal differs from nutrient limitation in a crucial aspect. Limiting the number of active nitrogenase enzymes in the environment by limiting metal (Mo, V, and Fe) availability only limits the rate at which cyanobacteria produce O₂, requiring other factors to impose limits upon the final O₂ partial pressure. Nitrogenase feedback inhibition regulates the O₂ partial pressure directly, independently of the rate of photosynthesis, and generates a value that corresponds to the geochemical observation.

An O₂ overshoot 2.3 billion years ago is suggested by an isotopic anomaly called the Lomagundi excursion. At 2.3 to 2.2 Ga ago, the isotopic record first reported from the Lomagundi formation in Zimbabwe indicates burial of heavy (¹³C enriched) carbon³. This ¹³C increase is interpreted³, though not universally², as indicating the presence of large amounts of O₂ on a global scale. If that interpretation is correct, its least explicable aspect is that following the Lomagundi excursion, oxygen levels drop once again³. Yet they do not drop to pre-cyanobacterial levels, rather they drop to oxygen levels very near 2% O₂, the oxygen partial pressure that nitrogenase feedback inhibition generates. If the Lomagundi excursion is taken as a valid proxy for high global O₂ levels, the following situation prevailed at the GOE. O₂ is a strong oxidant. Its contribution to metabolic evolution was not just new metabolic pathways, but more complete oxidation of existing organic substrates²⁸. O₂ mobilized organic nitrogen and carbon that had been sequestered in biomass. By liberating sequestered nitrogen (and carbon as CO₂) that had previously been inaccessible to anaerobes, the onset of O₂ accumulation at the GOE initiated a positive growth feedback loop for aerobic autotrophs that were not reductant limited: cyanobacteria. When anaerobically deposited nitrogen reserves had been liberated, nitrogenase feedback inhibition set in, driving O₂ levels down to Proterozoic levels, and keeping them low for a billion years thereafter. Our proposal does not hinge upon the Lomagundi excursion, yet if the excursion is interpreted as evidence for transiently high global O₂ levels then our proposal can account both for its emergence

(nitrogenase independent N availability during the excursion) and for its decline in the subsequent return to low O₂.

When and why did feedback inhibition at nitrogenase cease to keep O₂ low? At the origin of land plants, the nature of biomass changed and O₂ production by upright terrestrial plants became physically separated from N₂ fixation in aquatic environments and soil. Deposition by land plants of nitrogen-depleted cellulose, billions of tonnes of it, became a massive sink for CO₂ without exerting similar effects on nitrogen availability, thus allowing O₂ to increase through the standard mechanism of carbon burial, bypassing control by aquatic nitrogenase feedback.

Critics might interject that O₂ levels began to rise before the first fossil occurrence of land plants²⁹. We point out that nitrogenase limitation determines the maximum O₂ partial pressure near the water surface for nitrogenase-limited oxygen production. This limit does not identify the timepoint at which the deep ocean becomes fully oxic, since that depends upon other factors such as reductant load, ocean mixing, or both, independently of photic zone nitrogenase limitation. Stolper and Keller⁴ report that deep ocean oxygenation became complete 540 million years ago. If so, that was the first time (or possibly the first time since the Lomagundi excursion) that N-rich organic ocean floor sediment came into widespread contact with oxygenated water. This contact released organic N, leading to atmospheric O₂ increase, after which O₂ levels dropped once again^{29,30} to the value imposed by the nitrogenase limit. Nitrogenase inhibition returns O₂ to low levels following O₂ increases, thus explaining an otherwise puzzling aspect of Proterozoic O₂ variation.

In conclusion, oxygen inhibition of any ecosystem's cornerstone enzyme activity, nitrogenase, created a bottleneck for oxygenic primary production that is sufficient to account for low oxygen levels throughout the boring billion. Nitrogenase feedback inhibition could directly account for Proterozoic low oxygen stasis. It would have driven down transiently higher O₂ levels ensuing from nitrogenase-independent N availability, and it would have ceased at the origin of land plants. Our model requires light, CO₂ and N₂ in the photic zone and hence accommodates local and global variation in geochemical conditions while remaining robust to their effects. We propose that the factor limiting Proterozoic O₂ accumulation was not geochemical. It was biological, and the attribute of a single enzyme, nitrogenase, contained within and synthesized by living cells.

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1. Fischer, W. W., Hemp, J. & Johnson, J. E. Evolution of oxygenic photosynthesis. *Ann. Rev. Earth Planet Sci.* **44**, 647–683 (2016).
2. Holland, H. D. The oxygenation of the atmosphere and oceans. *Phil. Trans. Roy. Soc. Lond. B* **361**, 903–915 (2006).
3. Lyons, T. W., Reinhard, C. T. & Planavsky, N. J. The rise of oxygen in Earth's early ocean and atmosphere. *Nature* **506**, 307–315 (2014).
4. Stolper, D. A. & Keller, B. A record of deep-ocean dissolved O₂ from the oxidation state of iron in submarine basalts. *Nature* **553**, 323–327 (2018).
5. Lenton, T. M. *et al.* Earliest land plants created modern levels of atmospheric oxygen. *Proc. Natl. Acad. Sci. USA* **113**, 9704–9709 (2016).
6. Anbar, A. D. & Knoll, A. H. Proterozoic ocean chemistry and evolution: A bioinorganic bridge? *Science* **297**, 1137–1142 (2002).
7. Bar-On, Y. M., Phillips, R. & Milo, R. The biomass distribution on Earth. *Proc. Natl. Acad. Sci. USA* **115**, 6506–6511 (2018).
8. Poulton, S. W., Fralick, P. W. & Canfield, D. E. The transition to a sulphidic ocean ~1.84 billion years ago. *Nature* **431**, 173–177 (2004).
9. Canfield, D. E. & Farquhar, J. Animal evolution, bioturbation, and the sulfate concentration of the oceans. *Proc. Natl. Acad. Sci. USA* **106**, 8123–8127 (2009).
10. Olson, S. L., Reinhard, C. T. & Lyons, T. W. Cyanobacterial diazotrophy and Earth's delayed oxygenation. *Front. Microbiol.* **7**, 1526 (2016).
11. Fuchs, G. Alternative pathways of carbon dioxide fixation: Insights into the early evolution of life? *Annu. Rev. Microbiol.* **65**, 631–658 (2011).
12. Fay, P. Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiol. Rev.* **56**, 340–373 (1992).
13. Hoffman, B. M., Lukoyanov, D., Yang, Z. Y., Dean, D. R. & Seefeldt, L. C. Mechanism of nitrogen fixation by nitrogenase: The next stage. *Chem. Rev.* **114**, 4041–4062 (2014).

14. Hu, Y. & Ribbe, M. W. Biosynthesis of the metalloclusters of nitrogenases. *Annu. Rev. Biochem.* **85**, 455–483 (2016).
15. Anderson, J. S., Rittle, J. & Peters, J. C. Catalytic conversion of nitrogen to ammonia by an iron model complex. *Nature* **501**, 84–87 (2013).
16. Berman-Frank, I., Lundgren, P. & Falkowski, P. Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. *Res. Microbiol.* **154**, 157–164 (2003).
17. Steunou, A.-S. *et al.* Regulation of nif gene expression and the energetics of N₂ fixation over the diel cycle in a hot spring microbial mat. *ISME J.* **2**, 364–378 (2008).
18. Allen, J. F. A redox switch hypothesis for the origin of two light reactions in photosynthesis. *FEBS Lett.* **579**, 963–968 (2005).
19. Raven, J. A. Contributions of anoxygenic and oxygenic phototrophy and chemolithotrophy to carbon and oxygen fluxes in aquatic environments. *Aquat. Microb. Ecol.* **56**, 177–192 (2009).
20. Mitsui, A., Kumazawa, S., Takahashi, A., Ikemoto, H. & Arai, T. Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically. *Nature* **323**, 720–722 (1986).
21. Kumar, K., Mella-Herrera, R. A. & Golden, J. W. Cyanobacterial heterocysts. *Cold Spring Harb. Perspect. Biol.* **2**, a000315 (2010).
22. Sandh, G., Xu, L. & Bergman, B. Diazocyte development in the marine diazotrophic cyanobacterium *Trichodesmium*. *Microbiology* **158**, 345–352 (2012).
23. Dagan, T. *et al.* Genomes of stigonematalean cyanobacteria (subsection V) and the evolution of oxygenic photosynthesis from prokaryotes to plastids. *Genome Biol. Evol.* **5**, 31–44 (2013).
24. Esteves-Ferreira, A. A. *et al.* Cyanobacterial nitrogenases: Phylogenetic diversity, regulation and functional predictions. *Genet. Mol. Biol.* **40**, S261–275 (2017).
25. Butterfield, N. J. Proterozoic photosynthesis – A critical review. *Palaeontology* **58**, 953–972 (2015).
26. Tomitani, A., Knoll, A. H., Cavanaugh, C. M. & Ohno, T. The evolutionary diversification of cyanobacteria: molecular-phylogenetic and paleontological perspectives. *Proc. Natl. Acad. Sci. USA* **103**, 5442–5447 (2006).
27. Pang, K. *et al.* Nitrogen-fixing heterocystous cyanobacteria in the Tonian period. *Curr. Biol.* **28**, 616–622 (2018).
28. Sousa, F. L., Nelson-Sathi, S. & Martin, W. F. One step beyond a ribosome: The ancient anaerobic core. *Biochim. Biophys. Acta* **1857**, 1027–1038 (2016).

29. Wallace, M. W. *et al.* Oxygenation history of the Neoproterozoic to early Phanerozoic and the rise of land plants. *Earth Planet. Sci. Lett.* 466, 12–19 (2017).
30. Zhang, K. *et al.* Oxygenation of the Mesoproterozoic ocean and the evolution of complex eukaryotes. *Nat. Geosci.* 11, 345–350 (2018).