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# Nitrogenase inhibition limited oxygenation of the Proterozoic atmosphere

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**Cyanobacteria produced the atmospheric $O_2$ that began accumulating 2.4 billion years ago[1], leading to Earth's Great Oxidation Event (GOE)[2]. For nearly 2 billion years following the GOE, $O_2$ production was restricted and atmospheric oxygen remained low[2–5]. Oxygen rose again sharply with the advent of land plants roughly 450 million years ago, which increased atmospheric $O_2$ through carbon burial[4–5]. Why did the $O_2$ content of the atmosphere remain constant and low for more than a billion years despite the existence of $O_2$-producing cyanobacteria? While geological limitations have been explored[2–7], the limiting factor may have been biological, and enzymatic. Here we propose that $O_2$ was kept low by oxygen inhibition of nitrogenase activity. Nitrogenase is the sole $N_2$-fixing enzyme on Earth, and is inactive in air containing 2% or more $O_2$ by volume[8]. No $O_2$-resistant nitrogenase enzyme is known[9–12]. We further propose that nitrogenase inhibition by $O_2$ kept atmospheric $O_2$ low until upright terrestrial plants physically separated $O_2$ production in aerial photosynthetic tissues from $N_2$ fixation in soil, liberating nitrogenase from inhibition by atmospheric $O_2$.**

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Current views of oxygen in Earth history (Fig. 1) depict the first traces of $O_2$ appearing in the atmosphere starting about 2.7 to 2.5 Gy ago[1–5]. During the Great Oxidation Event, or GOE, roughly 2.4 billion years ago[2], $O_2$ rose to about 10% of its present atmospheric level (PAL), corresponding to an atmosphere of roughly 2% $O_2$ by volume[2], or even less[3]. Isotopic studies indicate that for roughly 1.5 billion years following the comparatively sudden GOE, further net $O_2$ accumulation ceased, with atmospheric levels remaining stable and below 10% PAL[2–4].

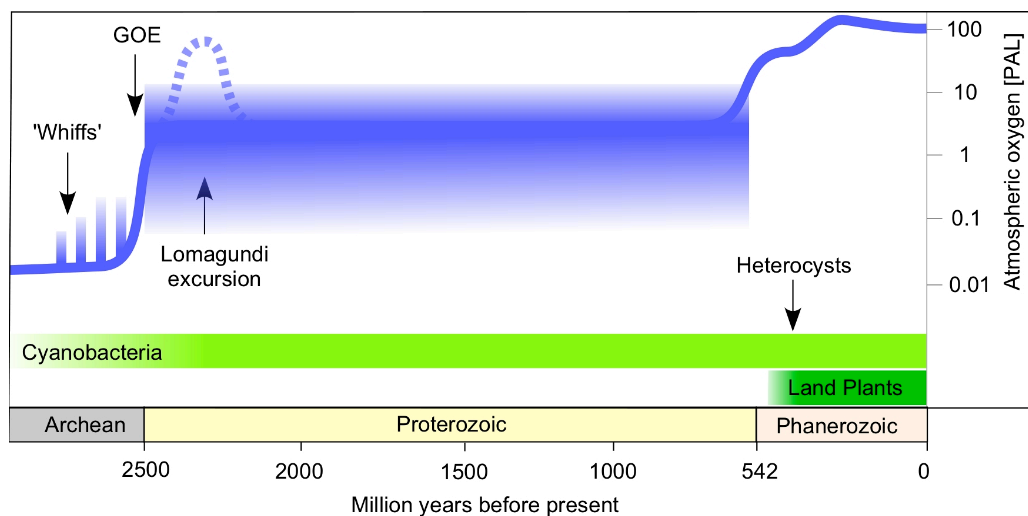


**Fig. 1 | Schematic summary of $O_2$ accumulation in Earth history.** Modified after refs. 1-5. For most of the Proterozoic eon, free $O_2$ was much less abundant than it is today. Lyons et al. (2014) estimate Proterozoic $O_2$ in the atmosphere as low as 0.001 PAL while Holland[2] estimates atmospheric $O_2$ at around 0.1 to 0.2 PAL. Stolper and Keller[4] estimate mid-Proterozoic deep ocean dissolved $O_2$ 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 $O_2$ 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 $O_2$. Their relevance is that cyanobacteria have an ancient fossil record, but the oldest fossil heterocysts[26] are younger than land plants, suggesting that cyanobacteria evolved this mechanism of $O_2$ protection in response to Phanerozoic $O_2$ accumulation. PAL; Present Atmospheric Level. GOE; Great Oxidation Event.

With atmospheric $O_2$ low, marine $O_2$ stayed low as well. Geochemical evidence suggests that the oceans remained largely anoxic throughout the Proterozoic[1–5], with a rapid rise to roughly

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modern oxygen levels starting around 580 My ago, perhaps as recently as only 430 My ago[4,5] (Fig. 1). Late increases in atmospheric $O_2$ 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 biomass[7]. Their ecological success has been linked to the rise in $O_2$ because terrestrial sequestration of organic carbon as fibrous biomass protects it from reoxidation to $CO_2$, curbing $O_2$ consumption[4–5].

What limited oxygen accumulation? A major puzzle of $O_2$ history is why $O_2$ rose so late, that is, why atmospheric and marine $O_2$ levels stayed low for almost 2 billion years despite the existence of cyanobacteria, which were capable of continuous light-driven $O_2$ production. What held cyanobacteria back, why did $O_2$ stop accumulating after the GOE and why did it remain low during the Proterozoic, or the "Boring Billion" as it is sometimes called[3].

Geochemists have long recognized that Proterozoic $O_2$ 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 $Fe^{2+}$ or $S^{2-}$, reductants that continuously consumed the $O_2$ produced by cyanobacteria, keeping $O_2$ low[8]. 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 activity[9]. 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 reason[6]. These proposals and others[2–5] 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 $O_2$ low for almost 2 billion years is yet unresolved[10].

Nitrogenase regulates oxygen levels. We propose that $O_2$-dependent feedback inhibition of a single enzymatic activity limited $O_2$ accumulation during the boring billion: inhibition of nitrogenase by $O_2$ gas. Carbon and nitrogen enter the biosphere in distinct chemical reactions catalysed by specific enzymes. For carbon there are six pathways of $CO_2$ assimilation that differ in age, oxygen tolerance, and key $CO_2$ reducing enzymes[11]. For $N_2$ there is only one entry point into metabolism: nitrogenase[12–14]. Nitrogenase is widespread among cyanobacteria[12]. There are Mo, Fe and V containing isoforms of the enzyme that all share a common ancestor and homologous active sites[13–15].

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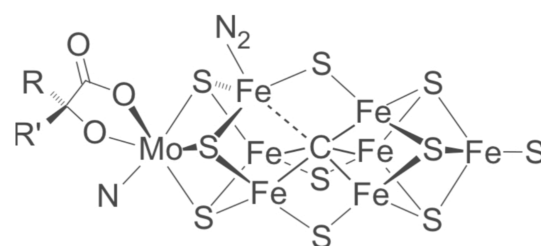


**Fig. 2 | Model of the oxygen-sensitive active site of nitrogenase.** Redrawn from ref.[15] with the proposed binding site for $N_2$.

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 far[14]. Like a blacksmith, nitrogenase uses ancient but robust technology. Nitrognease has an obligatory $H_2$ producing side reaction, and it requires 8 electrons and 16 ATP per $N_2$ fixed, the ATP being consumed at steps that alter the redox potential of FeS clusters via conformational change[13]. Nitrogenase requires numerous assembly factors[14], and has been neither replaced nor improved during evolution, which reveals that the solution that life found to fix $N_2$ is the only one readily attainable in 4 billion years of physiological engineering by microorganisms. Nitrogenase is a limiting factor. It is inhibited by $O_2$ in a feedback loop (Fig. 3), and this simple property alone could limit $O_2$ accumulation over geological time.

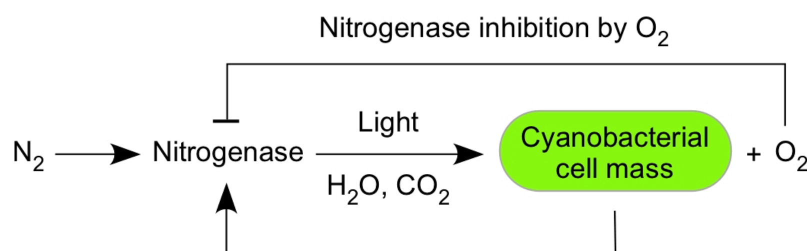


**Fig. 3 | Inhibitory feedback at nitrogenase.** $O_2$ inhibits nitrogenase, which is required for $O_2$ production in photosynthesis. A steady state is reached at environmental $O_2$ levels not exceeding 10% PAL.

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 $CO_2$

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fixation, but, for net growth to occur, $N_2$ 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 $CO_2$ and light, and with $N_2$ as the sole N source, then they grow and accumulate no more than 2% oxygen in their culture atmosphere[16]. The 2% $O_2$ remains constant during prolonged culture growth because this is the $O_2$ partial pressure beyond which nitrogenase activity becomes inhibited. With greater $O_2$, nitrogenase is inactivated and there is no fixed N to support further biomass accumulation. With less $O_2$, nitrogenase outpaces $CO_2$ fixation until the latter catches up, returning $O_2$ to 2% in the culture. In cyanobacteria, $CO_2$ fixation means $O_2$ production.

In microbial mats, oxygen inhibits nitrogenase activity and nitrogenase gene transcription following the onset of illumination during the natural diel cycle[17]. 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, $O_2$ concentration becomes inhibitory to nitrogen fixation[17]. Because there is no biochemical alternative to nitrogenase for fixing $N_2$, because there are no $O_2$ tolerant nitrogenases known, and because reductant for $CO_2$ 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 $H_2S$ instead of $H_2O$ as in *Oscillatoria limnetica*[18] is also subject to $O_2$-feedback inhibition, it would have been limited via the availability of reductant at $O_2$ levels far below those created by of oxygenic photosynthesis [19], and would not have impacted $O_2$ accumulation. Nitrogenase is an $O_2$ inhibited sensor that kept environmental $O_2$ low throughout the Proterozoic.

Cyanobacteria have evolved mechanisms to avoid nitrogenase inhibition by oxygen[12], including $N_2$ fixation in the dark[20], heterocysts[21] or filament bundles as in *Trichodesmium*[22]. Critics might counter that any one of those mechanisms could have bypassed $O_2$ feedback inhibition. There are three problems with this objection. First, evolution operates without foresight. Second, the mechanisms that cyanobacteria use to deal with modern $O_2$ 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

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Devonian in age[25] (Fig. 1), suggesting that heterocysts arose late in evolution, probably in response to levels of $O_2$ 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 activity[6] is an element of many proposals to account for low Proterozoic $O_2$. 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 $O_2$, requiring other factors to impose limits upon the final $O_2$ partial pressure. Nitrogenase feedback inhibition regulates the $O_2$ partial pressure directly, independently of the rate of photosynthesis, and generates a value that corresponds to the geochemical observation.

An $O_2$ 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 ($^{13}C$ enriched) carbon[3]. This $^{13}C$ increase is interpreted[3], though not universally[2], as indicating the presence of large amounts of $O_2$ on a global scale. If that interpretation is correct, its least explicable aspect is that following the Lomagundi excursion, oxygen levels drop once again[3]. Yet they do not drop to pre-cyanobacterial levels, rather they drop to oxygen levels very near 2% $O_2$, the oxygen partial pressure that nitrogenase feedback inhibition generates. If the Lomagundi excursion is taken as a valid proxy for high global $O_2$ levels, the following situation prevailed at the GOE. $O_2$ is a strong oxidant. Its contribution to metabolic evolution was not just new metabolic pathways, but more complete oxidation of existing organic substrates[28]. $O_2$ mobilized organic nitrogen and carbon that had been sequestered in biomass. By liberating sequestered nitrogen (and carbon as $CO_2$) that had previously been inaccessible to anaerobes, the onset of $O_2$ 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 $O_2$ 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 $O_2$ levels then our proposal can account both for its emergence

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(nitrogenase independent N availability during the excursion) and for its decline in the subsequent return to low $O_2$.

When and why did feedback inhibition at nitrogenase cease to keep $O_2$ low? At the origin of land plants, the nature of biomass changed and $O_2$ production by upright terrestrial plants became physically separated from $N_2$ fixation in aquatic environments and soil. Deposition by land plants of nitrogen-depleted cellulose, billions of tonnes of it, became a massive sink for $CO_2$ without exerting similar effects on nitrogen availability, thus allowing $O_2$ to increase through the standard mechanism of carbon burial, bypassing control by aquatic nitrogenase feedback.

Critics might interject that $O_2$ levels began to rise before the first fossil occurrence of land plants[29]. We point out that nitrogenase limitation determines the maximum $O_2$ 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 Keller[4] 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 $O_2$ increase, after which $O_2$ levels dropped once again[29,30] to the value imposed by the nitrogenase limit. Nitrogenase inhibition returns $O_2$ to low levels following $O_2$ increases, thus explaining an otherwise puzzling aspect of Proterozoic $O_2$ 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 $O_2$ levels ensuing from nitrogenase-independent N availability, and it would have ceased at the origin of land plants. Our model requires light, $CO_2$ and $N_2$ 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 $O_2$ 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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