

Review

Enigmatic evolution of microbial nitrogen fixation: insights from Earth's past

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The evolution of nitrogen fixation undoubtedly altered nearly all corners of the biosphere, given the essential role of nitrogen in the synthesis of biomass. To date, there is no unified view on what planetary conditions gave rise to nitrogen fixation or how these conditions have sustained it evolutionarily. Intriguingly, the concentrations of metals that nitrogenases require to function have changed throughout Earth's history. In this review, we describe the interconnection of the metal and nitrogen cycles with nitrogenase evolution and the importance of ancient ecology in the formation of the modern nitrogen cycle. We argue that exploration of the nitrogen cycle's deep past will provide insights into humanity's immediate environmental challenges centered on nitrogen availability.

Like the spinning gears of a clock, the various biogeochemical cycles on Earth occur in tandem with one another. As a result, the continuation of one biogeochemical process can be impacted by the progression of a separate yet connected cycle. Such feedback can be observed in the nitrogen cycle. Nitrogen is essential for life as a fundamental unit of nucleic acids and proteins. However, the largest reservoir of nitrogen, in the form of atmospheric N₂, is not available to be used as a nitrogen source for most life, outside of a small amount of nitrogen which is dissociated by high energy phenomena. Nitrogenase, the only enzyme capable of fixing nitrogen into the more bioavailable form, ammonia, and a central player in the nitrogen cycle in both terrestrial and marine environments, remains a key innovation for establishing the biosphere of modern Earth [1]. Therefore, all life throughout every environment is either directly or indirectly dependent on nitrogenase for the fixed N₂ produced via nitrogen fixation, life's singular solution for ready access to atmospheric nitrogen [2,3].

Biological nitrogen fixation (BNF) via nitrogenase has long had a critical role in the expanding Earth biosphere due to the limited nature of nitrogen as a nutrient over geological timescales [2]. An industrial workaround for fixed nitrogen limitations in nitrogen fertilizer production, the Haber-Bosch process, is an energetically costly endeavor to supplement a 3-billion-year-old molecular innovation [4]. As a metalloenzyme, nitrogenase utilizes several iron-sulfur clusters that, depending on the specific isozyme, will variably incorporate molybdenum, vanadium, or only iron [5]. The factors in Earth's history that determined the evolution of BNF remain largely unknown. For example, it is not yet known how the first nitrogenase evolved, to what degree earliest nitrogen fixation differed from extant processes, what selective pressures would have been needed to utilize alternative metals, and to what degree the sequence diversity of nitrogen fixation across extant organisms may evidence variations in past ecological conditions.

To date, there is no unified view on what planetary conditions gave rise to nitrogen fixation or how these conditions have sustained it evolutionarily. Intriguingly, however, the concentrations of metals that nitrogenases are dependent on have changed throughout Earth's history and vary

Highlights

Nitrogenase, the key enzyme for biological nitrogen fixation, is an evolutionary singularity, as only one mechanism capable of catalyzing the reduction of atmospheric N₂ into fixed nitrogen is known to have evolved.

Nitrogenase has three metal isoforms (iron, molybdenum, and vanadium) and is thus connected and dependent upon biogeochemical cycles for metal availability.

Molybdenum is abundant in modern oceans, was very scarce in ancient oceans and remains scarce in modern terrestrial environments. Despite its scarcity, it is proposed that early nitrogenases first utilized Mo-cofactors given its catalytic advantages over abundant Fe.

To understand the evolution and diversification of the nitrogen cycle, we must first understand the paleoecological and planetary conditions that give rise to biological nitrogen fixation.

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in different environments [6]. In this review, we describe the interconnection of the metal and nitrogen cycles with nitrogenase and the planetary impact of nitrogenase-mediated nitrogen fixation by discussing: (i) the temporal and spatial availability of relevant metals, (ii) the origins of microbial nitrogen fixation and its various metal isoforms, and (iii) the importance of ancient ecology in the formation of the modern, multitiered occupant nitrogen cycle. Finally, we argue that exploration of the nitrogen cycle's deep past may provide insights into our immediate problems centered on nitrogen availability [7,8].

Early earth and availability of potential metal cofactors

Just as N_2 gas dominates the current atmosphere of Earth, N_2 was also highly abundant in the Precambrian atmosphere but perhaps at a lower partial pressure than modern values [9,10]. Before the evolution of BNF via nitrogenase, fixed forms of nitrogen could only be produced through abiotic reactions that require high energy to break the triple bond of N_2 (Equation 1). Abiotic reactions would have used energy from lightning, meteor impacts, or UV-photocatalysis with Fe-S mineral catalysts to reduce N_2 [11–13]. These reactions alone likely could not meet the growing demand for fixed nitrogen as life began to evolve and expand, therefore providing selective pressure for the evolution of a biotic mechanism for reducing N_2 [13].



Understanding nitrogenase's impact on life at a planetary scale necessitates understanding the conditions of early Earth, such as atmospheric conditions and thereby metal availability, which may have shaped its emergence and evolution [14]. Using nitrogen isotope ratios from Archean sediments, nitrogenase is estimated to have originated by 3.2 billion years ago [15]. This estimate places the origin of nitrogenase, or a nitrogenase-like ancestor, within the Archean, before the Great Oxidation Event (GOE). Before the GOE, the atmosphere was largely devoid of oxygen, although small amounts of oxygen would have begun to accumulate in shallow marine environments millions of years before the GOE [16–18].

Prior to the rise of oxygen in the atmosphere, the Archean open ocean conditions were significantly reducing, which allowed iron, in the form of Fe^{2+} , to be abundant as a dissolved constituent with a concentration in the range of 10^{-7} mM [19]. For most of the Archean, the concentration of Fe^{2+} remained high throughout the water column. As oxygenic photosynthesis began to evolve, there were 'whiffs' of oxygen being produced, resulting in the oxidation of Fe^{2+} into Fe^{3+} , primarily near the surface [16]. The GOE, as its name implies, resulted in the shift of the ocean's redox chemistry to a more oxidized state. The oxidization of the oceans had a significant impact on the availability of iron, since Fe^{3+} forms iron (III) oxides that are highly insoluble in water. Thus, the origin of nitrogenase dating back before the GOE means that the enzyme evolved in an environment rich in bioavailable iron. As such, iron is an integral part of the nitrogenase complex, being found in both components in the form of an iron-sulfur cluster and as a metal cofactor. In one isoform of nitrogenase, Fe-nitrogenase, the metal cofactor contains only iron.

Unlike iron, molybdenum was particularly scarce in the Archean Ocean. One possible source of molybdenum in the Archean may have been seafloor hydrothermal vents [20]. As oxygen levels increased, oxidative weathering of terrestrial sulfide minerals would have mobilized the molybdenum enriched in these minerals into rivers and ultimately the oceans [21] (Figure 1). Molybdenum is now the most abundant trace metal in the oceans [22]. Despite the concentrations of molybdenum being significantly lower before the GOE, phylogenetic reconstruction with inferred ancestral sequences has revealed that the earliest nitrogenases were likely Mo-nitrogenases (Nif), rather than V- or Fe-nitrogenases [23]. Vanadium, which is less abundant

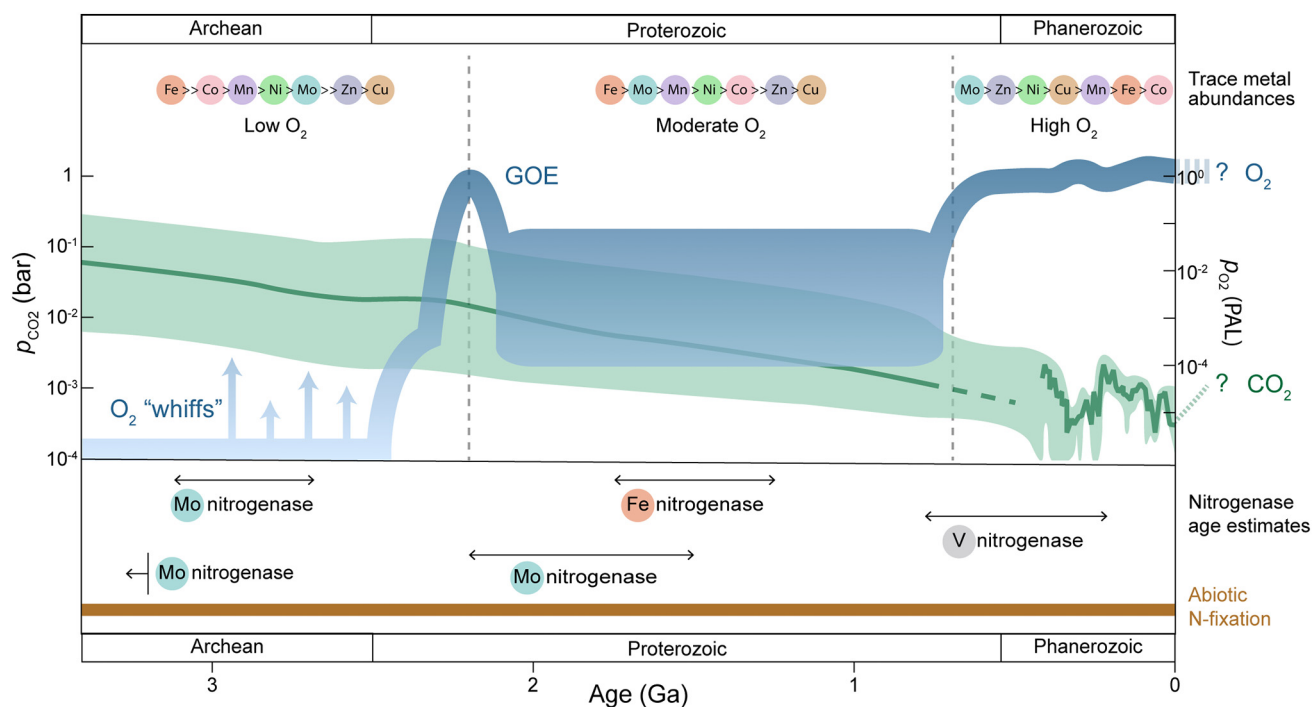


Figure 1. Timeline of Earth geochemistry and nitrogen fixation. Partial pressures of atmospheric oxygen (pO₂) from Lyons *et al.* [21] and of carbon dioxide (pCO₂) from [73]. Trace metal availabilities from Zerkle *et al.* [30], Saito *et al.* [74], and Moore *et al.* [24]. Mo-, V-, and Fe-nitrogenase age estimates from geochemical nitrogen isotope [15], evolutionary rate analyses of nitrogenase proteins [75], and reconciliation of nitrogenase gene trees and species chronograms [34]. Abiotic nitrogen fixation was likely ongoing throughout geologic history [11–13]. Abbreviation: GOE, Great Oxidation Event.

on modern Earth than molybdenum, was also biologically available in the Archean oceans in more acidic environments [24]. Today, vanadium is the second most abundant trace element in the ocean [25].

Origin of nitrogenase

Fixed or biologically available nitrogen is crucial for life and is found in important molecules such as nucleotides and amino acids. Therefore, an enzyme that could catalyze the conversion of N₂ into ammonia was an important innovation in the evolutionary history of life. Nitrogenase is an example of an evolutionary singularity, in which life evolved only one enzyme capable of catalyzing a crucial metabolic reaction [26]. It remains unknown if nitrogenase evolved due to the pressures of natural selection or if nitrogenase's origin was due to stochastic chance or historical contingency.

Recent paleomolecular analysis of the origin of nitrogenase suggests that nitrogenase originated from a maturase-like predecessor (also referred to as assembly scaffold) that was unable to fix N₂ [23,27]. The NifEN proteins of maturase act as scaffolds for the formation of a mature metal iron-sulfur cluster that is then transferred to the nitrogenase complex in the NifD active site. Whether or not the maturase precursor could incorporate molybdenum and, if so, whether such process had occurred through a similar mechanism as observed in extant NifEN, is unknown. There is also debate over which domain of life the first nitrogenase or maturase precursor originated in. While it has previously been accepted that nitrogenases originated in archaea [28], recent work has speculated that earliest nitrogenases may have existed in bacterial ancestors [29].

One method that is beginning to be used to decipher the enigmatic origins of nitrogenase and probe at questions of metal selection is ancestral sequence reconstruction (ASR), utilized to trace back protein sequences through time [27,30]. While ASR can be a valuable tool for studying evolutionary hypotheses, it is important to consider the overestimation of certain sequences due to survivor bias [31]. Ancient protein reconstruction is fundamentally limited to reconstructing the ancestors of survivor-descendent enzymes. For example, recent ASR of the substrate channel residues of nitrogenase has shown that the inferred ancestor of nitrogenase likely had a distinct substrate channel sequence that differs from extant sequences [32]. These residue differences may be a result of selective pressure for early nitrogenases to reduce an alternative substrate, such as HCN. It is therefore important to be cautious with evolutionary interpretations of sequence analysis based on extant sequences and current enzymatic activity alone. The power of the ASR approach is in extending functional investigations beyond the sequence space occupied by extant enzymes and identifying patterns in their functional variation through their evolutionary histories [30,33].

When nitrogenase did evolve, potentially from a maturase-like protein, it likely utilized molybdenum before incorporating alternative Fe- or V-cofactors. Phylogenetic analysis has indicated that the earliest nitrogenase ancestors exhibit sequence features consistent with molybdenum dependence [23,34]. Geological evidence (i.e., isotope fractionation values) also supports the early presence of Mo-nitrogenases dating back to the mid-Archaeon, well before the GOE [15]. The alternative nitrogenases have a distinct and highly negative $\delta^{15}\text{N}$ signature, which is not found in Archaeon sediments [15]. Additionally, all diazotrophs possess Mo-nitrogenase, while only a smaller fraction harbor the alternative nitrogenases as well.

There remains uncertainty as to why molybdenum, vanadium, and iron were selected as metal cofactors for nitrogenase. Despite the scarcity of molybdenum prior to the GOE, the kinetic advantage of Mo-nitrogenase may have favored its use as a cofactor over more abundant but less efficient alternative metals. In addition to being more common, Mo-nitrogenases are also more efficient (i.e., requiring less ATP per mol of N_2) and also catalyze the reduction reaction at a faster rate [35,36]. Knowledge of minimum molybdenum concentration thresholds that still support modern nitrogen fixation may reveal how molybdenum was first selected as a cofactor over Fe. Philippi *et al.* investigated the effects of low molybdenum concentrations in an anoxic, Proterozoic analog system, Lake Cadagno, and found that purple sulfur bacteria exclusively used Mo-nitrogenases despite the limited molybdenum availability (<10 nM) [37], thus supporting the ‘function over availability’ model of molybdenum. The use of vanadium as a cofactor is surprising, given that vanadium is one of the least utilized metals in prokaryotic metallomes [30]. While vanadium is an abundant trace metal today, it is difficult to constrain vanadium concentrations in deep time, resulting in uncertainty that vanadium would have been bioavailable during the Archean [24]. An outstanding question is why nitrogenase utilized vanadium over manganese, cobalt, or nickel, which were much more abundant in the Archean and are more commonly used in metalloenzymes [30] (Figure 1).

In order to better understand why certain metals were selected over others, and to what degree other metal factors could have been used, we need to decipher the evolutionary mechanisms that determine the selection of elements by reconstructing ancient Earth conditions in the laboratory [38] as well as by using environmental analogs of ancient Earth [39]. These rules of selection have impacts at the protein and mechanism level, as well as the organismal and even biogeochemical cycle levels. Therefore, it is critical to incorporate the selection of elements and changes in elemental abundance over time when considering the evolution of metabolisms that use these elements, such as nitrogen fixation. Whether or not bulk metal concentrations of the Archean

ocean, for example, accurately reflect the metal abundance of local niches where the earliest nitrogenases may have emerged, such as hydrothermal vents [20], is unknown and therefore important to consider. With a better understanding of how evolution has shaped nitrogenase in the past, we become better equipped to understand how this enzyme may continue to evolve in the future.

Modern nitrogen cycle

Insights from the past

Given the age of nitrogen fixation via nitrogenase and the drastically different environmental conditions in which it arose, it is intriguing to consider how modern nitrogen fixation has been shaped by the evolution of ancient nitrogenase. There are several outstanding questions that can be asked in order to better understand how the ancient past has defined modern processes. First, considering that BNF evolved in anoxic conditions, what enabled this process to proliferate in a variety of oxic modern environments? Second, how has the evolution of alternative nitrogenases enabled diazotrophs to expand into niches that would be inaccessible without these isozymes? Lastly, how and when did microbes responsible for much of modern nitrogen fixation, such as cyanobacteria, first obtain nitrogenases? With these questions in mind, we will discuss modern nitrogen fixation with particular focus on habitat, metal usage, and adaptations to environmental stressors, such as O₂ and nutrient limitations.

Marine environments

Nitrogen fixation remains a crucial process in modern oceans. Compared with terrestrial environments, nitrogen fixation is well-studied in marine environments for several reasons. First, oceans cover over 70% of the Earth's surface, making them a much larger biome to study the impacts of nitrogen fixation and the overall nitrogen cycle. Mixing also creates more homogeneity on a larger scale in the oceans than we see for soil communities, in which the physiochemical environment can change drastically within a few centimeters. N₂ is the dominant form of nitrogen in the oceans (94%), with all other nitrogen species being very low in relative abundance (6%) [40]. Of these secondary nitrogen species, the largest reservoir is fixed nitrogen in the form of nitrate (88%), followed by dissolved organic nitrogen. Particulate organic nitrogen, nitrite, and ammonia are all very minor nitrogen species in marine environments (less than 1%). In general, both marine and terrestrial environments are deficient in N sources, making the role of diazotrophs in creating bioavailable nitrogen sources all the more critical in these environments [41].

The cyanobacteria genus *Trichodesmium* is perhaps the most significant nitrogen fixer in marine ecosystems. Of the 100–200 Tg of reactive nitrogen produced annually via nitrogen fixation in the oceans, *Trichodesmium* is estimated to contribute 60–80 Tg N year⁻¹ [42]. Given the oxygenation of modern oceans, aerobic nitrogen fixers like *Trichodesmium* must employ mechanisms for protecting nitrogenase from O₂, which will otherwise inactivate the enzyme [42]. Unlike many other cyanobacteria genera, *Trichodesmium* species do not have heterocysts but rather protect their nitrogenase enzymes from oxygen via zones of cells called diazocytes [42]. These diazocytes lack the glycolipid layer that surrounds heterocysts, allowing for increased gas flux into the cells [43]. *Trichodesmium* is able to make the trade-off between increased N₂ flux into its diazocytes for nitrogen fixation at the cost of increased O₂ flux, as the overall flux of O₂ is decreased in saline waters versus freshwater, likely resulting in the dominance of *Trichodesmium* in marine environments over many other cyanobacteria genera [43]. However, a recent model has shown how *Trichodesmium* could maintain low intracellular O₂ levels without needing diazocyte spatial segregation, specifically through protection afforded by respiration of accumulated fixed carbon [43]. In addition to this mechanism of protecting nitrogenase from intracellular O₂, nitrogen

fixation in *Trichodesmium* is temporally regulated, with maximum activity occurring mid-day when oxygen production is lower [42,44].

In addition to having to develop strategies for dealing with oxygen inhibition, marine nitrogen fixers such as *Trichodesmium* have to cope with nutrient limitations. As discussed earlier, while molybdenum is readily available in modern oceans, iron is a major limiting nutrient, directly impacting primary production and oxidoreductase enzymes such as nitrogenase [45] (Figure 2). Atmospheric deposition of iron in the form of continental dust can supply this biolimiting nutrient to the open ocean (Figure 2). While *Trichodesmium* does not possess any siderophore encoding genes and therefore cannot dissolve iron from aeolian dust, *Trichodesmium* colonies utilize siderophores from other bacteria in exchange for providing a favorable microenvironment for dust capture [46]. However, not all surface waters receive substantial aeolian dust inputs, with approximately 30–40% of surface waters being classified as high-nutrient, low chlorophyll (HNLC) regions. Due to the lack of an iron source, these HNLC regions are characterized by low productivity (i.e., chlorophyll), despite the abundance of macronutrients [47–49]. Iron can also limit productivity in low-nutrient, low chlorophyll regions (LNLC) typically associated with subtropical gyres, in addition to limitations of other macro- and micronutrients [50].

Terrestrial environments

Nutrient and micronutrient availability in terrestrial environments are distinct from modern marine environments, as molybdenum is one of the scarcest biometals in soils and crusts [36]. While iron is a key limiting nutrient in oceans, iron is a highly abundant metal in the Earth's crust. Vanadium is also much more abundant than molybdenum in soils [36] (Figure 2). Given the molybdenum deplete conditions of terrestrial soils and crusts, alternative nitrogenases likely contribute significantly to BNF [36,51,52]. V-nitrogenase alone has been found to contribute to up to 50% of BNF rates in boreal forests [51]. However, the contributions of alternative nitrogenases to global BNF are generally understudied [53]. When assessing the impacts of alternative nitrogenases in terrestrial environments, it is important to note that terrestrial diazotrophs can be separated into two groups: symbiotic heterotrophs and free-living diazotrophs. Of the symbiotic heterotrophs, which have historically been more robustly studied for their agricultural significance than free-living diazotrophs, no alternative nitrogenase is found [36]. While the free-living diazotrophs have

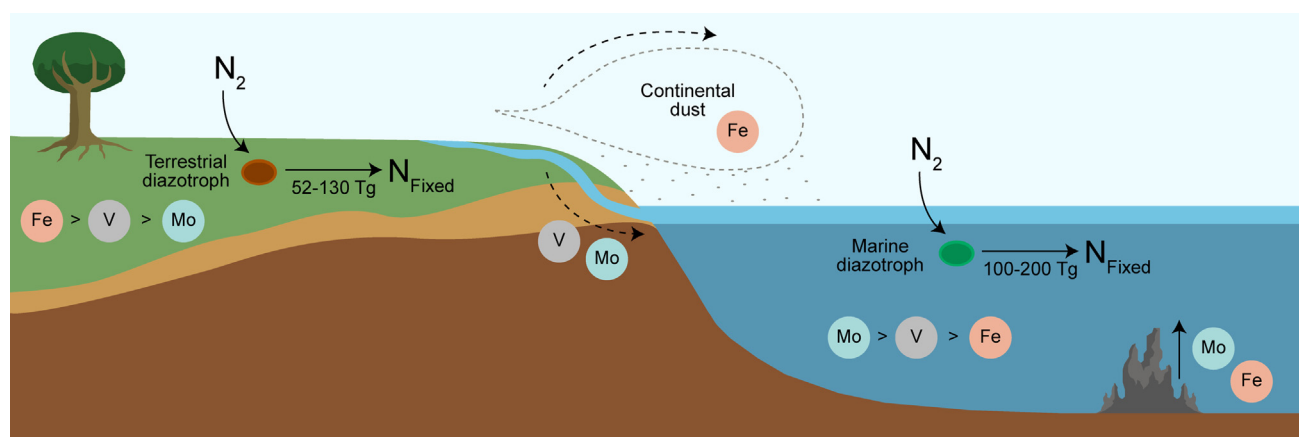


Figure 2. Schematic of modern biological nitrogen fixation rates. The abundance and sources of key metals for nitrogenase activity are highlighted. The size of the arrow indicates relative abundance as compared with the alternate biome.

alternative nitrogenases, our understanding of terrestrial alternative nitrogenases is limited by the focus on agriculturally important symbiotic diazotrophs rather than free-living species.

Heterotrophic, diazotrophic bacteria such as *Rhizobia* and *Frankia* form symbiotic relationships with legumes and actinorhizal plants, respectively [54]. These symbiotic diazotrophs are found mostly in low- to mid-latitude systems, corresponding to high-productivity environments for their plant hosts [55]. When nitrogen availability in the soil is low, the symbiont bacteria is recruited to the plant roots and nodules. The bacteria begin to fix nitrogen for the plant in exchange for organic acid nutrients [56]. While the diazotrophic symbiont is beneficial to the plant when nitrogen is scarce, it becomes unfavorable to support the symbiont when nitrogen is available given the energy needed to create organic carbon for the symbiont [56]. Therefore, there is a threshold of fixed nitrogen concentration above which symbiosis is disadvantageous. Adding nitrogen was found to inhibit BNF in all biomes across a variety of substrates, such as soils, leaf litter, and plant nodules [54].

Free-living, asymbiotic diazotrophs, while less studied than their symbiotic counterparts, contribute to a third of BNF [55]. Free-living diazotrophs are able to contribute to nitrogen fixation when vegetation is sparse, such as polar environments, by incorporating into cryptogamic covers [55]. These cryptogamic communities are made up of mosses, lichens, fungi, cyanobacteria, and/or algae [57]. Since free-living diazotrophs are not restricted to environments where host plants can survive, these asymbiotic bacteria are likely more important for BNF in cold and arid environments [55].

Global nitrogen fixation rates

Quantifying the amount of fixed nitrogen produced via terrestrial BNF is inherently challenging given the scope, but recent bottom-up meta-analysis estimates that terrestrial ecosystems produce 52–130 Tg N per year, with a median global value of 88 Tg N year⁻¹ [55]. Industrial nitrogen fixation via the Haber-Bosch process (120 Tg N year⁻¹) produces equal amounts to double the amount of fixed nitrogen as terrestrial biomes [58,59]. Both terrestrial and industrial nitrogen fixation rates are less than marine nitrogen fixation rates, 100–200 Tg N year⁻¹ (Figure 2).

Future of the nitrogen cycle

With the modern climate of Earth changing due to human activity, it is important to predict how biogeochemical cycles that are crucial for supporting life on Earth, such as the nitrogen cycle, will respond to changes in atmospheric composition, ocean pH, etc. Exactly how climate change will affect nitrogen fixation is still unclear, as some effects of climate change are predicted to stimulate nitrogen fixation while others will repress the process. First, we will consider the impacts of increasing atmospheric CO₂ on diazotrophs. As photosynthesis increases in response to high CO₂, the demand for fixed N also increases in both terrestrial and marine environments [60,61]. Some argue that diazotrophs would thrive under increased pCO₂ conditions, with nitrogen fixation estimated to be enhanced by 29% [62]. In high pCO₂ conditions, carbon-concentrating mechanisms (CCM) are downregulated to allow for energy to be directed toward N₂ fixation [62]. However, another study calculated that while doubling pCO₂ would result in a 30% decrease in CCM energy usage, this would only affect total cellular energy utilization by 3% [63]. Based on these calculations, the benefits of high CO₂ stimulation on nitrogen fixation may not be significant enough to override the negative impacts of other climate change parameters on nitrogen fixation rates.

Increased flux of atmospheric CO₂ to the ocean would ultimately lead to a decrease in oceanic pH, known as ocean acidification. Iron becomes more soluble at lower pH, but the bioavailability of iron may decrease as dissolved iron becomes incorporated by different organic ligands [64]. In already severely iron-limited environments, such as HNLC and LNLC regions, ocean acidification

will severely impact nitrogen fixation. *Trichodesmium* grown in iron-limited conditions downregulate photosystem I and chlorophyll *a* synthesis genes, which allows them to divert iron from these processes towards nitrogenase [65]. In this same study, iron limitation combined with ocean acidification conditions resulted in an overall decrease in nitrogen fixation rates of *Trichodesmium erythraeum*, suggesting that the increased iron solubility effect of ocean acidification will not be enough to override the suppression of nitrogen fixation as ocean acidification worsens. Recent projections suggest that nitrogen fixation via *Trichodesmium* will decrease by 27% by the end of the century with continued CO₂ emissions [63]. As *Trichodesmium* are the major nitrogen fixers in marine environments, this decrease will likely have significant impacts on the entire marine nitrogen cycle.

Global temperature changes, however, would have a stimulatory effect on diazotrophs up to their thermal maxima. In tropical regions, BNF species have a higher optimal temperature range, between 29°C and 37°C, than temperate regions, and would therefore respond better to increasing temperature [66,67]. Nitrogen fixation rates in Fe-limited *Trichodesmium* cultures were stimulated by increasing temperatures from 22°C to 32°C by a factor of 308% [68]. A similar increase in CO₂ fixation rates under Fe-limited conditions was also observed as temperature increased from 22°C to 32°C [68]. Increasing temperature decreased the cellular iron content of these cultures, presenting one way to alleviate iron limitations. In this same study, the authors predict that increasing temperatures would increase BNF by 76% by the year 2100 [68]. In marine environments, *Trichodesmium* and *Crocospaera* already live in warm waters (~30°C), which puts these genera at risk for climate change, increasing local ocean temperatures above their thermal maxima (36°C). Given the variety of changes in BNF rates when studying the effects of individual parameters of climate change, it is unclear how ecosystems and the global nitrogen cycle will be impacted as a result. As the nitrogen cycle is a key biogeochemical cycle for all life on Earth, it is becoming increasingly important to develop more complex climate models to incorporate the variety of responses that BNF may exhibit in response to the changing environment.

Implications for life in the universe

As life on Earth is dependent on the nitrogen fixation activity of nitrogenases, this makes understanding the past and future evolution of these enzymes critical for informing the extraterrestrial search for 'life as we know it'. Working under the constraints of nitrogenase on Earth, we would begin the search for extraterrestrial BNF by looking for planets or moons with a source of N₂ that can be fixed, along with available metal cofactors in the environment. Currently, N₂ on its own is very difficult to detect spectroscopically in the atmosphere of exoplanets, as it has no significant absorption features [69]. Within our solar system, the potential for BNF can be assessed with satellite instrumentation (e.g., ion or mass spectrometry) by tracking both nitrogen and known, alternative detectable substrates of nitrogenase. For example, several substrates that nitrogenase can reduce, such as N₂, acetylene, and CO₂, have been detected in Enceladus' geysers [70]. A recent model has shown that ancient Mars likely had abiotic, lightning-induced N₂ fixation, which could produce very low amounts (nM) of fixed nitrogen sources [71]. This abiotic process would not produce sufficient amounts of fixed nitrogen to sustain the modern Earth biosphere, therefore indicating that the evolution of an enzyme capable of nitrogen fixation would be favorable on early Mars as it was on early Earth (assuming these microbes have similar nitrogen requirements as terrestrial counterparts). From studying the evolution of nitrogen fixation on Earth, there are two evolutionary factors to consider as to the likelihood of BNF evolving. First, that nitrogenase evolved early in Earth's history may bode well for similar processes to evolve on other planets. Secondly, despite the abundance of N₂ as a substrate and the need for fixed nitrogen sources, terrestrial life is limited in how it can fix nitrogen to one single enzyme. Whether or not life elsewhere would evolve a singular mechanism for nitrogen fixation as quickly as life on Earth did remains unknown.

Current remote detection studies and missions have focused on the search for biomarkers, which are by definition organic compounds of proposed biological origin used for life detection studies. Potential inorganic biomarkers, such as metals involved in key metalloenzymes on Earth, remain largely overlooked. Many of the biogeochemical cycles on Earth that are fundamental to life as we know it are directly connected to different metal cycles, as discussed throughout this review for nitrogen fixation and the nitrogen cycle. Both biological and industrial nitrogen fixation (i.e., Haber-Bosch) utilize metal catalysts to reduce N_2 . Therefore, the importance of metals in catalyzing nitrogen fixation may be ubiquitous across all life. Thus, it would be important to know the metal availability on other planets or moons in the search for nitrogenase-like enzymes. It is also possible that an extraterrestrial nitrogenase-like enzyme could utilize other metal cofactors or substrates available in its geochemical environment.

Concluding remarks and future perspectives

A fundamental understanding of extant biomolecular and microbiological processes requires understanding the historical circumstances under which these processes first arose and were shaped. The reconstruction of past events largely relies upon the earliest fossil record, including indirect traces such as isotope signatures. Many of these critical processes arose billions of years ago. The roles of proteins, specifically the interrelated ways that they may underlie jumps in complexity that define extant life on our planet, are poorly resolved with the available record. This includes the origin of nitrogen fixation, a significant singular event in the history of life on Earth. A recent study suggests that complex animals could not have existed without nitrogen fixation [72]. In these ways, human civilization is directly attributable to singular biological innovations that happened billions of years ago. In order to understand how nitrogen fixation originated, persisted, and proliferated across diverse ecological niches over billions of years, we must take an integrated approach. By analyzing extant sequence diversity in genomic databases, probing extinct protein sequence diversity and function through evolutionary and *in silico* models, and laboratory reconstruction proxy studies looking backward in time, we might begin to access the traces of a lost molecular history of our planet, the finer contours of which are largely missing in the geochemical record. An arsenal of innovative responses to upcoming ecological and climatological challenges may yet be revealed by an accounting of past innovations spurred by planetary upheaval (see [Outstanding questions](#)).

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Outstanding questions

Some elements are more utilized in biology than others, in ways that do not always neatly comport with broad environmental availability. What drives the natural selection of the chemical elements in biology: function or availability? Is this dependence an inevitable consequence of the utility of those elements? Or is it an accident of history that might have worked out very differently if we ‘replayed the tape’ of evolution under slightly different conditions?

Nitrogen fixation arose under planetary conditions that are very different from those of modern Earth. A notable example is the oxygenation of the atmosphere, which drastically changed the availability of metals. How have changes in metal availability influenced the origin, evolution, and dependence of nitrogen fixation via nitrogenase?

The effects of climate change may stimulate or diminish biological nitrogen fixation, especially in marine settings. Ultimately, in which direction will climate change push nitrogen fixation, and thus the nitrogen cycle, on Earth in the next century?

The biosphere as we know it cannot exist without nitrogen and nitrogen fixation is essential for the diversity and scale of the modern biosphere. Today, obtaining N from the atmosphere most commonly relies on Mo. While Mo is the most abundant trace metal in modern oceans, it was more scarce in early Earth's oceans when this biochemical mechanism first evolved. Do these facts imply that certain metals are so essential for life as we know it that evolution selected for these elements despite their scarcity and, hence, that the availability of these elements should be a consideration in searching for life? Or does the coevolutionary history of life and environment on Earth reveal other possibilities?

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