



Biological nitrogen fixation by alternative nitrogenases in terrestrial ecosystems: a review

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Abstract Biological nitrogen fixation (BNF), a key reaction of the nitrogen cycle, is catalyzed by the enzyme nitrogenase. The best studied isoform of this metalloenzyme requires molybdenum (Mo) at its active center to reduce atmospheric dinitrogen (N_2) into bioavailable ammonium. The Mo-dependent nitrogenase is found in all diazotrophs and is the only nitrogenase reported in diazotrophs that form N_2 -fixing symbioses with higher plants. In addition to the canonical Mo nitrogenase, two alternative nitrogenases, which use either vanadium (V) or iron (Fe) instead of Mo are known to fix nitrogen. They have been identified in ecologically important groups including free-living bacteria in soils and freshwaters and as symbionts of certain cryptogamic covers. Despite the discovery of these alternative isoforms more than 40 years ago, BNF is still believed to primarily rely on Mo. Here, we review existing studies

on alternative nitrogenases in terrestrial settings, spanning inland forests to coastal ecosystems. These studies show frequent Mo limitation of BNF, ubiquitous distribution of alternative nitrogenase genes and significant contributions of alternative nitrogenases to N_2 fixation in ecosystems ranging from the tropics to the subarctic. The effect of temperature on nitrogenase isoform activity and regulation is also discussed. We present recently developed methods for measuring alternative nitrogenase activity in the field and discuss the associated analytical challenges. Finally, we discuss how the enzymatic diversity of nitrogenase forces a re-examination of existing knowledge gaps and our understanding of BNF in nature.

Keywords Biological nitrogen fixation · Terrestrial ecosystems · Nitrogenase · Alternative nitrogenases · Molybdenum · Vanadium · Iron-only

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Introduction

Nitrogen (N) is a main limiting nutrient for primary production in unmanaged terrestrial ecosystems (Wang et al. 2010). In addition, reduced N bioavailability to primary producers strongly shapes the response of ecosystems to climate change (Luo et al. 2004; Reich et al. 2006; Sigurdsson et al. 2013). Biological nitrogen fixation (BNF), which converts

the abundant but inert dinitrogen (N_2) molecule into ammonia, is the primary route for entry of new reactive, biologically available N (or ‘fixed’ N) in the absence of human activities (Galloway et al. 2004; Vitousek et al. 2013). The energetically demanding and oxygen (O_2) sensitive reaction is catalysed by the metalloenzyme nitrogenase which is found in prokaryotes living in symbiotic or mutualistic associations (e.g., with higher plants and lichens), in association with bryophytes or living freely (e.g., in soils) (Gaby and Buckley 2011). The nitrogenase enzyme occurs in three different isoforms; the molybdenum (Mo)-nitrogenase found in all diazotrophs and the only one known in diazotrophs forming symbioses with higher plants, and two alternative nitrogenases, the vanadium (V) and iron-only (Fe) nitrogenases, found in free-living bacteria and cyanobacteria. While all nitrogenases require large amount of Fe, the three isoforms differ chiefly by the metal (Mo, V or Fe) at a critical active site position (see section “[The nitrogenase isoforms](#)”). BNF is usually considered to rely primarily on the Mo isoform of the enzyme (Mo-Nase). Until recently, the importance and contribution of the other two nitrogenase isoforms, the V and Fe-only nitrogenases (V-Nase and Fe-Nase), to N_2 fixation in natural ecosystems (Bishop et al. 1980; Chisnell et al. 1988; Robson et al. 1986) had been little studied. Yet over the last decade, several findings suggest that alternative N fixation (i.e., nitrogen fixation by the V- and Fe-Nases) is an important ecological feature of BNF in terrestrial ecosystems and can contribute up to 70% of total BNF in some diazotrophs (e.g. cyanolichens) (Darnajoux et al. 2017, 2019; McRose et al. 2017b; Zhang et al. 2016).

This article provides a comprehensive overview of recent developments regarding the environmental significance of alternative N fixation and how it impacts our understanding of terrestrial BNF. First, we briefly present the Mo, V and Fe-only isoforms of the nitrogenase enzyme. Then we discuss and compare methods currently available for detecting the presence and activity of the alternative V and Fe-only nitrogenases in the field, including some of the limitations inherent to each method. We also provide a review of existing studies, most of which have been published in the last 5 years, on the characterization and quantification of alternative nitrogenase activity in tropical, temperate and boreal ecosystems, including forests and coastal environments. Finally, we discuss how

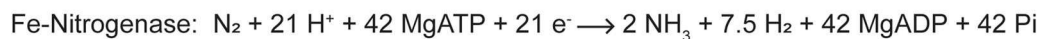
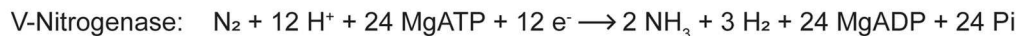
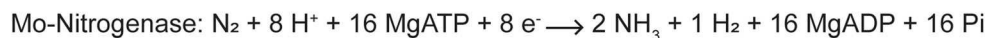
alternative nitrogenases affect our understanding of BNF and highlight the necessity of filling existing knowledge gaps on their role, importance, and activity in nature.

The nitrogenase isoforms

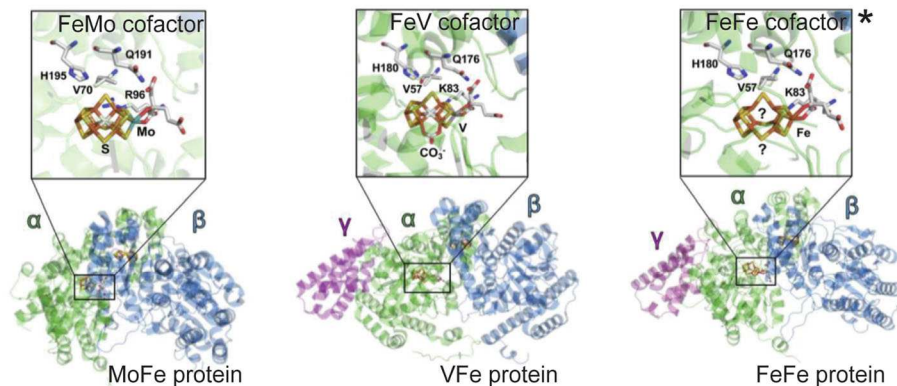
Three isoforms of the prokaryotic enzyme nitrogenase have been identified in *Archaea* and *Bacteria*, and across many bacterial phyla, including *Cyanobacteria*, *Proteobacteria*, *Firmicutes* (Bishop et al. 1982; Boisson et al. 2006; Chien et al. 2000; Chisnell et al. 1988; Eady 1996; Fallik et al. 1991; Hales 1990; Loveless and Bishop 1999; McRose et al. 2017b; Thiel 1993). These three nitrogenase isoforms, encoded by separate genes (Boyd et al. 2011; Chien et al. 2000; Dixon and Kahn 2004; Eady 1996; Hamilton et al. 2011; Raina et al. 1988; Schuddekopf et al. 1993; Thiel 1993; Werner Klipp 2004; Wolfinger and Bishop 1991; Zinoni et al. 1993, see section “[Nitrogenase genes](#)” for details), are structurally and phylogenetically related and are mainly distinguished by their metal cofactor; the molybdenum nitrogenase (Mo-Nase) with its FeMo cofactor, the vanadium nitrogenase (V-Nase) with its FeV cofactor and the iron nitrogenase (Fe-Nase) with its Fe-only cofactor (Bishop and Premakumar 1992; Joerger and Bishop 1988) (Fig. 1a).

In the process of reducing N_2 to ammonium (NH_4^+), the nitrogenase enzyme also reacts with H^+ to produce H_2 . H_2 production, which consumes both electrons and ATP, seems to be tightly coupled to N_2 reduction at the enzyme active site (Harris et al. 2019). One H_2 is produced for every reduced N_2 by the Mo-nitrogenase, but the H_2 to N_2 ratio appears to be much higher for the V- and the Fe-only nitrogenase, at least in vitro (Fig. 1a). The nitrogenase enzyme also reacts with a suite of doubly and triply bonded small molecules such as acetylene, cyanide, nitrous oxide, and others (Burgess and Lowe 1996). Reaction kinetics and substrate specificity are usually isozyme-dependent and have been extensively used to gain insight into enzyme mechanisms. For example, unlike Mo-nitrogenase, the V-nitrogenase can reduce carbon monoxide into small hydrocarbons (Lee et al. 2010) and both Fe- and V-nitrogenase have been found to reduce carbon dioxide into methane (Zheng et al. 2018). The stable isotopic composition of certain

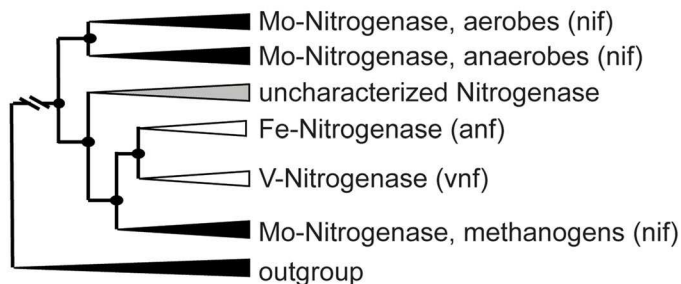
A. Stoichiometry



B. Structure



C. Phylogeny



D. Distribution in diazotroph genomes

Phylum	No. Genera	Nases represented	Environment
Bacteroidetes	2	Mo, Fe	Pathogen, Rice, Coastal sediments
Chlorobi	1	Mo, Fe	Coastal sediments
Cyanobacteria	2	Mo, V	Freshwater, cyanolichen
Firmicutes	7	Mo, V, Fe	Soil, Sediment, Clay, Spoiled Beer, Sewagew, Animal-associated
Proteobacteria	19	Mo, V, Fe	Freshwater, Aquaculture, Baltic Sea, Soils, Sediments, Mangroves, Plant-associated, Termite gut, Digestors
Verrucomicrobia	2	Mo, Fe	Termite gut
Euryarchaeaota	2	Mo, V, Fe	Sediments, Digestors, Lakes

nitrogenase products can also distinguish different isoforms (Zhang et al. 2014, 2016; Luxem et al. 2020a).

The evolution of the three nitrogenase isozymes over geological times, and which isozyme may have evolved first, have been extensively discussed (Anbar

Fig. 1 Overview of the nitrogenase isoforms. **a** Stoichiometry of N fixation by the three Nase isoforms in vitro (Eady 1996; Wall 2004). **b** Structural differences in Mo-Fe, V-Fe, and Fe-Fe proteins. Electrons travel from the [4Fe-4S] cluster in the Fe protein (not shown) to the P-cluster, located between the α - and β -subunits (green and blue, respectively), to the FeMo/FeV/FeFe-cofactor in the α subunit, where N_2 reduction occurs. The role of γ -subunit (purple) in alternative nitrogenases is not well understood. *The structure for the FeFe protein is a model based on the FeV protein adapted from (Mus et al. 2018). **c** Summary of maximum likelihood phylogenetic relationships between concatenated HDK protein sequences for *nif/vnf/anf* and uncharacterized forms of nitrogenase calculated by Boyd et al. (2011) and adapted from Mus (2018), Boyd et al. (2011), Mus et al. (2018). Filled circles at nodes indicate bootstrap values > 80. Adapted from (Mus et al. 2018). **d** Distribution of alternative nitrogenases in sequenced genomes of organisms across diverse phyla and locations of isolation. Based on (McRose et al. 2017b)

and Knoll 2002; Boyd et al. 2011; Peters and Boyd 2015). The canonical Mo-Nase is found in all N_2 -fixing bacteria and archaea (diazotrophs) and is the only nitrogenase reported in diazotrophs that form N_2 -fixing symbioses with higher plants (de Bruijn 2015; Dilworth and Loneragan 1991; Fallik et al. 1991). The so-called “alternative” nitrogenases (i.e., V-Nase and Fe-only Nase) are found in a limited, but diverse, number of diazotroph species, including ecologically important groups such as free-living soil bacteria and archaea (Bishop et al. 1980, 1982; Bishop and Premakumar 1992; Chien et al. 2000; Chisnell et al. 1988; Fallik et al. 1991; Hales 1990; Joerger and Bishop 1988; Joerger et al. 1989; McRose et al. 2017b; Pau et al. 1993; Robson et al. 1986), cyanobacteria (Boison et al. 2006; Kentemich et al. 1988; Masukawa et al. 2009; Ni et al. 1990; Thiel 1993; Thiel and Pratte 2013), and cyanolichens (Darnajoux et al. 2017; Gagunashvili and Andresson 2018; Hodgkinson et al. 2014; Zhang et al. 2016) (Fig. 1b).

Biological nitrogen fixation (BNF) has long been considered to rely primarily on the Mo isoform for several reasons. N_2 -fixing symbionts with higher plants (*Rhizobium* and *Frankia*), believed to be the major contributors to BNF, do not possess alternative nitrogenase genes. In addition, at room temperature, the Mo-Nase isoform fixes N_2 more efficiently than the alternative nitrogenases: the Mo-Nase requires “only” 16 mol of ATP per mole of N_2 , vs. 24 and 40 mol of ATP per mole of N_2 for the V- and Fe-only Nases, respectively, as assessed with in vitro assays (Fig. 1a).

In the laboratory, diazotrophs that possess more than one nitrogenase isoform preferentially express the Mo-nitrogenase when conditions, such as adequate environmental Mo, allow the synthesis of a functional enzyme (Boyd et al. 2011; Jacobitz and Bishop 1992; Jacobson et al. 1986; Maynard et al. 1994; Oda et al. 2005; Peters and Boyd 2015). Consequently, alternative nitrogenases appear to be “back up” enzymes for maintaining the N anabolism of diazotrophs (see section “Where have alternative nitrogenases been found so far?”).

Nonetheless, there is mounting evidence that alternative nitrogenases are significant contributors to BNF on a global scale. First, several recent reports highlight the importance of non-symbiotic N_2 fixation, epiphytic N_2 fixation associated with bryophytes and symbiotic N_2 fixation with fungi for global N input, especially in high latitude ecosystems, to which alternative nitrogenases could contribute (Chapin et al. 1990; DeLuca et al. 2002; Elbert et al. 2012; Michelsen et al. 2012; Rousk et al. 2013). The widespread occurrence of diazotrophs harboring alternative nitrogenase genes across many different environments (Betancourt et al. 2008; McRose et al. 2017b) suggests that these genes provide a significant competitive advantage to their hosts and possibly play important ecological and biogeochemical roles. These findings force a reassessment of the relative contribution of symbiotic N fixation in higher plants to terrestrial BNF and concomitantly, of the predominance of the Mo-isoform in the same process (Reed et al. 2011).

Experimental methods for the detection of alternative nitrogenases in nature

Nitrogenase genes

The synthesis of a functional nitrogenase enzyme is a complex process involving as many as 21 genes for regulation, electron transfer, chaperone and stability functions, protein maturation, synthesis of metal cofactors, and of course protein synthesis (Rubio and Ludden 2005). The inventory of genes required for N fixation varies amongst different diazotrophs, although a minimal core of 6 structural and cofactor biosynthetic genes is conserved (Dos Santos et al. 2012). The nitrogenase enzyme is made of two

proteins: the MoFe protein, a heterodimer containing the active site of the enzyme, and the Fe protein, a homodimer sometimes referred to as the nitrogenase reductase, which transfers electrons to the active site for reduction of N_2 to NH_3 (Peters et al. 1995). The MoFe protein is encoded by the *nifDK* gene while the Fe protein is encoded by the *nifH* gene in the Mo-nitrogenase (Seefeldt et al. 2009). Alternative nitrogenases are encoded by similar sets of genes, referred to as *vnfDK* and *vnfH* (for the V-nitrogenase) and *anfDK* and *anfH* (for the Fe-only nitrogenase). Alternative nitrogenases require an additional gene, *vnfG* (or *anfG*), which is necessary for processing aponitrogenase to functional nitrogenase (Chatterjee et al. 1997). Unlike *nifH*, *vnfH* and *anfH* genes, which are the most widely used molecular markers to identify diazotrophs from natural environments (Zehr et al. 2003), *nifD*, *vnfD* and *anfD* sequences cluster according to the nitrogenase isozyme.

A few studies have taken advantage of this robust clustering to study the presence of alternative nitrogenase genes in various natural environments, including soils, coastal sediments, rice fields, and lichens (Betancourt et al. 2008; Boison et al. 2006; Hodgkinson et al. 2014; McRose et al. 2017b). Next generation molecular biology tools, such as meta-transcriptomics, have been used to investigate the expression of alternative nitrogenases in pure culture, soil microcosms and sediment mesocosms (Bellenger et al. 2014; Bowen et al. 2014; Hamilton et al. 2011). To the best of our knowledge, it exists no report of alternative nitrogenase transcripts from environmental samples. However, while these approaches provide valuable genomic and transcriptomic information, reliable estimates of the relative contributions of alternative nitrogenases to BNF rates require direct measurement of nitrogenase activity.

Quantification of alternative nitrogenase rates in environmental samples

There are currently four primary methods available for the quantification of alternative BNF. Three of them (R ratio, ethane production, ISARA) are based on the reduction of acetylene by nitrogenase, a well-known reaction that has been used to assay nitrogenase activity since 1968 (Hardy et al. 1968). The fourth one is based on nitrogen stable isotope fractionation during N fixation by nitrogenase (^{15}N fractionation). A fifth

method, which has yet to be tested in the field and is thus not described in detail here, is based on the large hydrogen isotope fractionation during CO_2 reduction to methane by alternative nitrogenases (Luxem et al. 2020a).

R ratio

In addition to reducing N_2 into ammonium, the nitrogenase enzyme can reduce acetylene to ethylene (Hardy et al. 1968) and the Acetylene Reduction Assay (ARA), which measures the rate of acetylene reduction (AR) as a proxy for nitrogenase activity, has been commonly used in the laboratory and in the field for the last 50 years. ARA is non-destructive, sensitive and requires only low cost equipment (a gas chromatograph equipped with a flame ionization detector). The conversion of acetylene reduction rates into N_2 fixation rates requires correction by a conversion ratio R, defined as $R = (\text{acetylene reduction rate})/(\text{N}_2 \text{ fixation rate})$. The R ratio has a theoretical value ($R = 3-4$) based on the respective number of electrons needed to reduce acetylene and N_2 by the Mo-Nase (Hardy et al. 1973). This value was also validated in pure cultures (Eady and Robson 1984). Due to the extra production of H_2 by alternative nitrogenases, these isoforms have distinctive R ratios: ~ 0.5 and 1 for the Fe- and V-Nase respectively (Bellenger et al. 2014). Thus the R ratio can potentially be used to detect the presence of active alternative nitrogenases. The R ratio can be measured experimentally by performing incubations with either acetylene or a $^{15}N_2$ atmosphere, simultaneously on duplicate samples or consecutively on the same sample, and measuring both the acetylene reduction rate and the rate of ^{15}N incorporation into the sample (Hardy et al. 1973). Precision on R ratio measurements is thus negatively affected by both the error and bias from the ARA and ^{15}N incubation methods, which could be particularly important for low-activity samples. In addition, because of the inhibition of acetylene on $^{15}N_2$ reduction, both methods cannot be undertaken on the same samples at the same time, which also increases the uncertainty of the R ratio determination. Measurements of R ratios also suffer from potential artifacts such as the presence of easily absorbed forms of ^{15}N in commercial $^{15}N_2$ stocks (Dabundo et al. 2014), or/and the slow dissolution kinetics of $^{15}N_2$ gas (Mohr et al. 2010). Several factors can further

complicate the interpretation of R ratio measurements in environmental samples. In soils, heterogeneity in the natural abundance of ^{15}N in a given sample can result in significant errors on the rates of ^{15}N incorporation. In addition, processes such as BNF-independent ethylene cycling by microbes and plants in soils can affect the value of the R ratio, complicating interpretation of such data (Bleecker and Kende 2000; Nagahama et al. 1994). Finally, the R ratio has been shown to be variable in marine sediments and in cyanobacteria, a highly relevant group of diazotrophs worldwide, with values ranging from 1.5 to as high as 100 (for review, see Bellenger et al. 2014; Mohr et al. 2010; Seitzinger and Garber 1987). Some of these low values may reflect the ability of some cyanobacteria to release a substantial fraction of their fixed nitrogen in the extracellular media (Mulholland and Bernhardt 2005; Mulholland et al. 2004). Excretion of newly fixed nitrogen results in artificially low rates of N_2 fixation, since these rates are measured on the basis of ^{15}N incorporation into biomass. Thus, while measurements of the R ratio can sometimes be useful (Bellenger et al. 2014), additional methods are required for the reliable detection of alternative nitrogen fixation activity.

Ethane production during ARA

The alternative V-Nase and Fe-Nase produce a significant amount of ethane (C_2H_6) in addition to ethylene (C_2H_4) during acetylene reduction (typically 3–8% of ethane relative to ethylene (Dilworth et al. 1987; Dilworth et al. 1988)). In contrast, the Mo-Nase produces very small amount of ethane that have only been measured at high temperatures (above 40 °C) (Dilworth et al. 1993). Accordingly, the production of ethane has been used as a reliable proxy for alternative nitrogenase activity in many laboratory studies (Attridge and Rowell 1997; Chakraborty and Samaddar 1995; Davis et al. 1996; Fallik et al. 1991; Schneider et al. 1991). This method is difficult to use in the field, however, as acetylene reduction rates, which reflect a mixture of Mo-Nase and alternative nitrogenase activity, are typically an order of magnitude lower than in pure cultures, resulting in ethane yields that are near or below the detection limit of standard gas chromatographs. For this reason, the detection of ethane in environmental samples may require the pre-concentration of the sample headspace before

measurement by gas chromatography. The cycling of ethane by microbes in processes unrelated to BNF (Chen et al. 2019; Fukuda et al. 1984; Singh et al. 2017), the temperature dependency of the ethane to ethylene electron flux ratio in V-Nase (Dilworth et al. 1988), and laboratory studies of nitrogenase mechanism which show that mutant Mo-Nases can also produce ethane (Scott et al. 1992) are additional limitations on interpreting ethane signals as indicators of alternative BNF. Nonetheless, our studies have shown that measurements of ethane production as a proxy for alternative nitrogenase activity can be highly successful in systems with relatively low biological complexity and high BNF activity (e.g. ethane production in the ppm range) such as cyanolichens (Darnajoux et al. 2019).

Isotopic acetylene reduction assay (ISARA)

This new method uses the ^{13}C isotope fractionation of acetylene reduction to ethylene to estimate the contribution of alternative nitrogenases to activity within the acetylene reduction assay. The three nitrogenase isoforms are characterized by distinct ^{13}C fractionations ($^{13}\epsilon_{\text{AR}} = \delta^{13}\text{C}_{\text{acetylene}} - \delta^{13}\text{C}_{\text{ethylene}}$) during acetylene reduction to ethylene: ~ 13 –15% for Mo-Nase, ~ 7 –9% for V-Nase, and ~ 6 –7% for Fe-Nase (Zhang et al. 2016). In this assay, the $\delta^{13}\text{C}$ of the source acetylene and the produced ethylene are measured using a gas chromatograph-combustion-isotope ratio mass spectrometer. The total rate of acetylene reduction is also measured. The contribution of alternative Nases versus Mo-Nase to acetylene reduction can then be estimated by comparing the ^{13}C fractionation of the sample versus isoform-specific values: $f_{\text{altARA}} = (^{13}\epsilon_{\text{AR MoNase}} - ^{13}\epsilon_{\text{AR sample}}) / (^{13}\epsilon_{\text{AR MoNase}} - ^{13}\epsilon_{\text{AR altNase}})$. The use of Fe-Nase $^{13}\epsilon_{\text{AR}} (= 5.8$ – 6.5% , Zhang et al. 2016) for the value of $^{13}\epsilon_{\text{AR altNase}}$ in the above calculation produces the most conservative estimates of the fractional contribution of alternative nitrogenase activity to acetylene reduction. Independent evidence for V-Nase or Fe-Nases (e.g., DNA or RNA sequences, ethane/ethylene ratios from ARA and/or metal content) can be used to choose the appropriate value of $^{13}\epsilon_{\text{ARaltNase}}$ for calculations. Finally, total and isoform specific N_2 reduction rates can be estimated by correcting acetylene reduction rates with the relevant R ratios for different isoforms as determined by ISARA (i.e., total N_2 fixation

rate = AR rate $\times (1 - f_{\text{altARA}})/R_{\text{MoNase}} + \text{AR rate} \times f_{\text{altARA}}/R_{\text{altNase}}$. Although this method is still in its infancy and requires further testing in the laboratory and in the field to establish its full potential, it is to date the most reliable way to evaluate the contribution of alternative nitrogenases to environmental N_2 fixation rates.

¹⁵N fractionation

As shown in Zhang et al. (2014), the three nitrogenase isozymes when reducing N_2 also produce distinct organism-scale ¹⁵N isotope fractionations: $\epsilon_{\text{fix}} = \sim 2\text{‰}$ for Mo-Nase, $\sim 6\text{‰}$ for V-Nase and $\sim 8\text{‰}$ for Fe-Nase, where $\epsilon_{\text{fix}} = \delta^{15}\text{N}_{\text{dissolved N}_2} - \delta^{15}\text{N}_{\text{Nfixer biomass}}$ and $\delta^{15}\text{N} = [({}^{15}\text{N}/{}^{14}\text{N}_{\text{sample}}/{}^{15}\text{N}/{}^{14}\text{N}_{\text{air}}) - 1] \times 1000$ in per mil (‰) units. Thus, the ¹⁵N/¹⁴N isotope ratios of biomass, typically expressed as $\delta^{15}\text{N}$ values, can in theory be used to evaluate the contribution of alternative nitrogenases to N input in environmental samples with N_2 -fixing activity. This approach has been successfully used to demonstrate the role of alternative nitrogenases in past ocean anoxia (Zhang et al. 2014). The presence of multiple N sources with varying and often unknown $\delta^{15}\text{N}$ along with the existence of complex and variable N cycle processes (e.g., ammonium uptake, nitrification, denitrification) that impart their own $\delta^{15}\text{N}$ signal to the bulk biomass, makes the interpretation of field samples from modern environments inherently challenging. To properly constrain alternative BNF based on biomass $\delta^{15}\text{N}$, we recommend focusing on samples with biomass containing primarily newly fixed N from BNF, along with the use of other independent measurements of alternative nitrogenase activity (see above).

In summary, several methods currently exist to assess the contribution of alternative nitrogenases to

BNF with their specific strengths and weaknesses (Table 1). Cross-calibration of the four methods on the same samples (i.e. cyanolichens) shows the most robust correlations between ISARA and Ethane production data (Fig. 2a). ¹⁵N fractionation data measured for samples primarily comprised of new N from BNF also show good correlation with ethane production (Fig. 2b). Not surprisingly perhaps, the R ratio method appears to be the least reliable (Fig. 2c). The high detection limits of these methods are currently one of the major hurdles for the assessment of alternative nitrogenase activity in natural samples. The ¹⁵N fractionation approach requires BNF to be the primary driver of sample ¹⁵N/¹⁴N, limiting the application of this method to samples primarily comprised of newly fixed N. Measurements of ethane production for the detection of alternative nitrogenases can only be achieved on samples with high acetylene reduction rates because of two compounding factors: the production of ethane is only a few % of the production of ethylene and the limit of detection of most GC methods used to measure C_2H_6 production is in the high ppbv range. ISARA analyses require a minimum of ~ 300 ppmv ethylene for accurate detection of nitrogenase isoform activity. In both cases (ethane and ISARA), sensitivity can be improved with a pre-concentration step, which requires extra handling and increases costs.

We note that these high detection limits, which often require several-hour incubations to accumulate measurable amount of analyte in the incubation chambers, currently preclude kinetic studies on short time scales (i.e., a few minutes) to understand how diazotrophs modulate the expression and use of nitrogenase isoforms in response to environmental conditions (e.g., metal availability, temperature, energy resources).

Each method requires different levels of expertise and equipment. The acetylene reduction method

Table 1 A comparison of advantages and disadvantages of current methods to assess alternative nitrogenase contributions to BNF

	Reliability	User-friendly	Cost	Destructive
R-ratio	3 (lowest)	++	\$\$	Yes
C_2H_6	1 (high)	++++	\$	No
ISARA	1 (high)	+	\$\$	No
$\delta^{15}\text{N}$ of N_2 fixer biomass	2 (low)	++	\$\$	Yes

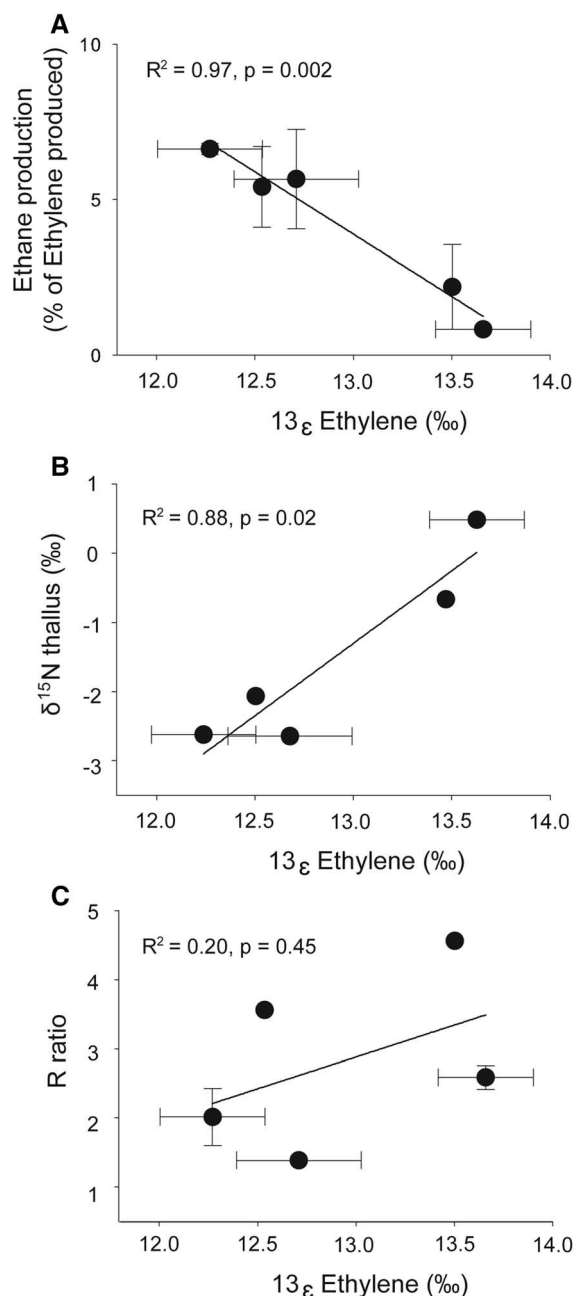


Fig. 2 Comparison of current methods for the assessment of alternative nitrogenase activity. Correlation between the $^{13}\epsilon$ (ethylene) as measured by ISARA and: **a** ethane production, **b** ^{15}N fractionation, and **c** the R ratio. Each data point corresponds to the same sample analyzed with two different methods. All data were collected in *Peltigera cyanolichens*

(ethylene and ethane production) is the most user friendly (Table 1). It only requires simple and affordable GC equipment, available in many research

facilities with limited training. Methods based on $\delta^{15}\text{N}$ measurements (R ratio and ^{15}N fractionation) require more specialized equipment and expertise. ISARA is a more difficult method to implement as most fee-for-service stable isotope facilities have yet to offer the option of ^{13}C measurements of acetylene and ethylene (Table 1). With regard to analytical cost, methods requiring the analysis of stable isotope fractionation (^{13}C for ISARA and ^{15}N for ^{15}N fractionation and R-ratio) are more expensive than methods solely relying on GC analysis (C_2H_6 production), even though numerous facilities now provide cost effective stable isotopes analyses (Table 1).

In the absence of a one-fits-all method, the use of complementary approaches to estimate the contribution of alternative nitrogenase to BNF is recommended. In the future, new methods, such as ISARA and ^{15}N fractionation, need to be implemented in a systematic manner to: (i) better evaluate the contribution of alternative nitrogenases in a wide range of ecosystems and biomes, and (ii) refine data collected using more classical approaches (i.e., ethane production and R ratio).

Where have alternative nitrogenases been found so far?

Because some of the methods for the detection of alternative nitrogenases are still relatively new, and the data on alternative nitrogenase activity are still sparse, we have included in this section habitats that have been shown to have low Mo content, or where Mo limitation of N fixation has been demonstrated, as places likely to harbor alternative nitrogenases activity.

Terrestrial environments—soils

Soils are a particularly favorable environments to look for alternative nitrogenases because Mo is one of the least abundant biometals in the earth crust, soils and plants (Kabata-Pendias 2010; Wedepohl 1995). Accordingly, Mo limitation of BNF has been reported in both agrosystems (Gupta 1997; Hafner et al. 1992; Srivastava et al. 1998; Vieira et al. 1998a, b) and in unmanaged ecosystems (see below and Fig. 3). In soils, the first evidence of Mo limitation was provided by Silvester and coworkers in tree litter, decaying

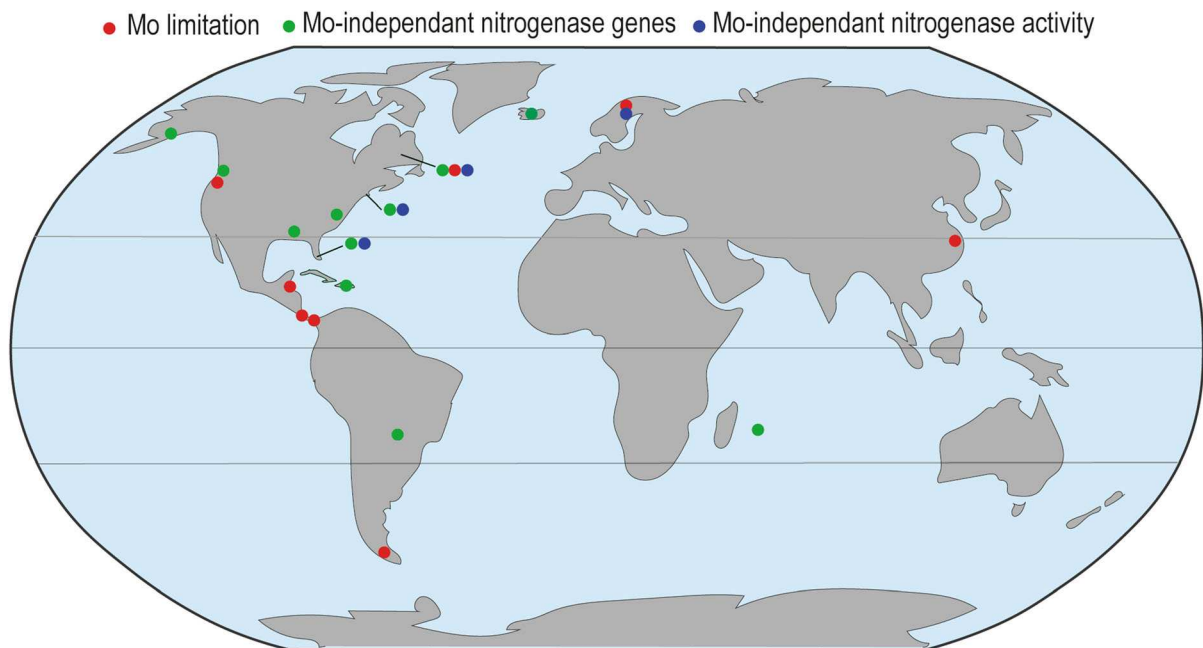


Fig. 3 Global map of alternative BNF distribution. The map indicates locations where Mo limitation of BNF (red dots), alternative nitrogenase genes (green dots), and activity of alternative BNF (blue dots) have been reported in environmental samples (Barron et al. 2009; Betancourt et al. 2008;

Gagunashvili and Andresson 2018; Hodkinson et al. 2014; Jean et al. 2013; Ma et al. 2019; McRose et al. 2017b; Reed et al. 2013; Rousk et al. 2017; Silvester 1989; Winbourne et al. 2017; Wurzbürger et al. 2012; Zhang et al. 2016)

wood and cyanolichen (*Lobaria* sp.) within Pacific northwestern forests (Silvester 1989; Horstmann et al. 1982). Other studies on the effects of trace metal and phosphorus (P) additions in Hawaiian soils indicated that Mo additions could promote higher BNF rates (Crews et al. 2000; Vitousek 1999; Vitousek and Hobbie 2000). Two other studies in the tropical forests of Panama reported Mo limitation of asymbiotic N_2 fixation in leaf litter (Barron et al. 2009; Wurzbürger et al. 2012). A study in the Belize rainforest showed Mo limitation of BNF in volcanic soils, but not in limestone soils (Winbourne et al. 2017). Mo limitation of BNF in leaf litter has also been reported in cold, temperate latitudes (Jean et al. 2013). Other studies reported no effect of Mo additions on BNF in soils and litter (Dynarsky and Houlton 2018), showing that Mo limitation is common but not ubiquitous. More details on the results of nutrient addition (N, P and Mo) on free-living nitrogen fixation in leaf litter and soils can be found in a recent meta-analysis by Dynarski and Houlton (2018). Mo limitation of BNF in soils has thus been observed over a wide range of latitudes, ranging from tropical to cold temperate environments.

In sharp contrast to Mo, Fe is the most abundant biometal in the earth crust and total V concentrations are on average 2 to 200 times higher than Mo concentrations in soils and leaf litter (Kabata-Pendias 2010), suggesting that V and/or Fe could be used to relieve Mo limitation in soils. The first studies on alternative nitrogenase genes in the environment showed that they were present in a variety of terrestrial environments such as soils, wood chips, coastal sediments, and rice fields (Betancourt et al. 2008; Boison et al. 2006; Loveless et al. 1999) (Fig. 3). More recently, we demonstrated the contribution of alternative nitrogenases to BNF in the rhizosphere and soil habitats, using R ratio values reported in the literature for tropical, temperate and boreal forests (Bellenger et al. 2014). In spite of a few exceptions (Sellstedt et al. 1986; Batterman et al. 2018), this analysis showed that R ratios reported in the literature for plant root nodules, known to own only the Mo-Nase, were usually within the theoretical 3–4 range with little variability. R ratios reported for free-living bacteria in soils were highly variable and ranged from less than 1 to more than 6 (Graham et al. 1980; Liengen 1999;

Martensson and Ljunggren 1984; Minchin et al. 1983; Moisaner et al. 1996; Nohrstedt 1983; Schwintzer and Tjepkema 1994; Wilson et al. 2012). This variability in soil R ratios has often been attributed to the difficulty of measuring ^{15}N incorporation in environmental samples with low BNF rates. Nonetheless, a careful analysis of the soil data revealed two distinct peaks in R ratio distribution: one centered on R ratios of $\sim 3\text{--}4$, values consistent with the Mo-isoform, and a second peak centered on R ratios of $\sim 1\text{--}2$, values characteristic of alternative nitrogenases (Bellenger et al. 2014). Variability in R ratios reported in soils thus reflects, at least in part, the contribution of different nitrogenase isoforms to BNF. In the same study (Bellenger et al. 2014), the variability of the R ratio was used successfully to evaluate the contribution of alternative nitrogenases to BNF in temperate forest soil microcosms. Nonetheless, as detailed in section “Quantification of alternative nitrogenase rates in environmental samples”, the R ratio has proven to be the least reliable method to characterize alternative BNF.

Cyanolichens and mosses in boreal and subarctic regions.

Mo limitation of BNF has been observed in cryptogamic species, such as mosses and cyanolichens (Fig. 2), which are major contributors to N cycling in high latitude ecosystems. The first evidence of Mo limitation in cryptogams was published by Silvester and coworkers in *Lobaria* spp. cyanolichens in Oregon, US (Horstmann et al. 1982). However, a more recent study on *Lobaria* and *Usnea* cyanolichens from the same region (Oregon) reported no effect of Mo addition on BNF (Marks et al. 2015b). High spatiotemporal variability in Mo limitation, which has been reported in leaf litter and cryptogams (Jean et al. 2013; Rousk et al. 2017; Wurzbürger et al. 2012), may explain some of the discrepancies between these two studies. More recently, Mo limitation of BNF has been demonstrated in cryptogams (bryophytes and cyanolichens) from high latitude and altitude regions (Eastern Canada, Northern Europe and Chile) (Darnajoux et al. 2014, 2017; Perez et al. 2017). Interestingly, studies on the cyanolichen *Peltigera aphthosa* showed that V levels are 2–5 times higher than Mo in cyanolichens with high rates of N_2 fixation, indicating that V may be used to relieve Mo limitation.

The presence of the V-Nase gene was recently reported in the *Peltigera* genera, which is ubiquitous in boreal and subarctic regions (Hodkinson et al. 2014). Two additional studies with boreal *Peltigera* cyanolichens collected in Sweden and Eastern Canada demonstrated the presence and activity of alternative nitrogenases using ISARA measurements and natural abundance ^{15}N isotope signatures (Darnajoux et al. 2017; Zhang et al. 2016). The contribution of alternative nitrogenases to BNF in these cyanolichens ranged from 20 to 60% in laboratory incubations at 10°C . The contribution of alternative BNF to N_2 fixation in *Peltigera* cyanolichens was ultimately confirmed in a more recent, ecosystem scale study across a 600 km latitudinal transect in Eastern Canada (Darnajoux et al. 2019). In this study, where the authors conducted a wide-ranging assessment across 10 species of *Peltigera* cyanolichens, V-Nase BNF was found to contribute between 0% of BNF at Mo-rich southern sites and $\sim 50\%$ of BNF at Mo-limited northern sites (Darnajoux et al. 2019). This study identified Mo availability as the dominant control for spatial and temporal variations in V-Nase activity across all sites and over the growing season. Mo limitation was more prominent at higher latitudes along the transect. This was most likely due to lower atmospheric deposition of both N and Mo (Darnajoux et al. 2015), resulting in a higher need for BNF to sustain biological N demand concomitant with Mo scarcity. Because Mo and V are contaminants in fossil fuels (Vouk and Piver 1983), atmospheric deposition might also play an important, but underexplored, role in controlling environmental metal distributions and alleviating Mo limitation in some areas.

Mo limitation of BNF by cyanobacteria associated with feather mosses in boreal forests in Canada and subarctic tundra in Sweden has been recently reported (Rousk et al. 2017). Other studies reported no effect of Mo addition on moss BNF (Scott et al. 2018; Smith 1984). The extent and spatiotemporal variability of Mo limitation remains difficult to predict for moss BNF due to the scarcity of data and the lack of direct evidence of the presence and activity of alternative nitrogenases in feather mosses. Nonetheless, the report of Mo limitation of moss BNF is of particular significance considering the critical role of cryptogamic species like mosses for BNF in boreal and subarctic ecosystems (DeLuca et al. 2002) and calls for further research.

Coastal environments

In contrast to terrestrial ecosystems, Mo is one of the most abundant micronutrient in marine waters, with concentrations around 100 nM (Collier 1985a), while V is found at concentrations ranging from 35 to 45 nM (Collier 1985b) and Fe is hardly detectable in dissolved inorganic form (Rijkenberg et al. 2014). Unsurprisingly, alternative nitrogenases have yet to be found in the open oceans (McRose et al. 2017b). However, under sulfidic conditions ($> 11 \mu\text{M H}_2\text{S}_{(\text{aq})}$), such as those sometimes found in marine and coastal sediments, Mo can react with sulfides to form thiomolybdates ($\text{MoO}_x\text{S}_{(4-x)}^{2-}$, with $1 < x < 4$) (Erickson and Helz 2000; Helz et al. 1996). Thiomolybdates readily react with particles such as Fe phases, Mn-oxides, and organic matter (Wagner et al. 2017), potentially reducing Mo availability to N_2 -fixers. This chemistry may account for recent findings of alternative nitrogenase genes (McRose et al. 2017b) and alternative nitrogenase enzyme activity (Zhang et al. 2016) in salt marsh microbial mats and sediments (Cape Cod, MA) (Fig. 3). Estimated using ISARA, the contribution of alternative nitrogenases to BNF in these sulfide-rich samples ranged from 20 to 55% (Zhang et al. 2016). In a similar study examining BNF in a brackish mangrove environment within the Florida Everglades, alternative nitrogenases were found to contribute over 24% of total BNF in leaf litter samples (McRose et al. 2017b). These findings are consistent with previous reports on alternative nitrogenase genes in N_2 -fixing isolates from mangrove sediments in Puerto Rico and North Carolina creek sediment (Betancourt et al. 2008). The presence of alternative nitrogenase genes was also recently reported in anoxic coastal waters off the coast of Peru (Christiansen and Loscher 2019), again suggesting that alternative nitrogen fixation in marine and coastal ecosystems may be more prevalent than usually recognized.

What factors favor the expression and activity of alternative nitrogenases over the canonical Mo-nitrogenase?

Low Mo availability

Molybdenum is known to prevent the synthesis of functional alternative nitrogenases in the model N_2 fixing bacteria, *Azotobacter vinelandii* and *Rhodobacter capsulatus* (Jacobitz and Bishop 1992; Jacobson et al. 1986; Kutsche et al. 1996; Masepohl et al. 2002) and in the freshwater heterocystous cyanobacterium *Anabaena variabilis* (Pratte et al. 2013; Thiel and Pratte 2013, 2014). In terrestrial environments, low Mo availability likely favors alternative nitrogenases use by diazotrophs. But alternative nitrogenases may also play a role when Mo appears to be sufficient. Abundant Mo does not automatically preclude alternative nitrogenase expression as shown in *A. vinelandii* grown at low temperatures (Walmsley and Kennedy 1991), in solid medium (Maynard et al. 1994) and in a strain of the N_2 -fixing, alpha-proteobacterium *Rhodopseudomonas palustris* (Oda et al. 2005) (also see section “[How robust are current BNF estimates?](#)”). In addition, the use of the V- or Fe-only nitrogenases to relieve Mo limitation must be understood in the context of the strategies and costs of acquiring Mo, V, and Fe in diazotrophs.

The acquisition of Mo, V and Fe in the natural environment is the result of complex and potentially expensive processes. Alternative nitrogenases are a way to relieve Mo limitation of BNF without requiring Mo uptake, thereby providing a competitive advantage to their hosts. In the top soil layer, Mo and V are found mainly as the oxoanions molybdate and vanadate, complexed by organic matter (phenolic and tannin-like compounds) and/or adsorbed on oxide surfaces (Goldberg et al. 1996; Marks et al. 2015a; Reddy and Gloss 1993; Wichard et al. 2009b). As expected, the chemical speciation of Mo in the soil is influenced by vegetative cover and organic matter decomposition (Wichard et al. 2009b). While organic matter binding prevents the leaching of metals away from the soil, it also reduces their bioavailability (Reddy and Gloss 1993; Wichard et al. 2009b). At the same time, Fe availability in oxic environments is low due to the insolubility of Fe oxides. Many microorganisms have evolved efficient strategies to recruit metals from their surroundings by producing small organic ligands, known as metallophores.

Metallophores bind metals in the extracellular environment, allowing a better control of metal speciation and thus of metal uptake by the metallophore-producing microorganisms (Kim et al. 2005; Kraepiel et al. 2009; Neilands 1995). Specific metallophore-based uptake systems have been characterized for Mo and V in diazotrophs (Bellenger et al. 2008b; Kraepiel et al. 2009; Liermann et al. 2005; Wichard et al. 2008, 2009a). Metallophore-mediated acquisition of metal is expensive, as shown in a recent study of the free-living soil N₂-fixer *A. vinelandii* which invests up to 35% of its newly fixed N into metallophore production to support metal acquisition (McRose et al. 2017a). In most laboratory experiments in which Mo and V are provided in readily available forms (molybdate and vanadate salts), V is acquired and the V-Nase is activated only after total depletion of Mo in the medium (Bellenger et al. 2011). Jouogo Noumsi et al. showed that the presence of tannic acids (used here as analogs of natural organic matter) significantly affected *A. vinelandii* metal acquisition and nitrogenase use strategy; metallophore production significantly increased to compete with tannic acids for Mo and V. Mo and V were taken up simultaneously and the V-Nase was activated at lower cell densities than in control cultures without tannic acid (Jouogo Noumsi et al. 2016). This suggests that in the presence of tannic acid, it was more beneficial for the cells to reduce metallophore production and use the V isoform of the nitrogenase than to increase metallophore production to fulfill their nitrogen requirements exclusively with the Mo-nitrogenase. Alternative nitrogenases may thus provide diazotrophs with additional flexibility in the management of their intracellular metal quotas in the natural environment.

Low temperatures

Low temperatures are known to alter nitrogenase isoform expression and activity. In at least one model organism (*Azotobacter vinelandii*), the alternative nitrogenase genes are not repressed by Mo at temperatures below 14–20 °C (Walmsley and Kennedy 1991). In addition, while the efficiencies of all nitrogenases decrease with decreasing temperatures, the efficiency of the V-Nase declines less than its Mo counterpart. This has been shown by in vitro studies on purified enzymes almost three decades ago (Miller and Eady 1988) and it has been confirmed more recently in vivo using the

model organism *Anabaena variabilis* (Darnajoux et al. unpublished). At temperatures below 10–12 °C, the Mo and the V isoforms of the nitrogenase showed comparable acetylene reduction activity and *A. variabilis* cultures grown in Mo-only and V-only media achieved similar growth rates at temperature below 20 °C. Thus, there may not be a growth advantage for diazotrophs using the Mo-Nase over the V-Nase at low temperatures. This has important, and yet still understudied, consequences for BNF in natural and managed ecosystems since Mo limitation appears to be widespread (see sections “Where have alternative nitrogenases been found so far?” and “Low Mo availability”) and the Earth annual average temperature is $\sim +14$ °C.

To what extent does nitrogenase diversity affect our understanding of biological nitrogen fixation?

The active contribution of alternative nitrogenase to BNF across a wide range of contrasting ecosystems raises several important questions regarding our current understanding of BNF.

Changes in acetylene reduction rates: do they reflect changes in N₂ fixation rates or a switch to a different nitrogenase isozyme?

As described above, Mo limitation of BNF has been demonstrated across a wide range of ecosystems from low to high latitudes (Barron et al. 2009; Jean et al. 2013; Rousk et al. 2017; Silvester 1989; Wurzbürger et al. 2012). Most, if not all, of these studies used the acetylene reduction assay, ARA, as a proxy for N₂ fixation and documented Mo limitation as an increase of AR rates in response to Mo additions. It is conceivable that in these Mo-limited habitats, alternative nitrogenases are responsible for the bulk of N₂ fixation prior to Mo-addition. Because the Mo-Nase is more efficient at reducing acetylene than the alternative nitrogenases (Bellenger et al. 2014), the observed increase in acetylene reduction rates may be driven, at least partially, by an increased contribution of the Mo-Nase in response to Mo additions. Diazotrophs can switch from one nitrogenase to another in a matter of few hours (Bellenger et al. 2011) and a switch from alternative to canonical (i.e. Mo-nitrogenase) N₂ fixation is certainly possible in field experiments where samples are usually pre-incubated for 12–18 h

before being assayed for acetylene reduction. The increase in N_2 fixation rates in response to Mo addition could thus be vastly overestimated.

The increased contribution of Mo-Nase after Mo addition was recently illustrated in a study on the effect of Mo addition to feather moss BNF in the arctic tundra (Rousk et al. 2017). In this study BNF by mosses was measured at field temperatures using both ARA and ^{15}N incorporation. Control mosses were characterized by variable R ratios (ARA/ N_2), suggesting the contribution of more than one nitrogenase isoform to BNF. In contrast, after 24 h incubations, the Mo-treated mosses yielded R ratio values (3–4) characteristic of the Mo-isoform.

In addition, the high seasonal and spatial variability in acetylene reduction rates often measured in field samples may partially reflect variable contributions of the different nitrogenase isoforms to BNF. In the future, particular attention to the contribution of alternative nitrogenases to N fixation in Mo-limited systems is warranted. In addition to the use of the R ratio, as illustrated by Rousk et al. (2017), a direct measurement of alternative nitrogenase activity using the ethane method or ISARA may also be required to understand ARA spatial and temporal variability. In these systems, N fixation rates must be measured using the ^{15}N incorporation method rather than ARA due to uncertainties in the value of the R ratio. Independently of the method, careful quantification of alternative nitrogenase activity is critical for our understanding of ARA spatial and temporal variability and thus for improving N_2 fixation estimates in the future.

How robust are current BNF estimates?

There is currently renewed interest in N_2 fixation in locales where surface-normalized rates are low (such as forest soils, tree canopies and moss-covered tundra), following the recognition that low rates over large areas may be significant for N inputs on local and global scales (DeLuca et al. 2002; Elbert et al. 2012; Lagerstrom et al. 2007; Lindo and Whiteley 2011; Menge and Hedin 2009; Sullivan et al. 2014). Our results, along with others (Betancourt et al. 2008; Boison et al. 2006; Darnajoux et al. 2014, 2017, 2019; Rousk et al. 2017; Zhang et al. 2016), show that alternative nitrogenases contribute to BNF in some of these important N_2 fixing habitats, including

cryptogamic covers that contribute up to half of BNF on land (Elbert et al. 2012).

This finding raises questions regarding the reliability of some existing BNF estimates. ARA is often the preferred method to assess BNF in environmental samples. The conversion of AR rates into N_2 fixation rates requires using the R ratio, which is dependent on the type of nitrogenase. Unfortunately, the R ratio is not always measured in field studies, partly because accurate measurements of both ARA and ^{15}N incorporation in samples with low BNF activity can be challenging. Even in field studies where the R ratio is measured, measurements are often carried out under laboratory conditions that can be poorly representative of the field (particularly for temperature (DeLuca et al. 2002)). Overall, studies that performed both ARA and ^{15}N incubations on the same samples in the field (Bellenger et al. 2014; Hedin et al. 2009; Vile et al. 2014) suggest that variations in the R ratio due to nitrogenase isozyme diversity have been underestimated.

Poorly calibrated ARA (and thus potentially overestimated R ratios) in systems where alternative nitrogenases are important may result in inaccurate estimates of N_2 fixation rates. For instance, in a study on cyanolichens, Darnajoux et al. reported that improper consideration of alternative nitrogenase activity in environmental samples leads to severe underestimation (up to $\sim 50\%$) of BNF activity (Darnajoux et al. 2017). This is in agreement with the large underestimates of BNF (by $\sim 30\%$) measured for microbial mats, leaf litter and sulfidic sediments (Zhang et al. 2016; McRose et al. 2017b).

Quantification of alternative BNF and a systematic revision of N_2 fixation rates based on ARA is required to refine N input estimates in habitats where alternative nitrogenases are present and active. We cannot stress enough the need to calibrate ARA with ^{15}N incorporation on a large number of samples (not only a small subset as often reported) and under environmentally relevant conditions for the accurate determination of N_2 fixation rates based on acetylene reduction rates.

High latitude ecosystems, which are currently experiencing rapid warming, and where cryptogamic covers significantly contribute to BNF, are of particular interest. In these low N input ecosystems, primary production and its response to global climate change are strongly constrained by N (Lebauer and Treseder

2008; Wang et al. 2010; Heimann and Reichstein 2008; Sigurdsson et al. 2013).

Perspectives and future research directions

Nitrogenase metal co-factor biogeochemistry

The use and activity of the various nitrogenase isoforms in terrestrial ecosystems is intertwined with the biogeochemistry of Mo, V and Fe. We highlight below important knowledge gaps that need to be addressed.

Inclusion of trace metals in organic matter analyses

Litter decomposition is a critical process that controls nutrient cycling at the forest scale. Nutrient dynamics during litter decomposition was recently proposed to play an important role in the emergence of P and Mo limitation of BNF in cold temperate forests (Pourhasan et al. 2016). Reed et al. reported that a leaf litter matrix with a Mo content $< 200 \text{ ng g}^{-1}$ was prone to Mo limitation of BNF in tropical forests (Reed et al. 2013). A similar Mo threshold was recently reported in high-latitude cyanolichens, where alternative nitrogenases significantly contributed to BNF in samples with a Mo content below 250 ng g^{-1} (Darnajoux et al. 2019). These new findings open the possibility to screen ecosystems worldwide for Mo limitation by mining literature data on the elemental composition of soil, litter and cryptogams. Unfortunately, trace metals are not routinely included in soil and litter analyses, which often focus on C and N. When trace metals are reported, Mo and especially V are rarely considered, except in studies on contaminated soils (Johnson and Hale 2004; Lawrey 1978). In addition, datasets on trace metal concentrations in terrestrial samples must be examined carefully, as sample preparation for elemental analysis must avoid metal-based equipment, such as steel Wiley mills and roller grinders, which can be a source of metal contamination (Marks et al. 2015b). Mo is particularly sensitive to contamination during sample preparation due to its low environmental concentrations and its presence in numerous metal alloys.

The importance of including Mo and V in soil analyses is further illustrated by studies on symbiotic N_2 -fixation in plants. Hungate et al. reported a long-

term decline in N_2 fixation by the leguminous vine *Galactia elliottii* under elevated atmospheric CO_2 . The CO_2 -induced decline was attributed to a decrease in Mo availability to the plant due to either a decrease in pH or an increase in soil organic matter content (Hungate et al. 2004). The effect, if any, of these changes in soil Mo availability on heterotrophic N_2 fixers was not tested. In a more recent study, Perakis et al. reported coupled soil accumulation of C, P and Mo in forests shaped by legacies of symbiotic N_2 -fixing trees. This nutrient accumulation was proposed as a mean to alleviate nutrient limitation for heterotrophic N_2 -fixers and promote soil BNF (Perakis et al. 2017).

The ongoing and anticipated effects of global climate change on both primary production (litter biomass) and organic matter decomposition are likely to strongly affect phosphorus and metal limitations, as well as alternative nitrogenases activity at the forest scale. A more systematic analysis of trace metals in environmental samples is the first step to build the global database needed to disentangle the complex interactions between major nutrients and trace metal dynamics, and BNF.

Proper assessment of Mo bioavailability to diazotrophs

A better evaluation of Mo bioavailability to diazotrophs is needed to refine the Mo threshold reported by Reed et al. (2013) and Darnajoux et al. (2019), because Mo limitation of BNF (and associated alternative nitrogenase expression) may be more directly dependent on Mo bioavailability than on total Mo content. Current methods to characterize Mo bioavailability were originally designed for higher plants using soft extraction approaches (e.g. resin binds, ammonia oxalate) (Lang and Kaupenjohann 1999; Liu et al. 1996; Poledniok and Buhl 2003). The extent to which these methods measure Mo availability to free living diazotrophs remains to be validated. Diazotrophs possess specialised high affinity uptake systems for Mo and V (e.g., metallophores) allowing them to dynamically affect metal speciation in their microenvironment to support their growth. New extraction methods mimicking metallophore efficiency for metal retrieval (i.e., Fe, Mo, V) from different environmental sources (e.g., organic matter, oxides) may provide better estimates for nitrogenase metal cofactor

bioavailability to diazotrophs than the soft extraction methods currently used. The use of synthesized or culture-purified metallophores as metal extractants could be explored.

Consider costs of metal acquisition in studies on trace metal limitation of BNF

There is increasing evidence that metallophores play an important role in nitrogenase metal cofactor acquisition and homeostasis. Research under environmentally relevant conditions, i.e., with organic matter and iron oxides present in the medium, are required to understand how the use of alternative nitrogenases is modulated by the cost of the metallophore-assisted acquisition of nitrogenase metal cofactors.

Physiology of diazotrophs

The constraints imposed by trace metal bioavailability on the use of alternative nitrogenases depend on physiology of diazotrophs, many aspects of which remain poorly understood. We describe below a few areas that require further study in this context.

Characterize metal acquisition and homeostasis in a greater diversity of diazotrophs

Our conceptual understanding of how diazotrophs manage cellular metal budgets for N_2 fixation is largely based on studies of the soil-dwelling, aerobic heterotroph *Azotobacter vinelandii* (Bellenger et al. 2008a, 2011; Bishop et al. 1986; Jacobitz and Bishop 1992; Pau et al. 1993) and the freshwater heterocystous cyanobacterium *Anabaena variabilis* (Darnajoux et al. 2014; Thiel et al. 2002; Thiel and Pratte 2013; Zahalak et al. 2004). While these organisms have provided mechanistic insights on important metal thresholds and acquisition strategies, they represent only a dismal fraction of the environmental and physiological diversity of terrestrial diazotrophs, which span soil, freshwater, and coastal habitats as well as aerobic and anaerobic metabolisms. Interestingly, in the facultative anaerobe *Rhodospseudomonas palustris*, the expression of alternative nitrogenases is not repressed by Mo (Oda et al. 2005) as transcription of alternative nitrogenase genes was observed in Mo-replete medium by a diazotrophic strain unable to synthesize active Mo nitrogenase. Thus, alternative

nitrogenase expression may not always be controlled by Mo availability as is usually assumed. In addition, beyond *R. palustris*, the strategies used by anaerobic diazotrophs to manage their metal quotas remain largely unexplored. More research is required to fully establish the occurrence of Mo regulation over alternative nitrogenase expression in diazotrophs.

Constraints on metabolic cost of canonical vs alternative nitrogenases

The metabolic costs of using different nitrogenase isoforms for BNF has been little explored beyond comparisons of growth and BNF rates. Studies carried out at room temperature (20–30 °C) report that growth based on V-Nase is 20 to 40% slower than Mo-Nase (Bellenger et al. 2011; McRose et al. 2017a; Thiel and Pratte 2013; Zhang et al. 2014). However, a recent study (Luxem et al. 2020b) shows that the type of carbon source utilized by *R. palustris* grown anaerobically under photoheterotrophic conditions at ~ 20 °C re-orders the relative growth rates of Mo-Nase and V-Nase based growth. Use of a more reduced carbon source like acetate rather than succinate led to slightly faster growth based on V-Nase than on Mo-Nase due to changes in biomass composition. In addition, a much lower H_2 production: N_2 reduction ratio for V-Nase was measured in vivo than previously observed for this enzyme within in vitro assays (i.e., Fig. 1a). The paradigm of intrinsically slow growth based on alternative nitrogenases has also been challenged by findings that the V-Nase and Mo-Nase-based growth rates of the cyanobacterium *Anabaena variabilis* are similar at cold temperatures (< 20 °C) (Darnajoux et al. unpublished). Thus changes in cellular metabolism (Luxem et al. 2020b), differences in the temperature dependence of the isoform specific activities (Miller and Eady 1988; Darnajoux et al. unpublished), and complexity of cellular nitrogenase regulation suggest that alternative BNF may be favored in specific environments. We suggest that future work considers the influence of metal management, temperature, energy availability, and metabolic N demands holistically in developing a mechanistic framework for how and why different nitrogenase isoforms are used in nature.

Conclusion

Three main factors may explain why the contribution of alternative nitrogenases to BNF has been overlooked for the last 40 years. (1) They are absent in bacteria that form symbiosis with higher plants, which have long been considered the major contributors to BNF worldwide. (2) The assessment of alternative nitrogenases activity in environmental samples was impeded by methodological constraints. (3) The unwarranted extrapolation of laboratory experiments performed on a limited number of model organisms to the field may have led to misconceptions regarding the uptake and intracellular management of Mo, V and Fe, as well as the regulation of nitrogenase isoforms by diazotrophs. However, an increasing body of evidence points to the importance of alternative nitrogenases to BNF in nature. It is now possible and urgent to investigate nitrogenase diversity in the field and quantify the contribution of alternative nitrogenases to BNF by updating traditional methods and implementing complementary new methods such as ISARA. Nitrogenase diversity needs to be included in our conceptual models of BNF, particularly because of its potentially impact on BNF estimates. It is also important to improve our understanding of how trace metal dynamics, temperature, and diazotroph physiology influence nitrogenase diversity and BNF in unmanaged ecosystems. Since semantics matter, we suggest that it is now time to stop referring to the V- and Fe-only nitrogenases as “alternative nitrogenases”, as this qualifier unconsciously confines them to a secondary role in terrestrial BNF, and start calling and considering them for what they really are—“complementary nitrogenases”—used by a wide range of prokaryotes to sustain N fixation under challenging environmental conditions.

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