



25TH ANNIVERSARY PAPER

Exploring the Role of Cryptic Nitrogen Fixers in Terrestrial Ecosystems: A Frontier in Nitrogen Cycling Research

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ABSTRACT

Biological nitrogen fixation represents the largest natural flux of new nitrogen (N) into terrestrial ecosystems, providing a critical N source to support

net primary productivity of both natural and agricultural systems. When they are common, symbiotic associations between plants and bacteria can add more than 100 kg N ha⁻¹ y⁻¹ to ecosystems. Yet, these associations are uncommon in many terrestrial ecosystems. In most cases, N inputs derive from more cryptic sources, including mutualistic and/or free-living microorganisms in soil, plant litter, decomposing roots and wood, lichens, insects, and mosses, among others. Unfortunately, large gaps remain in the understanding of cryptic N fixation. We conducted a literature review to explore rates, patterns, and controls of cryptic N fixation in both unmanaged and agricultural ecosystems. Our analysis indicates that, as is com-

Received 24 August 2022; accepted 25 October 2022

Supplementary Information: The online version contains supplementary material available at <https://doi.org/10.1007/s10021-022-00804-2>.

Author Contribution: All authors contributed to discussions about the ideas expressed in this paper. C.R.G.R. and S.P. led the development of the database, and all authors participated in the literature search and provided data to the database. C.C.C. and K.A.D. led the writing, and all co-authors provide text and revisions to the submitted version of the manuscript.

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Published online: 18 November 2022

mon with N fixation, rates are highly variable across most cryptic niches, with N inputs in any particular cryptic niche ranging from near zero to more than 20 kg ha⁻¹ y⁻¹. Such large variation underscores the need for more comprehensive measurements of N fixation by organisms not in symbiotic relationships with vascular plants in terrestrial ecosystems, as well as identifying the factors that govern cryptic N fixation rates. We highlight several challenges, opportunities, and priorities in this important research area, and we propose a conceptual model that posits an interacting hierarchy of biophysical and biogeochemical controls over N fixation that should generate valuable new hypotheses and research.

Key words: agriculture; asymbiotic; free-living; global change; N₂ fixation; nitrogen cycle; symbiotic; terrestrial ecosystems.

HIGHLIGHTS

- Biological nitrogen (N) fixation provides substantial N inputs to terrestrial ecosystems.
- A few N-fixing niches are conspicuous (for example, nodulated fixers) or well-studied (for example, soil, litter), but understanding of N fixation in most niches remains poor.
- We synthesize understanding of N fixation by “cryptic” N-fixers in natural and managed ecosystems.

INTRODUCTION

Biological nitrogen (N) fixation, the reduction of atmospheric di-nitrogen (N₂) to biologically available N (ammonia; NH₃) by the enzyme nitrogenase, accounts for much of the new N that enters unmanaged terrestrial ecosystems annually, and provides a key source of N that supports agricultural production (Cleveland and others 1999; Herridge and others 2022; Ladha and others 2022). N fixation represents a critical flux that can replenish lost N (for example, via fire, leaching, or gaseous forms) or add N to newly developing ecosystems. In addition, future N fixation inputs will strongly influence the biosphere’s responses to climate warming, land-use change, increasing atmospheric CO₂, N deposition, and fluctuating disturbance regimes, among other perturbations (Cleveland and others 2013; Wieder and others 2015; Reis and

others 2020). Yet, despite the importance of N fixation, the understanding of this fundamental process is far from complete.

Over the past several decades, there have been multiple efforts to quantify large-scale N fixation inputs in terrestrial ecosystems by up-scaling plot-level measurements (Cleveland and others 1999; Davies-Barnard and Friedlingstein 2020; Herridge and others 2022). As a group, those studies typically point to N fixation that occurs in nodules of vascular plants (via mutualisms with *Rhizobium* or *Frankia*) as the dominant pathway in some terrestrial ecosystems. In addition, studies increasingly show the importance of other forms of N fixation (hereafter referred to as *cryptic sources*), commonly called *free-living*, *asymbiotic*, or *non-symbiotic* N-fixers, but the group includes some niches that fix N via mutualisms (for example, soil, litter, lichens, moss, mycorrhizae-rhizosphere bacteria) (Dawson 1983). For example, a synthesis by Reed and others (2011) showed that N fixation via a combination of measured cryptic N-fixers can add 1–20 kg N ha⁻¹ y⁻¹ to terrestrial ecosystems, similar to mean or median reported symbiotic rates. Two more recent syntheses claimed that up to 2/3 of N fixation might occur via cryptic pathways in some ecosystems at regional-to-global scales (Reis and others 2020; Davies-Barnard and Friedlingstein 2020), and cryptic N fixation has been identified as the possible missing source in accounting for 13–22% of cereal N budgets in agricultural ecosystems globally (Ladha and others 2016). However, the relative paucity of measurements of many cryptic sources, as well as emerging evidence showing that a number of rarely measured cryptic pathways generate sizable N inputs, suggest that cryptic N fixation might be even higher than is currently recognized (for example, Pinto-Tomas and others 2009; Stewart and others 2011; Roley and others 2018).

Some cryptic N-fixing niches have been well studied. For example, many published studies have thoroughly described N fixation in soil and plant leaf litter, including multiple published syntheses on those two niches (Cleveland and others 1999; Reed and others 2011; Davies-Barnard and Friedlingstein 2020; Reis and others 2020). As a group, they show that: (1) both temperature and moisture (biophysical variables) exert strong control over N fixation in most, if not all niches; (2) that light strongly regulates N fixation carried out by autotrophs (for example, cyanobacteria associated with mosses and lichens) (Jackson and others 2011; Reed and others 2011; Gundale and others 2012a; b); and (3) that biogeochemistry (for example, N,

phosphorus [P], molybdenum [Mo], and oxygen availability) often regulates N fixation rates (synthesized in Reed and others 2011; Dynarski and Houlton 2017).

Yet, many other cryptic N-fixing niches have received far less attention, and little is known about their potential rates, their importance to the N budgets of the ecosystems where they exist, and what factors might regulate fixation in these niches (Figure 1). Here, we review the literature describing a number of cryptic N-fixing niches that remain relatively poorly understood. Specifically, we focus on those for which a paucity of field-based measurements (that is, reported as mass N/area/time) has limited the ability to either confidently generate large-scale rate estimates, or to perform a robust, quantitative analysis of their rates. Given the relatively good understanding of N fixation in soil and plant litter (for example, Reed and others 2011), we do not address those two relatively well-studied niches here. Instead, the objectives of this review are to: (1) present the current understanding of the rates, patterns, and controls of N fixation by many of the poorly understood cryptic niches in natural and agricultural ecosystems; (2) identify several emerging ideas and research priorities from a synthesis of published research on cryptic N fix-

ation; and (3) propose a new conceptual model in an attempt to provide insight into how the known controls on N fixation might influence where and when cryptic fixation is likely to be ecologically relevant.

METHODS

As part of a larger synthesis of global N fixation rates, patterns, and controls in terrestrial ecosystems (Reis and others forthcoming), we located papers published between 1970 and 2020 within the ISI Web of Science, SCOPUS, and Google Scholar databases using a single systematic search term (Appendix 1). We also used a snowballing technique to screen previous literature reviews and papers containing literature-derived rates to identify additional papers that might not have been located in the primary search. That is, the references cited in relevant papers were studied in an attempt to identify more papers to include in the study (for example, Wohlin 2014). The literature search initially returned more than 70 k papers, but we only selected publications that reported N fixation rates in some form for inclusion in the database. This additional screening resulted in about 900 publications that met the criteria for final inclusion. The overall database includes studies conducted in managed/disturbed and undisturbed/unmanaged natural ecosystems, including data from ecosystems regenerating from disturbance and at different successional stages. The database also includes studies from plantations and non-intensively managed pasturelands exposed to grazing, fire, and other management practices. However, for this review, we only used published papers that included scaled rates of N fixation (reported on an annual basis) to generate rate statistics (for example, rates reported in $\text{kg ha}^{-1} \text{y}^{-1}$, $\text{g m}^{-2} \text{y}^{-1}$, or others for which only a simple unit conversion was required).

For some of the cryptic niches described here (for example, endophytes and insects), there were insufficient data to generate even provisional N fixation rate estimates. However, for the niches where at least a handful of upscaled rate estimates were available, we report ranges, means and standard deviations of published N fixation rates. As is common in data syntheses, the numbers we report come from the published literature and might include potential biases. For example, as has been noted for symbiotic N fixation, studies on cryptic N fixation are likely biased toward locations where it is likely to be high or important, or in geographic regions that are relatively well-studied (Cleveland

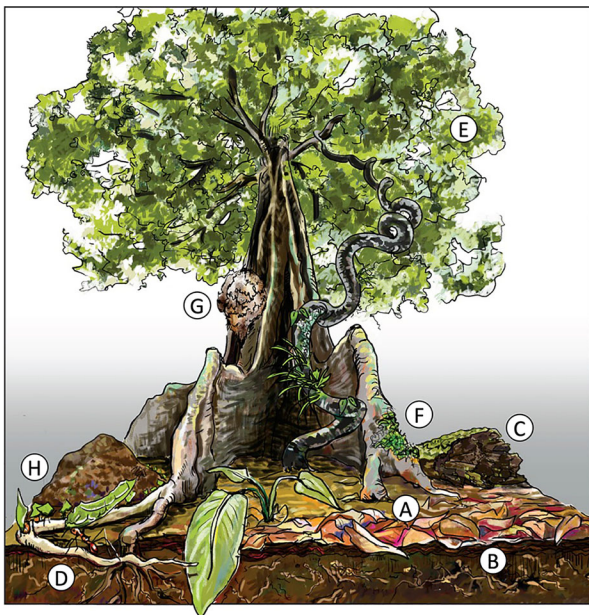


Figure 1. Artist's representation of a tropical forest illustrating many of the cryptic N-fixing niches present in terrestrial ecosystems, including: **a** leaf litter; **b** soil; **c** decaying wood; **d** roots and rhizosphere; **e** plant epiphytes; **f** cryptogamic covers, including mosses and lichens; **g** termites; and **h** leafcutter ant mound. Illustration by E. Harrington.

and others 1999; Staccone and others 2020; Soper and others 2021). Finally, our values include data reported in all published studies we found, some of which are quite high. All data used in the analysis are reported in Appendix 2.

CRYPTIC N FIXATION NICHES: PATTERNS, RATES, AND CONTROLS

N Fixation in Decomposing Wood and Aboveground Woody Residues

Decaying wood represents a potentially important but rarely quantified N fixation source in most of the world's forested ecosystems. The majority of measurements have been conducted in temperate forests (Son 2001), and the total number of N fixation studies in this niche remains relatively low (Figure 2a). Early efforts to quantify N fixation in wood suggested relatively modest but non-trivial rates, with higher rates occurring in forests with large quantities of woody debris. Based on our analysis of 45 published rate estimates of N fixation in decomposing wood and woody debris in temperate, tropical, and high latitude forest sites (Figure 2), we estimate that N fixation in this niche contributes $0\text{--}2.5\text{ kg N ha}^{-1}\text{ y}^{-1}$ (mean $\pm$ SD = $0.8 \pm 0.7\text{ kg N ha}^{-1}\text{ y}^{-1}$) to forested terrestrial ecosystems (Table 1).

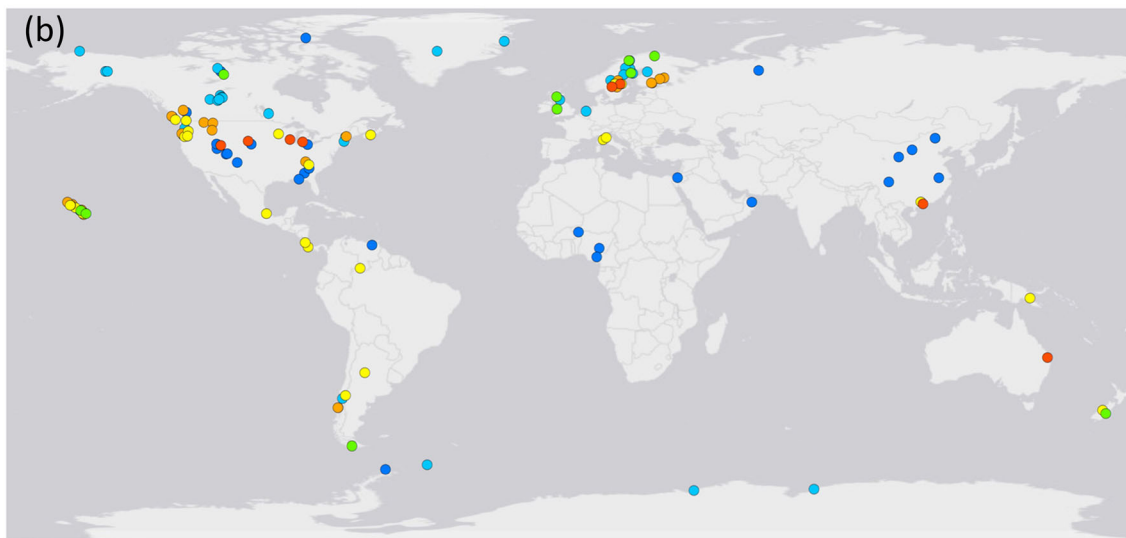
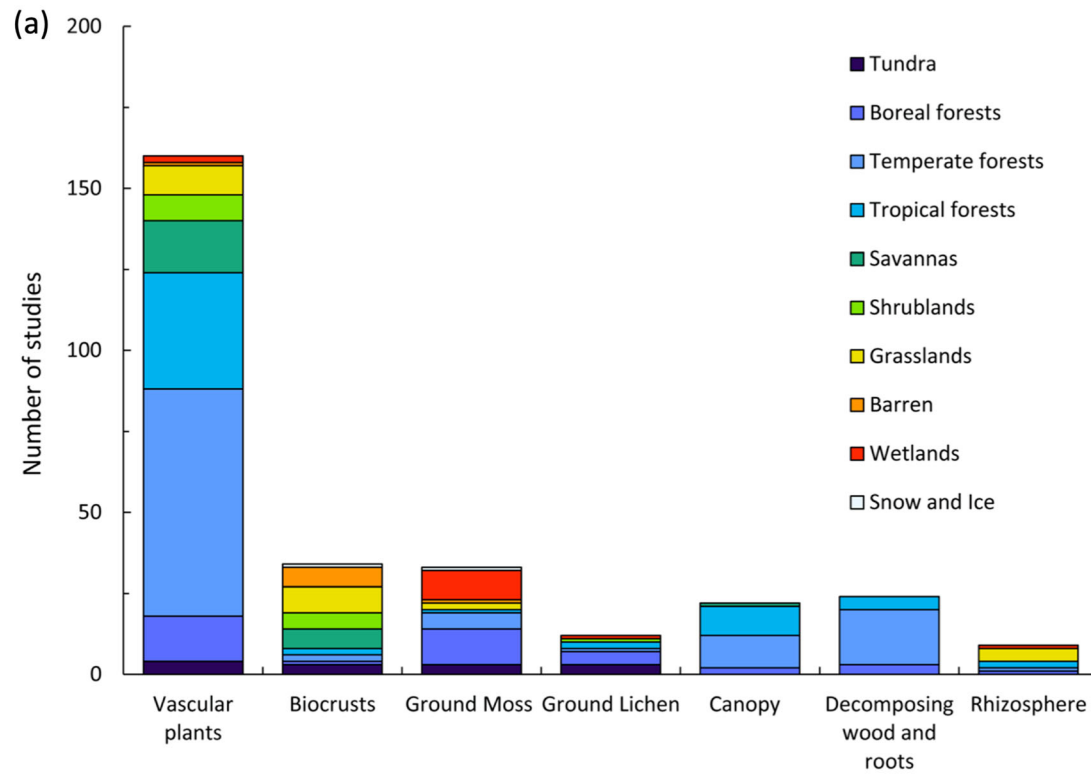
Woody tissues have relatively high C:N ratios (> 150 ; Weedon and others 2009), creating an N supply–demand imbalance for many decomposers, which favors fixation (Todd and others 1978). Abiotic factors (for example, moisture and temperature) strongly control N fixation in wood, including a nitrogenase activity temperature optimum near $25\text{ }^{\circ}\text{C}$ and a positive relationship between N fixation and wood moisture content (Hicks and others 2003). N fixation rates are generally higher in older wood, perhaps reflecting changes in substrate chemistry (for example, C:N, lignin:N) as decomposition progresses (Griffiths and others 1993), and/or delayed decomposer colonization rates in woody substrates (for example, in the interior of decomposing logs; Hyvönen and Ågren 2001). Work from a tropical forest in Hawaii also showed that N fixation rates were higher in decaying wood with relatively low N:P ratios (Matzek and Vitousek 2003), and Mo can sometimes limit N fixation in wood (Silvester 1989). Tropical and boreal forests are estimated to contain up to 87% of the world's decaying woody biomass (Pan and others 2013). Yet, with only one estimate from tropical rainforests of which we are aware (Matzek and Vitousek 2003) and one very recent

estimate from boreal forests (Benoist and others 2022), comprehensive measurements of N fixation rates in more forest types are needed (Figure 2).

N Fixation in Decomposing Plant Roots

A handful of studies have investigated N fixation in decomposing roots in temperate and high latitude forests (for example, Chen and Hicks 2003; Mäkipää and others 2018) (Figure 2), but we know of no measurements from other ecosystems. Yet, the belowground environment in many ecosystems often supports favorable conditions for N fixation (for example, high moisture, potentially high C:N ratios, proximity to other nutrients) (Hicks and Chen 2011). Reported rates of N fixation associated with decomposing roots are quite variable. From 15 published estimates, we estimate that N fixation on root litter can contribute $0\text{--}6.3\text{ kg N ha}^{-1}\text{ y}^{-1}$ (mean $\pm$ SD = $0.5 \pm 1.6\text{ kg N ha}^{-1}\text{ y}^{-1}$) (Table 1). Most measurements of N fixation in this niche have been conducted in forested ecosystems (Figure 2).

Controls on N fixation in decomposing roots are not well understood, but rates per unit root mass appear to be higher in coarse ($> 2\text{ mm}$) versus fine ($< 2\text{ mm}$) roots (Chen and Hicks 2003), perhaps reflecting higher C:N ratios in coarse roots (McCormack and others 2015). Root size classes are often used to organize both sampling effort and functional characterization in belowground studies, thus the collection of new data on N fixation rates as a function of root size class and decomposition status could be coupled with existing knowledge of root biomass and turnover patterns to better estimate ecosystem N inputs via this pathway (Smithwick and others 2014). For example, large, disturbance-driven pulses of N fixation in dead roots might continue for decades in ecosystems possessing high biomass of coarse roots that decompose slowly (Chen and Hicks 2003). Roughly 1/3 of global net primary productivity (NPP) goes to producing fine roots (Jackson and others 1997), which typically exhibit high rates of mortality and turnover (Gill and Jackson 2000). Thus, even low mass-specific N fixation rates in dead fine roots could yield relatively high annual and cumulative inputs in undisturbed ecosystems (Gill and Jackson 2000). The improved representation of belowground processes in biogeochemical models, coupled with evidence that dead-root N fixation is governed by many of the same environmental correlates as aboveground detritus (Hicks and Chen 2011), could lead to enhanced understanding of N fixation in decomposing roots.



Niches

- Biocrusts
- Ground Lichen
- Decomposing Wood and Roots
- Ground Moss
- Canopy
- Rhizosphere

Esri, HERE, Garmin, (c) OpenStreetMap contributors, and the GIS user community

Figure 2. **a** Histograms showing the number of published studies reporting total annual N fixation rates by niche for different ecosystem types, compared to the number of studies of vascular plants. *Vascular plants* include studies of N fixation that occurs in root nodules. *Ground moss* and *Ground lichen* include N fixation members of those groups not known to form biocrusts. *Canopy* includes studies of N fixation by epiphytes (lichen and moss) and epiphylls. Vascular plant data from Reis and others (forthcoming). **b** Global map showing the distribution of sites where data were collected.

Table 1. Estimated N Fixation Rates, Reported Controls, and Level of Understanding for Cryptic N-fixing Niches

N-fixing niche	N	N fixation rate (kg N ha ⁻¹ y ⁻¹)			Reported controls	Level of understanding
		Range	Mean	Median		
Decomposing wood and woody residue	45	0–2.5	0.8 ± 0.7	0.5	Moisture, temperature, nutrients, substrate stoichiometry	Intermediate
Decomposing plant roots	15	0–6.3	0.5 ± 1.6	0.1	Temperature, substrate stoichiometry	Very low
Plant endophytes	NA	NA	NA	NA	NA	Very low
Epiphytic lichens	27	0–3.5	0.7 ± 1.0	0.2	Temperature, moisture, nutrients, light	Low
Epiphytic bryophytes	13	0.7–9.6	1.8 ± 3.1	0.7	Temperature, moisture, nutrients, light	Very low
Rhizosphere	9	0–47.0	7.0 ± 13.6	1.2	Unknown	Very low
Ground moss	50	0–36.0	6.0 ± 10.5	1.0	Temperature, moisture, nutrients, light	High in boreal/Arctic, low/intermediate elsewhere
Ground lichen	16	0.4–100.1	11.1 ± 24.4	1.7	Temperature, moisture, nutrients, light	Low
Ground biocrust	100	0–100.0	5.0 ± 13.6	0	Temperature, moisture, nutrients, light	High in deserts, intermediate/low elsewhere
Termites, ants, and other insects	NA	NA	NA	NA	Unknown	Very Low

For each niche, we report the number of studies (N) used to calculate the range, mean (± 1 standard deviation), and median N fixation rate. For canopy bryophytes, values reflect some reported rates that included a combination of ground + canopy fixation rates.

N Fixation by Plant Endophytes

Plant bacterial endophytes commonly colonize plant intercellular spaces in leaves, roots, and stems (Kandel and others 2017). N-fixing endophytes and nitrogenase genes have been detected in the roots of some crops (Boddey and others 2003; Ladha and Reddy 2003) and natural grassland species (Rout and others 2013; Ritchie and Raina 2016; Gupta and others 2019), and stem tissue and foliage of several tree species (Bal and Chanway 2012; Carrell and Frank 2014; Doty and others 2016; Moyes and others 2016). Scaled rates of N fixation by bacterial endophytes are rare (Figure 2a), and upscaling such rates is difficult. The few scaled estimates that exist suggest a wide range of potential importance. Work in Brazilian sugar cane has documented that up to 40 kg ha⁻¹ y⁻¹ might be derived from endophyte N fixation (Urquiaga and others 2012). Such high rates have not been found in other countries where growers apply much higher rates of N fertilizer to sugar cane. In one grassland study, N-fixing root endophytes were estimated to contribute 10–15 kg N ha⁻¹ y⁻¹ (Ritchie and Raina

2016). On the other hand, another study in montane limber pine ecosystems measured much lower N fixation rates (0.02 kg N ha⁻¹ y⁻¹) by foliar endophytes (Moyes and others 2016; Wurzbarger 2016). The paucity of measurements makes it difficult to conclude whether N fixation in this niche is important.

N Fixation by Plant Epiphylls and Epiphytes

For most forested biomes, the understanding of N fixation by canopy epiphylls is poor (Lindo and Gonzalez 2010) (Figure 2). N fixation by epiphylls growing on canopy leaves has also been observed in a handful of tropical forest sites (Cusack and others 2009; Reed and others 2013; Stanton and others 2019). However, scaled estimates are rare, likely reflecting challenges of scaling small-scale measurements (for example, estimating the spatial extent or biomass of epiphytes), and surprisingly little is known about where epiphylls/epiphytes are present.

Most N-fixing epiphytes consist of lichens and/or bryophytes (mosses and liverworts) living on the trunks or in the canopies of forests (Lindo and Gonzalez 2010). Lichens are composite organisms of algae or cyanobacteria living among fungal filaments in a mutualistic relationship (Nash 1996), and many fix N via cyanobacterial symbionts (cyanolichens). All three divisions of bryophytes contain at least some species that associate with N₂-fixing cyanobacteria either endophytically or epiphytically. Early estimates suggested that in mature forests, N fixation via canopy mosses and cyanolichens could contribute up to 19 kg N ha⁻¹ y⁻¹ (Todd and others 1978; Antoine 2004; Lindo and Whiteley 2011; Jordan and others 1983; Benner and others 2007; Cusack and others 2009; Benner and Vitousek 2012). Based on measurements from both temperate and tropical forest ecosystems, we estimate that when present, epiphytic mosses/liverworts contribute 0.7–9.6 kg N ha⁻¹ y⁻¹ (mean ± SD = 1.8 ± 3.1 kg N ha⁻¹ y⁻¹) and epiphytic lichens contribute 0–3.5 kg N ha⁻¹ y⁻¹ (mean ± SD = 0.7 ± 1.0 kg N ha⁻¹ y⁻¹) (Table 1). However, particularly in forests where epiphytic moss and/or lichen biomass is high, more (and more rigorous) assessments of the role of canopy mosses and lichens as N fixers are critically needed. We predict this research frontier will eventually show they are important N-fixers in many ecosystems, especially forests, but robust estimates will not be possible until more rate measurements are available.

Research indicates that N fixation by epiphylls and epiphytes varies substantially by site, tree species, canopy position, and micronutrient availability (Benner and others 2007; Reed and others 2008; Antoine 2004; Cusack and others 2009). For epiphytic cyanolichens, light is hypothesized to be important, though conditions that favor rapid lichen growth in forest canopies do not always coincide with areas of high biomass, suggesting that competition, moisture, and other factors are also important (Antoine and McCune 2004). Thallus water content and temperature have also been shown to strongly regulate N fixation in epiphytic canopy lichens, with relatively wet conditions favoring higher rates (Denison and others 1979; Liu and others 2018). Nutrients have been shown to regulate epiphyte fixation, but some work suggests that N demand alone does not control the abundance or N fixation rates of canopy epiphytes as strongly as it does for some other niches (Matzek and Vitousek 2003). Some work has more clearly shown that N-fixing leaf epiphylls might be limited by rock-derived nutrients such as P and Mo (Reed

and others 2008; Matson and others 2015; Stanton and others 2019). For example, mosses and lichens growing in tree canopies appear particularly susceptible to P limitation, with the degree of limitation varying by local P availability (Marks and others 2015).

N Fixation in the Rhizosphere

Smercina and others (2019) argued that the majority of N fixation in soil is likely to be fixed in the immediate vicinity of plant roots (the rhizosphere) rather than in the surrounding soil, reflecting relatively greater C accessibility near roots. However, actual measured rates of rhizosphere N fixation are rare, with most work simply showing the *potential* for N fixation (for example, scanning electron microscopy or nifH genes showing the presence of N-fixing bacteria). In agricultural ecosystems, rhizosphere N fixation is frequently observed in grass crops such as miscanthus and switchgrass (Davis and others 2010; Roley and others 2018), and N-fixing bacteria have been isolated from the rhizosphere of rice and maize (Dommergues and others 1973; Hirota and others 1978; Ding and others 2019; Ladha and others 2022). In unmanaged ecosystems, N-fixing rhizosphere bacteria have been identified in mangroves (Holguin and others 1992), cacti (Aguirre-Garrido and others 2012), woody shrubs (Kaplan and others 2013), numerous flowering wetland species (Wickstrom and Garono 2007), and oak trees (Cobo-Díaz and others 2015).

Recent research has also shown that a suite of “mycorrhiza helper bacteria” associated with ectomycorrhizal fungal (EMF) root tips can fix N (for example, orders *Burkholderiales* and *Rhizobiales*) (Nguyen and Bruns 2015). Putative N fixation has been reported in bacteria associated with tuberculate ectomycorrhizae (TEM) on young lodgepole pines in British Columbia, Canada, but rates and controls on this form of N fixation remain unknown (Paul and others 2007). Measurements of rhizosphere N fixation are rare (Figure 2a), but those that do exist suggest that rates might be high in some sites. In agricultural soils, a greenhouse study using ¹⁵N dilution estimated that together, rhizosphere and endophytic N fixation could supply up to 30% of maize N requirements (Kuan and others 2016). A modeling study estimated potential rhizosphere N fixation inputs of 0.2–4 kg N ha⁻¹ y⁻¹ (Jones and others 2003). We estimate that rhizosphere N fixation ranges from 0 to 47 kg N ha⁻¹ y⁻¹ (mean ± SD = 7.0 ± 13.6

kg N ha⁻¹ y⁻¹) in unmanaged grassland and forest ecosystems (Table 1).

Smercina and others (2019) speculate about the controls on rhizosphere fixation, but very little is known. From the few studies that have been published, N availability has been shown to influence rates, with N additions causing declines in both N fixation and diazotroph abundance in the rhizosphere bacterial community (Smercina and others 2019). The C:N ratio of root exudates has consequently been hypothesized as a potentially significant control (Kolb and Martin 1988).

N Fixation by Cryptogamic Ground Covers, Ground Lichens, and Ground Mosses

Many surfaces in terrestrial ecosystems are covered by communities of cryptic organisms (cryptogamic covers) that consist of combinations of bryophytes, lichens, cyanobacteria, algae, and fungi, among others, and most include N-fixing organisms (Elbert and others 2012). Particularly in high-latitude ecosystems, ground bryophytes including feather-mosses and *Sphagnum* mosses are of particular importance, as they can reach substantial biomass (up to 4 Mg ha⁻¹; DeLuca and others 2008; Gundale and others 2013). In boreal and arctic ecosystems, annual rates of N fixation via *Sphagnum* have been reported to range from 1 to 35 kg N ha⁻¹ y⁻¹ (Larmola and others 2014, Rousk and others 2016, Vile and others 2014), though the majority of rates reported are < 5 kg N ha⁻¹ y⁻¹. Reported rates of N fixation associated with feather-mosses tend to be lower, ranging from 0.01 to 5.8 kg N ha⁻¹ y⁻¹ (Gundale and others 2013; Jean and others 2018). Based on 50 published rates of N fixation by ground mosses, we estimate that in forests where they are present, ground mosses fix 0–36 kg N ha⁻¹ y⁻¹ (mean ± SD = 6.0 ± 10.5 kg N ha⁻¹ y⁻¹) (Table 1).

Ground lichens are widely distributed and can be common in many different ecosystems (for example, forests, grasslands, shrublands) (Figure 2b). Based on 16 published measurements, we estimate that fixation by ground lichens contributes 0.4–100.1 kg N ha⁻¹ y⁻¹ (mean ± SD = 11.1 ± 24.4 kg N ha⁻¹ y⁻¹) to ecosystems where they are present (Table 1). The large uncertainty in published estimates highlights both their potential importance, as well as the need for more measurements to constrain N fixation rates by this niche.

In many arid ecosystems, biocrusts, bryophytes, and lichens contribute substantially to ecosystem N stocks by forming N-fixing biocrusts (Cornelissen

and others 2007). Generally, biocrusts tend to be most common in areas where soil is exposed to sunlight (Belnap and Lange 2003). N-fixing soil crusts have also been found in agricultural ecosystems, including the inter-row space of wheat fields (Witty and others 1979). Globally, N fixation by cryptogamic covers, including epiphytes, biocrusts, and rock cover, has been estimated to be 49 Tg N yr⁻¹ (Elbert and others 2012), which by itself is near the lower end of estimated total global terrestrial N fixation estimates (reviewed in Davies-Barnard and Friedlingstein 2020). Previously published estimates of annual N inputs via biocrusts at smaller scales are highly variable, ranging from 0.7 to 100 kg N ha⁻¹ y⁻¹ in natural ecosystems (Barger and others 2016) and from 0.8 to 25 kg N ha⁻¹ y⁻¹ in temperate cropping systems (Witty and others 1979). Our analysis also shows that biocrust rates are highly variable, with higher rates generally observed in grasslands and shrublands (5.0 ± 13.6 kg N ha⁻¹ y⁻¹) (Table 1). The ubiquity of cryptogamic covers in many ecosystems globally, and their frequent associations with cyanobacterial symbionts, underscores their potential importance as a cryptic source of N fixation in many ecosystems (Cornelissen and others 2007; Lindo and Gonzalez 2010). However, the consistently low rates of biocrust fixation measured by Tierney and others (2019) suggests that even when biocrusts are present in forests, they might be fixing at the lower end of reported rates for biocrusts as a group (See Appendix 2).

As in most other cryptic niches, temperature and moisture strongly control N fixation rates in biocrusts, lichens, and mosses, and because of the autotrophic nature of N fixation from these sources, light also plays a key role. Moisture strongly controls N fixation in biocrusts, as they are only physiologically active when wet (Nash 1996; Belnap 2002; Belnap and Lange 2003; Barger and others 2016). Nutrients also strongly regulate N fixation in these niches; observational and experimental studies have found downregulation of N fixation in both boreal mosses and biocrusts in response to N deposition (Hartley and Schlesinger 2002; DeLuca and others 2008; Gundale and others 2011; Gundale and others 2013). Mo and P have been shown to regulate N fixation rates in these niches, though their influence varies among organisms and biomes (Hartley and Schlesinger 2002; Benner and others 2007; Rousk and others 2017). Recent evidence also shows that functional traits of the host (for example, moss) determine the environmental conditions (for example, moisture and substrate chemistry) that associated

cyanobacteria experience and can strongly influence cyanobacteria colonization and N fixation (Liu and Rousk 2022).

N Fixation by Termites, Ants, and Other Insects

N fixation occurs in the hindguts of dozens of insect species, including termites, flies, beetles, wasps and ants—particularly leaf-cutter ants (Breznak 1973; Nardi and others 2002; Pinto-Tomas and others 2009; Bar-Schmuel and others 2019) (Figure 1). Although previous research confirms the *presence* of putative N-fixing bacteria in many groups of insects (for example, by detecting *nifH* genes), there are few scaled N fixation estimates for this niche. With some exceptions, direct measurements indicate modest inputs of 0.02–0.5 kg N ha⁻¹ y⁻¹ for termites, millipedes, and beetles across tropical and subtropical forests and deserts, accounting for up to 10–20% of total estimated free-living N fixation in those ecosystems (Nardi and others 2002; Yamada and others 2006). For colony-forming insects like leaf-cutter ants, N fixation is likely to be spatially patchy and focused around structures like downed logs or ant colonies (Ulyshen 2015) resulting in hot spots of N inputs (Bar-Schmuel and others 2019; Pinto-Tomas and others 2009).

N fixation in some termites varies in response to diet stoichiometry (C:N), consistent with the idea that N fixation in insects is driven by a need for dietary N supplementation (Breznak 1973; Nardi and others 2002; Bar-Schmuel and others 2019). Yet, beyond the apparent links between insect N fixation and the stoichiometry of their food source, little is known about the controls on N fixation by insects.

Other Emerging Cryptic N Fixers

As research into N fixation patterns continues, many previously unknown cryptic niches continue to be revealed. N fixation has been demonstrated on surfaces from fiddler crab shells (Zilius and others 2020) to arctic glacial ice (Telling 2011) to mammal droppings (Li and Master 1986). Not surprisingly, these studies found evidence of nitrogenase activity, but beyond the initial discoveries of the capacity for fixation, the patterns, prevalence, magnitude, and controls have not been rigorously explored for many of these emerging N-fixing niches. In addition, the role that these novel forms of cryptic fixation play in the N balance of ecosystems is still completely unknown.

CRYPTIC N FIXATION: SYNTHESIS, EMERGING IDEAS, AND NEXT STEPS

Resolving the patterns, rates, and controls of the many recognized but rarely investigated forms of cryptic N fixation represents a crucial next step in our understanding of the N cycle in both unmanaged and managed terrestrial ecosystems. Yet, only in rare cases have comprehensive measurements of N fixation via multiple cryptic sources been undertaken within any given ecosystem (for example, Crews and others 2001; Matzek and Vitousek 2003; Reed and others 2008; Cusack and others 2009; Menge and Hedin 2009; Tierney and others 2019). More often, measurements are limited to one or two niches. Thus, we argue that future studies should more comprehensively assess N inputs via a broader range of cryptic sources using rigorous methodologies and experimental protocols (Soper and others 2021) to establish the magnitude of N inputs via different sources. While N inputs via any one unmeasured niche might be relatively small, the sum of N fixation via multiple unmeasured cryptic sources could be sizeable—at least as high as estimated N deposition inputs in many natural ecosystems, and sometimes much higher (Reed and others 2011). Ultimately, the only way to assess the potential role of any given cryptic N fixation source is to measure it. As is common in many ecosystem studies, we also note the strong geographical bias of measurements from North America and Europe, with some parts of the world poorly or entirely unrepresented (for example, Africa). We argue for the urgent need to support and conduct future N fixation research in regions and ecosystems that have not been studied.

The large number of potential N-fixing niches implies that cryptic N fixation could significantly contribute to the N economy of many ecosystems, as suggested by several circumstantial examples. For example, Turner and others (2019) measured large increases in ecosystem N capital between 15 and 25 years after high-severity wildfire in lodgepole pine forests, and hypothesized that the large increases in N stocks came largely from what would be sizeable inputs via cryptic N fixation sources. By contrast, low-severity fires in longleaf pine savannas result in relatively modest post-fire N fixation inputs, yet cryptic N fixation might still account for up to 78% of total N fixation (Tierney and others 2019). Synthesis of 114 long-term continuous agricultural experiments from around the world (maize, rice, and wheat) showed that N inputs via fertilizer, atmospheric deposition, and *known* cryptic sources—including N fixation in soil organic

matter and manures—were insufficient to balance the amount of N harvested in any of these major cereal crops (Ladha and others 2016). The authors hypothesized that unmeasured cryptic forms of N fixation could be contributing $\sim 13 \text{ kg N ha}^{-1} \text{ y}^{-1}$ for maize and wheat and $22 \text{ kg N ha}^{-1} \text{ y}^{-1}$ for rice. While these examples focus on disturbed or managed ecosystems, we predict that cryptic forms of N fixation are similarly critical for replenishing N lost through gaseous or dissolved pathways in mature ecosystems—especially those with a paucity of symbiotic N-fixers. However, more research across a broad range of mature ecosystems is needed to address this hypothesis.

Given the incomplete understanding of the distribution and importance of cryptic N-fixers in many ecosystems, it isn't surprising that the understanding of the controls on cryptic N fixation rates is still evolving. Yet, several consistent relationships are emerging. First, as with many fundamental biogeochemical processes, biophysical variables (for example, light for autotrophic fixers, temperature, and water availability for all fixers) exert strong control over N fixation rates in many—if not all—cryptic niches (Li and others 2018; Gundale and others 2012b; Smercina and others 2019). For N-fixing autotrophs, the role of light is obvious: It provides the reducing power (energy) in the form of organic C needed to carry out fixation. For the vast majority of fixers, evidence for strong temperature control on fixation is also robust. Houlton and others (2008) found that across a broad range of species, strains, latitudes and environments, nitrogenase showed maximum activity at an average of $\sim 25^\circ\text{C}$, with individual studies showing a range of optimum temperatures (Prévost and others 1987; Gundale and others 2012b). Many studies have also shown strong moisture controls on N fixation (Nash 1996; Hofmockel and Schlesinger 2007; Jackson and others 2011; Rousk and others 2018), but in situ N fixation rates likely reflect strong interactions between temperature and water availability (Voroney 2007). We argue that biophysical conditions (and their interactions) strongly influence N fixation rates in all ecosystems, help explain strong temporal variation in N fixation rates within and among sites, and offer an opportunity to more robustly estimate variation in N fixation rates through time (Tierney and others 2019; Soper and others 2021).

In addition to strong biophysical controls, N often regulates N fixation. In both managed and unmanaged ecosystems, observational studies and manipulative experiments have shown that in many niches (for example, bulk and rhizosphere

soil, leaf litter, moss, biocrusts), N fixation tends to decline in response to increasing N availability (Reed and others 2007; Reed and others 2011; Dynarski and Houlton 2017; Perakis and others 2017; Roley and others 2018; Zheng and others 2018; but see Dynarski and others 2019). However, other factors (for example, light, moisture, carbon availability) have been shown to regulate N fixation rates more strongly than N availability in some environments (Reed and others 2007; Dynarski and others 2019).

Nutrients other than N also regulate cryptic N fixation. Manipulative experiments often show elevated N fixation rates in multiple niches in response to P and/or Mo additions (for example, Barron and others 2009; Wurzbarger and others 2012; Dynarski and Houlton 2017). Iron (Fe) is also a key component of nitrogenase, and Fe limitation of leaf litter N fixation has been observed in a lowland tropical forest on calcareous parent material (Winbourne and others 2017). The expression of alternative Fe- and vanadium (V)-based nitrogenases has been observed in soils in laboratory microcosms (Bellenger and others 2014) and boreal cyanolichens (Darnajoux and others 2019). However, like with Mo, the importance of Fe, V, copper (Cu), and sulfur (S) as controls of cryptic N fixation across different niches and biomes remains poorly studied.

For many of the cryptic niches described here, substrate C quality and/or nutrient stoichiometry also emerge as common controls. For example, high substrate C:N ratios tend to favor higher rates of N fixation, likely reflecting increased N demand in high C and low N substrates. Similarly, N fixation tends to be higher in substrates with relatively high-quality C (Vitousek and Hobbie 2000). Low N:P ratios often favor fixation in the niches where nutrient controls have been assessed (predominantly soil and leaf litter) (Eisele and others 1989; Reed and others 2007). The emergence of nutrient stoichiometry (that is, nutrient ratios, rather than simply the concentrations of individual nutrients) as a control over cryptic N fixation is consistent with recent work showing the importance of interactions among nutrient acquisition strategies, and is consistent with the theory that relative rather than absolute amounts of nutrients determine N fixation activity (Menge and others 2009). Some of these interactions (for example, between N and P acquisition) have been explored for symbiotic N-fixers (for example, Treseder and Vitousek 2001; Batterman and others 2013a; Nasto and others 2014) but have not been well characterized in cryptic niches.

Finally, top-down ecological controls (for example, herbivory) could be important in regulating cryptic N-fixers. Vitousek and Howarth (1991) hypothesized that grazing by herbivores could influence the distribution and abundance of N-fixing organisms in marine ecosystems, and Chan and others (2004) showed that grazing by zooplankton could influence cyanobacteria blooms in freshwater ecosystems. In a review of marine N fixation, Zehr and Capone (2020) speculated about the role of “food web interactions” on N fixation, noting that *Trichodesmium* blooms respond to components of the upper food web in marine ecosystems (Bonnet and others 2016). It is conceivable that herbivory might also regulate cryptic fixers on land, and we view this as an important N fixation research gap that should be addressed.

CRYPTIC N FIXATION CONTROLS: A NEW CONCEPTUAL MODEL

Given clear similarities in N fixation controls among many cryptic N fixation niches, we propose a conceptual model of the hierarchy of interacting controls on cryptic N fixation (Figure 3). We hypothesize that cryptic N fixation depends on a suite of factors (temperature, water), energy (light for autotrophs, C supply for heterotrophs), and nutrients (P, Mo, and so on), and that N fixation also depends on the availability of these resources (energy and nutrients) compared to N availability and the relative demand for each. Importantly, each of these is required for N fixation, so the absence of any one of them would limit N fixation. However, whereas some of the biophysical factors are frequently at levels that yield negligible N fixation (for example, too cold or too dry), energy and nutrients are typically available at levels sufficient to support at least some N fixation. Thus, we hypothesize that biophysical controls are stronger than biogeochemical controls.

In general, we hypothesize cryptic N fixation rates will respond unimodally to both temperature and moisture, with niche- and biome-specific N fixation optima, and likely with important interactions between temperature and moisture (Gundale and others 2012a) (Figure 3a). Under favorable biophysical conditions, we hypothesize that the balance of energy supply and non-N nutrient availability vs. N availability (that is, element stoichiometry for heterotrophic niches) determines N fixation. Specifically, under favorable biophysical conditions, we would generally expect that rates of N fixation decline with increasing N

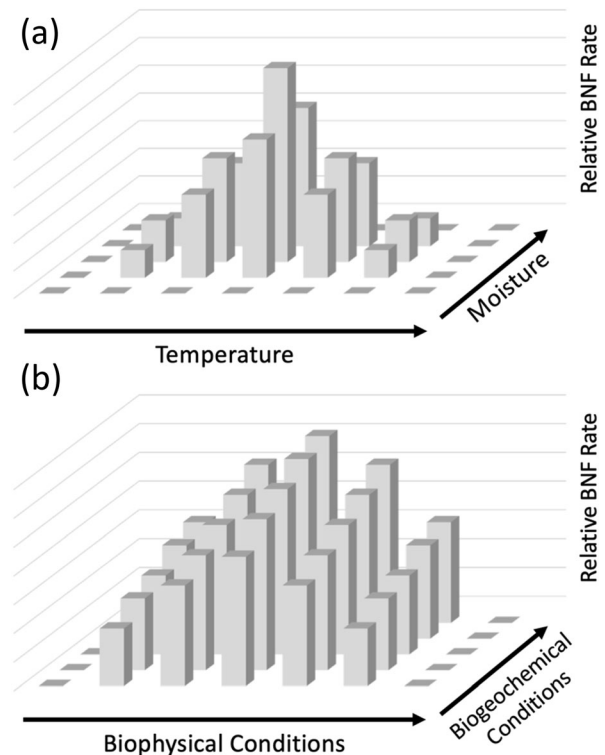


Figure 3. Conceptual illustration showing the proposed hierarchy of controls on cryptic N fixation. We hypothesize that N fixation rates are most strongly governed by **a** biophysical controls (for example, temperature and moisture), and that niche-specific relative fixation rates will vary unimodally with temperature and moisture, with rates increasing as temperature and moisture approach more optimal conditions. Secondly, as biophysical conditions become less limiting to N fixation, we posit that biogeochemical controls **b** will more strongly regulate rates. We hypothesize that under more optimal biophysical conditions, relative N fixation rates decrease with N availability, but increase with the availability of potentially limiting critical nutrients (for example, P, Mo).

availability and increase with P availability (Reed and others 2011) (Figure 3b). Numerous studies have found strong seasonal variation in cryptic N fixation rates, with nutrient effects on N fixation rates secondary to seasonal effects (Reed and others 2007; Winbourne and others 2017), and other studies have shown much more modest N fixation rates during dry conditions (for example, Gundale and others 2012a). These are all consistent with our conceptual model of a hierarchy of controls, where biophysical drivers range more widely than biogeochemical drivers.

This conceptual model of N fixation controls provides insight into where and when cryptic N

fixation rates are most important. Biophysical and biogeochemical controls (individually or in combination) can vary over different spatial and temporal scales, leading to heterogeneous rates of cryptic N fixation across landscapes and through time. For example, in most biomes, moisture and temperature exhibit strong seasonal patterns, but availability of nutrients such as N, P and Mo tends to be more spatially variable and/or disconnected from some N fixation niches (Wurzburger and others 2012; Perakis and others 2017). In other cryptic niches (for example, litter and decomposing wood), nutrient availability and stoichiometry might instead exhibit strong temporal patterns (for example, as stoichiometry shifts throughout organic matter decomposition).

Disturbances and other drivers (for example, climate change) that alter ecosystem properties like soil moisture, soil nutrients, carbon, light availability, and microbial community composition can also influence cryptic N fixation rates. Our model of interacting controls could help explain why measured patterns of cryptic N fixation after disturbance and throughout succession sometimes appear context dependent. For example, symbiotic N fixation typically peaks in early succession and then declines (for example, Batterman and others 2013b; Taylor and others 2019; Wurzburger and others 2021). Patterns and rates of cryptic N fixation following disturbance might be more complex, given the high taxonomic and metabolic diversity of cryptic fixers, variation in potential niche-specific physiological and biogeochemical optima, and/or small-scale heterogeneity in biophysical and biogeochemical factors and microsites (DeLuca and others 2008; Menge and Hedin 2009; Taylor and others 2019; Tierney and others 2019). On the other hand, our conceptual model, which reflects our current understanding of the controls on cryptic N fixation, implies that fixation might also be predictable following disturbances. We argue that the search for successional trends of cryptic fixers is a valuable pursuit, and that our conceptual model might be useful for future studies addressing both spatial and temporal patterns of cryptic N fixation.

Our conceptual model predicts the highest rates of fixation in places (and at times) when biophysical factors are favorable, when N is relatively scarce, and when other essential nutrients (for example, P and Mo) are relatively abundant (Figure 3). “Optimum” conditions for cryptic fixers likely vary among ecosystems because of microbial adaptation to local conditions. For example, several warming experiments in arctic and boreal ecosys-

tems have found neutral or negative effects of warming on moss-associated N fixation (Sorensen and others 2012; Rousk and others 2018; Carrell and others 2019). However, this framework is relevant for identifying understudied niches that could provide substantial N inputs. For example, N fixation in decomposing wood has not been robustly quantified in tropical forests, but might be significant because of favorable biophysical conditions and high substrate C:N ratios.

CONCLUSIONS

Whether or not a set of unidentified or unmeasured N fixation sources are “missing” from the N budgets of terrestrial ecosystems has been debated for decades (Binkley and others 2000). Regardless of the N balance of any particular site, cryptic N-fixers likely play an underappreciated, and in some cases completely unrecognized role in the N cycle. Nitrogen availability helps regulate critical processes such as food production, ecosystem productivity, biosphere responses to climate change, and many others, which makes understanding the patterns, rates, and controls on N inputs from cryptic N fixation more critical than ever. Moving forward, we suggest that field-based studies of N cycling in both natural and agricultural ecosystems should explicitly consider N fixation inputs via the suite of potential N fixers described here. In addition, continued efforts to explore the energetic (for example, light and carbon), biophysical (for example, temperature and moisture), biogeochemical (for example, nutrients) and ecological (for example, herbivory and competition) controls and their interactions are essential for the development of improved N fixation response functions that could be implemented in large-scale models. Currently, despite the importance of N fixation for accurately predicting ecosystem-to-global responses to environmental change, few earth system models represent cryptic forms of N fixation. Some that do use somewhat crude phenomenological relationships between biome-scale N fixation estimates and coarse predictors like actual evapotranspiration (Wieder and others 2015), and some more complex model representations of N fixation do not include cryptic fixation (Braghiere and others 2022). Further research establishing how cryptic N fixation rates vary with a suite of potential controls would greatly advance efforts to more accurately represent and scale this fundamental process.

ACKNOWLEDGEMENTS

This paper is a contribution from a working group on biological nitrogen fixation supported by the U.S. Geological Survey John Wesley Powell Center and Ecosystems Mission Area to S.S.P., S.C.R., D.N.L.M., and C.C.C. C.C.C. acknowledges support from grants from the National Science Foundation to investigate nutrient cycling in terrestrial ecosystems (DEB-1754126, DEB-1556643, DEB-2027263). S.A.B. acknowledges support from the United Kingdom Natural Environment Research Council (NE/M019497/1, NE/S009663/1) and The Leverhulme Trust. M.G.T. acknowledges support from the Vilas Trust of the University of Wisconsin-Madison and the National Science Foundation (DEB-2027261). V.G.S. acknowledges support by the US Department of Energy, Office of Science, Biological and Environmental Research (BER) under contract DE-AC05-00OR22725. M.J.G. acknowledges support from the Swedish Research Council VR. We thank S. Jovan and A. Staccone for contributing to the development of the database. The paper also benefitted from useful discussions with B. Weber and E. Rodriguez-Caballero. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government. We are grateful for constructive feedback from the editors, E. Rastetter, and two anonymous reviewers.

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