To Fix or Not To Fix: Controls on Free-Living Nitrogen-Fixation in the Rhizosphere

Article *in* Applied and Environmental Microbiology · January 2019

DOI: 10.1128/AEM.02546-18

CITATIONS
4

READS
354

4 authors:

Darian Smercina
Michigan State University
4 PUBLICATIONS **4** CITATIONS
SEE PROFILE

Sarah E Evans
Michigan State University
28 PUBLICATIONS **1,453** CITATIONS
SEE PROFILE

Maren L Friesen
Michigan State University
89 PUBLICATIONS **984** CITATIONS
SEE PROFILE

L. K. Tiemann
University of New Hampshire
20 PUBLICATIONS **592** CITATIONS
SEE PROFILE

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

MINIREVIEW

To Fix or Not To Fix: Controls on Free-Living Nitrogen Fixation in the Rhizosphere

Darian N. Smercina,[a] Sarah E. Evans,[b] Maren L. Friesen,[c] Lisa K. Tiemann[a]

[a]Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, Michigan, USA
[b]W. K. Kellogg Biological Station, Department of Integrative Biology, Michigan State University, Hickory Corners, Michigan, USA
[c]Department of Plant Pathology and Department of Crop and Soil Sciences, Washington State University, Pullman, Washington, USA

ABSTRACT Free-living nitrogen fixation (FLNF) in the rhizosphere, or N fixation by heterotrophic bacteria living on/near root surfaces, is ubiquitous and a significant source of N in some terrestrial systems. FLNF is also of interest in crop production as an alternative to chemical fertilizer, potentially reducing production costs and ameliorating negative environmental impacts of fertilizer N additions. Despite this interest, a mechanistic understanding of controls (e.g., carbon, oxygen, nitrogen, and nutrient availability) on FLNF in the rhizosphere is lacking but necessary. FLNF is distinct from and occurs under more diverse and dynamic conditions than symbiotic N fixation; therefore, predicting FLNF rates and understanding controls on FLNF has proven difficult. This has led to large gaps in our understanding of FLNF, and studies aimed at identifying controls on FLNF are needed. Here, we provide a mechanistic overview of FLNF, including how various controls may influence FLNF in the rhizosphere in comparison with symbiotic N fixation occurring in plant nodules where environmental conditions are moderated by the plant. We apply this knowledge to a real-world example, the bioenergy crop switchgrass (*Panicum virgatum*), to provide context of how FLNF may function in a managed system. We also highlight future challenges to assessing FLNF and understanding how FLNF functions in the environment and significantly contributes to plant N availability and productivity.

KEYWORDS free-living nitrogen fixation, diazotrophs, environmental controls, rhizosphere, rhizosphere-inhabiting microbes

Biological nitrogen fixation (BNF), the process by which gaseous N_2 is converted into ammonia (NH_3) via the enzyme nitrogenase, is crucial for the availability of nitrogen (N) in terrestrial ecosystems (1). BNF includes symbiotic (i.e., nodule formation) and free-living N fixation (FLNF), defined as N fixation occurring without a formal plant-microbe symbiosis. In 2011, Reed et al. reviewed FLNF, covering topics from carbon (C), N, and oxygen controls on FLNF to ecosystem scale responses of FLNF (2). In this review, we build on and update that body of work, focusing on mechanistic controls of FLNF, including the influence of diazotroph diversity, and place particular emphasis on FLNF in the rhizosphere (at or near root surfaces), which has implications for FLNF supporting crop production. In some cases, FLNF has been regarded as a subcategory of symbiotic N fixation, due to its close proximity to roots and, thus, is considered to have similar environmental constraints (3). However, we argue that FLNF, particularly in the rhizosphere, is an important process distinct from symbiotic N fixation, is carried out by wholly different bacterial species, and, thus, warrants its own investigation (Fig. 1).

Surprisingly, FLNF actually evolved long before symbiotic N fixation, appearing between 1.5 and 2.2 billion years ago (4) compared with only 59 million years ago for symbiotic N fixation (5). Because of dynamically fluctuating conditions in the rhizosphere, FLNF occurs under more diverse and variable conditions than symbiotic N

Citation Smercina DN, Evans SE, Friesen ML, Tiemann LK. 2019. To fix or not to fix: controls on free-living nitrogen fixation in the rhizosphere. Appl Environ Microbiol 85:e02546-18. https://doi.org/10.1128/AEM.02546-18.

Editor Isaac Cann, University of Illinois at Urbana-Champaign

Address correspondence to Darian N. Smercina, marinisd@msu.edu.

Accepted manuscript posted online 18 January 2019
Published 6 March 2019

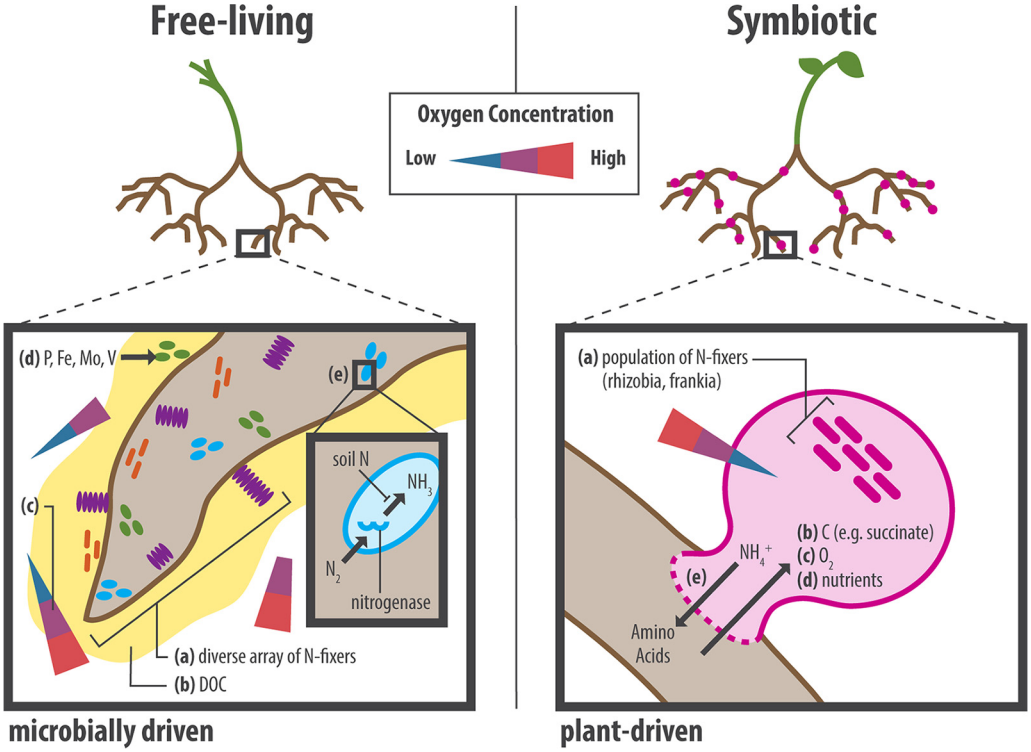

FIG 1 Contrasting habitats of free-living and symbiotic nitrogen fixation. (a) FLNF is carried out by a diverse array of N fixers living in a community, while symbiotic N fixation is performed only by a few bacteria (e.g., rhizobia and *Frankia*) living in a population. (b) FLNF is supported by dissolved organic carbon (DOC) in the soil, a variable and complex C source, while symbiotic N fixers receive a constant supply of simple C compounds (i.e., succinate) directly from the host plant. (c) Oxygen concentration in the rhizosphere is highly variable and driven by soil structure and texture and respiration by microbes and roots. Conversely, symbiotic N fixers are supplied oxygen at low concentrations by their host plant. (d) Nutrients necessary to support FLNF (e.g., P, Fe, Mo, and V) must be acquired by the diazotroph. However, these nutrients are delivered to symbiotic N fixers by the host plant. (e) Diazotrophs in the rhizosphere can access N from soil and FLNF, while all symbiotically fixed N is delivered to the plant.

fixation, making it difficult to draw conclusions about FLNF based on research of symbiotic N fixation (Fig. 2). The lack of research focused on FLNF is surprising considering that the process is ubiquitous in terrestrial systems and can provide significant inputs of N equal to or greater than symbiotic N fixation (2, 6, 7). For example, Cleveland et al. estimate via a modeling approach that FLNF, including in the rhizosphere, bulk soil, on leaf litter and decaying wood, and on plant and leaf surfaces, contributes 6 kg N ha^{-1} $year^{-1}$ on average to tropical forest systems (ranging from 2.4 to 14 kg N ha^{-1} $year^{-1}$), while symbiotic BNF was estimated at only 4.5 kg N ha^{-1} $year^{-1}$ (6). Similarly, Reed et al. show that FLNF rates fall within the range of symbiotic BNF rates for all biomes (2). Summing the FLNF rates for each biome (accounting for land area of each biome), as estimated by Reed et al., FLNF contributes ~76 Tg N $year^{-1}$ globally (falling within Vitousek et al. estimated range of 40 to 100 Tg N fixed $year^{-1}$) (7), which far exceeds inputs from lightning (5 Tg N $year^{-1}$) (8) and is more than half the N fixed industrially via Haber-Bosch reactions (120 Tg N $year^{-1}$) (2, 8). Of this N contributed by FLNF, the majority is likely to be fixed in the rhizosphere because of C accessibility (discussed below), making understanding rhizosphere FLNF key to understanding this important N input.

FLNF in the rhizosphere has been of particular interest in low-input crop production because this source of N could reduce reliance on chemical fertilizers. This is especially important in biologically based agriculture (organic agriculture) and in low-input agricultural systems in developing countries. In these cases, a greater reliance on FLNF could ameliorate some of the negative environmental impacts associated with chemical N additions (i.e., nitrate leaching and greenhouse gas N_2O efflux). One area in which FLNF has been documented and could provide these benefits is in perennial bioenergy

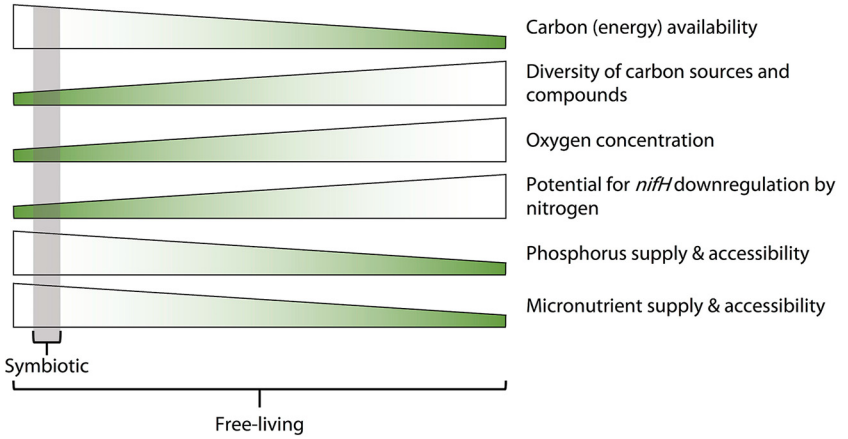

FIG 2 Environmental factors known to impact FLNF presented with triangles representing a theoretical range for each factor, low (narrow, dark-colored) to high (broad, light-colored). In contrast, symbiotic N fixation, represented by vertical hatched bar, only occurs in a narrow range of each of the environmental conditions. For example, FLNF can occur over a wide range of oxygen concentrations from low to high, while symbiotic N fixation occurs only at low oxygen concentrations.

cropping systems, such as miscanthus (*Miscanthus giganteus*) (9) and switchgrass (*Panicum virgatum*) (10). For example, Davis et al. showed that FLNF, associated with miscanthus, rhizomes, and isolated root-associated bacteria, could supply the N which had been missing from the miscanthus N budget (9). Ruan et al. demonstrated a lack of response in switchgrass crop yields with increasing N addition rates, suggesting that switchgrass can obtain at least some N from FLNF, supporting crop yields at low N that match those at high N fertilizer addition (10). Switchgrass is known to support the growth of free-living N fixers (11–13). In our own work, we have observed the diazotroph *Azotobacter vinelandii* successfully colonizing switchgrass roots (Fig. 3). FLNF has also been directly observed in association with switchgrass in our own work (Fig. 4) and by others (14, 15).

Despite interest in FLNF and its demonstrated potential to support food and bioenergy crop production, we still know surprisingly little about the environmental controls on FLNF and how they differ from symbiotic N fixation. We know rhizosphere diazotrophs face different challenges compared with the symbiotic N fixers, who are provided with a relatively stable environment as pH, energy, nutrients, and oxygen are all optimized for them by their plant host (Fig. 1). As diazotrophs face the challenges associated with a fluctuating climate (soil moisture and temperature) and acquiring

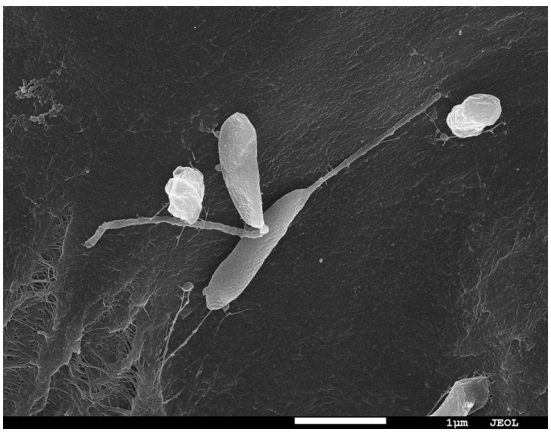

FIG 3 Scanning electron micrograph (×20,000) showing the free-living nitrogen-fixer *Azotobacter vinelandii* living on a switchgrass root. Cave-in-rock variety switchgrass seedlings were grown in sterile jars and inoculated with *A. vinelandii* (ATCC BAA-1303).

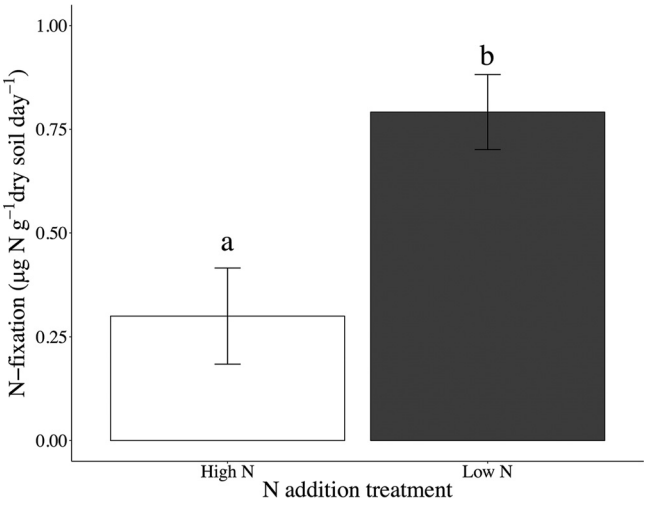

FIG 4 Preliminary N-fixation rates from switchgrass rhizosphere soils receiving high N additions (High N; +125 kg Urea-N ha^{-1} $year^{-1}$) and low N additions (Low N; +25 kg Urea-N ha^{-1} $year^{-1}$). Sterile switchgrass (var. Cave-in-Rock) seeds were planted into a sterile sand and vermiculite mixture (50:50 vol/vol) containing a core of field soil as root inoculum. Field soils were collected from marginal land sites managed by the Great Lakes Bioenergy Research Center (GLBRC) in southern Michigan. Plants received one addition of N at planting and a one-half Hoagland's nutrient solution (N free). Plants were grown in the greenhouse for 4 months prior to harvest. N-fixation rates were measured on 2-g root/rhizosphere samples via $^{15}N_2$ enrichment method (35). Samples (n = 6 per treatment) were placed in 10-ml gas vials and adjusted to 60% water holding capacity using a 4 mg C ml^{-1} glucose solution. Vials were sealed, evacuated, and adjusted back to atmospheric pressure by adding 1 ml of $^{15}N_2$ gas, 10% equivalent volume of oxygen, and balanced with helium. Vials incubated for 7 days and were then dried and ground for ^{15}N analysis. Final values were calculated following Warembourg (80). N additions did not significantly impact N-fixation rates (P = 0.1585).

resources for growth outside a symbiotic relationship, their responses to a highly variable environment must also be more flexible and evolutionarily more diverse. In this review, we will discuss what is known about diazotrophs, potential controls on the activity of diazotrophs and rates of FLNF in the rhizosphere, and highlight gaps in our knowledge that limit our ability to optimize rhizosphere conditions in order to promote FLNF in managed systems. Finally, as an example of a managed system where FLNF could be critically important for productivity, yields, and sustainability, we will apply what is known about FLNF to predict the impacts of FLNF in switchgrass bioenergy cropping systems.

The diversity of free-living N fixers. The ability to synthesize nitrogenase and fix N is exclusively prokaryotic (16). While N-fixing organisms are predominantly bacteria, some methanogenic archaea have been observed to fix N (17). N-fixing organisms are found across a wide range of bacterial phyla, including, *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria*, *Firmicutes*, *Cyanobacteria*, and green sulfur bacteria (17). Furthermore, soils are home to high diazotroph diversity, containing over 50% more operational taxonomic units (OTUs) than marine systems (17). This diversity can be observed even within rhizosphere communities. For example, diazotrophs isolated from the switchgrass rhizosphere represented at least 52 different bacterial phylotypes across multiple phyla, including *Firmicutes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria* (13). Overall, the diversity of diazotrophs actively fixing N in the rhizosphere at any given time is likely to be high.

Despite the high diversity of diazotrophs, nitrogenase, the enzyme involved in BNF, has only three known forms. Nitrogenase consists of two metalloproteins, an iron (Fe) protein responsible for ATP synthesis, and, most commonly, a molybdenum-iron (Mo-Fe) protein responsible for substrate (i.e., N_2) and proton reduction (18). Molybdenum nitrogenase (Mo-nitrogenase) is the most ubiquitous isozyme synthesized by organisms from bacterial phyla *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gam-*

maproteobacteria, *Firmicutes*, and *Cyanobacteria*. Many diazotrophs from *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Firmicutes* can also synthesize alternative forms of nitrogenase that substitute the Mo-Fe cofactor with vanadium-iron (V-nitrogenase) and/or iron-iron (Fe-nitrogenase) cofactors under Mo-limited conditions (18, 19). These slight variations in enzyme structure may influence FLNF and its responses to environmental conditions.

It has been shown that the different forms of nitrogenase vary in substrate affinity, efficiency, and temperature sensitivity, all of which influence FLNF rates. For example, Bellenger et al. demonstrate that alternative forms of nitrogenase exhibit lower R ratios (the ratio between FLNF rates measured by acetylene reduction, an indirect measure of N fixation, and rates measured via fixation of $^{15}N_2$) than Mo-nitrogenase (20). *Azotobacter vinelandii* R ratios for Mo-nitrogenase were found to 3.5 $\pm$ 1.1, while R ratios for V-nitrogenase and Fe-nitrogenase were 1.2 $\pm$ 0.4 and 0.5 $\pm$ 0.3, respectively (20). This indicates that alternative nitrogenase enzymes have a lower affinity for acetylene gas than the Mo-nitrogenase. V-nitrogenase also expresses higher isotopic discrimination against $^{15}N_2$ with a fractionation factor of −4‰ versus −1‰ for Mo-nitrogenase (21). Electron allocation varies among the different forms of nitrogenase as well (22). Mo-nitrogenase allocates the majority (~75%) of its electrons to N_2 reduction, while Fe-nitrogenase allocates the majority of its electrons to proton reduction (22). V-nitrogenase electron allocation approaches a 50:50 exchange between N_2 reduction and proton reduction (22). These results seem to suggest that the Mo-nitrogenase is the most efficient nitrogenase at converting N_2 to NH_3; however, other work has shown that temperature influences the relative efficiencies of these isozymes, complicating this issue. V-nitrogenase has been shown to be more effective than Mo-nitrogenase at low temperatures (~5°C), as illustrated by a 40-fold versus 400-fold decrease in activity, respectively, as temperature decreased from 30°C to 5°C (23). At higher temperatures (e.g., 30°C), Mo-nitrogenase is more efficient, most likely due to its higher affinity for N_2 than V-nitrogenase, as indicated by differences in K_m for the reduction of N_2 to NH_3 (19 and 29 kPa for Mo-nitrogenase and V-nitrogenase, respectively) (24). These functional differences illustrate the potential for different forms of nitrogenase to respond differentially to various environmental conditions, such as metal availability (see below) and temperature, and highlight the need for further research into how replacement of Mo with V or Fe influences nitrogenase function.

Carbon controls on free-living N fixation. It is well known that N fixation is an energetically and, therefore, carbon (C) expensive process. This was shown in early studies of FLNF, where the free-living N fixer *Clostridium pasteurianum* was incapable of fixing N unless supplied with adequate availability of C substrate (i.e., sucrose or pyruvate) (25). This substrate requirement is driven by the high demand for ATP by nitrogenase (26); 16 ATP and 8 electrons are required for the conversion of 1 N_2 molecule to 2 NH_3 molecules (27). Such high energy demands limit FLNF such that diazotrophs can only fix N when adequate supplies of C are available. In fact, FLNF was previously overlooked as a significant source of N because it was thought that soil organic matter could not provide enough energy, in the form of accessible C, to support N fixation (28). However, plant root exudates, C-rich secretions consisting of low-molecular-weight compounds such as sugars, organic acids, and mucilage (i.e., polysaccharides) (29), are a potential source of C capable of meeting diazotroph energy demands. Root exudation makes the rhizosphere a hot spot for microbial activity (30) and a key area for FLNF in the soil.

Plants allocate a significant portion of their fixed C belowground; on average, ~40% of a plant's photosynthate is translocated into belowground biomass (31). Of this translocated C, ~12% is typically recovered in the soils as root exudates, root exudate-derived metabolites, and microbial biomass C (31). For some prairie grasses, the portion of fixed C recovered from soils can be as much as 15% (32). Switchgrass is known to allocate a significant portion of its fixed C belowground. Switchgrass C allocation to

TABLE 1 Root exudate chemistry of switchgrass[a]

Compound group	Identified compound(s)	Concentration ± SD (μM)
Alcohol	Methanol	2.654 ± 0.629
Amino acid/sugar	Alanine, glycine, lysine, valine, betaine, methylamine, trimethylamine	6.144 ± 11.126
Carbohydrate	Arabinose, fructose, glucose	77.589 ± 51.494
Ketone	Acetone	12.083 ± 7.789
Nucleic acid	Thymine, thymidine, uracil	1.939 ± 3.657
Organic acid	Acetate, acetoacetate, allantoin, benzoate, formate, gallate, lactate, succinate, tartrate	13.169 ± 22.014
Quinone	Quinone	2.947 ± 3.291

[a]Exudate samples were collected from switchgrass seedlings grown hydroponically under sterile conditions. Plant growth medium was snap-frozen on liquid nitrogen, freeze-dried, and sent to the Pacific Northwest National Lab Environmental Molecular Sciences Laboratory for analysis. Exudate chemistry was determined by collection of 1D nuclear magnetic resonance (NMR) spectra for each sample following standard Chenomx (Edmonton, Alberta, Canada) sample preparation and data collection guidelines (81). Samples were analyzed on a Varian Direct Drive (VNMRS) spectrometer (Agilent Technologies, Santa Clara, CA) equipped with a Varian triple resonance salt-tolerant cold probe with a cold carbon preamplifier and a Dell Precision T3500 Linux workstation running VNMRJ 3.2.

roots and soil was measured at 40% and 6%, respectively, 1 day after a ^{13}C-CO_2 pulse-chase labeling (33). Of the 6% fixed C recovered from soils, 92% was found in microbial biomass (33), indicating that C recently fixed by switchgrass was quickly assimilated into the rhizosphere microbial community. This highlights the potential for switchgrass to support FLNF in its rhizosphere. Furthermore, as discussed in a recent review by Bowsher et al., both quality and quantity of root exudation responds to N availability, highlighting the interplay between plant C inputs and soil N availability (34). While we are unaware of any studies which have directly explored the response of FLNF to additions of root exudates, it is well established that C additions typically stimulate N fixation, with additions of C being an integral part of the methods used to measure rates of rhizosphere FLNF (35). There is great need for studies that elucidate the linkages and feedbacks between N availability, plant C exudation rates, and FLNF.

Although rhizosphere-focused studies are limited, work on FLNF in other regions, particularly bulk soil and litter, suggest that quality (i.e., form) of C substrates may be just as important as the quantity of C in regulating FLNF (1). Glucose has been used as a C source for methods assessing N fixation in the rhizosphere and bulk soil (35), but methods for isolating and culturing diazotrophs often use other forms of C, such as malate, mannitol, and sucrose (36). Other C compounds, including acetate, have been shown to inhibit nitrogenase activity; nitrogenase activity of *Azotobacter paspali* was completely inhibited in pure cultures grown on acetate and reduced by 50% on root surfaces exposed to acetate (37). Furthermore, there can be differential responses to C sources depending on environmental conditions. When in association with grass roots, N fixation by *A. paspali* increased with additions of citrate, but when in pure culture, citrate additions reduced N fixation rates by half (37). These studies provide some insight on the influence of specific C compounds on FLNF, but root exudates are a complex mixture of low-molecular-weight compounds (29). Switchgrass exudates, for example, were found to contain over 30 different compounds (L. K. Tiemann, A. W. Bowsher, M. L. Friesen, S. E. Evans, and D. W. Hoyt, unpublished data) (Table 1), suggesting that diazotrophs in the switchgrass rhizosphere have access to a diverse range of C compounds. Carbon form, particularly the diverse C forms in the rhizosphere, may be an important control on FLNF; however, it is very difficult to draw any concrete conclusions about the influence of C form on FLNF because of a lack of rhizosphere studies that explore this topic. This clear hole in our understanding of rhizosphere FLNF highlights the need for studies assessing how C compounds found in plant root exudates influence FLNF of individual diazotrophs as well as complex microbial communities (e.g., rhizosphere communities).

The form of C available to FLNF can also drive the efficiency and productivity of N fixation and determines the growth strategy of the organisms. Although little is known

about the efficiency and productivity of rhizosphere FLNF specifically, we can make inferences based on general FLNF research. Surprisingly, FLNF may be more productive (i.e., greater N fixed per unit biomass) than symbiotic N fixation. Although symbiotic N fixers can fix more N per gram of cellular material (1.0 to 2.5 versus 0.1 g N_2 g^{-1} cellular material for diazotrophs) (38), free-living diazotrophs live much shorter lives (on the order of hours versus weeks for symbionts) and have 10 times higher nitrogenase activity (25 to 50 mg N fixed g^{-1} protein h^{-1} versus 2 to 5 mg N fixed g^{-1} protein h^{-1} for symbiotic N fixation) (38). If these rates are averaged over the lifetime of the organism, rhizosphere diazotrophs may match or even exceed fixation by symbionts (38). Thus, if plants are supporting a large and active diazotroph community, they may be benefiting from highly productive N fixation, with N becoming available as the diazotroph biomass rapidly turns over. Although these data highlight the potential importance of FLNF as a plant N source, it is important to note that these FLNF rates are based on growth under optimal conditions. As discussed in subsequent sections, variations in other environmental conditions (e.g., oxygen availability and nutrient availability) are likely to influence the productivity of FLNF.

Oxygen controls on free-living N fixation. Oxygen concentrations in the rhizosphere are dynamic and extremely difficult to measure; therefore, most available research on rhizosphere oxygen concentration has been conducted in saturated systems (e.g., wetlands or sediments). For example, studies we could find were conducted using wetland plants grown in peat or sand (39, 40), agar (41), or using seagrass (*Cymodocea rotundata*) grown in saturated sediments (42). In these studies, rhizosphere oxygen concentration ranged from near 0% (anaerobic) to 20% (ambient). While these studies indicate a wide range of oxygen concentrations that occur in the rhizosphere, it is unclear if this variability in oxygen concentration is similar in nonsaturated rhizospheres such as that of switchgrass. We also note that soil texture (including particle size and compaction) is likely to impact rhizosphere oxygen concentrations; this topic is discussed further in the "Other environmental controls on free-living nitrogen fixation" section below.

Rhizosphere oxygen concentration also likely follows a diel pattern that could partially control the activity of rhizosphere-associated microbes (42). Modeling efforts suggest that diel changes in water flow through the rhizosphere (43) would have a big impact on oxygen concentration. Active root and microbial growth in the rhizosphere may also create oxygen depletion zones within the rhizosphere (44). Microsites of very low or relatively high oxygen concentration may also form in the rhizosphere, as occurs with roots of aquatic systems (45). We are unaware of any studies which have specifically examined oxygen as a control on FLNF in the rhizosphere; however, we know oxygen is a strong inhibitor of nitrogenase activity, and we can discuss physiological responses of diazotrophs to oxygen using research derived predominately from pure culture studies.

Oxygen irreversibly inhibits nitrogenase, even in aerobic organisms (46). Therefore, diazotrophs must employ protection mechanisms to maintain N fixation when oxygen is present. This includes avoidance of oxygen via growth strategy, spatial and/or temporal isolation of nitrogenase from oxygen, and production of biofilms as oxygen diffusion barriers (2, 19). Diazotrophs can also remove oxygen by increasing substrate utilization, which increases respiration rates, thereby decreasing oxygen concentrations (19, 38). This particular mechanism is likely at work in the switchgrass rhizosphere, as switchgrass has been shown to stimulate microbial growth in the rhizosphere via exudation and, thereby, substrate utilization (47). A rapidly growing rhizosphere community, regardless of diazotroph presence, is likely to reduce oxygen concentrations around the root to FLNF-favorable levels.

Oxygen management requires energy investment and so can greatly influence the efficiency of FLNF, or the amount of N fixed per unit C. Microaerophilic organisms are the most efficient N fixers, fixing an estimated 26 mg N_2 g^{-1} C metabolized (27 and references therein). In contrast, anaerobes can fix 11 mg N_2 g^{-1} C, while aerobes can

only fix 7 mg N_2 g^{-1} C (27 and references therein). One study found that upward of 60% of the energetic costs of N fixation are indirect costs associated with combating oxygen (48). This may be especially true when organisms use increased respiration to remove oxygen (49). Patra et al. found a negative relationship between substrate-induced respiration and rates of FLNF in both rhizosphere and bulk soil, suggesting that as diazotrophs increase respiration to combat oxygen damage, there is less C available for FLNF (50). However, even under high oxygen pressure, if carbohydrate availability is sufficiently high, diazotrophs may still carry out N fixation (49).

Under optimal oxygen concentrations, N fixation can actually be an energetically favorable mechanism for NH_3 acquisition, having a slight energetic advantage over assimilatory nitrate reduction (48). However, because of the diversity of diazotrophs present and potentially active in the rhizosphere, it is difficult to pinpoint one optimal oxygen concentration for FLNF. For example, Inomura et al. found the oxygen optima of *Azotobacter vinelandii* to be 3%, while Großkopf and LaRoche demonstrated that oxygen concentrations around 5% resulted in significantly higher nitrogenase activity and lower respiration by *Crocosphaera watsonii*, a marine cyanobacteria, than ambient oxygen concentrations (20%) (48, 49). It is likely that diazotrophs with different growth strategies, oxygen protection mechanisms, and C demands will also have different oxygen optima. Overall, work assessing the response of FLNF to different oxygen concentrations is sparse, and there is no information available about how whole communities of diazotrophs may respond to oxygen availability or if a community-level oxygen optimum exists.

Free-living N fixation and N availability and form. As discussed by Reed et al., diazotrophs can access N via N fixation or through uptake of externally available N sources (2), which can include both low- and high-molecular-weight organic N sources (51). Given the energy costs of FLNF, it is generally downregulated by increasing N availability, as diazotrophs use external N in favor of fixed N (2). This is corroborated by several field studies which demonstrate that environments with low N availability, whether soil, rhizosphere, or moss, typically have greater FLNF rates than sites with high N availability (50, 52, 53). The switchgrass rhizosphere is likely to be one such environment as root N uptake creates an N deplete zone in the rhizosphere (44) and switchgrass is thought to be particularly skilled at scavenging N (54). In fact, we confirm in our own work that unfertilized switchgrass rhizospheres exhibit greater FLNF rates than fertilized rhizospheres (Fig. 4). However, as different N sources will require variable amounts of energy for uptake and utilization, N form is likely to play an important role in how N availability influences FLNF.

Ammonium, the direct product of N fixation, is well known to inhibit N fixation (2) and has been shown to inhibit nitrogenase synthesis at the genetic level through the regulation of *nifA* gene transcription (19). However, it does not inhibit the activity of already synthesized nitrogenase in most organisms (38). In fact, there are only a few diazotrophs that regulate nitrogenase posttranslationally, including *Azospirillum brasilense* (19). This posttranslational regulation is carried out by dinitrogenase reductase ADP-ribosyltransferase (DraT) in response to ammonium and is reversed by dinitrogenase reductase-activating glycohydrolase (DraG) (19). Organisms with DraG-DraT regulation are likely to be more responsive to increases in ammonium, shutting down nitrogenase activity as soon as ammonium becomes available, while organisms without this posttranslational regulation may cease enzyme synthesis in response to ammonium but will continue to have functioning nitrogenase in their cells. Characterizing the presence of posttranslational regulation systems is important to understanding diazotroph response to changes in N availability, particularly ammonium. Huergo et al. suggest that DraT may be present in many diazotrophs (55), but to date, this regulatory system has only been well studied in the photosynthetic bacteria *Rhodospirillum rubrum* (56).

Glutamine also downregulates nitrogenase synthesis at the genetic level (*nif*A) via a pathway similar to ammonium regulation (19). In fact, glutamine may influence am-

monium regulation of N fixation as intracellular glutamine levels regulate *glnD* modification of the P_{II} protein, an important cellular N sensor (55). Glutamine and glutamate were found to downregulate N fixation of *Herbaspirillum seropedicae* (57). However, in the same study, histidine, lysine, and arginine had no effect on nitrogenase activity. In a similar study, ammonium, glutamine, and nitrate reduced N fixation in *A. brasilense* (58). Conversely, nitrate concentrations of up to 800 μmol had no inhibitory effect on growth or N fixation by *C. watsonii* (48).

Overall, the availability of external N sources generally reduces rates of FLNF. Although, few studies have targeted rhizosphere diazotrophs to confirm these responses to N availability and the role of N form. Furthermore, the magnitude of this response in the rhizosphere (i.e., complete shutoff FLNF or reduced rates of fixation) which is likely to depend both on the concentration and the form of external N, is not known.

Controls of phosphorus and micronutrients on free-living N fixation. The availability of phosphorus (P) and micronutrients, including Fe, Mo, and V, is known to influence N fixation (2). Yet, to the best of our knowledge, there are no studies on the controls of P, Fe, Mo, and/or V specifically targeted to rhizosphere FLNF. There have been many studies examining the importance of these nutrients on FLNF, mostly summarized by Reed et al., but these have been almost exclusively conducted in bulk soil and leaf litter (2). Here, we summarize more recent findings that may help us better understand the nutrient constraints on rhizosphere FLNF.

Phosphorus is a key nutrient in energy production and has been frequently documented as a control on N fixation (59, 60). FLNF associated with the nonlegume tree *Eucalyptus urophylla* was ~27% higher in soils receiving additions of P than in the "no-added P" control (61). Furthermore, additions of N and P resulted in similarly boosted FLNF rates (61), suggesting that P limitation was a stronger driver of FLNF than N availability in this system. A meta-analysis examining FLNF responses to nutrient additions found P fertilization to significantly increase FLNF but only in tropical forest systems (62). We may expect a similar response in the rhizosphere where root uptake of P can result in a P-depleted rhizosphere (44); thus, P may be a particularly important limiter of rhizosphere FLNF.

The response of FLNF to P availability is highly variable and can be further complicated when other nutrients, including Fe, Mo, and V, which are all essential components of nitrogenase, are also limiting. For example, Wurzburger et al. found that the limitation of FLNF by Mo and P varied along a P gradient of Panamanian soils (63). In P-rich soils, Mo was most limiting, but this shifted to a colimitation of Mo and P in P-poor soils (63). However, P alone never limited N fixation in this system (63). Conversely, a study of Costa Rican soils found that P availability was the dominant control on N fixation, while Mo concentrations did not correlate with soil FLNF rates (60). These contrasting findings may be the result of differences in soil organic matter. Wichard et al. describe how binding of Mo to organic matter can prevent Mo limitation (64). This suggests that Mo may be more available in organic soils and, therefore, a less important control on FLNF in organic-rich soils. Overall, the majority of studies indicate that increased P, Fe, Mo, and V availability generally have positive effects on FLNF. However, the most limiting nutrient and, therefore, the dominant control on FLNF is variable. This could be particularly relevant in the rhizosphere where recent advances in two-dimensional (2D) and three-dimensional (3D) element mapping have revealed connections between root growth and exudation and micronutrient concentrations, particularly metals, that can control microbial community composition and physiology (65). For example, high concentrations of available iron found at root tips (66) could be important for diazotrophs, as iron plays such a crucial role in nitrogenase construction and functioning. This highlights the need to explore how availability of these nutrients, specifically in the rhizosphere, may influence diazotroph community composition and the potential for FLNF.

Other environmental controls on free-living N fixation. The rhizosphere habitat poses an extra set of challenges to FLNF not experienced by symbiotic N fixation, as diazotrophs are more directly influenced by variation in the soil environment. For example, soil texture may influence how diazotrophs manage oxygen because of the relationship between texture and diffusion of substrate (i.e., C) and oxygen. Increasing clay content of soils can create microaerophilic and anaerobic microsites where bacteria can be protected from oxygen exposure (67), thus, potentially supporting larger populations of N fixers and/or more efficient N fixers. Indeed, Gupta and Roper found more rhizosphere N fixation in soils with greater clay content, which shifted to greater N fixation along root surfaces as clay content decreased (67). While soil texture is known to influence soil microbial community activity, there is little research exploring how soil texture may influence FLNF in the rhizosphere.

Soil pH, which is also likely to be highly variable in the rhizosphere, may also be an important environmental control on FLNF. The rhizosphere is a dynamic environment in which root growth is continuously altering the pH of the surrounding soil (39); therefore, pH may have a different effect on rhizosphere FLNF. For example, pH can change 0.5 to 1 pH units when moving just 1 mm away from the root surface (68). We also know that at the field scale, acidic soil pH has been shown to decrease N-fixation rates in aerobic soils (1). Furthermore, a study of alpine meadow soils, with pH values ranging from 5 to 8, found lower richness and diversity of diazotroph communities at acidic pH (69) suggesting that lower N fixation in acidic soils may be due to reduced community redundancy. Wang et al. also examined the relative abundances of the three dominant genera in their soils (*Azospirillum*, *Bradyrhizobium*, and *Mesorhizobium*) across the pH gradient (69). *Azospirillum* (a free-living N fixer) abundance did not vary significantly with pH, but *Bradyrhizobium* (a free-living and symbiotic N fixer) abundance increased with decreasing pH and *Mesorhizobium* (a symbiotic N fixer) abundance was reduced at acidic pH (69). This work provides some insight into response of rhizosphere FLNF to soil pH and suggests it may depend heavily on the dominant diazotroph in the community. But, with the lack of *in situ* rhizosphere studies, it is difficult to draw any strong conclusions about the magnitude or direction of rhizosphere FLNF response to changes in soil pH.

FLNF is also known to be temperature and moisture sensitive, increasing as both temperature and soil moisture increase, and these responses are well summarized by Reed et al. (2). However, rhizosphere-focused studies are lacking, highlighting a need for both mechanistic and field-scale studies aimed at addressing this knowledge gap. Soil moisture may be of particular interest, as the roots are likely to exhibit strong control over rhizosphere water availability, creating water accumulation and depletion zones according to uptake and consumption (44), which could dramatically alter oxygen dynamics.

Methodological considerations for studying free-living N fixation. The assessment of FLNF rates and diazotroph diversity across environmental gradients and ecosystems is crucial to furthering our understanding of FLNF. However, there are methodological issues that should be considered in the assessment of both rates and diversity. FLNF rates are most commonly measured either via acetylene reduction or ^{15}N isotope enrichment. Acetylene reduction takes advantage of the ability of nitrogenase to reduce triple bonded molecules other than N_2. In this method, diazotrophs are supplied acetylene (C_2H_2) which is reduced to ethylene (C_2H_4) via nitrogenase (70). Concentrations of ethylene can then be measured over time to obtain a proxy for FLNF rates. Unfortunately, there are multiple issues that should be accounted for when using this method. First, this is an indirect measure of FLNF and, as such, requires a conversion constant that relates acetylene reduction rates to N_2 reduction rates. While a conversion factor of 3 is commonly used to transform acetylene reduction rates to FLNF rates (70), the actual conversion value can vary from 30 to 1 (20). As discussed above (see "The diversity of free-living nitrogen fixers"), the different forms of nitrogenase have different affinities for acetylene which can result in highly variable ratios between acetylene

reduction rates and FLNF rates. Moreover, this method relies on the measurement of ethylene production over time. It is assumed that this ethylene production is the direct result of acetylene reduction; however, ethylene has been shown to evolve from soils independent of acetylene (71). Ethylene is an important compound in plant growth and signaling and is produced by both plants and bacteria (72). This production is not associated with the presence of acetylene. Controls that account for background ethylene production can be included with the analysis; however, they have been shown to result in misrepresentative final rates of acetylene reduction (71). Therefore, we recommend that acetylene reduction not be used for the assessment of rhizosphere FLNF rates.

The ^{15}N isotope incorporation method represents a good alternative to acetylene reduction. This method supplies diazotrophs with $^{15}N_2$ in place of atmospheric N_2, which is dominated by ^{14}N (35). As diazotrophs fix N, the ^{15}N label is incorporated into the soil and can be measured to determine FLNF rates (35). A reference sample which provides ^{15}N content in a given sample prior to $^{15}N_2$ fixation is required to obtain FLNF rates. ^{15}N isotope enrichment is a direct measure of FLNF rates and is, therefore, more accurate than acetylene reduction. However, this method is costlier in time and funds than acetylene reduction. The contribution of N via N fixation has also been frequently assessed isotopically by assuming all fixed N found in soils will have a value of 0‰ (73, 74). In other words, it is assumed that there is no fractionation during N fixation. Although symbiotic N fixation does not result in any fractionation, this is not true of FLNF which has an average fractionation factor of −2.5‰ (21). However, the true fractionation factor of a sample will depend on the relative abundance of different nitrogenase forms and may actually range from −1‰ if Mo-nitrogenase dominated to −4‰ if V-nitrogenase dominated (21). When trying to assess the relative contribution of N fixation to soil N pools, this fractionation must be accounted for in order to accurately assess the contribution of FLNF as well as symbiotic N fixation.

The assessment of diazotroph diversity also comes with challenges and we direct you to Gaby and Buckley for a detailed description of some of these challenges (75). In brief, Gaby and Buckley discuss two major concerns surrounding diazotroph diversity assessment (75). First, PCR primer selection for the *nifH* gene can lead to bias in measures of both diversity and relative abundance of amplified organisms (75). If using universal PCR primers, it is important to select primer pairs with high coverage but low phylogenetic bias (76). Second, paralogs of *nifH* can lead to a false-positive detection of the *nifH* gene (75). Lastly, as discussed earlier, there are several forms of nitrogenase. These isozymes are regulated by different genes, namely, *nifH*, *anfH*, and *vnfH* for Mo-nitrogenase, Fe-nitrogenase, and V-nitrogenase, respectively (77). Thus, it may also be prudent to assess the diversity of the *anfH* and *vnfH* genes alongside *nifH*.

Conclusions. FLNF likely occurs predominately in the rhizosphere where C from root exudates can support the energy demands of N fixation. Throughout this review, we have tried to put FLNF in the context of cropping systems, using switchgrass as an example of where the reduction or elimination of fertilizer inputs due to FLNF could significantly improve system sustainability (78). However, there is still much we do not know. We know that switchgrass allocates >40% of its fixed C belowground and over 90% of exudate C released directly to the soil is incorporated into microbial biomass and that switchgrass exudates are also very diverse (L. K. Tiemann, A. W. Bowsher, M. L. Friesen, S. E. Evans, and D. W. Hoyt, unpublished data) (Table 1). However, very little is understood about how different exudate compounds may promote or inhibit FLNF. It is also not clear whether C quality or quantity plays a larger role in FLNF. In the switchgrass rhizosphere, ample C supply and stimulated microbial growth likely result in reduced oxygen concentrations favorable to FLNF, but at the same time, this often results in reduced efficiency of FLNF (i.e., more C use for less N fixed), suggesting that C and oxygen availability may interact to control FLNF.

Nitrogen and phosphorus availability are likely strong controls on rhizosphere FLNF. For example, we can predict that uptake of N and P by switchgrass roots likely creates a nutrient-depleted rhizosphere, and yet we do not know how diazotrophs respond to these conditions. Lastly, it is important to consider the medium in which FLNF is occurring, namely, the soil. Little is known about how soil texture, pH, temperature, and moisture availability influence rhizosphere FLNF rates. Available research suggests clay soils with neutral pH and moderate temperatures and moisture availability are likely to be most favorable. However, roots exert strong control over rhizosphere conditions, including altering soil pH and moisture availability, and may ultimately prove more influential than soil properties alone. For example, different varieties of switchgrass exhibit different root architecture that has been shown to drive changes in microbial community structure and function (79).

Overall, the controls on FLNF in the rhizosphere are poorly understood. The rhizosphere is a dynamic environment, heterogenous both in resource and oxygen availability which makes it difficult to not only relate FLNF to symbiotic N fixation but also to predict both the direction and magnitude of FLNF response to the discussed controls. More research is needed at the mechanistic, ecosystem, and global level in order to better understand the role of rhizosphere FLNF in terrestrial systems and its controls.

ACKNOWLEDGMENTS

We thank James Runde for his graphics work on Fig. 1.

This work was conducted under the MMPRNT project, funded by the DOE BER Office of Science award DE-SC0014108. This work was also funded, in part, by the DOE Great Lakes Bioenergy Research Center (DOE BER Office of Science DE-SC0018409).

We declare no conflict of interest.

REFERENCES

1. Vitousek PM, Cassman K, Cleveland C, Crews T, Field CB, Grimm NB, Howarth RW, Marino R, Martinelli L, Rastetter EB, Sprent JI. 2002. Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry 57:1–45. https://doi.org/10.1023/A:1015798428743.
2. Reed SC, Cleveland CC, Townsend AR. 2011. Functional ecology of free-living nitrogen fixation: a contemporary perspective. Annu Rev Ecol Evol Syst 42:489–512. https://doi.org/10.1146/annurev-ecolsys-102710-145034.
3. Mus F, Crook MB, Garcia K, Garcia Costas A, Geddes BA, Kouri ED, Paramasivan P, Ryu M-H, Oldroyd GED, Poole PS, Udvardi MK, Voigt CA, Ané J-M, Peters JW. 2016. Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. Appl Environ Microbiol 82:3698–3710. https://doi.org/10.1128/AEM.01055-16.
4. Boyd E, Peters JW. 2013. New insights into the evolutionary history of biological nitrogen fixation. Front Microbiol 4:201. https://doi.org/10.3389/fmicb.2013.00201.
5. Sprent JI, James EK. 2007. Legume evolution: where do nodules and mycorrhizas fit in? Plant Physiol 144:575–581. https://doi.org/10.1104/pp.107.096156.
6. Cleveland CC, Houlton BZ, Neill C, Reed SC, Townsend AR, Wang Y. 2009. Using indirect methods to constrain symbiotic nitrogen fixation rates: a case study from an Amazonian rain forest. Biogeochemistry 99:1–13. https://doi.org/10.1007/s10533-009-9392-y.
7. Vitousek PM, Menge DNL, Reed SC, Cleveland CC. 2013. Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. Philos Trans R Soc Lond Sci 368:20130119. https://doi.org/10.1098/rstb.2013.0119.
8. Fowler D Coyle M, Skiba U, Sutton MA, Cape JN, Reis S, Sheppard LJ, Jenkins A, Grizzetti B, Galloway JN, Vitousek P. 2013. The global nitrogen cycle in the twenty-first century. Philos Trans R Soc Lond B Biol Sci 368:20130164. https://doi.org/10.1098/rstb.2013.0164.
9. Davis SC, Parton WJ, Dohleman FG, Smith CM, Del Grosso S, Kent AD, DeLucia EH. 2010. Comparative biogeochemical cycles of bioenergy crops reveal nitrogen-fixation and low greenhouse gas emissions in a Miscanthus × giganteus agro-ecosystem. Ecosystems 13:144–156. https://doi.org/10.1007/s10021-009-9306-9.
10. Ruan L, Bhardwaj AK, Hamilton SK, Robertson GP. 2016. Nitrogen fertilization challenges the climate benefit of cellulosic biofuels. Environ Res Lett 11:064007. https://doi.org/10.1088/1748-9326/11/6/064007.
11. Tjepkema JD, Burris RH. 1976. Nitrogenase activity associated with some Wisconsin prairie grasses. Plant Soil 45:81–94. https://doi.org/10.1007/BF00011131.
12. Morris DR, Zuberer DA, Weaver RW. 1985. Nitrogen fixation by intact grass-soil cores using 15N2 and acetylene reduction. Soil Biol Biochem 17:87–91. https://doi.org/10.1016/0038-0717(85)90094-X.
13. Bahulikar RA, Torres-Jerez I, Worley E, Craven K, Udvardi MK. 2014. Diversity of nitrogen-fixing bacteria associated with switchgrass in the native tallgrass prairie of Northern Oklahoma. Appl Environ Microbiol 80:5636–5643. https://doi.org/10.1128/AEM.02091-14.
14. Rodrigues RR, Moon J, Zhao B, Williams MA. 2017. Microbial communities and diazotrophic activity differ in the root-zone of Alamo and Dacotah switchgrass feedstocks. GCB Bioenergy 9:1057–1070. https://doi.org/10.1111/gcbb.12396.
15. Roley SS, Duncan DS, Liang D, Garoutte A, Jackson RD, Tiedje JM, Robertson GP. 2018. Associative nitrogen fixation (ANF) in switchgrass (Panicum virgatum) across a nitrogen input gradient. PLoS One 13:e0197320. https://doi.org/10.1371/journal.pone.0197320.
16. Postgate JR. 1982. Biology of nitrogen fixation: fundamentals. Philos Trans R Soc Lond B Biol Sci 296:375–385. https://doi.org/10.1098/rstb.1982.0013.
17. Gaby JC, Buckley DH. 2015. Assessment of nitrogenase diversity in the environment, p 209–216. *In* Frans J. de Bruijn (ed), Biological nitrogen fixation. John Wiley & Sons, Inc, Hoboken, NJ.
18. Rees DC, Tezcan F, Haynes C, Walton MY, Andrade S, Einsle O, Howard JB. 2005. Structural basis of biological nitrogen fixation. Philos Trans A Math Phys Eng Sci 363:971–984. https://doi.org/10.1098/rsta.2004.1539.
19. Dixon R, Kahn D. 2004. Genetic regulation of biological nitrogen fixation. Nat Rev Microbiol 2:621–631. https://doi.org/10.1038/nrmicro954.
20. Bellenger JP, Xu Y, Zhang X, Morel FMM, Kraepiel AML. 2014. Possible contribution of alternative nitrogenases to nitrogen fixation by asymbiotic N 2-fixing bacteria in soils. Soil Biol Biochem 69:413–420. https://doi.org/10.1016/j.soilbio.2013.11.015.

21. Unkovich M. 2013. Isotope discrimination provides new insight into biological nitrogen fixation. New Phytol 198:643–646. https://doi.org/10.1111/nph.12227.
22. Schneider K, Müller A. 2004. Iron-only nitrogenase: exceptional catalytic, structural and spectroscopic features. p 281–307. *In* Smith BE, Richards RL, Newton WE (ed). Catalysts for nitrogen fixation. Springer Netherlands, Heidelberg, Germany.
23. Miller RW, Eady RR. 1988. Molybdenum and vanadium nitrogenases of Azotobacter chroococcum Low temperature favors N2 reduction by vanadium nitrogenase. Biochem J 256:429–432. https://doi.org/10.1042/bj2560429.
24. Dilworth MJ, Eldridge ME, Eady RR. 1993. The molybdenum and vanadium nitrogenases of Azotobacter chroococcum: effect of elevated temperature on N2 reduction. Biochem J 289:395–400. https://doi.org/10.1042/bj2890395.
25. Carnahan JE, Mortenson LE, Mower HF, Castle JE. 1960. Nitrogen fixation in cell-free extracts of Clostridium pasteurianum. Biochim Biophys Acta 44:520–535.
26. Mortenson LE. 1964. Ferredoxin and ATP, requirements for nitrogen fixation in cell-free extracts of Clostridium pasteurianum. Proc Natl Acad Sci U S A 52:272–279. https://doi.org/10.1073/pnas.52.2.272.
27. Hill S. 1992. Physiology of nitrogen fixation in free-living heterotrophs, p 87–134. *In* Stacey G, Burris RH, Evans HJ (ed), Biological nitrogen fixation. Chapman & Hall, New York, NY.
28. Stewart WDP. 1969. Biological and ecological aspects of nitrogen fixation by free-living micro-organisms. Proc R Soc Lond B Biol Sci 172:367–388.
29. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57:233–266. https://doi.org/10.1146/annurev.arplant.57.032905.105159.
30. Kuzyakov Y, Blagodatskaya E. 2015. Microbial hotspots and hot moments in soil: concept & review. Soil Biol Biochem 83:184–199. https://doi.org/10.1016/j.soilbio.2015.01.025.
31. Jones DL, Nguyen C, Finlay RD. 2009. Carbon flow in the rhizosphere: carbon trading at the soil–root interface. Plant Soil 321:5–33. https://doi.org/10.1007/s11104-009-9925-0.
32. Roper MM, Fillery IRP, Jongepier R, Sanford P, Macdonald LM, Sanderman J, Baldock JA. 2013. Allocation into soil organic matter fractions of 14C captured via photosynthesis by two perennial grass pastures. Soil Res 51:748–759. https://doi.org/10.1071/SR12375.
33. Chaudhary DR, Saxena J, Lorenz N, Dick RP. 2012. Distribution of recently fixed photosynthate in a switchgrass plant-soil system. Plant Soil Environ 58:249–255.
34. Bowsher AW, Evans S, Tiemann LK, Friesen ML. 2018. Effects of soil nitrogen availability on rhizodeposition in plants: a review. Plant Soil 423:59–85. https://doi.org/10.1007/s11104-017-3497-1.
35. Gupta VVSR, Kroker SJ, Hicks M, Davoren CW, Descheemaeker K, Llewellyn R. 2014. Nitrogen cycling in summer active perennial grass systems in South Australia: non-symbiotic nitrogen fixation. Crop Pasture Sci 65:1044–1056. https://doi.org/10.1071/CP14109.
36. Baldani JI, Reis VM, Videira SS, Boddey LH, Baldani VLD. 2014. The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. Plant Soil 384:413–431. https://doi.org/10.1007/s11104-014-2186-6.
37. Dobereiner JO, Day JM. 1975. Nitrogen fixation in the rhizosphere of tropical grasses. *In* Stewart WDP (ed), Nitrogen fixation by free-living microorganisms. Cambridge University Press, London, United Kingdom.
38. Mulder EG. 1975. Physiology and ecology of free-living, nitrogen-fixing bacteria, p 3–29. *In* Nitrogen fixation by free-living micro-organisms. Cambridge University Press, London, United Kingdom.
39. Blossfeld S, Gansert D, Thiele B, Kuhn AJ, Lösch R. 2011. The dynamics of oxygen concentration, pH value, and organic acids in the rhizosphere of Juncus spp. Soil Biol Biochem 43:1186–1197. https://doi.org/10.1016/j.soilbio.2011.02.007.
40. Minett DA, Cook PL, Kessler AJ, Cavagnaro TR. 2013. Root effects on the spatial and temporal dynamics of oxygen in sand-based laboratory-scale constructed biofilters. Ecol Eng 58:414–422. https://doi.org/10.1016/j.ecoleng.2013.06.028.
41. Tschiersch H, Liebsch G, Borisjuk L, Stangelmayer A, Rolletschek H. 2012. An imaging method for oxygen distribution, respiration and photosynthesis at a microscopic level of resolution. New Phytol 196:926–936. https://doi.org/10.1111/j.1469-8137.2012.04295.x.
42. Pedersen O, Borum J, Duarte CM, Fortes MD. 1998. Oxygen dynamics in the rhizosphere of Cymodocea rotundata. Mar Ecol Prog Ser 169:283–288. https://doi.org/10.3354/meps169283.
43. Espeleta JF, Cardon ZG, Mayer KU, Neumann RB. 2017. Diel plant water use and competitive soil cation exchange interact to enhance NH 4+ and K+ availability in the rhizosphere. Plant and Soil 414:33–51. https://doi.org/10.1007/s11104-016-3089-5.
44. York LM, Carminati A, Mooney SJ, Ritz K, Bennett MJ. 2016. The holistic rhizosphere: integrating zones, processes, and semantics in the soil influenced by roots. J Exp Bot 67:3629–3643. https://doi.org/10.1093/jxb/erw108.
45. Brune A, Frenzel P, Cypionka H. 2000. Life at the oxic–anoxic interface: microbial activities and adaptations. FEMS Microbiol Rev 24:691–710. https://doi.org/10.1111/j.1574-6976.2000.tb00567.x.
46. Robson RL, Postgate JR. 1980. Oxygen and hydrogen in biological nitrogen fixation. Annu Rev Microbiol 34:183–207. https://doi.org/10.1146/annurev.mi.34.100180.001151.
47. Liang C, Jesus EDC, Duncan DS, Quensen JF, Jackson RD, Balser TC, Tiedje JM. 2016. Switchgrass rhizospheres stimulate microbial biomass but deplete microbial necromass in agricultural soils of the upper Midwest, USA. Soil Biol Biochem 94:173–180. https://doi.org/10.1016/j.soilbio.2015.11.020.
48. Großkopf T, LaRoche J. 2012. Direct and indirect costs of dinitrogen fixation in Crocosphaera watsonii WH8501 and possible implications for the nitrogen cycle. Front Microbiol 3:236. https://doi.org/10.3389/fmicb.2012.00236.
49. Inomura K, Bragg J, Follows MJ. 2017. A quantitative analysis of the direct and indirect costs of nitrogen fixation: a model based on Azotobacter vinelandii. ISME J 11:166–175. https://doi.org/10.1038/ismej.2016.97.
50. Patra AK, Le Roux X, Abbadie L, Clays-Josserand A, Poly F, Loiseau P, Louault F. 2007. Effect of microbial activity and nitrogen mineralization on free-living nitrogen fixation in permanent grassland soils. J Agron Crop Sci 193:153–156. https://doi.org/10.1111/j.1439-037X.2006.00247.x.
51. Norman JS, Friesen ML. 2017. Complex N acquisition by soil diazotrophs: how the ability to release exoenzymes affects N fixation by terrestrial free-living diazotrophs. ISME J 11:315. https://doi.org/10.1038/ismej.2016.127.
52. Hobbs NT, Schimel DS. 1984. Fire effects on nitrogen mineralization and fixation in mountain shrub and grassland communities. J Range Manage 37:402–405. https://doi.org/10.2307/3899624.
53. Kox MAR, Lüke C, Fritz C, van den Elzen E, van Alen T, Op den Camp HJM, Lamers LPM, Jetten MSM, Ettwig KF. 2016. Effects of nitrogen fertilization on diazotrophic activity of microorganisms associated with Sphagnum magellanicum. Plant Soil 406:83–100. https://doi.org/10.1007/s11104-016-2851-z.
54. Fike JH, Parrish DJ, Wolf DD, Balasko JA, Green JT, Jr, Rasnake M, Reynolds JH. 2006. Long-term yield potential of switchgrass-for-biofuel systems. Biomass Bioenergy 30:198–206. https://doi.org/10.1016/j.biombioe.2005.10.006.
55. Huergo LF, Pedrosa FO, Muller-Santos M, Chubatsu LS, Monteiro RA, Merrick M, Souza EM. 2012. PII signal transduction proteins: pivotal players in post-translational control of nitrogenase activity. Microbiology 158:176–190. https://doi.org/10.1099/mic.0.049783-0.
56. Halbleib CM, Ludden PW. 2000. Regulation of biological nitrogen fixation. J Nutr 130:1081–1084. https://doi.org/10.1093/jn/130.5.1081.
57. Klassen G, Pedrosa FO, Souza EM, Funayama S, Rigo LU. 1997. Effect of nitrogen compounds on nitrogenase activity in Herbaspirillum seropedicae SMR1. Can J Microbiol 43:887–891. https://doi.org/10.1139/m97-129.
58. Steenhoudt O, Vanderleyden J. 2000. Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol Rev 24:487–506. https://doi.org/10.1111/j.1574-6976.2000.tb00552.x.
59. Reed SC, Seastedt TR, Mann CM, Suding KN, Townsend AR, Cherwin KL. 2007. Phosphorus fertilization stimulates nitrogen fixation and increases inorganic nitrogen concentrations in a restored prairie. Appl Soil Ecol 36:238–242. https://doi.org/10.1016/j.apsoil.2007.02.002.
60. Reed SC, Cleveland CC, Townsend AR. 2013. Relationships among phosphorus, molybdenum and free-living nitrogen fixation in tropical rain forests: results from observational and experimental analyses. Biogeochemistry 114:135–147. https://doi.org/10.1007/s10533-013-9835-3.
61. Zheng M, Li D, Lu X, Zhu X, Zhang W, Huang J, Fu S, Lu X, Mo J. 2016. Effects of phosphorus addition with and without nitrogen addition on biological nitrogen fixation in tropical legume and non-legume tree

plantations. Biogeochemistry 131:65–76. https://doi.org/10.1007/s10533-016-0265-x.
62. Dynarski KA, Houlton BZ. 2018. Nutrient limitation of terrestrial free-living nitrogen fixation. New Phytol 217:1050–1061. https://doi.org/10.1111/nph.14905.
63. Wurzburger N, Bellenger JP, Kraepiel AM, Hedin LO. 2012. Molybdenum and phosphorus interact to constrain asymbiotic nitrogen fixation in tropical forests. PLoS One 7:e33710. https://doi.org/10.1371/journal.pone.0033710.
64. Wichard T, Mishra B, Myneni SC, Bellenger JP, Kraepiel AM. 2009. Storage and bioavailability of molybdenum in soils increased by organic matter complexation. Nature Geosci 2:625–629. https://doi.org/10.1038/ngeo589.
65. Oburger E, Schmidt H. 2016. New methods to unravel rhizosphere processes. Trends Plant Sci 21:243–255. https://doi.org/10.1016/j.tplants.2015.12.005.
66. Williams PN, Santner J, Larsen M, Lehto NJ, Oburger E, Wenzel W, Glud RN, Davison W, Zhang H. 2014. Localized flux maxima of arsenic, lead, and iron around root apices in flooded lowland rice. Environ Sci Technol 48:8498–8506. https://doi.org/10.1021/es501127k.
67. Gupta VVSR, Roper MM. 2010. Protection of free-living nitrogen-fixing bacteria within the soil matrix. Soil Tillage Res 109:50–54. https://doi.org/10.1016/j.still.2010.04.002.
68. Youssef RA, Chino M. 1989. Root-induced changes in the rhizosphere of plants. I. pH changes in relation to the bulk soil. Soil Sci Plant Nutr 35:461–468. https://doi.org/10.1080/00380768.1989.10434779.
69. Wang Y, Li C, Kou Y, Wang J, Tu B, Li H, Li X, Wang C, Yao M. 2017. Soil pH is a major driver of soil diazotrophic community assembly in Qinghai-Tibet alpine meadows. Soil Biol Biochem 115:547–555. https://doi.org/10.1016/j.soilbio.2017.09.024.
70. Hardy RWF, Holsten RD, Jackson EK, Burns RC. 1968. The acetylene–ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol 43:1185–1207. https://doi.org/10.1104/pp.43.8.1185.
71. Nohrstedt HÖ. 1983. Natural formation of ethylene in forest soils and methods to correct results given by the acetylene-reduction assay. Soil Biol Biochem 15:281–286. https://doi.org/10.1016/0038-0717(83)90072-X.
72. Wang KL, Li H, Ecker JR. 2002. Ethylene biosynthesis and signaling networks. Plant Cell 14:s131–s151. https://doi.org/10.1105/tpc.001768.
73. Shearer G, Kohl DH. 1986. N_2-fixation in field settings: estimations based on natural ^{15}N abundance. Aust J Plant Physiol 13:699–756.
74. Boddey RM, Polidoro JC, Resende AS, Alves BJR, Urquiaga S. 2001. Use of the ^{15}N natural abundance technique for the quantification of the contribution of N_2 fixation to sugar cane and other grasses. Aust J Plant Physiol 28:889–895.
75. Gaby JC, Buckley DH. 2011. A global census of nitrogenase diversity. Environ Microbiol 13:1790–1799. https://doi.org/10.1111/j.1462-2920.2011.02488.x.
76. Gaby JC, Buckley DH. 2012. A comprehensive evaluation of PCR primers to amplify the nifH gene of nitrogenase. PLoS One 7:e42149. https://doi.org/10.1371/journal.pone.0042149.
77. Zehr JP, Jenkins BD, Short SM, Steward GF. 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ Microbiol 5:539–554. https://doi.org/10.1046/j.1462-2920.2003.00451.x.
78. Robertson GP, Stephen KH, Bradford LB, Bruce ED, R Cesar I, Randall DJ, Douglas AL, Scott MS, Kurt DT, James MT. 2017. Cellulosic biofuel contributions to a sustainable energy future: choices and outcomes. Science 356:eaal2324. https://doi.org/10.1126/science.aal2324.
79. Stewart CE, Damaris R, Karolien D, Elizabeth P, Louise H, Comas GS, Virginia LJ, Marty RS, Madhavan S. 2017. Seasonal switchgrass ecotype contributions to soil organic carbon, deep soil microbial community composition and rhizodeposit uptake during an extreme drought. Soil Biol Biochem 112:191–203. https://doi.org/10.1016/j.soilbio.2017.04.021.
80. Warembourg FR. 1993. Nitrogen fixation in soil and plant systems, p 127–156. *In* Knowles R, Blackburn H (ed), Nitrogen isotope techniques. Academic Press, San Diego, CA.
81. Weljie AM, Newton J, Mercier P, Carlson E, Slupsky CM. 2006. Targeted profiling: quantitative analysis of 1 H NMR metabolomics data. Anal Chem 78:4430–4442. https://doi.org/10.1021/ac060209g.