ENVIRONMENTAL AND BIOLOGICAL CONTROLS ON FREE-LIVING NITROGEN FIXATION

By

Darian Smercina

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Crop and Soil Sciences — Doctor of Philosophy

2020

ABSTRACT

ENVIRONMENTAL AND BIOLOGICAL CONTROLS ON FREE-LIVING NITROGEN FIXATION

By

Darian Smercina

Free-living nitrogen fixation (FLNF) is the biological conversion of gaseous N_2 into ammonia (NH_3) by heterotrophic bacteria and archaea not in symbiosis with plants. This energy intensive process occurs predominately where carbon (C) is readily available to support these high energy demands, such as in the rhizosphere where roots exude C into the soil environment. FLNF has continually gained attention as an important and ubiquitous N source to terrestrial systems and as a potential alternative to fertilizer N addition in crop production. In particular, it has gained interest for its potential to support production of bioenergy cropping systems, like switchgrass (*Panicum virgatum),* particularly when grown on marginal lands. Diverse communities of N-fixing organisms (diazotrophs) have been identified in the rhizospheres of these cropping systems as well as FLNF activity. But, to harness this potential N source it is important to understand the controls on FLNF. My dissertation work characterizes biological and environmental controls on FLNF associated with the switchgrass rhizosphere.

Though there are decades of research available on symbiotic N-fixation, the conditions under which FLNF occurs are quite distinct including dynamic C sources and availabilities, oxygen concentrations, and N availability. FLNF is also carried out by a diverse community of diazotrophs rather than a single population as with symbiotic N-fixation. To determine the important controls on FLNF, I first tested impacts of C source and oxygen concentration on FLNF through development of an optimized method for

measuring FLNF. I found that increased diversity of C sources and lower oxygen concentrations promoted the most FLNF. I then examined the effects of legacy and short-term N additions on both FLNF rates and the composition of the diazotroph community in the switchgrass rhizosphere. Surprisingly, I found no evidence for legacy or short-term N controls on FLNF rates or diazotroph community composition. However, I found a strong rhizosphere effect on diazotroph community composition, suggesting switchgrass selects for a distinct and consistent N-fixing community. Lastly, I determined controls on FLNF under field conditions and how these controls relate to plant available N, using a variety of field and molecular data. I found that soil N availability was the dominant control on FLNF, but the direction of this control depended on the soil N pool. Together, my work highlights several important environmental and biological controls on FLNF and ultimately improves our ability to understand and predict this important N source for terrestrial systems.

Lastly, my work adds to the growing body of evidence that FLNF occurs in many systems and can contribute largely to plant N demands. By extrapolating the average of my measured FLNF rates from μg N fixed g^{-1} dry soil day^{-1} to kg N ha^{-1} yr^{-1}, I found FLNF has the potential to contribute upwards of 11.0 kg N ha^{-1} yr^{-1}. These rates are 2x greater than the estimated contribution of N from symbiotic N-fixation in temperate grasslands and meet approximately 31% of the N deficit identified in switchgrass systems from previous work. Although these extrapolated rates are based on optimized conditions for potential FLNF rates and therefore are likely overestimates, they highlight the important role of FLNF in switchgrass cropping systems and its potential to contribute to improving the sustainability of bioenergy production.

This thesis is dedicated to Adam.

Thank you for supporting me through every step of this process and always encouraging me to follow my dreams.

And to Kiera whose unconditional love, wagging tail, and crazy antics fill my life with happiness.

And lastly, to my dad, Jarrett Marinis, who taught me to be unapologetically me…and to "kick ass and take names".

ACKNOWLEDGEMENTS

I would first like to thank my committee members for all their support throughout this process. Thank you to Sarah Evans for always being so positive and supportive – your belief in me and my work was always great appreciated! Thank you to Maren Friesen for sharing in my crazy methods ideas and always being on board to try anything – you allowed my PhD to be a creative as well as objective endeavor. Thank you to Brian Teppen for all your thoughtful ideas and questions – you challenged me to think outside the ecology of soils and remember the complex environment I study. Lastly, thank you to my PhD advisor, Lisa Tiemann – you were an excellent mentor and exactly the one I needed. Thank you for supporting me through this entire process, always challenging to try harder, and convincing me to stick it out.

I want to thank the many, many people who have been involved in the MMPRNT project over the last five years. This project has been integral to my work as well as my growth as a researcher and it couldn't have been successful without all of you and your hard work. I'd specifically like to thank a fellow graduate student on the project, Tayler Ulbrich – you've been a great friend, an excellent colleague, and some helpful, and friendly competition. Thank you for supporting and challenging me as we've grown into independent researchers!

Lastly, I want extend a sincere thank you to all the family, friends, teachers, and colleagues who have been a part of this journey with me. Thank you for all your love, kindness, and support. I wouldn't have made it this far without the love and support of my family. I want to thank my parents for always encouraging me to pursue my dreams,

even if they didn't understand why I wanted a PhD in the first place. Thank you to my little sister, Brianna – wanting to be a good role model for you has always pushed me to be better and try harder. And lastly, to my partner and best friend – thank you, Adam. We started this journey together and I know I could never have finished it without you. Thank you for sharing your enthusiasm for life and for discovery with me and for always supporting me.

TABLE OF CONTENTS

LIST OF TABLES ix

LIST OF FIGURES x

KEY TO ABBREVIATIONS xii

CHAPTER 1: INTRODUCTION 1
1.1 THE DIVERSITY OF FREE-LIVING N-FIXERS 6
1.2 CARBON CONTROLS ON FREE-LIVING N-FIXATION 8
1.3 OXYGEN CONTROLS ON FREE-LIVING N-FIXATION 12
1.4 FREE-LIVING N-FIXATION AND N AVAILABILITY AND FORM 15
1.5 CONTROLS OF PHOSPHORUS AND MICRONUTRIENTS ON FREE-LIVING N-FIXATION 17
1.6 OTHER ENVIRONMENTAL CONTROLS ON FREE-LIVING N-FIXATION 19
1.7 METHODOLOGICAL CONSIDERATIONS FOR STUDYING FREE-LIVING N-FIXATION 21
1.8 CONCLUSIONS 24
REFERENCES 26

CHAPTER 2: OPTIMIZATION OF METHODS FOR ASSESSING FREE-LIVING NITROGEN FIXATION 34
2.1 ABSTRACT 34
2.2 INTRODUCTION 35
2.3 MATERIALS AND METHODS 40
2.3.1 Sample Collection 40
2.3.2 Soil moisture and water holding capacity 42
2.3.3 Acetylene reduction 42
2.3.4 $^{15}N_2$ incorporation method 46
2.3.5 $^{15}N_2$ incorporation carbon source testing 47
2.3.6 $^{15}N_2$ incorporation C solution pH 48
2.3.7 $^{15}N_2$ incorporation oxygen concentration testing 48
2.3.8 $^{15}N_2$ incorporation incubation time testing 49
2.3.9 Data Analysis 49
2.4 RESULTS 50
2.4.1 Acetylene Reduction 50
2.4.2 $^{15}N_2$ Incorporation Carbon Source 53
2.4.3 $^{15}N_2$ incorporation Oxygen Concentration 55
2.4.4 $^{15}N_2$ incorporation Incubation Time 57
2.5 DISCUSSION 57
2.6 CONCLUSIONS 65
APPENDIX 67
REFERENCES 69

CHAPTER 3: SWITCHGRASS SELECTS FOR A DISTINCT AND CONSISTENT RHIZOSPHERE DIAZOTROPH COMMUNITY 75
3.1 ABSTRACT 75
3.2 INTRODUCTION 76
3.3 METHODS 80
3.3.1 Experimental Setup 80
3.3.2 Planting and growth 82
3.3.3 Aboveground biomass 84
3.3.4 Rhizosphere N-fixation 84
3.3.5 Rhizosphere diazotroph community composition 85
3.3.6 Data Analysis 87
3.4 RESULTS 89
3.4.1 Plant metrics 89
3.4.2 Free-living nitrogen fixation rates 90
3.4.3 Diazotroph community structure 91
3.4.4 Indicator species 94
3.5 DISCUSSION 96
3.5.1 Diazotroph community composition in response to N availability 97
3.5.2 FLNF in response to N availability across sites 99
3.5.3 Linking diazotroph community composition and FLNF rates 101
3.6 CONCLUSIONS 103
APPENDIX 105
REFERENCES 108

CHAPTER 4: TEMPORAL DYNAMICS OF FREE-LIVING NITROGEN FIXATION IN THE SWITCHGRASS RHIZOSPHERE 114
4.1 ABSTRACT 114
4.2 INTRODUCTION 115
4.3 METHODS 117
4.3.1 Field Site and Weather Data 117
4.3.2 Soil Collection and Nutrient Analyses 117
4.3.3 Free-living Nitrogen Fixation 118
4.3.4 Plant Available Inorganic Nitrogen 119
4.3.5 Soil Extracellular Enzyme Activities 120
4.3.6 Plant Metrics 121
4.3.7 Soil Diazotroph Community Composition 122
4.3.8 Data Analysis 124
4.4 RESULTS 126
4.4.1 N-fixation rates 126
4.4.2 Diazotroph community composition 127
4.4.3 Random Forest Regression 129
4.5 DISCUSSION 133
4.6 CONCLUSIONS 139
APPENDIX 141
REFERENCES 148

LIST OF TABLES

Table 2.1: Site characteristics including soil properties, climate, and land use history for Lux Arbor (LUX) and Lake City (LC)……41

Table 2.2: Table of experimental conditions for optimization tests……....43

Table 3.1: Site characteristics including soil properties, climate, and land use history for three field sites Lux Arbor (LUX), Lake City (LC), and Escanaba (ESC) …….82

Table 3.2: Average relative abundance and standard errors of diazotroph classes by sample type and site……..95

Table 3.3: Table of OTUs identified by *indicspecies* as associated with N-fixation rates of > 10 µg N per g rhizosphere per day. ……....96

Table 3.4: Table of OTUs whose presence appears to be associated with increased N-fixation rates……..……..96

Table 4.1: Table of OTUs associated with the measured pulse in N-fixation.....129

Table S4.1: Results of randomForest regression analysis for full 2017 field season data.....145

Table S4.2: Results of randomForest regression analysis for pulse-associated dates including diazotroph community composition (class-level).....146

LIST OF FIGURES

Figure 1.1: Contrasting habitats of free-living and symbiotic nitrogen-fixation.....2

Figure 1.2: Environmental factors known to impact FLNF.....3

Figure 1.3: Scanning electron micrograph of *Azotoabcter vinelandii*.....5

Figure 1.4: Preliminary N-fixation rates from switchgrass rhizosphere soils.....15

Figure 2.1: Acetylene reduction rates at different incubation times.....50

Figure 2.2: Acetylene reduction rates at different oxygen concentrations.....51

Figure 2.3: Acetylene reduction rates with addition of different C sources.....52

Figure 2.4: Background ethylene (A) production and (B) consumption.....52

Figure 2.5: $^{15}N_2$ incorporation rates with addition of different C sources.....53

Figure 2.6: Effect of buffering C cocktail on $^{15}N_2$ incorporation.....54

Figure 2.7: $^{15}N_2$ incorporation rates at different oxygen concentrations.....56

Figure 2.8: $^{15}N_2$ incorporation rates at different incubation times.....57

Figure S2.1: Images of AMS soil/turf probe used to obtain intact cores.....68

Figure 3.1: Experimental design of (A) N additions and (B) planting.....80

Figure 3.2: Plant aboveground biomass (mg) by site.....89

Figure 3.3: Nitrogen fixation rates of the rhizosphere for each site.....90

Figure 3.4: Principal coordinates analysis of (A) field soil and (B) rhizosphere samples.....91

Figure 3.5: Average relative abundance of diazotroph classes by site.....92

Figure 3.6: Average relative abundance of diazotroph classes by legacy N (Fert = Fertilized, Unfert = Unfertilized) and short-term N additions (High = High N addition, Low = Low N addition) for rhizosphere samples.....93

Figure S3.1: Average relative abundance of diazotroph classes by legacy N addition and site for soil samples.....106

Figure S3.2: Principal coordinates analysis of field soil samples and rhizosphere samples for (A) Lux Arbor, (B) Lake City, (C) Escanaba and (D) All samples.....107

Figure 4.1: Free-living nitrogen fixation rates across 2017 growing season.....126

Figure 4.2: Principal coordinate analysis (PCoA) of soil diazotroph communities.....128

Figure 4.3: Results of random forest regression analysis.....131

Figure 4.4: Correlations of important full season predictors of N-fixation.....132

Figure 4.5: Correlations of important pulse-associated predictors of N-fixation.....133

Figure S4.1: Alpha diversity metrics (A) Chao1, (B) Shannon Index, and (C) Inverse Simpson by collection date.....142

Figure S4.2: Relative abundance of diazotroph classes, based on *nifH* amplicon sequencing, by sample date.....143

Figure S4.3: Free-living nitrogen fixation rates across the 2017 growing season represented as boxplots for each sample date and field fertilizer treatment.....144

KEY TO ABBREVIATIONS

ANOVA	Analysis of Variance
ARA	Acetylene Reduction Assay
BNF	Biological Nitrogen Fixation
BSC	Biological Soil Crusts
C	Carbon
CO_2	Carbon Dioxide
CV	Coefficient of Variation
DOC	Dissolved Organic Carbon
DON	Dissolved Organic Nitrogen
ESC	Escanaba (field site)
Fe	Iron
FLNF	Free-Living Nitrogen Fixation
GC	Gas Chromatograph
GLBRC	Great Lakes Bioenergy Research Center
He	Helium
HMM	Hidden-Markov Models
LC	Lake City (field site)
LUX	Lux Arbor (field site)
Mo	Molybdenum
N	Nitrogen
N_2	Dinitrogen gas
N_2O	Nitrous Oxide

NAG	N-acetyl-β-D-glucosaminidase
NH_3	Ammonia
O_2	Oxygen
OTU	Operational Taxonomic Unit
P	Phosphorus
PCoA	Principal Coordinates Analysis
PERMANOVA	Permutational multivariate analysis of variance
TN	Total Nitrogen
UHP	Ultra High Purity
V	Vanadium

CHAPTER 1:

INTRODUCTION

Originally published as: Smercina, D. N., Evans, S. E., Friesen, M. L., & Tiemann, L. K. (2019). To fix or not to fix: controls on free-living nitrogen fixation in the rhizosphere. Appl. Environ. Microbiol., 85(6), e02546-18.

Biological N-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 (Vitousek et al. 2002). 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. I 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, considered to have similar environmental constraints (Mus et al. 2016). However, I argue that FLNF, particularly in the rhizosphere, is an important process distinct from symbiotic N-fixation and carried out by wholly different bacterial species, and thus warrants its own investigation (Fig. 1.1).

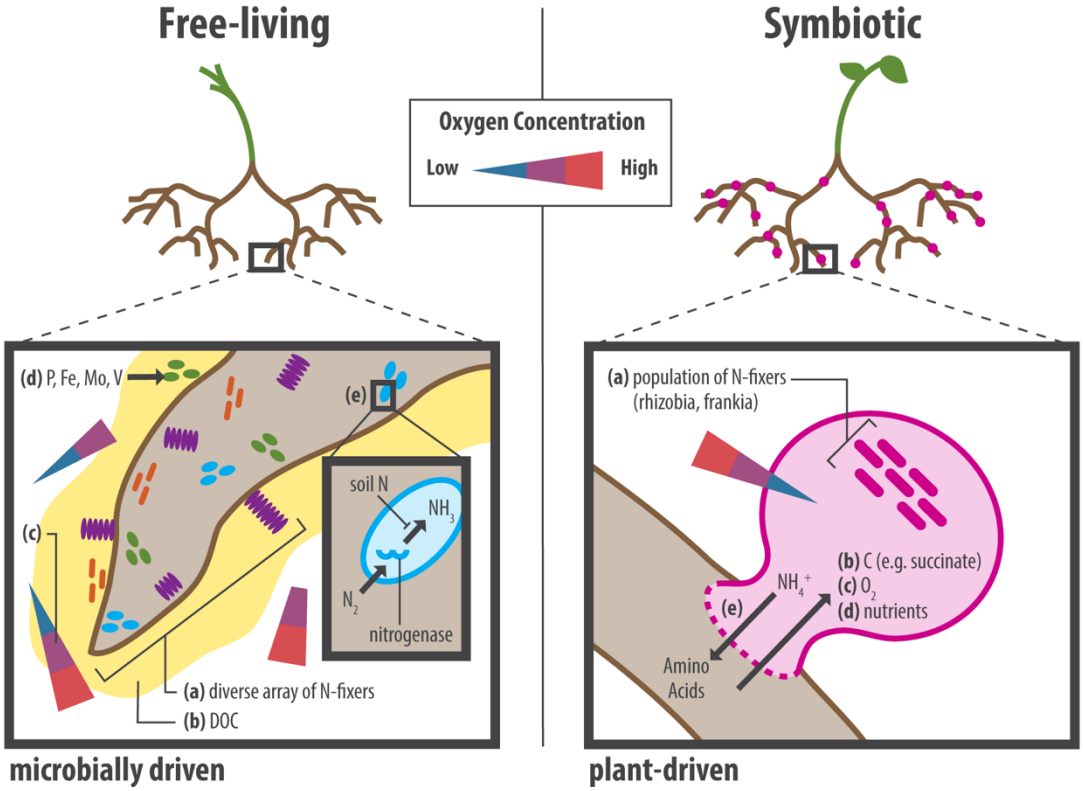

Figure 1.1: **Contrasting habitats of free-living and symbiotic nitrogen-fixation**

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. malate) 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, 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.

Surprisingly, FLNF actually evolved long before symbiotic N-fixation, appearing between 1.5 and 2.2. billion years ago (Boyd et al. 2013) compared to only 59 million years ago for symbiotic N-fixation (Sprent and James 2007). Because of dynamically

fluctuating conditions in the rhizosphere, FLNF occurs under more diverse and variable conditions than symbiotic N-fixation making it difficult to draw conclusions about FLNF based on research of symbiotic N-fixation (Fig. 1.2). 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 (Cleveland et al. 2009, Reed et al. 2011, Vitousek et al. 2013). For example, Cleveland *et al.* (2009) 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} yr^{-1} on average to tropical forest systems (ranging from 2.4 to 14 kg N ha^{-1} yr^{-1}) while symbiotic BNF was estimated at only 4.5 kg N ha^{-1} yr^{-1}. Similarly, Reed *et al.* (2011) show that FLNF rates fall within the range of symbiotic BNF rates for all biomes. Summing the FLNF rates for each biome (accounting for land area of each biome), as estimated by Reed *et al.* (2011), FLNF contributes ~76 Tg N yr^{-1} globally (falling within Vitousek *et al.* (2002) estimated range of 40-100 Tg N fixed yr^{-1}), which far exceeds

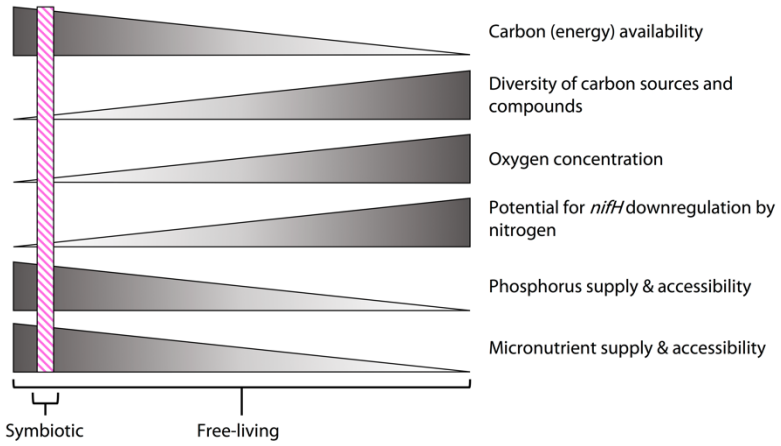

Figure 1.2: **Environmental factors known to impact FLNF** presented with triangles representing a theoretical range for each factor, low (narrow, light-colored) to high (broad, dark-colored). In contrast, symbiotic N-fixation, represented by vertical hatched bar, only occurs in a narrow range of each 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.

inputs from lightning (5 Tg N yr^{-1}; 8) and is more than half the N fixed industrially via Haber-Bosch reactions (120 Tg N yr^{-1}; Reed et al. 2011, Fowler et al. 2013). 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 cropping systems, like miscanthus (*Miscanthus* × *giganteus;* Davis et al. 2010) and switchgrass (*Panicum virgatum;* Ruan et al. 2016). For example, Davis *et al.* (2010) 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. Ruan *et al.* (2016) 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. Switchgrass is known to support the growth of free-living N-fixers (Tjepkema and Burris 1976, Morris et al. 1985, Bahulikar et al. 2014). In my own work, I have observed the diazotroph *Azotobacter vinelandii* successfully colonizing switchgrass roots (Fig. 1.3).

FLNF has also been directly observed in association with switchgrass in our own work and by others (Rodrigues et al. 2017, Roley et al. 2018).

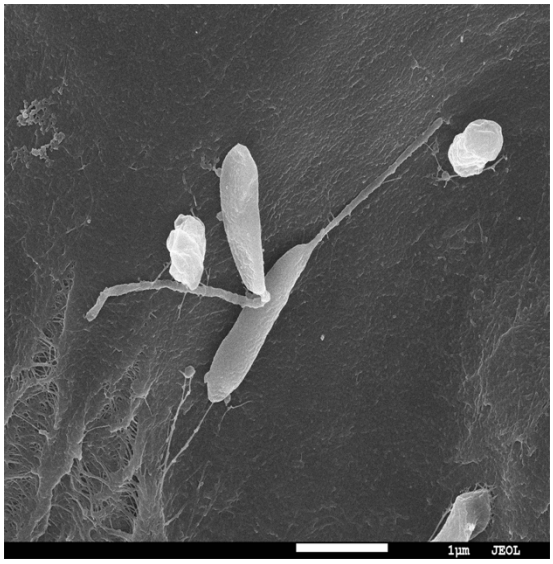

Figure 1.3: **Scanning electron micrograph of *Azotobacter vinelandii***, a free-living nitrogen-fixer living on a switchgrass root (x 20,000). Cave-in-rock variety switchgrass seedlings were grown in sterile jars and inoculated with *A. vinelandii* (ATCC BAA-1303).

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 to 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. 1.1). As diazotrophs face the challenges associated with a fluctuating climate (soil moisture, temperature) and acquiring resources for growth outside of a symbiotic relationship, their responses to a highly variable environment must also be more flexible and evolutionarily more diverse. In this review, I 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, I will apply what is known about FLNF to predict the impacts of FLNF in switchgrass bioenergy cropping systems.

1.1 THE DIVERSITY OF FREE-LIVING N-FIXERS

The ability to synthesize nitrogenase and fix N is exclusively prokaryotic (Postgate 1982). While N-fixing organisms are predominantly bacteria, some methanogenic archaea have been observed to fix N (Gaby and Buckley 2015). N-fixing organisms are found across a wide range of bacterial phyla including, Alpha-, Beta-, Delta-, and Gammaprotebacteria, Firmicutes, Cyanobacteria, and Green Sulfur Bacteria (Gaby and Buckley 2015). Further, soils are home to high diazotroph diversity, containing over 50% more OTUs than marine systems (Gaby and Buckley 2015). 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, Alpha-, Beta-, Delta- and Gamma-proteobacteria (Bahulikar et al. 2014). 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 (Rees et al. 2005). Molybdenum nitrogenase (Mo-nitrogenase) is the most ubiquitous isozyme

synthesized by organisms from bacterial phyla Alpha-, Beta-, Delta-, and Gammaprotebacteria, Firmicutes, and Cyanobacteria. Many diazotrophs from Alpha-, Beta-, and Gammaprotebacteria 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) co-factors under Mo-limited conditions (Rees et al. 2005, Dixon and Kahn 2004). 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.* (2014) 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 (Bellenger et al. 2014). *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 (Bellenger et al. 2014). This indicates that alternative nitrogenase enzymes have a lower affinity for acetylene gas compared to 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 (Unkovich 2013). Electron allocation varies among the different forms of nitrogenase as well (Schneider and Müller 2004). 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 (Schneider and Müller 2004). V-nitrogenase electron allocation approaches a 50:50 exchange between N_2 reduction and proton reduction (Schneider and Müller 2004).

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 (Miller and Eady 1988). 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; Dilworth et al. 1993). 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.

1.2 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; Carnahan et al. 1960). This substrate requirement is driven by the high demand for ATP by nitrogenase (Mortenson 1964); 16 ATP and 8 electrons are required for the conversion of one N_2 molecule to two NH_3 molecules (Hill 1992). 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 (Stewart 1969). However, plant root exudates, C-rich secretions consisting of low molecular weight compounds such as sugars, organic acids, and mucilage (i.e. polysaccharides) (Bais et al. 2006), are a potential source of C capable of meeting diazotroph energy demands. Root exudation makes the rhizosphere a hotspot for microbial activity (Kuzyakov and Blagodatskaya 2015) 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 (Jones et al. 2009). Of this translocated C, ~12% is typically recovered in the soils as root exudates, root exudate-derived metabolites, and microbial biomass C (Jones et al. 2009). For some prairie grasses, the portion of fixed C recovered from soils can be as much as 15% (Roper et al. 2014). Switchgrass is known to allocate a significant portion of its fixed C belowground. Switchgrass C allocation to roots and soil was measured at 40% and 6%, respectively, one day after a ^{13}C-CO_2 pulse-chase labeling (Chaudhary et al. 2012). Of the 6% fixed C recovered from soils, 92% was found in microbial biomass (Chaudhary et al. 2012) 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. Further, as discussed in a recent review by Bowsher *et al.*, (2018) both quality and quantity of root exudation responds to N availability highlighting the interplay between plant C inputs and soil N availability. 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 (Gupta et al. 2014). There is great need for studies that elucidate the linkages and feedbacks between N availability, plant C exudation rates and FLNF.

Though rhizosphere focused studies are limited, work on FLNF in other regions, particularly bulk soil and litter suggest quality (i.e. form) of C substrates may be just as important as the quantity of C in regulating FLNF (Vitousek et al. 2002). Glucose has been used as a C source for methods assessing N-fixation in the rhizosphere and bulk soil (Gupta et al. 2014) but, methods for isolating and culturing diazotrophs often use other forms of C, like malate, mannitol, and sucrose (Baldani et al. 2014). 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 (Dobereiner and Day 1975). 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 (Dobereiner and Day 1975). 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 (Bais et al. 2006). Switchgrass exudates, for example, were found to contain over 30 different compounds (Smercina et al. 2020) 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. Though 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. Though symbiotic N-fixers can fix more N per gram of cellular material (1.0-2.5 versus 0.1 g N_2 g^{-1} cellular material for diazotrophs; Mulder 1975), free-living diazotrophs live much shorter lives (on the order of hours versus weeks for symbionts) and have 10 times higher nitrogenase activity (25-50 mg N fixed g^{-1} protein hr^{-1} versus 2-5 mg N fixed g^{-1} protein hr^{-1} for symbiotic N-fixation; Mulder 1975). If these rates are averaged over the lifetime of the organism, rhizosphere diazotrophs may match or even exceed fixation by symbionts (Mulder 1975). 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, nutrient availability) are likely to influence the productivity of FLNF.

1.3 OXYGEN CONTROLS ON FREE-LIVING N-FIXATION

Oxygen concentrations in the rhizosphere are dynamic and extremely difficult to measure, therefore most available research of 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 (Blossfeld et al. 2011, Minett et al. 2013), agar (Tschiersch et al. 2012), or using seagrass (*Cymodocea rotundata*) grown in saturated sediments (Pedersen et al. 1998). 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 non-saturated 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 activity of rhizosphere associated microbes (Pedersen et al. 1998). Modeling efforts suggest that diel changes in waterflow through the rhizosphere (Espeleta et al. 2017) that 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 (York et al. 2016). Microsites of very low or relatively high oxygen concentration may also form in the rhizosphere as occurs with roots of aquatic systems (Brune et al. 2000). 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 can discuss physiological responses of diazotrophs to oxygen using research derived predominately from pure culture studies.

Oxygen irreversibly inhibits nitrogenase, even in aerobic organisms (Robson and Postgate 1980). 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 (Reed et al. 2011, Dixon and Kahn 2004). Diazotrophs can also remove oxygen by increasing substrate utilization, which increases respiration rates, thereby decreasing oxygen concentrations (Mulder 1975, Dixon and Kahn 2004). 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 (Liang et al. 2016). 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 (Hill 1992 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 (Hill 1992 and references therein). One study found that upwards of 60% of the energetic costs of N-fixation are indirect costs associated with combating oxygen (Großkopf and LaRoche 2012). This may be especially true when organisms use increased respiration to remove oxygen (Inomura et al. 2017). Patra *et al.* (2007) 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. However, even under high oxygen pressure, if carbohydrate availability is sufficiently high, diazotrophs may still carry out N-fixation (Inomura et al. 2012).

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 (Großkopf and LaRoche 2012). 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.* (2017) found the oxygen optima of *Azotobacter vinelandii* to be 3%, while Großkopf and LaRoche (2012) demonstrated that oxygen concentrations around 5% resulted in significantly higher nitrogenase activity and lower respiration by *Crocosphaera watsonii*, a marine cyanobacteria, compared to ambient oxygen concentrations (20%). 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.

1.4 FREE-LIVING N-FIXATION AND N AVAILABILITY AND FORM

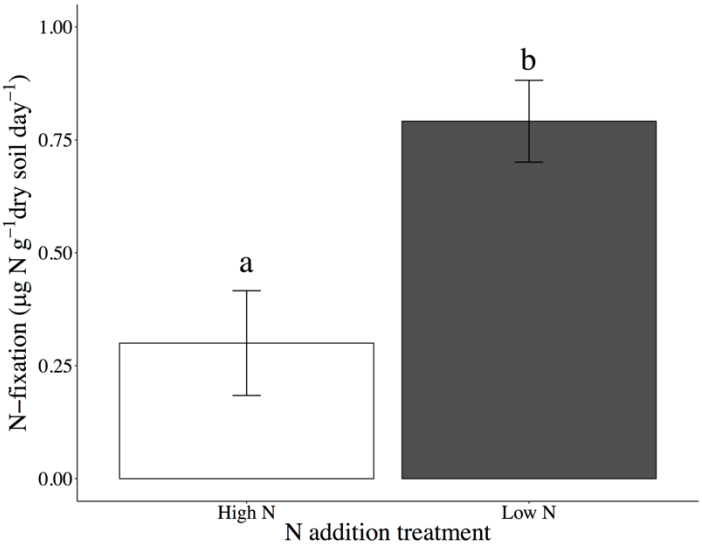

Figure 1.4: **Preliminary N-fixation rates from switchgrass rhizosphere soils** receiving high N additions (High N; +125 kg Urea-N ha^{-1} yr^{-1}) and low N additions (Low N; +25 kg Urea-N ha^{-1} yr^{-1}). Sterile switchgrass (var. Cave-in-Rock) seeds were planted into a sterile sand and vermiculite mixture (50:50 v/v) 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 ½ 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 (1993). N additions did not significantly impact N-fixation rates (p = 0.1585).

As discussed by Reed *et al.* (2011) diazotrophs can access N via N-fixation or through uptake of externally available N sources, which can include both low and high molecular weight organic N sources (Norman and Friesen 2016). Given the energy costs of FLNF, it is generally downregulated by increasing N availability as diazotrophs use external N in favor of fixed N (Reed et al. 2011). 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 (Hobbs and Schimel 1984, Patra et al. 2007, Kox et al. 2016). The

switchgrass rhizosphere is likely to be one such environment as root N uptake creates an N deplete zone in the rhizosphere (York et al. 2016) and switchgrass is thought to be particularly skilled at scavenging N (Fike et al. 2006). In fact, we confirm in our own work that unfertilized switchgrass rhizospheres exhibit greater FLNF rates than fertilized rhizospheres (Fig. 1.4). 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 (Reed et al. 2011) and has been shown to inhibit nitrogenase synthesis at the genetic level through regulation of *nifA* gene transcription (Dixon and Kahn 2004). However, it does not inhibit the activity of already synthesized nitrogenase in most organisms (Mulder 1975). In fact, there are only a few diazotrophs that regulate nitrogenase post-translationally including *Azospirillum brasilense* (Dixon and Kahn 2004). This post-translational regulation is carried out by DraT (dinitrogenase reductase ADP-ribosyltransferase) in response to ammonium and is reversed by DraG (dinitrogenase reductase activating glycohydrolase) (Dixon and Kahn 2004). 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 post-translational regulation may cease enzyme synthesis in response to ammonium, but will continue to have functioning nitrogenase in their cells. Characterizing the presence of post-translational 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 (Huergo et al.

2012), but to date this regulatory system has only been well studied in the photosynthetic bacteria, *Rhodospirillum rubrum* (Halbleib and Ludden 2000).

Glutamine also downregulates nitrogenase synthesis at the genetic level (*nif*A) via a pathway similar to ammonium regulation (Dixon and Kahn 2004). In fact, glutamine may influence ammonium regulation of N-fixation as intracellular glutamine levels regulate GlnD modification of the P_{II} protein, an important cellular N sensor (Huergo et al. 2012). Glutamine and glutamate were found to downregulate N-fixation of *Herbaspirllium serpedicae* (Klassen et al. 1997). 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* (Steenhoudt and Vanderleyden 2000). Conversely, nitrate concentrations of up to 800 µmol had no inhibitory effect on growth or N-fixation by *C. watsonii* (Großkopf and LaRoche 2012).

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

1.5 CONTROLS OF PHOSPHORUS AND MICRONUTRIENTS ON FREE-LIVING N-FIXATION

Availability of phosphorus (P) and micronutrients including Fe, Mo, and V is known to influence N-fixation (Reed et al. 2011). 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, most summarized by Reed et al. (2011), but these have been almost exclusively conducted in bulk soil and leaf litter. 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 (Reed et al. 2007, Reed et al. 2013). FLNF associated with the non-legume tree, *Eucalyptus urophylla* was ~27% higher in soils receiving additions of P compared to the "no-added P" control (Zheng et al. 2016). Further, additions of N and P resulted in similarly boosted FLNF rates (Zheng et al. 2016), 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 (Dynarski et al. 2017). We may expect a similar response in the rhizosphere where root uptake of P can result in a P depleted rhizosphere (York et al. 2016), 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.* (2012) found that limitation of FLNF by Mo and P varied along a P gradient of Panamanian soils. In P-rich soils, Mo was most limiting, but this shifted to a co-limitation of Mo and P in P-poor soils (Wurzburger et al. 2012). However, P alone never limited N-fixation in this system (Wurzburger et al. 2012). 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 (Reed et al. 2013). These contrasting findings may be the result of differences in soil organic matter. Wichard *et al.* (2009) describes how binding of Mo to organic matter can prevent Mo limitation. 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 2D and 3D element mapping have revealed connections between root growth and exudation and micronutrient concentrations, particularly metals, that can control microbial community composition and physiology (Oburger and Schmidt 2016). For example, high concentrations of available iron found at root tips (Williams et al. 2014) 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.

1.6 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 (Gupta and Roper

2010), thus potentially supporting larger populations of N-fixers and/or more efficient N-fixers. Indeed, Gupta and Roper (2010) found more rhizosphere N-fixation in soils with greater clay content, which shifted to greater N-fixation along root surfaces as clay content decreased. 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 (Blossfeld et al. 2011) and therefore, pH may have a different effect on rhizosphere FLNF. For example, pH can change 0.5-1 pH units when moving just 1 mm away from the root surface (Youssef and Chino 1989). We also know that at the field scale, acidic soil pH has been shown to decrease N-fixation rates in aerobic soils (Vitousek et al. 2002). Further, 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 (Wang et al. 2017) suggesting that lower N-fixation in acidic soils may be due to reduced community redundancy. Wang *et al.* (2017) also examined the relative abundances of the three dominant genera in their soils (*Azospirillum, Bradyrhizobium,* and *Mesorhizobium*) across the pH gradient. *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 (Wang et al. 2017). 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.* (2011). 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 (York et al. 2016), which could dramatically alter oxygen dynamics.

1.7 METHODOLOGICAL CONSIDERATIONS FOR STUDYING FREE-LIVING N-FIXATION

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 (Hardy et al. 1968). 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 (Hardy et al. 1968), the actual conversion value can vary from 30 to 1 (Bellenger et al. 2014). 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 (Nohrstedt 1983). Ethylene is an important compound in plant growth and signaling and is produced by both plants and bacteria (Wang et al. 2002). This production is not associated with the presence of acetylene. Controls that account for background ethylene production can be included with the analysis, however these have been shown to result in misrepresentative final rates of acetylene reduction (Nohrstedt 1983). Therefore, we recommend that acetylene reduction not be used for 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 (Gupta et al. 2014). As diazotrophs fix N, the ^{15}N label is incorporated into the soil and can be measured to determine FLNF rates (Gupta et al. 2014). 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. 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‰ (Shearer and Kohl 1986, Boddey et al. 2001). In other words, it is assumed that there is no fractionation during N-fixation. Though symbiotic N-fixation does not result in any fractionation, this is not true of FLNF which has an average fractionation factor of –2.5‰ (Unkovich 2013). 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 (Unkovich 2013). When trying to assess relative contribution of N-fixation to soil N pools, this fractionation must be accounted for in order to accurately assess contribution of FLNF as well as symbiotic N-fixation.

Assessment of diazotroph diversity also comes with challenges and I direct you to Gaby and Buckley (2011) for a detailed description of some of these challenges. In brief, Gaby and Buckley (2011) discuss two major concerns surrounding diazotroph diversity assessment. First, PCR primer selection for the *nifH* can lead to bias in measures of both diversity and relative abundance of amplified organisms (Gaby and Buckley 2011). If using universal PCR primers, it is important to select primer pairs with high coverage, but low phylogenetic bias (Gaby and Buckley 2012). Second, paralogs of *nifH* can lead to a false-positive detection of the *nifH* gene (Gaby and Buckley 2011). Lastly, as discussed earlier, there are several forms of nitrogenase. These isozymes are regulated by different genes – *nifH*, *anfH*, and *vnfH* for Mo-nitrogenase, Fe-nitrogenase, and V-nitrogenase, respectively (Zehr et al. 2003). Thus, it may also be prudent to assess diversity of the *anfH* and *vnfH* genes alongside *nifH*.

1.8 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 (Robertson et al. 2017). However, there is still much we don't 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. 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 results 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, yet we don't know how diazotrophs respond to these conditions. Lastly, it is important to consider the medium in which FLNF is occurring, 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 show to drive changes in microbial community structure and function (Stewart et al. 2017)

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.

REFERENCES

REFERENCES

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. Applied and environmental microbiology 80: 5636-5643.

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.

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 and soil 384: 413-431.

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 Biology and Biochemistry 69: 413-420.

Blossfeld, S., Gansert, D., Thiele, B., Kuhn, A. J., & Lösch, R. 2011. The dynamics of oxygen concentration, pH value, and organic acids in the rhizosphere of Juncus spp. Soil biology and biochemistry, 43(6), 1186-1197.

Boddey RM, Polidoro JC, Resende AS, Alves BJR, Urquiaga S 2001. Use of the 15N natural abundance technique for the quantification of the contribution of N2 fixation to sugar cane and other grasses. Aust J Plant Physiol 28:889–895

Bowsher, AW, Evans, S, Tiemann, LK, & Friesen, ML. 2018. Effects of soil nitrogen availability on rhizodeposition in plants: a review. Plant and Soil, 423(1-2), 59-85.

Boyd E, Peters JW. 2013. New insights into the evolutionary history of biological nitrogen fixation. Frontiers in microbiology 4:201.

Brune, A., Frenzel, P., & Cypionka, H. 2000. Life at the oxic–anoxic interface: microbial activities and adaptations. FEMS Microbiology Reviews, 24(5), 691-710.

Carnahan JE, Mortenson LE, Mower HF, Castle JE. 1960. Nitrogen fixation in cell-free extracts of Clostridium pasteurianum. Biochimica et biophysica acta 44: 520-535.

Chaudhary, D. R., Saxena, J., Lorenz, N., & Dick, R. P. 2012. Distribution of recently fixed photosynthate in a switchgrass plant-soil system. Plant Soil Environ, 58(6), 249-255.

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 99: 1–13.

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.

Dilworth MJ, Eldridge ME, Eady RR. 1993. The molybdenum and vanadium nitrogenases of Azotobacter chroococcum: effect of elevated temperature on N2 reduction. Biochemical journal 289: 395-400.

Dixon R, Kahn D. 2004. Genetic regulation of biological nitrogen fixation. Nature Reviews Microbiology 2: 621-631.

Dobereiner JO, Day JM. 1975. Nitrogen fixation in the rhizosphere of tropical grasses. Nitrogen Fixation by Free-living Micro-organisms (Ed. by WDP Stewart). Cambridge University Press, London.

Dynarski KA, Houlton BZ. 2017. Nutrient limitation of terrestrial free-living nitrogen fixation. New Phytologist 217: 1050-1061.

Espeleta, J. F., Cardon, Z. G., Mayer, K. U., & Neumann, R. B. 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(1-2), 33-51.

Fike, J. H., Parrish, D. J., Wolf, D. D., Balasko, J. A., Green Jr, J. T., Rasnake, M., & Reynolds, J. H. 2006. Long-term yield potential of switchgrass-for-biofuel systems. Biomass and bioenergy, 30(3), 198-206.

Fowler D. Coyle, M., Skiba, U., Sutton, M.A., Cape, J.N., Reis, S., Sheppard, L.J., Jenkins, A., Grizzetti, B., Galloway, J.N. and Vitousek, P. 2013. The global nitrogen cycle in the twenty-first century. Phil Trans R Soc B, 368: 1–13.

Gaby JC, Buckley DH. 2011. A global census of nitrogenase diversity. Environmental Microbiology 13:1790-1799.

Gaby JC, Buckley DH. 2012. A comprehensive evaluation of PCR primers to amplify the nifH gene of nitrogenase. PloS one 7: e42149.

Gaby JC, Buckley DH. 2015. Assessment of Nitrogenase Diversity in the Environment. Pages 209-216 in Frans J. de Bruijn, eds. Biological Nitrogen Fixation. John Wiley & Sons, Inc.

Großkopf T, LaRoche J. 2012. Direct and indirect costs of dinitrogen fixation in Crocosphaera watsonii WH8501 and possible implications for the nitrogen cycle. Frontiers in microbiology 3: 236.

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 and Pasture Science 65:1044-1056.

Gupta VVSR, Roper MM. 2010. Protection of free-living nitrogen-fixing bacteria within the soil matrix. Soil and Tillage Research 109: 50-54.

Halbleib CM, Ludden PW. 2000. Regulation of biological nitrogen fixation. The Journal of Nutrition 130: 1081-1084.

Hardy RWF, Holsten RD, Jackson EK, Burns RC. 1968. The acetylene–ethylene assay for N2 fixation: laboratory and field evaluation. Plant Physiol 43:1185–1207

Hill S. 1992. Physiology of nitrogen fixation in free-living heterotrophs. Biological nitrogen fixation: 87-134.

Hobbs NT, Schimel DS. 1984. Fire effects on nitrogen mineralization and fixation in mountain shrub and grassland communities. Journal of Range Management: 402-405.

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(1), 176-190.

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. The ISME Journal 11: 166-175.

Jones, DL, Nguyen, C, & Finlay, RD. 2009. Carbon flow in the rhizosphere: carbon trading at the soil–root interface. Plant and soil, 321(1-2), 5-33.

Klassen G, Pedrosa FO, Souza EM, Funayama S, Rigo LU. 1997. Effect of nitrogen compounds on nitrogenase activity in Herbaspirillum seropedicae SMR1. Canadian journal of microbiology 43: 887-891.

Kox MA, Lüke C, Fritz C, van den Elzen E, van Alen T, den Camp HJO, Ettwig KF. 2016. Effects of nitrogen fertilization on diazotrophic activity of microorganisms associated with Sphagnum magellanicum. Plant and Soil 406: 83-100.

Kuzyakov, Y., & Blagodatskaya, E. 2015. Microbial hotspots and hot moments in soil: concept & review. Soil Biology and Biochemistry, 83, 184-199.

Liang, C., Jesus, E. D. C., Duncan, D. S., Quensen, J. F., Jackson, R. D., Balser, T. C., & Tiedje, J. M. 2016. Switchgrass rhizospheres stimulate microbial biomass but deplete microbial necromass in agricultural soils of the upper Midwest, USA. Soil Biology and Biochemistry, 94, 173-180.

Miller RW, Eady RR. 1988. Molybdenum and vanadium nitrogenases of Azotobacter chroococcum Low temperature favors N2 reduction by vanadium nitrogenase. Biochemical journal 256: 429-432.

Minett, D. A., Cook, P. L., Kessler, A. J., & Cavagnaro, T. R. 2013. Root effects on the spatial and temporal dynamics of oxygen in sand-based laboratory-scale constructed biofilters. Ecological engineering, 58, 414-422.

Morris DR, Zuberer DA, Weaver RW. 1985. Nitrogen fixation by intact grass-soil cores using 15N2 and acetylene reduction. Soil Biology and Biochemistry 17: 87-91.

Mortenson LE. 1964. Ferredoxin and ATP, requirements for nitrogen fixation in cell-free extracts of Clostridium pasteurianum. Proceedings of the National Academy of Sciences 52: 272-279.

Mulder EG. 1975. Physiology and ecology of free-living, nitrogen-fixing bacteria Nitrogen fixation by free-living micro-organisms. Cambridge University Press: 3-29.

Mus F, Crook MB, Garcia K, Costas AG, Geddes BA, Kouri ED, Udvardi MK. 2016. Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. Applied and environmental microbiology 82: 3698-3710.

Nohrstedt, HÖ. 1983. Natural formation of ethylene in forest soils and methods to correct results given by the acetylene-reduction assay. Soil Biology and Biochemistry, 15(3), 281-286.

Norman JS, Friesen ML. 2016. Complex N acquisition by soil diazotrophs: how the ability to release exoenzymes affects N fixation by terrestrial free-living diazotrophs. The ISME Journal 11: 315.

Oburger, E., & Schmidt, H. 2016. New methods to unravel rhizosphere processes. Trends in plant science, 21(3), 243-255.

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. Journal of Agronomy and Crop Science 193: 153-156.

Pedersen, O., Borum, J., Duarte, C. M., & Fortes, M. D. 1998. Oxygen dynamics in the rhizosphere of Cymodocea rotundata. Marine Ecology Progress Series, 169, 283-288.

Postgate JR. 1982. Biology of nitrogen fixation: fundamentals. Philosophical Transactions of the Royal Society of London B: Biological Sciences 296: 375-385.

Reed SC, Cleveland CC, Townsend AR. 2011. Functional ecology of free-living nitrogen fixation: a contemporary perspective. Annual review of ecology, evolution, and systematics 42: 489-512.

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.

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. Applied Soil Ecology 36: 238-242.

Rees DC, Tezcan F, Haynes C, Walton MY, Andrade S, Einsle O, Howard JB. 2005. Structural basis of biological nitrogen fixation. Phil Trans R Soc A 363: 971-84.

Robertson, G. Philip, Stephen K. Hamilton, Bradford L. Barham, Bruce E. Dale, R. Cesar Izaurralde, Randall D. Jackson, Douglas A. Landis, Scott M. Swinton, Kurt D. Thelen, and James M. Tiedje. 2017. Cellulosic biofuel contributions to a sustainable energy future: Choices and outcomes. Science, 356(6345), eaal2324.

Robson RL, Postgate JR. 1980. Oxygen and hydrogen in biological nitrogen fixation. Annual Reviews in Microbiology 34: 183-207.

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.

Roley, S. S., Duncan, D. S., Liang, D., Garoutte, A., Jackson, R. D., Tiedje, J. M., & Robertson, G. P. 2018. Associative nitrogen fixation (ANF) in switchgrass (Panicum virgatum) across a nitrogen input gradient. PloS one, 13(6), e0197320.

Roper MM, Fillery IRP, Jongepier R, Sanford P, Macdonald LM, Sanderman J, Baldock, JA. 2014. Allocation into soil organic matter fractions of 14C captured via photosynthesis by two perennial grass pastures. Soil Research 51: 748-759.

Ruan L, Bhardwaj AK, Hamilton SK, Robertson GP. 2016. Nitrogen fertilization challenges the climate benefit of cellulosic biofuels. Environmental Research Letters 11: 064007.

Schneider K, Müller A. 2004. Iron-Only Nitrogenase: Exceptional Catalytic, Structural and Spectroscopic Features. Pages 281-307 In Catalysts for Nitrogen Fixation. Springer Netherlands.

Shearer G, Kohl DH 1986. N2-fixation in field settings: estimations based on natural 15N abundance. Aust J Plant Physiol 13:699–756

Smercina, D. N., Bowsher, A. W., Evans, S. E., Friesen, M. L., Eder, E. K., Hoyt, D. W., & Tiemann, L. K. (2020). Switchgrass Rhizosphere Metabolite Chemistry Driven by Nitrogen Availability. *Phytobiomes*

Sprent JI, James EK. 2007. Legume evolution: where do nodules and mycorrhizas fit in?. Plant Physiology 144:575-81.

Steenhoudt O & Vanderleyden J. 2000. Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS microbiology reviews, 24(4), 487-506.

Stewart WDP. 1969. Biological and ecological aspects of nitrogen fixation by free-living micro-organisms. Proceedings of the Royal Society of London Series B, Biological Sciences 172: 367-388.

Stewart, Catherine E., Damaris Roosendaal, Karolien Denef, Elizabeth Pruessner, Louise H. Comas, Gautam Sarath, Virginia L. Jin, Marty R. Schmer, and Madhavan Soundararajan. 2017. Seasonal switchgrass ecotype contributions to soil organic carbon, deep soil microbial community composition and rhizodeposit uptake during an extreme drought. Soil Biology and Biochemistry, 112, 191-203.

Tjepkema JD, Burris RH. 1976. Nitrogenase activity associated with some Wisconsin prairie grasses. Plant and Soil 45: 81-94.

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 Phytologist, 196(3), 926-936.

Unkovich M. 2013. Isotope discrimination provides new insight into biological nitrogen fixation. New Phytologist 198:643-6.

Vitousek PM, Cassman K, Cleveland C, Crews T, Field CB, Grimm NB, Sprent JI. 2002. Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry 57: 1-45.

Vitousek PM, Menge DNL, Reed SC, Cleveland CC. 2013. Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. Philosophical Transactions of the Royal Society of London Series B, Biological Sciences 368.

Wang KL, Li H, Ecker JR. 2002. Ethylene biosynthesis and signaling networks. The Plant Cell 14: s131-51.

Wang Y, Li C, Kou Y, Wang J, Tu B, Li H, Yao M. 2017. Soil pH is a major driver of soil diazotrophic community assembly in Qinghai-Tibet alpine meadows. Soil Biology and Biochemistry 115: 547-555.

Warembourg, F. R. 1993. Nitrogen fixation in soil and plant systems. Nitrogen isotope techniques, 127-156.

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.

Wichard T, Mishra B, Myneni SC, Bellenger JP, Kraepiel AM. 2009. Storage and bioavailability of molybdenum in soils increased by organic matter complexation. Nature Geoscience 2: 625-629.

Williams, P.N., Santner, J., Larsen, M., Lehto, N.J., Oburger, E., Wenzel, W., Glud, R.N., Davison, W. and Zhang, H. 2014. Localized flux maxima of arsenic, lead, and iron around root apices in flooded lowland rice. Environmental science & technology, 48(15), 8498-8506.

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.

York, L. M., Carminati, A., Mooney, S. J., Ritz, K., & Bennett, M. J. 2016. The holistic rhizosphere: integrating zones, processes, and semantics in the soil influenced by roots. Journal of experimental botany, 67(12), 3629-3643.

Youssef, R. A., & Chino, M. 1989. Root-induced changes in the rhizosphere of plants. I. pH changes in relation to the bulk soil. Soil Science and Plant Nutrition, 35(3), 461-468.

Zehr, JP, Jenkins, BD, Short, SM, & Steward, GF. 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environmental microbiology, 5(7), 539-554.

Zheng M, Li D, Lu X, Zhu X, Zhang W, Huang J, 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.

CHAPTER 2:

OPTIMIZATION OF METHODS FOR ASSESSING FREE-LIVING NITROGEN FIXATION

2.1 ABSTRACT

This work aimed to optimize assay conditions of two common methods for measuring potential free-living nitrogen-fixation (FLNF), acetylene reduction assay (ARA) and $^{15}N_2$-incorporation ($^{15}N_2$), for use with soil/rhizosphere samples. I tested the impact of different carbon (C) sources, oxygen concentrations (O_2), and incubation times on FLNF rates of two low-fertility Michigan soils via ARA and $^{15}N_2$. FLNF rates were greatest with addition of a C cocktail, at low O_2, and with 7-day incubations for both methods. FLNF via ARA was 1700x greater with a C cocktail versus glucose only and via $^{15}N_2$ was 17x greater with a C cocktail compared to other C sources and no-C controls. Specific O_2 optimum varied by method and site. A 7-day incubation was needed for the ARA, but a 3-day incubation was suitable for $^{15}N_2$. Lastly, I confirm previously identified issues with the ARA of acetylene-independent ethylene production/consumption resulting in potential FLNF measurement error of 1.3 – 52.3 μg N g^{-1} day^{-1}. I present an optimized method for measuring potential FLNF in soil/rhizosphere samples which will allow for consistent and comparable FLNF rate measurements. Researchers should account for C source, O_2, and incubation time when assessing FLNF and use the ARA method with caution.

Originally published as: Smercina, D. N., Evans, S. E., Friesen, M. L., & Tiemann, L. K. (2019). Optimization of the $^{15}N_2$ incorporation and acetylene reduction methods for free-living nitrogen fixation. Plant and Soil, 445(1), 595-611.

2.2 INTRODUCTION

Free-living nitrogen fixation (FLNF), N-fixation by soil organisms not in symbiotic associations with plants, is prevalent across all ecosystems and contributes a measurable portion of N to terrestrial systems annually (Brouzes et al 1969; Reed et al. 2011). Though FLNF can occur throughout the soil, limitations on carbon (C) availability likely constrain FLNF to C-rich regions such as the rhizosphere (Knowles 1965; Smercina et al. 2019). FLNF is of great interest in agricultural systems, especially low input cropping systems and bioenergy crop production (Roley et al. 2018), because of its potential to offset the need for external N inputs, potentially reducing or even eliminating waste N and the associated negative environmental impacts (e.g. eutrophication and N_2O production; Vitousek et al 1997).

While FLNF has been measured across natural and managed ecosystems (Reed et al. 2011; Roley et al. 2018), the methods employed have not been optimized to account for the wide diversity of diazotrophs (N-fixing organisms) and conditions present in soils and the rhizosphere. The most commonly employed method for measuring FLNF is the acetylene reduction assay (ARA; Hardy et al. 1968) because it is a relatively simple method with low analysis costs. This technique relies on the capacity of nitrogenase, the N-fixing enzyme, to reduce triple bonded molecules other than N_2; thus, acetylene is reduced to ethylene and ethylene concentrations can then be easily measured using a standard gas chromatograph (GC) equipped with a flame ionization detector (Hardy et al. 1968). However, previous work suggests there are challenges to successfully applying the ARA to FLNF, particularly in the rhizosphere (Witty 1979; van Berkum and Bohlool 1980; Boddey 1987; Giller 1987; Smercina et al. 2019).

Nitrogenase has a different affinity for acetylene than N_2, often outcompeting N_2 when in the same system (Brouzes and Knowles 1973), making measures of acetylene reduction only a proxy for N-fixation rates. To overcome this a conversion factor is employed, typically assumed to be three, to estimate N-fixation rates (Hardy et al. 1968). However, this conversion factor can actually range from less than 1 to over 30 depending on the form of nitrogenase (i.e. whether it is the typical nitrogenase with a molybdenum cofactor, or an alternative nitrogenase with a vanadium, or iron cofactor; Bellenger et al. 2014). In a soil, or more specifically the rhizosphere, the potential diversity of N-fixing bacteria, including diverse physiology and different forms of nitrogenase, can have a large impact on the difference between acetylene reduction rates and actual FLNF rates that no single conversion factor can capture (Smercina et al. 2019).

In addition, the measured product of the ARA, ethylene, can be both produced and consumed in soils independent of actual acetylene reduction (van Berkum and Bohlool 1980; Zechmeister-Boltenstern and Smith 1998). Ethylene is a plant hormone with wide-ranging effects and is naturally produced in soils by both plants and bacteria (Witty 1979; Nohrstedt 1983; Wang et al. 2002; Friesen et al. 2011). In fact, a study which added ^{14}C-labeled acetylene to the headspace of soil core incubations found that only 43% of recovered ethylene carried the label, indicating that over half of the ethylene was produced endogenously by soil organisms (Witty 1979). Methanogenic bacteria have been shown to oxidize ethylene via the enzyme methane monooxygenase (de Bont 1976; Boddey 1987; Xin et al. 2017) and though few recent studies have been published on the topic, our calculations of Gibb's free energy for the

oxidation of ethylene to ethylene oxide reveal a favorable and spontaneous reaction at room temperature ($\Delta G° = -81.4$ kJ mol^{-1}). Finally, acetylene may inhibit ethylene oxidation and actually result in overestimation of FLNF as endogenously produced ethylene is not consumed (Witty 1979; van Berkum and Bohlool 1980). Although concerns regarding ethylene production and consumption during the ARA have been raised previously, experimental controls that account for non-acetylene reduction associated ethylene fluxes are often lacking or misrepresentative, which leads to inaccurate estimates of acetylene reduction (Boddey 1987). Despite these issues, the ARA still remains the standard and most accessible method for assessing FLNF, but its use may be weakening our understanding of this process.

In order to truly understand the potential contribution of FLNF to total available N in natural and managed systems, it is important to use a method that measures FLNF directly and has been optimized to account for the diversity of N-fixing microbes and the wide range of environmental conditions they face. The ARA is an indirect method for assessing N-fixation that was originally designed and optimized for assessing symbiotic N-fixation by rhizobia in association with legumes. ARA is suited for measurement of symbiotic N-fixation because N is fixed at high rates so that measurement duration can be short and all rhizobia utilize the molybdenum cofactor form of nitrogenase under microaerobic metabolism. In contrast, the $^{15}N_2$ incorporation method directly assesses N-fixation by measuring the difference in ^{15}N abundance of samples exposed to $^{15}N_2$ and reference samples (Boddey 1987; Warembourg 1993; Weaver and Danso 1994). This method is more accurate and direct than the ARA (Myrold et al. 1999), though not without potential issues, such as contamination via ^{15}N-nitrate, ammonium, and nitrous

oxide of the $^{15}N_2$ gas source (Dabundo et al. 2014). This contamination results in assimilation of ^{15}N unrelated to FLNF and can occur at rates greater than or equal to $^{15}N_2$ incorporation, thus leading to overestimates of FLNF (Dabundo et al. 2014). While $^{15}N_2$ incorporation has predominately been used to determine the conversion factor needed for calculating FLNF rates from acetylene reduction rates (Myrold et al. 1999), it has not been optimized to assess FLNF of whole soil communities.

Optimization of either ARA or $^{15}N_2$ incorporation methods to assess FLNF requires consideration of the diversity in organisms and growth strategies of bacteria performing FLNF. In particular, there is need for a method that measures FLNF potential in an informative way, based on conditions which may be expected in the study system (e.g. rhizosphere). Previous studies have recommended providing glucose as a C source to optimize N-fixing conditions (Brouzes et al. 1969; Brouzes and Knowles 1973; Gupta et al. 2014; Roley et al. 2018), however the form of C which is optimal for FLNF likely varies by the organisms present (Smercina et al. 2019). Therefore, it is important to find a C source that can support whole diazotroph communities rather than specific populations. Potentially favorable C sources may include sucrose, malic acid or citric acid, all of which are used to isolate diazotrophs from the environment (Baldani et al. 2014), as well as mannitol and calcium lactate which have been used in previous studies when assessing FLNF (O'Toole and Knowles 1973; Patriquin and Knowles 1975). Additionally, soil niche spaces are extremely heterogenous with regards to water content, gas exchange and nutrient availability. This likely influences the diazotroph community composition and therefore, the optimal conditions for FLNF.

In this study, we optimize conditions which support the greatest potential acetylene reduction via the ARA and potential FLNF via the $^{15}N_2$ incorporation method. The goal of this work was to identify conditions under which FLNF can be routinely measured, relatively quickly and easily, with robust results that capture differences in the diazotroph community inked to edaphic factors. Specifically, we choose target conditions which mimic those that may be expected in the rhizosphere to provide "realistic" potential rates. We assess different C sources, known to support diazotroph growth, to determine which supports the most N-fixation. FLNF is also influenced by oxygen availability with nitrogenase being irreversibly inhibited by oxygen presence (Robson and Postgate 1980). Yet, many diazotrophs cannot grow under strictly anaerobic conditions and anaerobic incubations may not be appropriate (Silvester et al 1982; Smercina et al. 2019). Therefore, it is also necessary to determine optimal oxygen concentrations (O_2) for FLNF. Another important aspect of these assays is incubation time. Though no single incubation time is standard for assessing N-fixation rates, shorter incubations should be more favorable for limiting changes in microbial community structure (Weaver and Danso 1994; Goldfarb et al. 2011; Oliverio et al. 2017). In this work, we investigate different incubation lengths to determine the shortest incubation time that yields detectable FLNF rates. Lastly, we test for issues with the ARA including background ethylene production and consumption to determine its suitability for soil and rhizosphere samples. Overall, we present an optimized method of assessing potential FLNF rates which attempts to account for and incorporate the wide diversity of diazotrophs and their growth requirements. This is extremely important if we

want to systematically study FLNF across systems in order to better understand factors controlling diazotrophs and rates of FLNF.

2.3 MATERIALS AND METHODS

2.3.1 Sample Collection

Soils were collected from two Michigan field sites, Lux Arbor (LUX; 42.476365, -85.451887) and Lake City (LC; 44.296098, -85.199612), with different soil texture, climate, and land use history (Table 2.1). These field sites are maintained as part of the Great Lakes Bioenergy Research Center's marginal land experiment (https://www.glbrc.org/). Each field site has four replicate split plots of switchgrass (*Panicum virgatum* L.; cv. Cave-in-Rock) monoculture which have been maintained since 2013. Switchgrass is known to associate with diazotrophs (Bahulikar et al 2014) and FLNF has been measured in association with the switchgrass rhizosphere (Roley et al 2018; Smercina et al unpublished data). Split plots are divided into fertilized (+ 56 kg urea-N ha^{-1} yr^{-1}) and unfertilized (no added N) halves. We sampled both fertilized and unfertilized soils to allow us to optimize assay conditions for high and low N across different soil and climate conditions.

Because the ARA is relatively more accessible due to lower costs with regards to materials and analysis equipment, we conducted our initial optimization efforts using this method (described below). For ARA tests, soils were collected using a shovel to a depth of 10 cm from edges of switchgrass plots in July of 2016. Soils were also collected from plot edges in March of 2017 for comparison of ethylene production and consumption on fresh versus stored soils (see below). Soils were kept cool until

returning to the lab where they were stored at 4 °C until analysis. Soils were sieved (4 mm mesh) and homogenized prior to analysis.

Table 2.1: Site characteristics including soil properties, climate, and land use history for Lux Arbor (LUX) and Lake City (LC)

Site	Soil Taxonomy	pH	P (ppm)	Total N (%)	Total C (%)	30-yr avg. precipitation (mm)	30-yr avg. temp. (˚C)	Previous land use	Pre-settlement vegetation
LUX	Typic Hapludalfs (Alfisol)	5.8	12	0.06	0.77	842.0	9	Idle for 20 years	Oak-hickory forest
LC	Oxyaquic Haplorthod (Spodosol)	7.3	24	0.06	0.92	812.3	6.5	Unimproved pasture	Hemlock-white pine forest

Note: Data provided by Great Lakes Bioenergy Research Center (GLBRC) marginal land experiment (https://lter.kbs.msu.edu/research/long-term-experiments/marginal-land-experiment/) and Kasmerchak and Schaetzl (2018)

$^{15}N_2$ incorporation method optimization was conducted on intact soil cores collected using a 1.5 cm diameter turf corer to a depth of 5 cm (Fig. S2.1). Cores were collected in March of 2017 (incubation time test only, see below) and 2018 (C source and oxygen concentration tests, see below). These intact soil cores were sized to fit within 20 ml gas vials (Wheaton, DWK Life Sciences, Millville, NJ, USA), so the samples could be immediately transferred to their incubation vials at the time of collection in order to minimize soil disturbance between collection and analysis (Fig. S2.1). Ten replicate cores were collected from three randomly chosen points within each split plot for a total of 240 cores per field site. We also collected four reference cores, one per replicate block, per site. Cores were collected, immediately transferred to vials and placed in a cooler until returning to the lab. Cores were stored at 4 ˚C until analysis and all assays were started within 72 hours of sample collection. Extra soil was also collected for measurement of soil moisture and water holding capacity (WHC; described below).

2.3.2 Soil moisture and water holding capacity

Soils were sieved (4 mm mesh) prior to soil moisture and WHC analysis. Soil moisture was determined on all soils (2016, 2017, and 2018) using 5 g of soil dried at 60 ˚C for at least 24 hours. WHC was determined using 10 g of field moist soil. Soil was placed inside a funnel and saturated. Soils were covered with plastic wrap to prevent evaporation; three small holes were poked in the top of the plastic wrap to maintain atmospheric pressure within the funnel and flask. Soils were allowed to drain for 24 hours, then weighed to determine 100% WHC.

2.3.3 Acetylene reduction

Optimization of the ARA was conducted in January 2017 on soils collected in July of 2016 (Hardy et al. 1968; Myrold et al. 1999). Soils were stored at 4˚C from time of collection to time of analysis, for a total of 5 months. While this storage time is likely to reduce microbial activity and therefore may reduce ARA rates, methodological comparisons between samples which have experienced similar conditions should not be hindered and are still valid for this methods study. Ten grams of sieved soils, collected in July 2016, were weighed into 4 oz. (127 ml) mason jars (Ball® Corp., Broomfield, CO, USA). Jars were loosely capped and pre-incubated at 25 °C for one week prior to analysis to revive the microbial community after long cool-storage. During the pre-incubation, 100 µg glucose C g^{-1} dry soil were added each day, a rate of C input matching that expected in the rhizosphere (Baudoin et al. 2003), to support the microbial community. At the start of the analysis, 4 mg C ml^{-1} glucose solution was added to the soils in a ratio of 1 ml solution to 1 g dry soil, matching the rate used by Gupta et al. (2014) in a similar study of FLNF in grasses. This rate of C addition results

in the equivalent of ~570 µg of C per day over 7 days which is approximately 5x the amount which may be available *in situ*. Though greater than what might be expected *in situ*, these C additions ensure C limitation does not occur. After C addition, jars were then tightly sealed and 10% of the headspace was replaced with acetylene generated in the lab from additions of calcium carbide to nanopure water. Addition of the acetylene gas marked incubation start time. ARA optimization test variables included incubation time, oxygen concentration, C source, and checks of background ethylene production and consumption (Table 2.2).

Table 2.2: Table of experimental conditions for optimization tests

Optimization	Method	Incubation times	Oxygen concentration (%)	Carbon source	Corresponding Figure
Incubation time	ARA	1, 4, 6, 18, 24, 168 hours	0, 5, 10, 20	Glucose	Fig. 2.1
	$^{15}N_2$	1, 3, 7 days	10	C cocktail	Fig. 2.8
Oxygen concentration	ARA	1, 4, 6, 18, 24, 168 hours	0, 5, 10, 20	Glucose	Fig. 2.2
	$^{15}N_2$	3 days	0, 5, 10, 20	C cocktail	Fig. 2.7
Carbon source	ARA	18 hours	0, 5, 10, 20	Glucose, glucose + sucrose + malate	Fig. 2.3
	$^{15}N_2$	3 days	10	Water, sucrose, sucrose + malate, C cocktail	Fig. 2.5

To test incubation time, jars were sampled 1, 4, 6, 18, 24, and 168 (7 days) hours after incubation start for LUX soils and 6, 18, 24, and 168 hours after incubation start for LC soils. At each time point, 10 ml of gas were withdrawn from each jar and injected into pre-evacuated 10 ml gas vials (Thomas Scientific, Swedsboro, NJ, USA). We used

two jars per sample to ensure no more than three gas samples were taken from one jar. Thus, a total of 80 jars (5 replicates * 4 oxygen concentrations * 2 site * 2 jars) were used to measure ARA rates at each of the six time points representing a total of 40 samples. It is important to note that 30 ml of gas were removed at each sampling, without replacement. This represents a removal of ~24% of the total headspace. While this headspace removal does not affect the comparison of relative rates, as all samples were treated the same, measures of actual ARA may be negatively impacted. When headspace is removed and not replaced, resulting back pressure can cause an influx of air that dilutes ethylene and acetylene concentrations within the jars. When measuring actual ARA rates, researchers should be sure to replace the volume of sampled headspace with He or other appropriate gas to maintain pressure and account for the resulting dilution. Gas samples were analyzed for ethylene concentration on a Trace™ 1310 gas chromatograph equipped with a flame ionization detector (Thermo Fisher, Waltham, MA, USA). Acetylene reduction rates were calculated as the difference in ethylene gas concentration between each time point and the first sampling point divided by the incubation time (in days) and grams of dry soil (μg C_2H_4 g^{-1} dry soil day^{-1}).

The effect of oxygen concentration on acetylene reduction was tested on replicate jars at 0, 5, 10, and 20% oxygen. These oxygen concentrations were chosen with consideration to soil and rhizosphere conditions, microbial growth optima, and N-fixation optima. Soil and rhizosphere oxygen concentrations are highly variable, making it difficult to mimic soil/rhizosphere conditions with just one oxygen concentration. For example, some rhizospheres are oxygen-rich (Pedersen et al. 1989; Blossfeld et al. 2011), while others are oxygen-depleted (Tschiersch et al. 2012; Minett et al. 2013). It is

also difficult to optimize oxygen concentrations for microbial growth and N-fixation because nitrogenase is deactivated by oxygen, but many diazotrophs are aerobic organisms requiring at least some oxygen to grow (Bottomley and Myrold 2015). Therefore, the chosen oxygen concentrations span a range of potential oxygen optima from anaerobic to ambient. To create these concentrations, jars were evacuated via vacuum manifold and the headspace replaced with Ultra High Purity Helium (UHP-He). Then, 5.6, 11.2, and 22.4 ml of headspace gas were removed and replaced with identical amounts of pure O_2 to create 5, 10, and 20% oxygen concentrations, respectively. No headspace gas was replaced with O_2 in the 0% oxygen treatment. Finally, we replaced 10% of the jar headspace with acetylene. Gas samples were collected from jars at 1, 4, 6, 18, 24, and 168 hours after acetylene addition and analyzed as described above. Each oxygen concentration was replicated five times and acetylene reduction rates were calculated as described above.

We also tested the impact of C source on acetylene reduction rates. A second set of LUX soil jars receiving the oxygen concentration treatments described above were duplicated. These soils received a C cocktail containing glucose, sucrose, and malic acid represented in equal proportions based on C content. These C sources were chosen based on recommendations for isolating diazotrophs from soils (Baldani et al. 2014) and all represent compound groups which are found in the rhizosphere (i.e. carbohydrates and organic acids; Baudoin et al. 2003). Though many studies only use glucose as a C source (Gupta et al. 2014), diazotrophs are a diverse community and are isolated using a wide variety of C sources. We attempted to simulate the diversity of C sources available in the rhizosphere using a mixture of C compounds. The 4 mg C ml^{-}

[1] solution was added as described above and jars were incubated for 18 hours. Gas samples were collected and analyzed as described above.

Lastly, we examined the potential for background ethylene production and consumption in our soils from LUX. Ten grams of soil collected in July 2016 (stored) or March 2017 (fresh) were weighed into ten replicate jars. Jars containing soil from July 2016 were pre-incubated as described above, while jars containing soil from March 2017 were pre-incubated at room temperature for 24 hours prior to incubation start. After pre-incubation, a glucose solution was added as described above and jars were sealed. To test background production, five replicate jars were incubated without addition of acetylene gas. To test background consumption, 10% of the headspace in five replicate jars was replaced with an ethylene standard, but no acetylene was added. Gas samples were taken 6, 18, and 24 hours and analyzed as described above. Results from these samples are reported as μg C_2H_4 g^{-1} dry soil.

2.3.4 $^{15}N_2$ incorporation method

FLNF rates were measured based on net changes in ^{15}N of soil incubated with $^{15}N_2$ gas (Gupta et al. 2014). Cores were pre-incubated at room temperature for 24 hours prior to ^{15}N analysis to ensure a fully active microbial community at the time of $^{15}N_2$ addition. Following pre-incubation, C source (or water) was added to each core using a syringe and 21-gauge needle to bring the cores to 60% WHC. The syringe and needle allowed the solution to be more evenly distributed along the intact core. Vials were then capped with aluminum rings and septa and evacuated. Immediately following evacuation experimental vials received 1 mL of 98 atom% acid-washed $^{15}N_2$ gas (Sigma-Aldrich, Inc., St. Louis, MO, USA) and reference vials received UHP-N_2. This

results in a pN_2 of ~1.87 atm, more than sufficient to saturate N-fixing sites. Knowles (1980) recommends achieving 0.8 to 1.0 atm pN_2 and a minimum of 0.4 atm. $^{15}N_2$ was acid washed with 5% sulfuric acid prior to addition to vials to ensure no contamination with ^{15}N-NH_3 and other gases. Following N_2 addition, oxygen was added to the vials to achieve the appropriate concentration (see below). Vial atmospheres were balanced with UHP-He. Samples were incubated at room temperature for one, three, or seven days. After incubation, vials were uncapped and samples were placed in a 60 °C drying oven for 48 hours. After drying, samples were ground into a fine powder on a roller mill, weighed into tin capsules, and then analyzed following standard procedures at Washington State University's Stable Isotope Core Laboratory (Pullman, WA). Briefly, tinned samples were combusted to N_2 with an ECS 4010 elemental analyzer (Costech Analytical, Valencia, CA) and analyzed on a Delta PlusXP continuous flow isotope ratio mass spectrometer (Thermofinnigan, Bremen) equipped with a 3 m GC column. FLNF rates were calculated in μg N fixed g^{-1} dry soil day^{-1} as:

$$\frac{AE_i \times TN_i}{AE_{atm} \times t}$$

where AE_i represents atom percent access of sample against an unenriched reference sample, TN_i represents total nitrogen content in sample, AE_{atm} represents atom percent excess in the vial atmosphere (98 atom% in our case), and t is incubation time in days (Warembourg 1993; Roley et al. 2018).

2.3.5 $^{15}N_2$ incorporation carbon source testing

We tested the impact of three C sources including sucrose, sucrose plus malic acid and a C cocktail, and a no-C (water) control on FLNF rates on intact cores

collected in March of 2018 (Table 2.2). For these C source tests, we expanded on the ARA work by including both a no-C control and a 2-C source mixture in order to more thoroughly understand the role of C source in stimulating FLNF. In place of a glucose-only treatment, we chose to use sucrose as it is metabolized to glucose and is a favored C source for diazotrophs (Baldani et al. 2014). Our C cocktail contains glucose, sucrose, malic acid, and citric acid adjusted to a pH of 7 using potassium hydroxide. Each source in the C cocktail provides an equivalent amount of C (1 mg C g^{-1} dry soil). C sources were added at concentration of 4 mg C g^{-1} dry soil (Gupta et al. 2014). All solutions were added in a volume that brought soil moisture content to 60% water holding capacity.

2.3.6 $^{15}N_2$ incorporation C solution pH

Checks of C solution pH revealed significant decreases in pH following addition of organic acids (malate and citrate). Solution pH when unbuffered was ~3.0 (data not shown). We wanted to test the impact on FLNF of adding buffered versus unbuffered C cocktail to soils. Thirty-six extra intact cores were sampled from fertilized halves of split-plots (9 cores per split-plot) at LUX. Samples were divided into three treatments (n=12 per treatment): buffered C cocktail, unbuffered C cocktail, or water control. The pH of the buffered C cocktail was adjusted as described above, while unbuffered C cocktail pH was not altered. C cocktail or water was added to cores as described above and FLNF rates were assessed following the $^{15}N_2$ incorporation method described above.

2.3.7 $^{15}N_2$ incorporation oxygen concentration testing

We tested four oxygen concentrations including, anaerobic (0%), 5%, 10% and 20% on intact cores collected in March of 2018 (Table 2.2). UHP-O_2 was added to

evacuated vials following addition of N_2 to bring atmospheric oxygen concentrations to one of the four concentrations above. Oxygen concentration tests were conducted on soils provided a C cocktail as the C source and were incubated for three days.

2.3.8 $^{15}N_2$ incorporation incubation time testing

To determine how long samples should be exposed to $^{15}N_2$, we tested incubation times of one, three, and seven days on intact cores collected in March of 2017 (Table 2.2). Longer incubations can result in large microbial community shifts which can bias results (Weaver and Danso 1994). Further, longer incubations increase the probability of fixed ^{15}N being denitrified and thus lost from the soil. Alternatively, incubations that are too short may result in too little ^{15}N being incorporated into the soil and thus not obtaining measurable differences between enriched and reference soils. Incubation test cores were provided C cocktail as the C source and received 10% oxygen.

2.3.9 Data Analysis

Results from ARA testing were analyzed by one-way ANOVA followed by Tukey's post hoc with test variable (i.e. incubation time, oxygen concentration, C source) as a fixed effect using the R *stats* package (R core team 2018). Where applicable, test variables were analyzed individually by site. Differences between test variable groups were considered significant at $\alpha \leq 0.05$. Ethylene production and consumption results were analyzed by one-way ANOVA followed by Tukey's post hoc.

Results of ^{15}N optimization tests were analyzed by two-way ANOVA with Tukey's post hoc. Analysis was carried out by site with test variables (i.e. C source, oxygen concentration or incubation time) as a fixed effect and fertilizer treatment as a random effect nested within field block using the *nlme* R package (Pinheiro et al. 2018). Site

was a significant factor ($p < 0.05$), therefore all analyses of test variable were carried out separately for each site. Fertilizer treatment was not significant, therefore we pooled results from all samples within a site such that each test variable group is represented by n=24. Significant differences between test variable groups were considered significant at $\alpha \leq 0.05$. C cocktail buffering data was analyzed by one-way ANOVA with Tukey's post hoc with buffering treatment as a fixed effect.

Due to differences in collection date and sample handling, no statistical analyses were performed comparing ARA and $^{15}N_2$ results. We also did not perform statistical analyses comparing $^{15}N_2$ results for intact cores collected in March 2017 to March 2018.

2.4 RESULTS

2.4.1 Acetylene Reduction

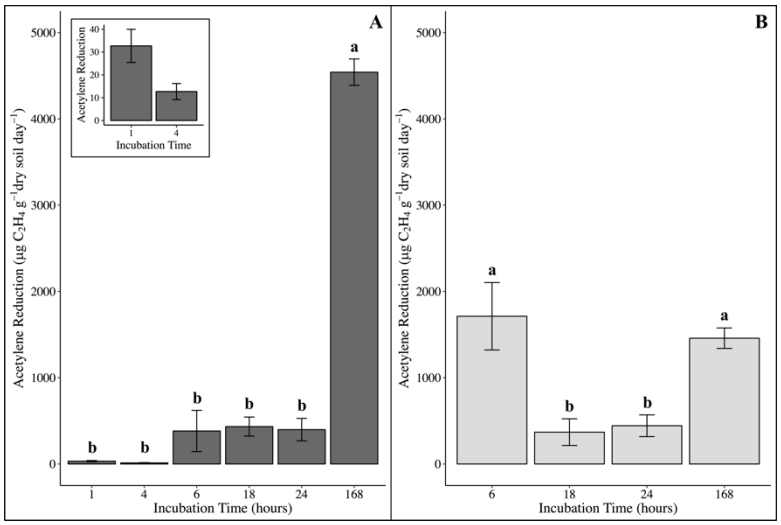

Figure 2.1: **Acetylene reduction at different incubation times** (hours) in µg ethylene (C_2H_4) g^{-1} dry day^{-1} rates for (A) LUX and (B) LC. Bars shows average acetylene reduction (n = 20) with standard error bars. Lowercase letters indicate significant differences between incubation times at $p \leq 0.05$. Inset figure provides a zoom in on 1- and 4-hour incubation time at LUX. Samples were incubated at 0, 5, 10, and 20% oxygen concentrations with glucose as a carbon source.

Optimization of the ARA included testing of incubation time, oxygen concentration, C source, and ethylene background controls. Acetylene reduction rates

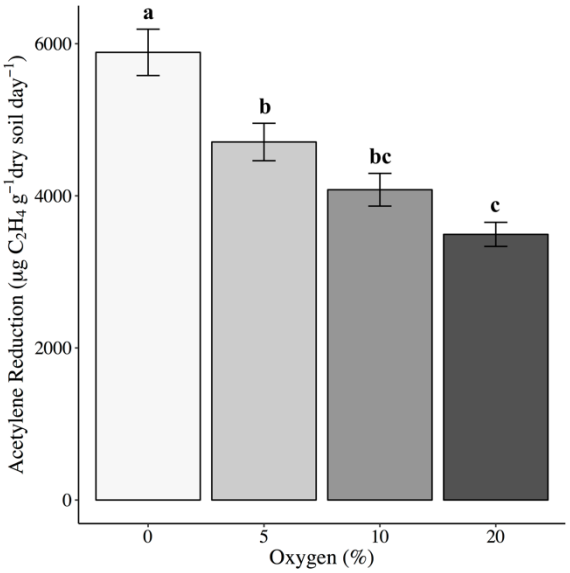

Figure 2.2: Acetylene reduction at different oxygen concentrations (%) in µg ethylene (C_2H_4) g^{-1} dry soil day^{-1} rates for LUX at 7-day incubation time point. Bars represent average acetylene reduction (n = 5) with standard error bars. Lowercase letters indicate significant differences at $p \leq 0.05$. Samples were incubated for 7 days with glucose as a carbon source.

were measured at 1, 4, 6, 18, and 24 hours and 7 days after incubation start (Fig. 2.1). Acetylene reduction rates did not differ significantly at LUX for the first 24 hours. After 7 days, acetylene reduction rates were significantly greater than all other time points for LUX ($P < 0.0001$). There was a spike in rates after 6 hours of incubation at LC. Acetylene reduction rates after 6 hours and 7 days of incubation were not significantly different and were both greater than all other measured time points. Acetylene reduction rates were also tested under oxygen concentrations of 0, 5, 10, and 20%. Testing of O_2 was done at all incubation times, but there was no relationship between oxygen concentration and acetylene reduction rates before 7 days of incubation. However, using a 7-day incubation, we found that acetylene reduction rates decreased steadily with increasing oxygen concentration with 0% having the greatest reduction rates and 20% have the lowest (Fig. 2.2). 10% oxygen concentration was not significantly different from 5% or 20%. C source tests were conducted at all tested oxygen concentrations

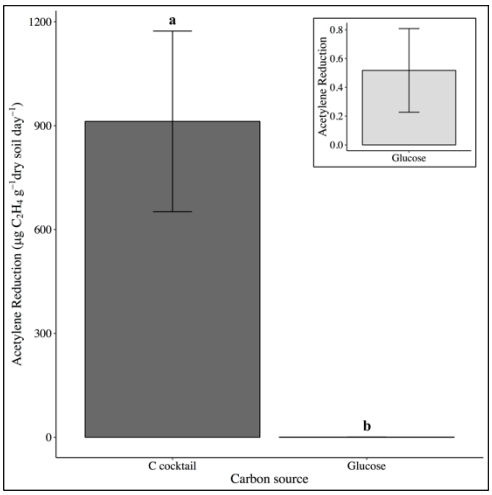

Figure 2.3: Acetylene reduction rates with addition of different C sources in μg ethylene (C_2H_4) g^{-1} dry soil day^{-1}. Bars represent average acetylene reduction (n = 20) with standard error bars. Lowercase letters indicate significant differences at $p \leq 0.05$. Inset figure provides zoom in on glucose addition treatment. Samples were incubated for 18 hours at 0, 5, 10, and 20% oxygen.

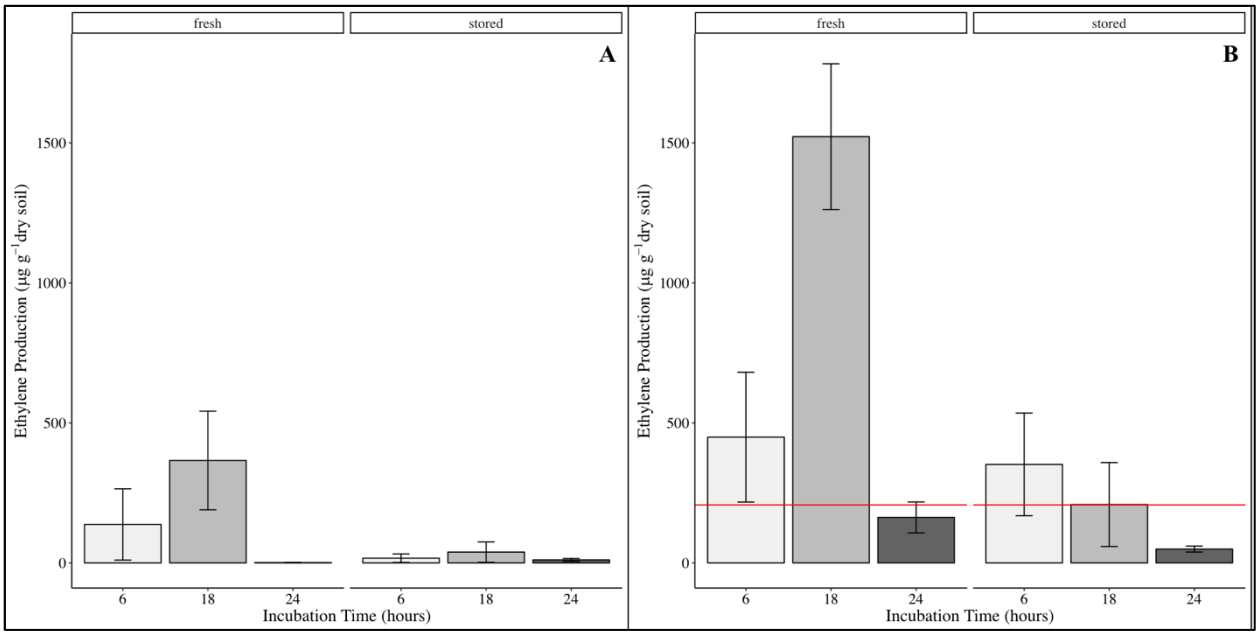

Figure 2.4: Background ethylene (A) production and (B) consumption of fresh and stored soil samples incubated with addition of ethylene standard. Bars represent average ethylene concentration in μg ethylene g^{-1} dry soil (n = 5) with standard error bars. Horizontal line represents initial ethylene concentration (206.8 μg ethylene g^{-1} dry soil; B only). Lowercase letters indicate significant differences at $p \leq 0.05$.

over an 18 h incubation. Because there was no effect of oxygen concentration, we report averages across all oxygen concentrations. Acetylene reduction rates with

glucose addition were very low, averaging ~0.5 μg C_2H_4 g^{-1} dry soil day^{-1} (Fig. 2.3). On average, rates were over 1700 times higher when C cocktail was added versus glucose alone (P = 0.00122). Lastly, we examined soil samples for background production or consumption of ethylene. Soils incubated without addition of acetylene still produced measurable concentrations of ethylene, and this was not significantly affected by incubation time (Fig. 2.4A). Incubations of soils that included additions of ethylene gas, but not acetylene demonstrated both production and consumption of ethylene, with significant differences in ethylene concentration over time (Fig. 2.4B). These changes in ethylene concentrations through time would equate to acetylene reduction rates ranging from 3.8 to 157.0 μg C_2H_4 g^{-1} soil day^{-1} or using 3 as a conversion factor, N-fixation rates of 1.3 to 52.3 μg N g^{-1} soil day^{-1}.

2.4.2 $^{15}N_2$ Incorporation Carbon Source

We tested the effect of four different C sources in the following combinations: sucrose, sucrose plus malate, and the C cocktail (glucose, sucrose, malic acid, and

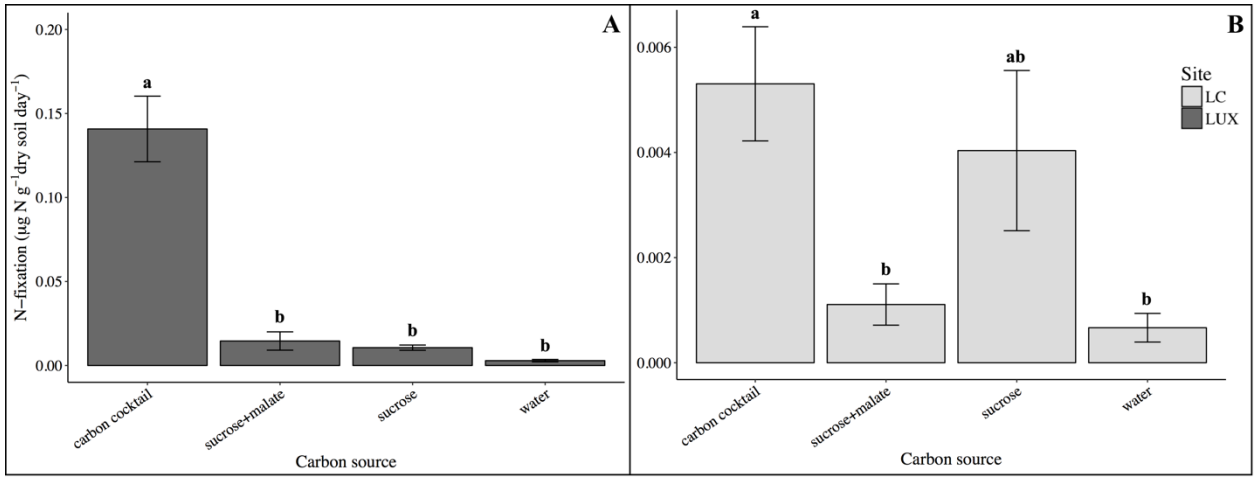

Figure 2.5: **$^{15}N_2$ incorporation rates with additions of different C sources** at (A) LUX and (B) LC. Bars represent average N-fixation in μg N g^{-1} dry soil day^{-1} (n = 24) with standard error bars. Lowercase letters indicate significant differences at $p \leq 0.05$. Samples were incubated at 10% oxygen for three days.

citric acid), on rates of FLNF over a 3-day incubation at 10% O_2 relative to a no-C control. Additions of C cocktail stimulated the greatest FLNF at both field sites.

FLNF rates were significantly greater under additions of the C cocktail ($P < 0.0001$), than other C sources or no-C controls additions at LUX (Fig. 2.5A). C cocktail additions were 13.3x and 9.7x greater than sucrose ($P < 0.0001$) or sucrose plus malate ($p < 0.0001$) additions respectively. FLNF rates under the C cocktail treatment were 48.7x greater than the no-C treatment ($P < 0.0001$). There were no significant differences in FLNF rates between no-C controls and sucrose or sucrose plus malate treatments at LUX.

In LC soils, additions of C cocktail resulted in 7.96x greater FLNF than no-C controls ($P = 0.00348$) and 4.8x greater FLNF than sucrose plus malate ($P = 0.01026$; Fig. 2.5B). FLNF rates with C cocktail additions were 1.3x greater than for sucrose

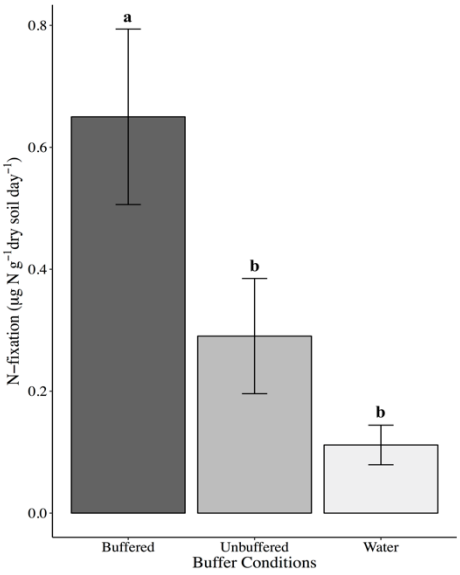

Figure 2.6: **Effect of buffering C cocktail on $^{15}N_2$ incorporation** at LUX. Bars represent average N-fixation in μg N g^{-1} dry soil day^{-1} (n = 12) with standard error bars. Lowercase letters indicate significant differences at $p \leq 0.05$. Samples were incubated at 10% oxygen for three days.

additions, but this increase was not significant. FLNF rates were not significantly different between sucrose, sucrose plus malate, and no-C treatments.

We also tested the importance of buffering the pH of the C cocktail before adding it to the soils. Checks of C cocktail solution pH revealed that the presence of malic acid decreased pH to acidic levels near 2-3 (*data not shown*). To test whether this pH change impacted FLNF rates, we measured the impact of buffered (pH ≈ 7.0) versus unbuffered (pH ≈ 3.0) C cocktail relative to no-C controls on FLNF for a subset of samples from LUX (n = 12 per treatment). Buffering the C cocktail resulted in 2.2x greater FLNF rates than unbuffered C cocktail (P = 0.04373) and 5.8x greater FLNF rates than no-C controls (P = 0.00185; Fig. 2.6). FLNF rates from unbuffered C cocktail additions were not significantly different from the no-C treatment (P = 0.4339).

2.4.3 $^{15}N_2$ incorporation Oxygen Concentration

We tested the effect of four oxygen concentrations on FLNF rates ranging from anaerobic (0% oxygen) to ambient (20%) during a 3-day incubation with C cocktail additions. Optimal oxygen concentration varied between sites, with 10% oxygen being most favorable at LUX and 5% oxygen being most favorable at LC (Fig. 2.7).

FLNF rates at LUX were greatest under 10% oxygen ($P < 0.0001$) followed by 20% oxygen (Fig. 2.7A). 10% oxygen concentrations resulted in 5.8x greater FLNF compared to 0% O_2 ($P < 0.0001$) and 5.6x greater FLNF compared to 5% O_2 ($P < 0.0001$). FLNF rates under 0% and 5% oxygen did not differ significantly from 20% oxygen.

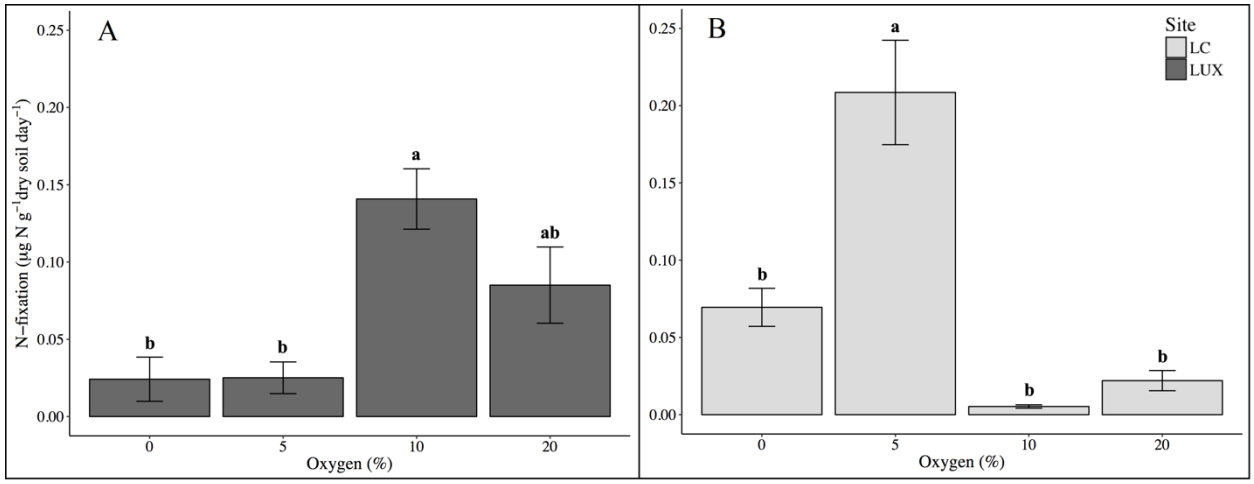

Figure 2.7: $^{15}N_2$ incorporation rates at different oxygen concentrations (%) at (A) LUX and (B) LC. Bars represent average N-fixation in μg N g^{-1} dry soil day^{-1} (n = 24) with standard error bars. Lowercase letters indicate significant differences at $p \leq 0.05$. Samples were incubated with carbon cocktail as the carbon source for three days.

5% oxygen resulted in significantly greater FLNF rates than all other test oxygen concentrations at LC (Fig. 2.7B). FLNF rates were 3x and 9.5x greater under 5% oxygen than 0% ($P < 0.0001$) and 20% ($P < 0.0001$) oxygen, respectively. 5% oxygen resulted in 39.3x greater FLNF rates than those measured under 10% oxygen ($P < 0.0001$) at LC. There were no significant differences in FLNF rates between 0%, 10%, or 20% oxygen concentrations.

2.4.4 $^{15}N_2$ incorporation Incubation Time

We examined FLNF rates at three different incubation times (1, 3, and 7 days). FLNF rates were greatest after 7 days of incubation at both LUX ($P < 0.0001$; Fig. 2.8A) and LC ($P < 0.0001$; Fig. 2.8B). At LUX, a 7-day incubation resulted in 164x greater

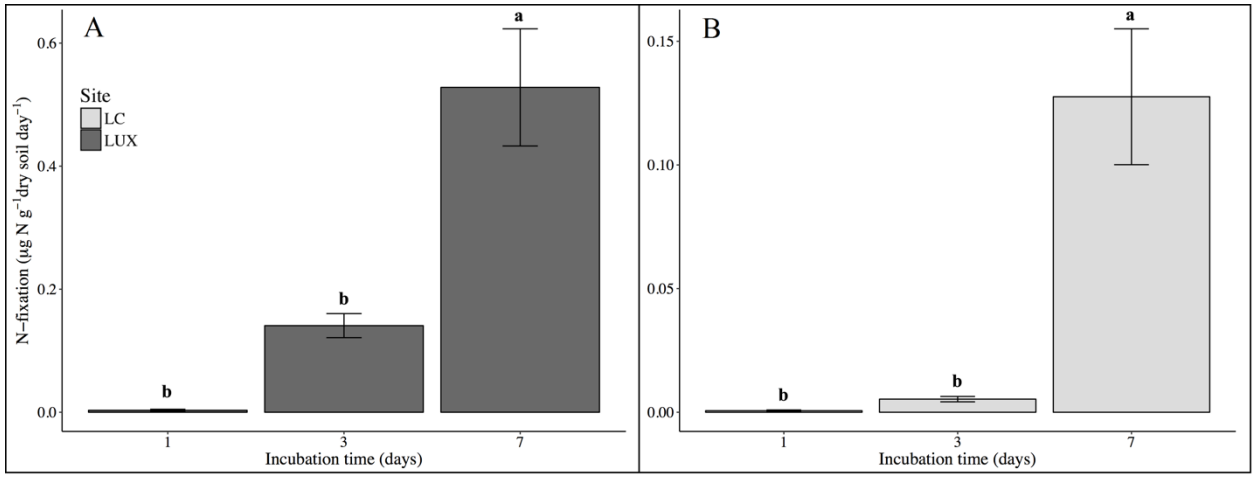

Figure 2.8: $^{15}N_2$ incorporation rates at different incubation times (days) at (A) LUX and (B) LC. Bars represent average N-fixation in μg N g^{-1} dry soil day^{-1} (n = 24) with standard error bars. Lowercase letters indicate significant differences at $p \leq 0.05$. Samples were incubated with carbon cocktail as the carbon source and at 10% oxygen.

FLNF rates than a 1-day ($P < 0.0001$) incubation and 3.8x greater FLNF rates than a 3-day ($P < 0.0001$) incubation. Though a 3-day incubation resulted in 43.8x greater FLNF rates than a 1-day incubation at LUX, this difference was not significant ($P = 0.194$). At LC, a 7-day incubation resulted in 201.2x greater FLNF rates than a 1-day ($P < 0.0001$) incubation and 24.1x greater FLNF rates than a 3-day ($P < 0.0001$) incubation.

2.5 DISCUSSION

In this study, we optimized conditions for measurement of potential FLNF via $^{15}N_2$ incorporation in bulk and rhizosphere soils. We also identified conditions which are optimal for measurement of potential acetylene reduction via the ARA. The $^{15}N_2$

incorporation method is free of the issues associated with the ARA and is the most direct method of assessing N-fixation rates (Warembourg 1993). Our work illustrates several issues with the ARA; we found significant amounts of endogenous ethylene production and evidence of ethylene consumption in our soils, thus confirming past work which suggested these processes are at work in soil systems (Witty 1979; Nohrstedt 1983; Boddey 1987; Zechmeister-Boltenstern and Smith, 1998). Ethylene production and consumption can complicate results of the ARA and the addition of ethylene positive and negative controls is often not adequate to account for these background processes (Witty 1979; van Berkum and Bohlool 1980; Nohrstedt 1983; Sloger and van Berkum 1988). Though we recommend avoiding use of the ARA, it may be preferred because it has lower cost and is relatively high-throughput with automated GC systems. The ARA may still be useful for identifying important drivers of FLNF at high temporal and spatial resolution if the proper controls are used and absolute quantification of FLNF or N-budgeting are not the primary research goals. ARA may also be appropriate for short-term measurements in simplified systems, but should be validated with the $^{15}N_2$ incorporation method. If the ARA is used to assess potential rates of FLNF, researchers can counter some of the inherent issues with the ARA by using high replication, including both positive and negative control samples to account for background ethylene production and consumption, and using the optimized conditions we suggest for measuring the maximum acetylene reduction potential.

Optimization of the ARA and $^{15}N_2$ incorporation included testing of incubation time. Because the ARA is highly sensitive, shorter incubation times are often recommended (Brouzes et al. 1971), however required incubation times may vary by

study system (Myrold et al. 1999). Therefore, we chose to assess acetylene reduction across a variety of incubation times to determine which allowed us to measure potential FLNF most precisely. At LUX, there were no significant differences in incubation time until the 7-day sampling, while at LC 6-hour and 7-day incubations resulted in the greatest acetylene reduction rates and 18-hour and 24-hour incubations had significantly lower rates. These results indicated that incubation time was an important determinant of potential FLNF rates and that optimal incubation time was likely site-dependent. Based on our study system, we found that longer incubation times (e.g. 7 days rather than the suggested range of 1 to 24 hour) were needed to achieve consistently detectable results when using the ARA. However, it is important to note that use of long incubation times, particularly when using the ARA, can result in large overestimates of FLNF (Silvester et al 1982). This is thought to be the result of de-repression by which inhibition of FLNF by acetylene causes bacterial N-deprivation which then stimulates increased FLNF activity or nitrogenase synthesis (Silvester et al 1982; Rai et al 1992). This lag in ARA activity may also result from a lag in diazotroph population growth as these organisms adapt to the incubation conditions (van Berkum and Bohlool 1980). Given this issue, we strongly encourage researchers to optimize incubation time for their own systems, reducing times whenever feasible.

FLNF rates measured via $^{15}N_2$ incorporation were also greatest after 7 days of incubation. We were surprised to find that differences in FLNF rates between 7 days and 3 days were so great, with FLNF rates being approximately 4 and 24 times greater at 7 days than at 3 days for LUX and LC, respectively. This may indicate that after 7 days of incubation, diazotroph communities have shifted to a dominance of organisms

well-suited to assay conditions. Others have shown that communities can change drastically over the course of these incubations (Weaver and Danso 1994; Goldfarb et al. 2011; Oliverio et al. 2017). Therefore, 7-day incubations, though demonstrating high FLNF rates, may not accurately predict the potential for the endemic diazotroph community to fix N.

At the other extreme, a 1-day incubation resulted in negligible incorporation of ^{15}N into the soil with values in the pg N g^{-1} dry soil day^{-1} range. Though a 1-day incubation is likely to have the least changed microbial community, it does not appear to be long enough to ensure that ^{15}N incorporation is great enough to be well above the background levels of a relatively large soil total N pool and well above measurement detection and sensitivity limits of the elemental analyzers and mass spectrometers routinely used for these analyses. Therefore, in our study system, a 3-day incubation is optimal, allowing for adequate incorporation of ^{15}N label into the soil while minimizing changes to the microbial community. In systems with potentially high FLNF rates, such as tropical ecosystems (Reed et al. 2011), shorter incubation times may be feasible. However, in systems which are likely to have lower FLNF rates, such as tundra and temperate forests (Reed et al. 2011), incubation times of up to 7 days may be required. In these cases, researchers should acknowledge the potential for (or ideally, measure) microbial community shifts. In summary, as with the ARA, we again recommend optimization of incubation times for each unique study system.

We optimized oxygen concentration for the ARA and $^{15}N_2$ incorporation by testing four concentrations ranging from anaerobic to ambient including 0, 5, 10, and 20% oxygen. Acetylene reduction rates were greatest under anaerobic conditions and

decreased as oxygen concentration increased. This finding is generally supported by previous studies showing anaerobic conditions to be more favorable than ambient O_2 concentrations for acetylene reduction of sieved soils (Chang and Knowles 1965; Brouzes et al 1969; Brouzes et al 1971). However, we were surprised that microaerobic conditions were not more favorable to ARA. Previous work has indicated that microaerobic conditions are optimal for supporting the diversity of oxygen needs within the diazotroph community (Brouzes et al 1971; Silvester et al 1982). These microaerobic conditions likely balance the need to protect nitrogenase from oxygen damage with aerobic growth. (Boyd et al. 2015; Smercina et al. 2019). There are several potential explanations for our results. First, acetylene can inhibit oxygen use in aerobic soils (Knowles et al 1973), though acetylene can also reduce growth of some anaerobic diazotrophs and would likely also suppress N-fixation in our anaerobic incubations (Knowles et al 1973). Second, it is also important to note that the ARA was performed on homogenized and sieved soils, therefore soil microsites were likely disturbed. Soil structure is a key factor influencing soil oxygen concentration (Tiedje et al. 1984) and microsites may be of particular importance in maintaining optimal oxygen concentrations for FLNF (Smercina et al. 2019). Anaerobic conditions may be more favorable for FLNF in disturbed soils because the microsites favorable to strict anaerobes and microaerophilic organisms may be exposed. Lastly, the ARA was performed on saturated soils which due to reduced oxygen diffusion tend to be oxygen depleted (Tiedje et al. 1984) and therefore more favorable to anaerobic organisms.

These results highlighted both the importance of maintaining soil structure and choosing an appropriate soil moisture content when assessing FLNF. Therefore, we

chose to use intact cores and adjust to 60% WHC, an optimal moisture content for microbial activity (Linn and Doran 1984), for $^{15}N_2$ incorporation optimization work. Following Weaver and Danso's (1994) ARA in grass systems, we advocate for the removal of soil homogenization from N-fixation assay protocols, using instead intact soil cores, in order to maintain the rich variety of niche space and activity of a diverse array of N-fixing organisms. However, if researchers conduct work on homogenized soils or with the ARA, we recommend low oxygen concentration or anaerobic conditions be used. Researchers should also be advised that ARA conducted under low oxygen availability has been associated with FLNF de-repression, particularly for longer incubation times (Silvester et al 1982; Rai et al 1992).

With intact soil cores, we again tested the same four oxygen concentrations using the $^{15}N_2$ incorporation method. We found that low, but not anaerobic oxygen concentrations are most favorable for potential FLNF when assessed on intact cores held at 60% WHC. Specific oxygen optima varied by site likely due to a variety of factors including differences in soil texture and soil moisture content (Tiedje et al. 1984). At LUX, potential FLNF was greatest around 10% oxygen while at LC 5% oxygen promoted the greatest potential FLNF. This may have been strongly influenced by soil texture and resulting integrity of intact cores from each site. While cores from LUX were likely to maintain their structure, cores from LC, where soils are sandier, frequently lost some structure once the C cocktail was added. As discussed above, loss of structure can expose microsites resulting in more exposure of anaerobic and microaerophilic diazotrophs to oxygen. Differences in oxygen optima may also be the result of differences in diazotroph community composition, though we did not assess community

composition in this study. Researchers should consider evaluating how oxygen concentration and water content impact FLNF in their study system before choosing the values of these parameters used in their assays.

Finally, we tested how C source impacts potential FLNF rates. We found that supplying a C source with more C forms supports greater potential FLNF than C sources with fewer compounds regardless of the method used to measure FLNF. ARA results indicated that addition of C cocktail increased potential acetylene reduction rates compared to glucose only. Using the $^{15}N_2$ incorporation method, we found C cocktail to increase potential FLNF by 8 to 48 times above no-C controls depending on site. It is not surprising that a C cocktail supports greater FLNF than single sources of C as diazotrophs are diverse and often isolated with C sources other than glucose (Baldani et al. 2014). Surprisingly, additions of a single C source (sucrose) did result in similar rates of FLNF to the C cocktail at LC. However, FLNF did not differ significantly between sucrose and no-C treatments at either site. In contrast, Roley et al. (2018) found additions of glucose solution to increase FLNF by nearly 5 times compared to no glucose additions. While this suggests that at some sampling times and sites one C source may sufficiently stimulate the microbial community, we recommended use of the C cocktail for both the ARA and $^{15}N_2$ incorporation methods as it is likely to stimulate FLNF across a wide range of conditions and study sites. We also note that it is important to adjust the pH of the C cocktail solution as we observed a decrease in FLNF rates when unbuffered, acidic C cocktail was used versus buffered, neutral C cocktail. Unbuffered C cocktail may place selective pressure on the diazotroph community as

both diversity and structure of diazotroph communities have been shown to be influenced by soil pH (Fan et al. 2018).

Lastly, our work adds to the growing body of evidence that FLNF occurs in many systems and can contribute largely to plant N demands (Borman et al. 1993; Reed et al. 2011; Ladha et al. 2016; Roley et al. 2018). Up to of 48% of N demand for grasses such as maize, rice, and wheat were found to come from sources other than soil and fertilizer N (Ladha et al. 2016), and others have shown N-fixation can contribute significantly (> 50% in some cases) to maize N demands (Chalk 2016; Kaun et al. 2016). In switchgrass systems, like those studied in this work, Roley et al. (2018) found N deficits at upwards of 35 – 58 kg N ha^{-1} yr^{-1} and extrapolated FLNF rates indicated this process could meet 80 to 100% of the deficit. By extrapolating our average FLNF rates from µg N fixed g^{-1} dry soil day^{-1} to kg N ha^{-1} yr^{-1}, we found FLNF at LUX has the potential to contribute 0.25 to 11.0 kg N ha^{-1} yr^{-1}. These rates are up to 2x greater than the estimated contribution of N from symbiotic N-fixation in temperate grasslands (Reed et al. 2011) and meet approximately 19 – 31 % of the N deficit identified by Roley et al. (2018). Extrapolation of FLNF rates at LC results in much lower annual N contributions from FLNF with potential contributions of only 0.04 to 0.35 kg N ha^{-1} yr^{-1}. These large differences in potential N contribution from FLNF across sites only serve to highlight the need for better estimation of FLNF and better understanding of controls on this important N source. Although these extrapolated rates are based on optimized conditions for potential FLNF rates, they are still useful for estimating potential contribution of FLNF to the overall N budget, as is done with other potential N-cycle

process rates (e.g. N-mineralization, nitrification, denitrification), with the understanding that budget numbers generated using these potential rates are likely overestimates.

2.6 CONCLUSIONS

As demonstrated in past work, we confirmed that issues with the ARA, such as background ethylene production and consumption, limit its applicability to measuring FLNF in soils. While we recommend avoiding use of the ARA, researchers could overcome some of its issues by including high sample replication and controls for background ethylene production and consumption. If ARA is chosen, we recommend longer incubation times, anaerobic conditions, and the addition of a C cocktail. In addition, we would caution against using the ARA results to estimate N budgets as it is not a direct measure of N-fixation or N accumulation, but of potential nitrogenase enzyme activity.

We strongly recommend that FLNF potential be measured using the $^{15}N_2$ incorporation method and be conducted on intact soil cores. To ensure optimal conditions for FLNF across a wide variety of diazotrophs, we recommend use of a C cocktail containing a mixture of glucose, sucrose, malic acid, and citric acid, and a 3-day incubation to allow for adequate incorporation of the ^{15}N label over the shortest incubation time. Researchers should consider testing incubation time in their own system as the time needed to achieve adequate ^{15}N incorporation will vary by activity of diazotroph communities at each site. In some cases, incubation times may be reduced, thereby reducing changes to the microbial community during the incubation. We also recommend incubating samples at low oxygen concentrations (between 5 and 10%) as this promoted the greatest potential FLNF compared to ambient or anaerobic conditions

in our study. However, because optimal oxygen concentration is variable across sites with different soil texture, we recommend that researchers optimize oxygen concentration for each new site tested.

The ARA has been used for decades and without proper positive and negative controls has likely hindered our ability to investigate FLNF across systems. While the ARA could be successfully employed in some cases – i.e. initial testing to reduce cost of optimization work, ultimately a more direct measurement method such as $^{15}N_2$ incorporation and consideration of the optimal conditions for a given study system are needed to move our understanding of this important process forward.

APPENDIX

SUPPLEMENTAL MATERIAL

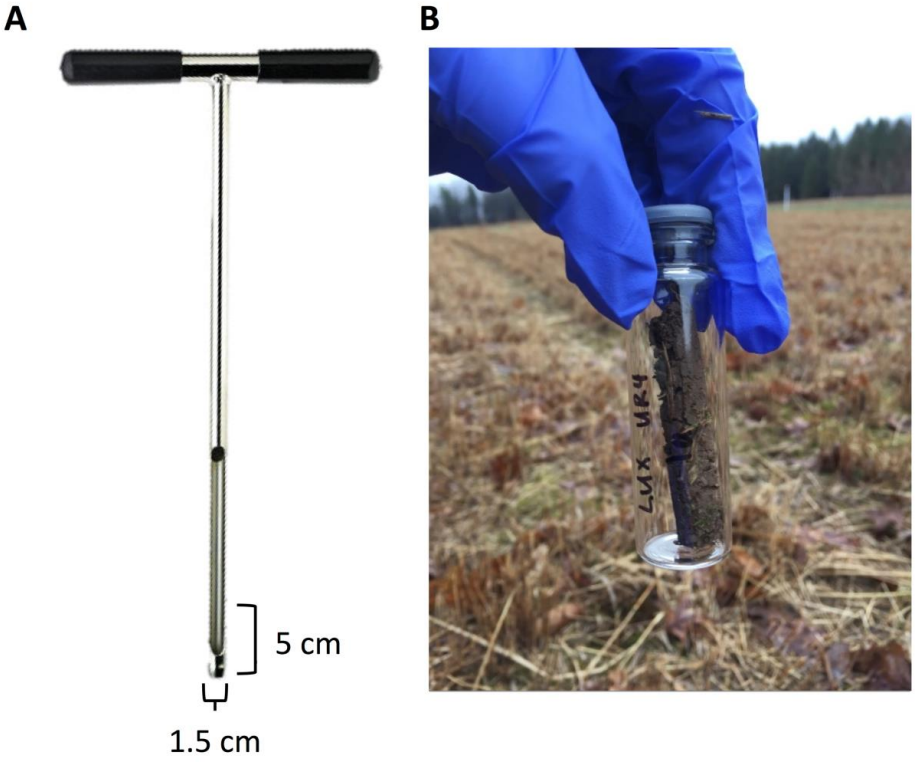

Figure S2.1: Images of AMS soil/turf probe used to obtain intact cores (A) and intact core collected in field and transferred to 20 ml gas vial (B). Soil/turf probe has an inside diameter of 1.5 cm and cores were collected to 5 cm depth. Intact cores were transferred in-field to sample vials, then stored in a cooler until transport to the lab.

REFERENCES

REFERENCES

Bahulikar, R. A., Torres-Jerez, I., Worley, E., Craven, K., & Udvardi, M. K. (2014) Diversity of nitrogen-fixing bacteria associated with switchgrass in the native tallgrass prairie of northern Oklahoma. Appl. Environ. Microbiol., 80(18), 5636-5643.

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 and soil 384: 413-431.

Baudoin, E, Benizri. E, Guckert, A. (2003). Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. Soil Biology and Biochemistry 35(9), 1183-1192.

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 Biology and Biochemistry 69: 413-420.

Blossfeld, S., Gansert, D., Thiele, B., Kuhn, A. J., & Lösch, R. (2011) The dynamics of oxygen concentration, pH value, and organic acids in the rhizosphere of Juncus spp. Soil biology and biochemistry, 43(6), 1186-1197.

Boddey, R. M. (1987) Methods for quantification of nitrogen fixation associated with gramineae. Critical reviews in plant sciences, 6(3), 209-266.

Bormann, B. T., Bormann, F. H., Bowden, W. B., Piece, R. S., Hamburg, S. P., Wang, D., ... & Ingersoll, R. C. (1993). Rapid N^ 2 Fixation in Pines, Alder, and Locust: Evidence From the Sandbox Ecosystems Study. *Ecology*, *74*(2), 583-598.

Bottomley, P. J., & Myrold, D. D. (2014) Biological N inputs. *Soil microbiology, ecology and biochemistry*, *3*, 365-388.

Boyd, E. S., Costas, A. M. G., Hamilton, T. L., Mus, F., & Peters, J. W. (2015) Evolution of molybdenum nitrogenase during the transition from anaerobic to aerobic metabolism. *Journal of bacteriology*, *197*(9), 1690-1699.

Bürgmann, H., Meier, S., Bunge, M., Widmer, F., & Zeyer, J. (2005) Effects of model root exudates on structure and activity of a soil diazotroph community. *Environmental Microbiology*, *7*(11), 1711-1724.

De Bont, J. A. M. (1976) Oxidation of ethylene by soil bacteria. *Antonie van Leeuwenhoek*, *42*(1-2), 59-71.

Brouzes, R., Lasik, J., & Knowles, R. (1969) The effect of organic amendment, water content, and oxygen on the incorporation of 15N2 by some agricultural and forest soils. Can.J.Microbiol.15(8):899-905.

Brouzes, R., & Knowles, R. (1973) Kinetics of nitrogen fixation in a glucose-amended, anaerobically incubated soil. Soil.Biol.Biochem.5:223-229.

Brouzes, R., Mayfield, C. I., & Knowles, R. (1971) Effect of oxygen partial pressive on nitrogen fixation and acetylene reduction in a sandy loam soil amended with glucose. Plant.Soil.(Special):481-494.

Chalk, P. M. (2016). The strategic role of 15 N in quantifying the contribution of endophytic N 2 fixation to the N nutrition of non-legumes. *Symbiosis*, *69*(2), 63-80.

Chang, P. C., & Knowles, R. (1965). Non-symbiotic nitrogen fixation in some Quebec soils. *Canadian Journal of Microbiology*, *11*(1), 29-38.

Fan, K., Weisenhorn, P., Gilbert, J. A., Shi, Y., Bai, Y., & Chu, H. (2018) Soil pH correlates with the co-occurrence and assemblage process of diazotrophic communities in rhizosphere and bulk soils of wheat fields. *Soil Biology and Biochemistry*, *121*, 185-192.

Friesen, M. L., Porter, S. S., Stark, S. C., von Wettberg, E. J., Sachs, J. L., & Martinez-Romero, E. (2011) Microbially mediated plant functional traits. *Annual review of ecology, evolution, and systematics*, *42*, 23-46.

Giller, K. E. (1987) Use and abuse of the acetylene reduction assay for measurement of “associative” nitrogen fixation. Soil biology and biochemistry, 19(6), 783-784.

Goldfarb, K. C., Karaoz, U., Hanson, C. A., Santee, C. A., Bradford, M. A., Treseder, K. K., ... & Brodie, E. L. (2011) Differential growth responses of soil bacterial taxa to carbon substrates of varying chemical recalcitrance. *Frontiers in microbiology*, *2*, 94.

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 and Pasture Science 65:1044-1056.

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

Knowles, R., (1965) The significance of nonsymbiotic nitrogen fixation. Soil.Sci.Soc.Am.Proc.29(2):223.

Knowles, R., Brouzes, R., & O'toole, P. (1973). Kinetics of nitrogen fixation and acetylene reduction, and effects of oxygen and of acetylene on these processes,

in a soil system. *Bulletins from the Ecological Research Committee*, (17), 255-262.

Knowles, R. (1980) Nitrogen fixation in natural plant communities and soils. Nitrogen fixation in natural plant communities and soils., 557-582.

Kuan, K. B., Othman, R., Rahim, K. A., & Shamsuddin, Z. H. (2016). Plant growth-promoting rhizobacteria inoculation to enhance vegetative growth, nitrogen fixation and nitrogen remobilisation of maize under greenhouse conditions. *PloS one*, *11*(3), e0152478.

Ladha, J. K., Tirol-Padre, A., Reddy, C. K., Cassman, K. G., Verma, S., Powlson, D. S., ... & Pathak, H. (2016). Global nitrogen budgets in cereals: A 50-year assessment for maize, rice, and wheat production systems. *Scientific reports*, *6*, 19355.

Linn, D. M., & Doran, J. W. (1984) Effect of water-filled pore space on carbon dioxide and nitrous oxide production in tilled and nontilled soils 1. *Soil Science Society of America Journal*, *48*(6), 1267-1272.

Minett, D. A., Cook, P. L., Kessler, A. J., & Cavagnaro, T. R. (2013) Root effects on the spatial and temporal dynamics of oxygen in sand-based laboratory-scale constructed biofilters. Ecological engineering, 58, 414-422.

Mirza, B.S. and Rodrigues, J.L. (2012) Development of a direct isolation procedure for free-living diazotrophs under controlled hypoxic conditions. *Appl. Environ. Microbiol.*, *78*(16), pp.5542-5549.

Myrold, D. D., R. Reuss, and M. J. Klug. (1999) Dinitrogen fixation. Pages 241-257 in G. P. Robertson, C. S. Bledsoe, D. C. Coleman, and P. Sollins, eds. Standard Soil Methods for Long-Term Ecological Research. Oxford University Press, New York, New York, USA.

Nohrstedt, H. Ö. (1983) Natural formation of ethylene in forest soils and methods to correct results given by the acetylene-reduction assay. *Soil Biology and Biochemistry*, *15*(3), 281-286.

Oliverio, A. M., Bradford, M. A., & Fierer, N. (2017) Identifying the microbial taxa that consistently respond to soil warming across time and space. *Global change biology*, *23*(5), 2117-2129.

O Toole, P., & Knowles, R. (1973) Oxygen inhibition of acetylene reduction (nitrogen fixation) in soil effect of glucose and oxygen concentrations. Soil.Biol.Biochem.5:783-788.

Patriquin, D. G., & Knowles, R. (1975) Effect of oxygen, mannitol and ammonium concentrations on nitrogenase (C2H2) activity in a marine skeletal carbonate sand. Marine.Biol.32:49-62.

Pedersen, O., Borum, J., Duarte, C. M., & Fortes, M. D. (1998) Oxygen dynamics in the rhizosphere of Cymodocea rotundata. Marine Ecology Progress Series, 169, 283-288.

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. (2018) nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-137, URL: https://CRAN.R-project.org/package=nlme

R Core Team. (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/.

Rai, A. N., Borthakur, M., & Bergman, B. (1992). Nitrogenase derepresssion, its regulation and metabolic changes associated with diazotrophy in the non-heterocystous cyanobacterium Plectonema boryanum PCC 73110. *Microbiology*, *138*(3), 481-491.

Reed SC, Cleveland CC, Townsend AR. (2011) Functional ecology of free-living nitrogen fixation: a contemporary perspective. Annual review of ecology, evolution, and systematics 42: 489-512.

Robson RL, Postgate JR. (1980) Oxygen and hydrogen in biological nitrogen fixation. Annual Reviews in Microbiology 34: 183-207.

Roley, S. S., Duncan, D. S., Liang, D., Garoutte, A., Jackson, R. D., Tiedje, J. M., & Robertson, G. P. (2018) Associative nitrogen fixation (ANF) in switchgrass (Panicum virgatum) across a nitrogen input gradient. *PloS one*, *13*(6), e0197320.

Silver, W.S. and Postgate, J.R., (1973) Evolution of asymbiotic nitrogen fixation. *Journal of Theoretical Biology*, *40*(1), pp.1-10.

Silvester, W. B., Sollins, P., Verhoeven, T., & Cline, S. P. (1982). Nitrogen fixation and acetylene reduction in decaying conifer boles: effects of incubation time, aeration, and moisture content. *Canadian Journal of Forest Research*, *12*(3), 646-652.

Sloger, C., & van Berkum, P. (1988). Endogenous ethylene production is a potential problem in the measurement of nitrogenase activity associated with excised corn and sorghum roots. *Plant physiology*, *88*(1), 115-118.

Smercina, D. N., Evans, S. E., Friesen, M. L., & Tiemann, L. K. (2019) To fix or not to fix: controls on free-living nitrogen fixation in the rhizosphere. *Appl. Environ. Microbiol.*, *85*(6), e02546-18.

Tiedje, J. M., Sexstone, A. J., Parkin, T. B., & Revsbech, N. P. (1984) Anaerobic processes in soil. *Plant and Soil*, *76*(1-3), 197-212.

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 Phytologist, 196(3), 926-936.

Van Berkum, P., & Bohlool, B. B. (1980). Evaluation of nitrogen fixation by bacteria inassociation with roots of tropical grasses. *Microbiol. Rev*, *44*, 491-517.

Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens, G. E., Matson, P. A., Schindler, D. W., ... & Tilman, D. G. (1997). Human alteration of the global nitrogen cycle: sources and consequences. *Ecological applications*, *7*(3), 737-750.

Wang KL, Li H, Ecker JR. (2002) Ethylene biosynthesis and signaling networks. The Plant Cell 14: s131-51.

Warembourg, F. R. (1993) Nitrogen fixation in soil and plant systems. *Nitrogen isotope techniques*, 127-156.

Weaver, R. W., & Danso, S. K. (1994) Dinitrogen fixation. *Methods of Soil Analysis: Part 2—Microbiological and Biochemical Properties*, (methodsofsoilan2), 1019-1045.

Witty, J. F. (1979) Acetylene reduction assay can overestimate nitrogen-fixation in soil. *Soil biology and biochemistry*.

Xin, J. Y., Xu, N., Ji, S. F., Wang, Y., & Xia, C. G. (2017) Epoxidation of Ethylene by Whole Cell Suspension of Methylosinus trichosporium IMV 3011. Journal of Chemistry, 2017.

Zechmeister-Boltenstern, S., & Smith, K. A. (1998) Ethylene production and decomposition in soils. *Biology and fertility of soils*, *26*(4), 354-36

CHAPTER 3:

SWITCHGRASS SELECTS FOR A DISTINCT AND CONSISTENT RHIZOSPHERE DIAZOTROPH COMMUNITY

3.1 ABSTRACT

Cellulosic bioenergy crops, like switchgrass (*Panicum virgatum*), represent an alternative to biofuels like maize (*Zea mays*), providing benefits such as climate mitigation with the potential to grow on marginal lands not suitable for food crop production. In order to optimize the sustainability of bioenergy crop production, there is a need to understand conditions which allow for these crops to produce high biomass yields while growing on marginal lands under low-input agricultural practices (e.g. limited nutrient and water additions). Free-living nitrogen fixation (FLNF) is a potentially important nitrogen (N) source for bioenergy crops like switchgrass, yet the contributions of FLNF to plant N demand are unclear. It is also not well understood how fertilizer N additions may influence the potential for FLNF to contribute plant available N. In this study, we used a greenhouse experiment to measure the response of the switchgrass rhizosphere N-fixing microbial community composition and its N-fixing potential to legacy and short-term N additions using soil from three Michigan marginal land sites. Surprisingly, we found no response of FLNF rates to either legacy or short-term N additions and no difference in FLNF rates across sites. We found no significant effect of site or N treatment on overall rhizosphere or field soil diazotroph communities, but there was a clear effect of the rhizosphere on community composition. While we found no direct link between diazotroph community composition and FLNF rates, we identified

several OTUs that were associated with greater FLNF and propose that these organisms warrant further study.

3.2 INTRODUCTION

Cellulosic bioenergy crops, including the C4 perennial grass switchgrass (*Panicum virgatum*), represent a renewable fuel source and a potential alternative to fuel production from crops like maize (*Zea mays*; Robertson et al. 2017). These perennial grasses have high climate mitigation potentials through both carbon (C) capture and belowground C allocation, resulting in soil C accrual and mitigation of ~9.5 Mg CO_2-eq ha^{-1} yr^{-1} (Robertson et al. 2017). However, the challenge of such cropping systems is in enabling sustainable plant growth on lands not suitable for food production (i.e. marginal lands) under low inputs of nutrients and water (Gelfand el al. 2013; Robertson et al. 2017).

Switchgrass is a particularly promising bioenergy crop, producing high biomass yields when grown on marginal lands even with minimal inputs of fertilizer nitrogen (N) or water (Gelfand et al. 2013; Mehmood et al. 2017; Robertson et al. 2017). In fact, switchgrass productivity on marginal lands is often unresponsive to fertilizer N additions; similar yields have been observed at N levels both above and below plant N demands (Ruan et al 2016; Wang et al. 2019). Furthermore, yields remain consistently high despite N removal via yearly harvest (Parrish and Fike 2005). An N mass balance suggests that switchgrass is accessing an unaccounted-for N source at rates of 35 – 58 kg N ha^{-1} yr^{-1} (Roley et al. 2018). Recent evidence points towards free-living nitrogen fixation (FLNF) as this unaccounted-for N source (Roley et al. 2019; Smercina et al. 2019a).

Free-living nitrogen fixation, here defined as N-fixation occurring in and around roots (rhizosphere) without direct plant symbiosis, is increasingly recognized for its potential to increase bioenergy crop sustainability by providing an alternative N source in lieu of fertilizer N additions (Reed et al 2011; Bloch et al 2020). This energy-intensive process, which transforms dinitrogen (N_2) gas to biologically-available ammonia, occurs under dynamic conditions and is carried out by a large diversity of bacteria living in soils and the rhizosphere (Smercina et al 2019a). FLNF readily occurs in the rhizospheres of many grasses, including maize (Chalk 2016; Kaun et al. 2016) and switchgrass (Roley et al 2018; Smercina et al 2019b), where roots exude easily accessible C that can support FLNF activity. FLNF in the rhizosphere of these grasses has the potential to contribute significantly to plant N demands (Borman et al. 1993; Reed et al. 2011; Ladha et al. 2016). However, in order to harness these N contributions, we need a better understanding of the controls on FLNF and the diazotrophic organisms which carry out this process.

Nitrogen has been shown to be a major control on FLNF (Hobbs et al 1984; Patra et al 2007; Reed et al. 2011; Kox et al 2016; Smercina et al. 2019b). Diazotrophs are not strictly reliant on fixed N to meet their N demands and can access external N sources, including organic and inorganic N (Reed et al. 2011; Norman and Friesen 2017). Because FLNF is energy intensive, it is often more energetically favorable to use external N than to fix N from the atmosphere (Reed et al. 2011; Smercina et al. 2019b). Consequently, high N availability generally down-regulates FLNF and low N availability, such as what might be observed in the switchgrass rhizosphere, often promotes FLNF

(Reed et al. 2011; Smercina et al. 2019b). Thus, N fertilization of switchgrass cropping systems is likely to impact the potential contributions of N from FLNF.

The magnitude of this response in FLNF to N is likely to be driven by the composition of the diazotroph community. The switchgrass rhizosphere-associated diazotroph community is very diverse, found to contain members of several different Phyla including Alpha, Beta, Gamma, and Deltaproteobacteria and Firmicutes (Bahulikar et al. 2014; Roley et al. 2019; White et al *in review*). These organisms represent diverse life histories and growth strategies, ranging from oligotrophs to copiotrophs and from strict anaerobes to aerobes, and produce multiple forms of nitrogenase, the enzyme involved in N-fixation, all of which influence a given organisms FLNF activity (Smercina et al. 2019b). Diazotrophs also differ in their response to N availability. Some diazotrophs, such as *Azospirillum brasilense*, can regulate the function of nitrogenase, the enzyme responsible for N-fixation, post-translationally thereby stopping FLNF activity when external N becomes available (Dixon and Kahn 2004). However, many diazotrophs only regulate nitrogenase transcription (Dixon and Kahn 2004), therefore FLNF activity via already synthesized nitrogenase may occur in these organisms even when external N is available. Overall, the relative representation of different growth strategies as well as enzyme forms and regulation present within the diazotroph community is likely to influence FLNF activity.

The composition of the diazotroph community and relative representation of different growth strategies is likely to be impacted by N fertilization. Long-term N fertilization, on order of 20 years of application, has been shown to cause evolutionary shifts in symbiotic N-fixers, resulting in declining mutualisms between rhizobia and their

hosts (Klinger et al 2016), though not necessarily reducing N-fixation function (Schmidt et al 2017). Similarly, N fertilization for seven to upwards of 30 years has also been observed to cause shifts in diazotroph communities (Wang et al 2016; Feng et al 2018; Fan et al 2019; Roley et al 2019). Under pressures of long-term, or legacy, N fertilization, diversity of diazotroph communities may decrease (Feng et al 2018), potentially shifting towards those organisms which can rapidly regulate FLNF activity and effectively compete for externally-available N through rapid growth (Fan et al 2019; Smercina et al 2019b). Such shifts in community composition will also likely influence the response of FLNF and diazotroph community composition to short-term N availability.

In this study, we explore the impact of legacy and short-term N additions on FLNF rates and the switchgrass-associated diazotroph community across marginal land sites. We posit three hypotheses: (1) Increasing N availability (legacy and short-term), moderated by site, will reduce FLNF rates and diazotroph community diversity (2) The magnitude of response to N for FLNF rates and diazotroph community composition will be more pronounced under short-term N additions (3) Diazotroph community structure will be linked to measured FLNF rates such that we will observe distinct community composition and presence of specific diazotrophs where we measure greater FLNF.

3.3 METHODS

3.3.1 Experimental Setup

We explored the response of the switchgrass (*Panicum virgatum*) diazotroph community composition and activity (i.e. FLNF) to legacy and short-term N addition treatments using a reciprocal treatment experimental design (Fig. 3.1A). To represent legacy N treatments, field soils were collected from fertilized and unfertilized plots (as described below) and used as inoculum for greenhouse pots. Short-term N treatments were created in the greenhouse where plants received additions of either high N (equivalent to 125 kg N ha^{-1} yr^{-1}) or low N (equivalent to 25 kg N ha^{-1} yr^{-1}). Collectively, this created four legacy by short-term N addition treatments including fertilized + low N (Fert), fertilized plus high N (Fert + N), unfertilized + low N (Unfert), and unfertilized + high N (Unfert + N).

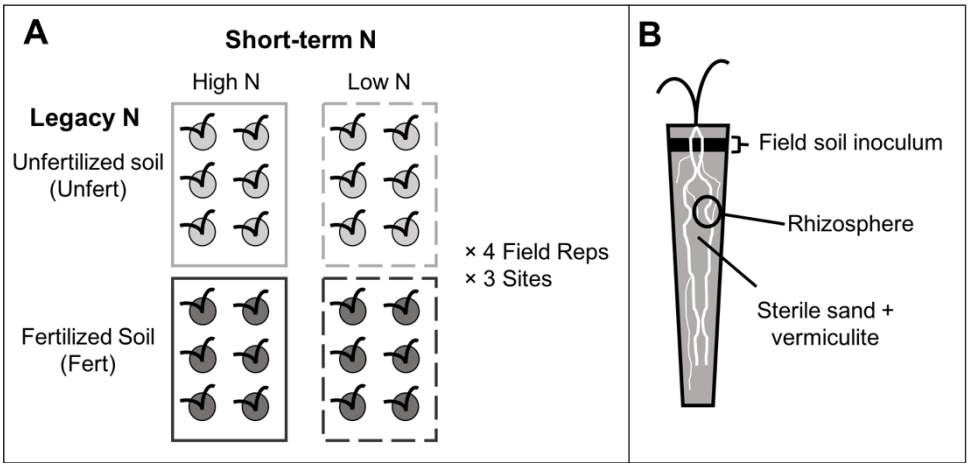

Figure 3.1: Experimental design of (A) N additions and (B) planting. There were 6 replicate pots per legacy x short-term N treatment combination. This was repeated for each field replicate (4 per site) and each field site (3 – Escanaba, Lake City, and Lux Arbor) for a total of 288 pots. Field soils were used to inoculate pots by adding a thin layer of soil near the surface of each pot.

In the greenhouse, switchgrass (var. Cave-in-Rock) plants were grown individually in large deepots (Stuewe and Sons, Tangent, OR, USA) filled with a 50:50

(v/v) mixture of autoclave sterilized sand and vermiculite. Each pot also received ~ 1 cm of field soil as an inoculum at a depth of ~1-2 cm from the top of the pot (Fig. 3.1B). The field soil was then covered with 50:50 sand-vermiculite mix, filling the remainder of the pot. Soils for pot inoculation were collected from three Michigan field sites, Lux Arbor (LUX; 42.476365, -85.451887), Lake City (LC; 44.296098, -85.199612), and Escanaba (ESC; 45.7627, -87.1877) with distinct climate and soil characteristics (Table 3.1; Kasmerchak and Schaetzl 2018). These field sites are maintained as part of the Great Lakes Bioenergy Research Center's Marginal Land Experiment (GLBRC MLE; https://www.glbrc.org/). Each site has four replicate split plots of switchgrass (*Panicum virgatum* L.; cv. Cave-in-Rock) monoculture which have been maintained since 2013. Main plots are divided into fertilized (+ 56 kg urea-N ha^{-1} yr^{-1}) and unfertilized (no added N) split-plots. We sampled soils from both fertilized and unfertilized split-plots to represent our legacy N treatments. In total, we had 24 distinct soil samples (3 field sites x 4 replicate blocks x 2 fertilizer N treatments). Soils were returned to the lab, on ice, and stored at 4 ˚C until further use. Each soil sample was sieved (2 mm) and homogenized before addition to the greenhouse pots.

Table 3.1: Site characteristics including soil properties, climate, and land use history for three field sites Lux Arbor (LUX), Lake City (LC), and Escanaba (ESC)

Site	Soil Taxonomy	Texture	% Sand	% Silt	% Clay	pH	Total N (%)	Total C (%)	P (ppm)	30-yr avg. precip. (mm)	30-yr avg. temp. (˚C)
LUX	Typic Hapludalfs (Alfisol)	Loam	51.1	31.7	17.2	5.8	0.06	0.77	12	842.01	9.0
LC	Oxyaquic Haplorthod (Spodosol)	Loamy sand	84.7	7.8	7.5	7.3	0.06	0.92	24	812.29	6.5
ESC	Inceptic Hapludalf (Alfisol)	Sandy Loam	57.1	27.7	15.2	7.0	0.15	1.73	14	728.22	5.3

Note: Data provided by Great Lakes Bioenergy Research Center (GLBRC) marginal land experiment (https://lter.kbs.msu.edu/research/long-term-experiments/marginal-land-experiment/) and Kasmerchak and Schaetzl (2018)

3.3.2 Planting and growth

Prior to planting, switchgrass seeds were scarified, stratified, and sterilized as follows. Approximately 500 seeds were acid scarified by shaking in 50 ml of 8M sulfuric acid for 15 minutes. Seeds were then washed 3 times with distilled water by shaking 5 minutes each time. Seeds were then stratified by plating onto a petri dish containing a sterile Whatman #1 filter paper (GE Healthcare Life Sciences, Chicago, IL, USA). Seeds were covered with a second filter paper and then received 5 ml of sterile 0.2% (m/v) potassium nitrate. The petri dish was sealed with parafilm and then stored at 4 ˚C in the dark for at least 4 days and up to 2 weeks. After stratification, seeds were bleach and vapor-phase sterilized. For bleach sterilization, seeds were transferred to a sterile specimen cup and shaken with 5% sodium hypochlorite for 15 minutes and then washed 3 times with sterile nanopure. Seeds were then plated onto a sterile petri dish and spread into a single layer and then placed in a desiccator for vapor-phase sterilization. Vapor-phase sterilization uses chlorine gas and all work was carefully

carried out in a fumehood. To generate chlorine gas, 3 ml of 8.25% sodium hypochlorite (household bleach) were added to 100 ml of concentrated hydrochloric acid in a beaker contained within a desiccator. Immediately following addition of the bleach, the desiccator was sealed for four hours. After four hours, the seeds were removed and the petri dish was quickly sealed. Seeds were stored in the dark at 4 ˚C until planting.

Sterility of seeds was confirmed by plating seeds on LB agar prior to planting. Sterile seeds were planted directly into the field soil, thus allowing the seedling and its roots to be inoculated with the soil microbial community. At planting, pots received 50 ml of autoclave sterilized ½ Hoagland's solution (2.5 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.024 mM H_3BO_3, 0.004 mM $MnCl_2*4H_2O$, 0.102 µM $CuSO_4*5H_2O$, 0.382 µM $ZnSO_4*7H_2O$, 0.248 µM $Na_2MoO_4*2H_2O$, 5.4 µM NaFeEDTA) with either high (28.3 mM; equivalent to 125 kg N ha^{-1}) or low (5.7 mM; equivalent to 25 kg N ha^{-1}) additions of N as urea according to their associated treatment. Each legacy by short-term N treatment combination was replicated six times per field site and field replicate for a total of 288 pots. Plants were grown in the greenhouse under mist irrigation for four months before harvest. After 4 months of growth, plants were harvested for determination of aboveground biomass, rhizosphere N-fixation, and rhizosphere diazotroph community composition. At harvest, 18 plants had died leaving 270 samples for harvest. Plant losses were distributed randomly across treatments with most treatments having at least 20 of the 24 plants surviving. The LUX Unfert treatment only had 17 surviving plants at harvest.

3.3.3 Aboveground biomass

Plants were carefully removed from their pots onto aluminum foil sterilized with 70% ethanol. All aboveground biomass for each plant was clipped from the roots and then dried at 60 ˚C for 48 hours. Aboveground biomass was weighed after drying to obtain total aboveground biomass in mg.

3.3.4 Rhizosphere N-fixation

Roots were shaken to remove any loose sand and vermiculite mixture. Any sand and vermiculite still adhering to the roots was considered part of the rhizosphere. Portions of root with adhering field soil were intentionally avoided for this assay. A subset of root material was clipped and placed in a 20 ml gas vial for measurement of FLNF via $^{15}N_2$ incorporation. Vials were immediately placed in a cooler until further analysis. Additionally, a second subset of roots from one plant per treatment group (N treatment * field rep * site) were collected as reference vials for ^{15}N analysis. Prior to ^{15}N analysis, all root material in each vial was weighed and then samples were stored at room temperature to equilibrate for 24 hours prior to analysis. Following methods described by Smercina et al. (2019a), we added a 4-carbon source cocktail containing glucose, sucrose, citrate, and malate at a ratio of 1 ml solution per g root material. After carbon addition, vials were sealed and evacuated. Vial headspace was replaced with 1 ml of $^{15}N_2$ (Sigma-Aldrich, Inc., St. Louis, MO, USA), 10% Ultra High Purity (UHP) oxygen, and balanced with UHP Helium. Reference samples received UHP-N_2 in place of $^{15}N_2$. Vials were incubated for three days at room temperature. Vials were then uncapped and samples were dried at 60 ˚C for 48 hours before grinding and weighing for ^{15}N analysis. Samples were analyzed following standard procedures at University of

California Davis's Stable Isotope Facility (Davis, CA). Rhizosphere FLNF rates were calculated in μg N fixed g^{-1} rhizosphere day^{-1} as:

$$\frac{AE_i \times TN_i}{AE_{atm} \times t}$$

where AE_i represents atom percent excess of sample against an unenriched reference sample, TN_i represents total nitrogen content in sample, AE_{atm} represents atom percent excess in the vial atmosphere (98 atom% in our case), and t is incubation time in days (Warembourg 1993; Roley et al. 2018).

3.3.5 Rhizosphere diazotroph community composition

A subset of root/rhizosphere material (described above) was clipped and immediately frozen on liquid N_2. Roots with field soil adhering were intentionally avoided for this assay. Samples were transferred to a -80 ˚C freezer until DNA extraction. Rhizosphere microbial DNA was extracted from 0.5 g of rhizosphere material per sample using the Qiagen DNeasy PowerSoil kit (QIAGEN, Hilden, Germany). Field soil inoculum (0.5 g soil per sample; 8 samples per site) was also extracted for sequencing of the initial diazotroph community. PCR reactions to amplify *nifH* were carried out in 25 μl reactions with 2 μl of DNA extract, 1X AmpliTaq Gold 360 Master Mix (Applied Biosciences, Foster City, CA), 0.027 μg T4 gene 32 Protein (New England Biolabs, Ipswich, MA, USA), and 500 nM concentrations of both the forward and reverse primer. We used the IGK3/DVV forward and reverse primer pair, recommended by Gaby and Buckley (2012) as optimal primer sets for capturing the widest diversity of diazotrophs, with Illumina linker sequences for library prep. IGK3 sequence (5' to 3') with linker sequence underlined was

ACACTGACGACATGGTTCTACAGCIWTHTAYGGIAARGGIGGIATHGGIAA and DVV

sequence (5’ to 3’) with linker sequence underlined was TACGGTAGCAGAGACTTGGTCTATIGCRAAICCICCRCAIACIACRTC. PCR reactions were carried out as follows: 95 ˚C start for 10 minutes, 34 cycles of denaturation at 95 ˚C for 30 secs, annealing at 58 ˚C for 30 secs, and extension at 72 ˚C for 30 secs, final extension at 72 ˚C for 7 minutes. Amplification of target gene was confirmed via gel electrophoresis (1.5% gel agar, 90 V, 45 minutes). Despite several attempts at amplification, only 200 of the 270 rhizosphere samples successfully amplified while 17 of the 24 field soil samples successfully amplified. PCR products were cleaned to remove <100 bp bands of potential primer dimers using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and then quantified via Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States). Samples were normalized to a 1– 6 ng μl^{-1} DNA concentration before library prep. Samples were submitted to the Michigan State University RTSF Genomics Core Facility (East Lansing, MI) for library prep and 2 x 250 bp paired-end read sequencing on the MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq standard reagent kit v.2..

Sequence processing was performed following a modified version of the NifMAP pipeline (Angel et al. 2018). Sequence data were received as demultiplexed fastq files. Forward and reverse reads were merged via USEARCH v. 10.0.240 fastq_mergepairs and then quality and length filtered to maximum expected errors of 1 and minimum length of 300 bp via USEARCH v. 10.0.240 fastq_filter. Sequences were then filtered for non-*nifH* reads using four Hidden-Markov Models (HMM) as described in Angel et al. (2018). Sequences were then dereplicated using USEARCH v. 10.0.240 fastx_uniques and clustered using USEARCH v. 10.0.240 cluster_otus resulting in 6896 representative

OTUs. The cluster_otus command also filters chimeras. Sequences were then mapped back to reference OTUs using USEARCH v. 10.0.240 usearch_global at 97% similarity. 96% of sequences successfully mapped to cluster reference OTUs. Sequences were then frameshift corrected and translated to protein sequences using Framebot (Wang et al. 2013) and then filtered for homologs using HMM as described in Angel et al. (2018) before generating a final OTU table. At the end, 3025 OTUs were identified and used for phylogenetic tree construction and taxonomic classification. A phylogenetic tree was constructed using an amino acid reference alignment (Angel et al. 2018). Sequences were aligned to the reference using MAFFT v. 7.305 and then used to generate a tree via FastTree v. 2.1.9. Finally, taxonomy was assigned to sequences using Blast+ v. 2.7.1 tblastn command which allows querying of protein sequences against a nucleotide database. We used Gaby and Buckley's (2014) *nifH* sequence database as the taxonomic reference database for our sequences. Taxonomy was assigned according to percent similarity using empirically derived cutoffs (Gaby et al. 2018) of 75% similarity for family, 88.1% for genus, and 91.9% for species. All other taxonomic assignments matching at <75% similarity were only assigned at the order level.

3.3.6 Data Analysis

Results from aboveground biomass and rhizosphere FLNF rates were analyzed by a three-factor ANOVA followed by Tukey's post hoc with site, legacy N, and short-term N as fixed effects using the R *stats* package (R core team 2018). Differences between treatment groups (site*legacy*short-term) were considered significant at $\alpha \leq 0.05$.

OTU counts were first rarefied to an even sampling depth of 800 using *rarefy_even_depth* in the R *phyloseq* package. This was chosen based on the rarefaction curves which indicated that most of the diversity was captured within this sample size, while limiting loss of samples due to low OTU counts. Six of the 217 samples were removed from downstream data analysis because they had too few OTU counts (i.e. < 800 sample size). These include five samples from ESC and one sample from LUX. All downstream analyses are based on the rarefied OTU counts.

To evaluate beta-diversity of field soil and rhizosphere samples, we used weighted UNIFRAC to generate distance matrices using the distance function in R *phyloseq* and then ordinated via principal coordinate analysis (PCoA) using the *ordinate* function in R phyloseq. We used *adonis* in R vegan to conduct PERMANOVA of the soil and rhizosphere distance matrices by site*legacy N (soils) and site*legacy N*short-term N (rhizosphere). Differences between treatment groups were considered significant at $\alpha \leq 0.05$. We also explored differences in the relative abundance for diazotroph classes by site, legacy, and short-term N additions using a three-factor ANOVA as described above.

Lastly, we explored diazotroph community data for OTUs potentially important to predicting FLNF. First, FLNF data was binned as follows: 0-1, 1-2, 2-4, 4-6, 6-10, > 10 μg N fixed g^{-1} rhizosphere day^{-1}. We then used *indicspieces* package in R to identify OTUs associated with each N-fixation bin, with a focus on those OTUs associated with the >10 FLNF rate bin. We also manually explored OTUs associated with FLNF using presence-absence data. First, we selected OTUs which were present in at least 10 samples where N-fixation had been measured (i.e. non-zero N-fixation). We then used a

one-way ANOVA to identify OTUs in which there was at least a marginally significant ($p < 0.1$) difference in FLNF rates when these OTUs were present versus when they were absent. We selected OTUs for further exploration where the mean of FLNF trended towards greater when those OTUs were present versus absent.

3.4 RESULTS

3.4.1 Plant metrics

Aboveground plant biomass differed significantly by site and N treatment (Fig. 3.2). Generally, short-term N additions of high N tended to result in greater aboveground biomass with the impact of legacy N effects varying by site. We were unable to assess total belowground biomass in this study.

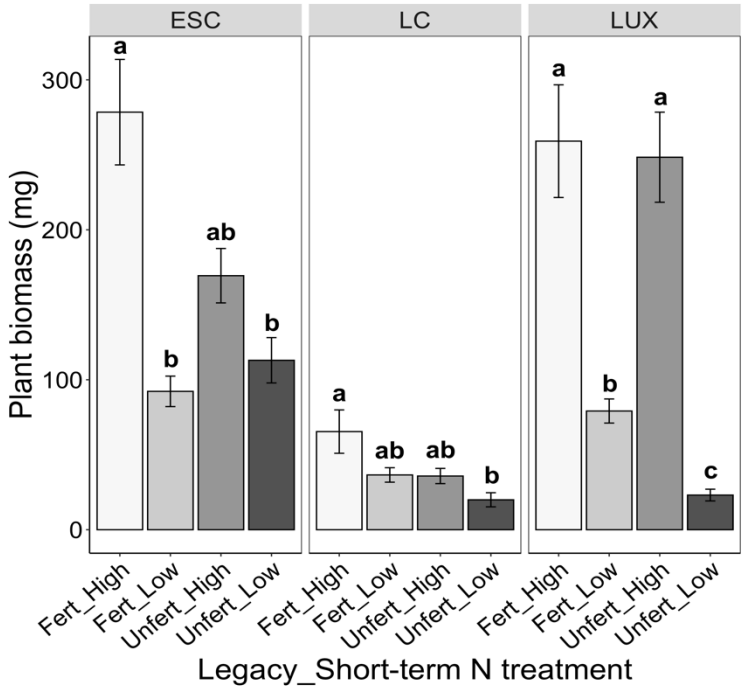

Figure 3.2: Plant aboveground biomass (mg) by site (ESC = Escanaba, LC = Lake City, LUX = Lux Arbor) and legacy by short-term N treatments (Fert_High = Fertilized field + High N addition in greenhouse, Fert_Low = Fertilized field + Low N greenhouse, Unfert_High = Unfertilized field + High N greenhouse, Unfert_Low = Unfertilized field + Low N addition). Each bar is representative of n = 24. Letters indicate significant differences within a site where $p < 0.05$.

3.4.2 Free-living nitrogen fixation rates

FLNF rates, measured in the rhizosphere, were not significantly impacted by site ($F = 1.309$, $p = 0.272$), legacy N ($F = 1.208$, $p = 0.273$), or short-term N addition ($F = 0.83$, $p = 0.363$). Rates were highly variable across nitrogen treatments (Fig. 3.3), even within sites, ranging from below detection to nearly 30 µg N fixed g^{-1} rhizosphere day^{-1}. Coefficients of variation (CV) for site, legacy N, and short-term N addition were often greater than the overall variation of all FLNF rates (CV = 299.7%). Legacy N treatments had CV of 275.6% and 333.8% for Fert and Unfert treatments, respectively, while CV of short-term N treatments were 300.2% and 285.7% for high and low N, respectively. CV

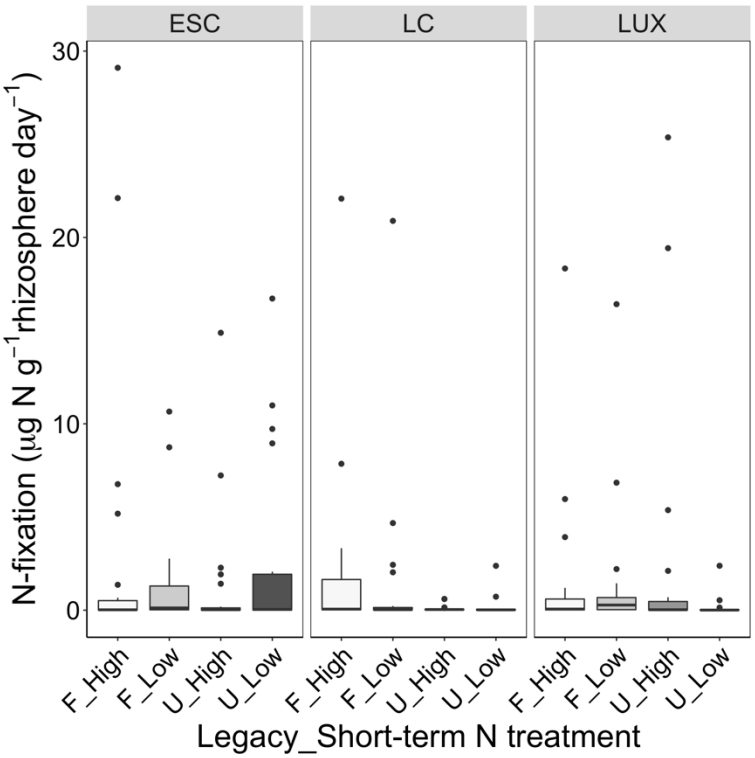

Figure 3.3: Nitrogen fixation rates of the rhizosphere for each site (ESC = Escanaba, LC = Lake City, LUX = Lux Arbor) by the legacy and short-term N treatments (F_High = Fertilized field + High N addition in greenhouse, F_Low = Fertilized field + Low N greenhouse, U_High = Unfertilized field + High N greenhouse, U_Low = Unfertilized field + Low N addition). Boxplots show full range of N-fixation values for each treatment with solid black horizontal bars representing average N-fixation (n = 24 per bar).

within each site were 253.8%, 376.9%, and 305.1% for ESC, LC, and LUX, respectively. N-fixation rates did not correlate significantly with aboveground plant biomass or any diazotrophs groups including phylum and class.

3.4.3 Diazotroph community structure

Initial diazotroph community composition, those organisms present in the soil inoculum collected from three different locations, differed significantly by site (Fig. 3.4A). Three groups appear to drive the observed differences in community composition between sites in the soil inoculum: Actinobacteria, Gammaprotebacteria, and Methanococci (Fig. 3.5 and Table 3.2). Actinobacteria had greater relative abundance in

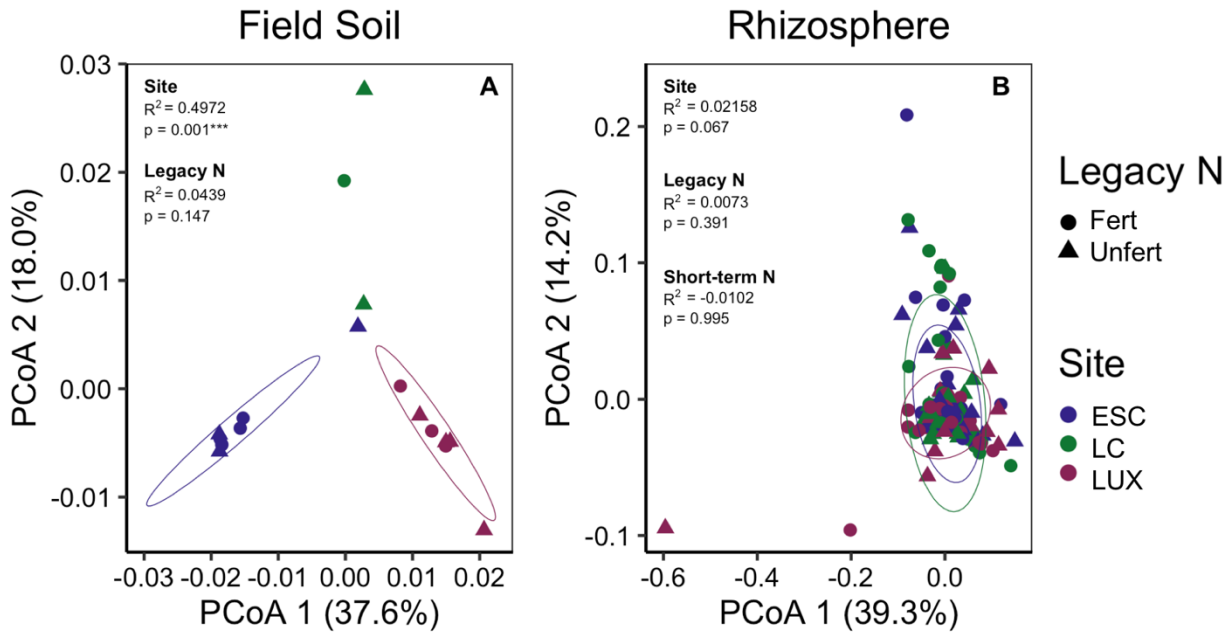

Figure 3.4: Principal coordinates analysis of (A) field soil and (B) rhizosphere samples. Points are based on a weighted Unifrac distance matrix of the relative abundance of rarefied OTU counts. Points are colored by site (ESC = Escanaba, LC = Lake City, LUX = Lux Arbor) with shape representing legacy N treatment (Fert = Fertilized, Unfert = Unfertilized). Each point represents one sample. Ellipses represent 95% confidence ellipse of the centroid for each site. Note that LC does not have an ellipse because only 3 soil samples (of the 8 total) amplified for *nif*H and were sequenced.

ESC soils than in LC or LUX soils. Gammaproteobacteria and Methanococci had greater relative abundance in LC soils than in ESC or LUX soils. Legacy N had no impact on overall diazotroph community composition (Fig. 3.4A) or community members (Fig. S3.1).

Differences in community composition by site were no longer apparent in the rhizosphere samples (Fig. 3.4B). Examining relative abundance of diazotroph community members by class across sites reveals strikingly similar community composition in the rhizosphere, with no significant differences in any classes between sites (Fig. 3.5). This response was mainly driven by just 1 or 2 classes of diazotrophs within each site. ESC saw decreases in Actinobacteria and increases in

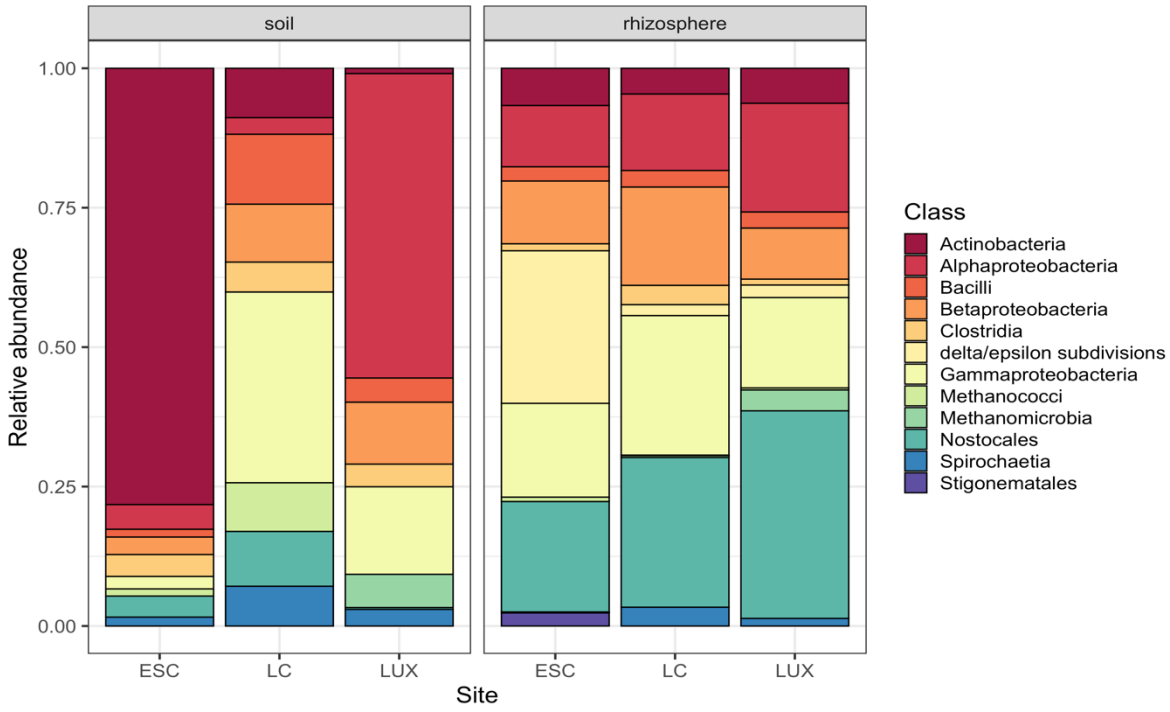

Figure 3.5: Average relative abundance of diazotroph classes by site (ESC = Escanaba, LC = Lake City, LUX = Lux Arbor) for soil (those soils used to inoculate the greenhouse pots) and rhizosphere samples. There were no significant differences between sites in rhizosphere samples. Bars represent the average of 8 samples for soil (only 3 for LC) and 96 samples for rhizosphere. Full results are presented in Table 3.1.

Alphaproteobacteria. LC saw a decrease in Methanococci, while LUX saw an increase in Nostocales (Table 3.1). N treatment, whether legacy or short-term, had minimal effects on the rhizosphere diazotroph community (Fig. 3.6). Legacy N was associated with greater relative abundance of Betaproteobacteria when fertilized ($F = 4.675$, $p = 0.0306$) and Actinobacteria when unfertilized ($F = 8.222$, $p = 0.0041$). Short-term additions of high N increased the relative abundance Alphaproteobacteria ($F = 5.676$, $p = 0.017$), while additions of low N increased the relative abundance of Nostocales ($F = 18.647$, $p < 0.0001$).

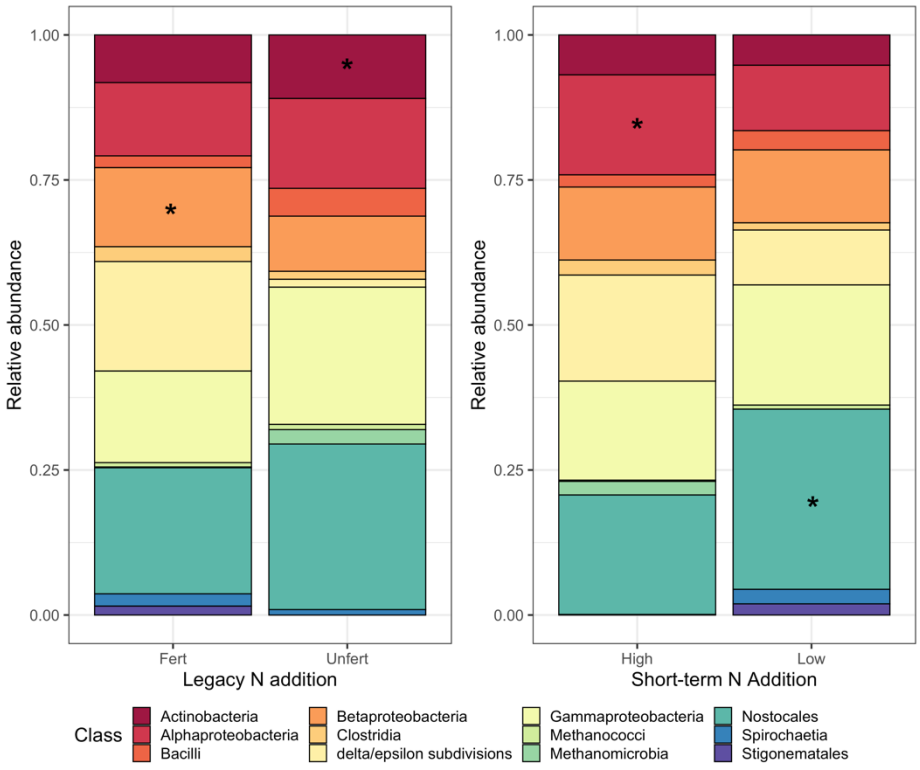

Figure 3.6: Average relative abundance of diazotroph classes by legacy N (Fert = Fertilized, Unfert = Unfertilized) and short-term N additions (High = High N addition, Low = Low N addition) for rhizosphere samples. Asterisks indicate significant difference in diazotroph class by N treatment at $p < 0.05$ with asterisk placed on the bar with greater relative abundance. Each bar represents the average of 144 samples.

3.4.4 Indicator species

We identified six diazotroph OTUs using the *indicspecies* function which were indicative of samples where we measured FLNF rates at greater than 10 μg N fixed g^{-1} rhizosphere day^{-1} and may be important for understanding FLNF (Table 3.3). These included three Cyanobacteria, two Proteobacteria, and one Actinobacteria. In a separate analysis, we also examined OTU presence and absence relative to FLNF to identify OTUs whose presence was associated with increased FLNF relative to when those OTUs were absent. We then looked for the presence of those OTUs in the top ten samples with the greatest fixation rates. We found 4 OTUs which were consistently present in the top ten samples (Table 3.4) with 8 of these 10 samples containing at least 1 of the identified OTUs and 6 of the 10 samples containing at least 3 of these OTUs. Though these OTUs were different than those identified with *indicspecies*, they share similar phylogeny. Both groups of OTUs contain members of the *Frankiaceae* and *Nostoc* families.

Table 3.2: Average relative abundance and standard errors of diazotroph classes by sample type and site (presented visually in Fig. 3.3). Lowercase letters indicate significant differences between sample type where $p < 0.05$. Asterisks indicate significant difference between sample type across sites where $p < 0.05$. Relative abundances with significant differences by sample type or across sites are bolded for ease of reading.

	Soil			Rhizosphere		
Class	**ESC**	**LC**	**LUX**	**ESC**	**LC**	**LUX**
Actinobacteria	**0.782 ± 0.028** a *	**0.088 ± 0.5** b	**0.009 ± 0.04** b	0.066 ± 0.095	0.046 ± 0.057	0.062 ± 0.008
Alphaproteobacteria	**0.044 ± 0.024** *	0.029 ± 0.084	0.545 ± 0.043	0.109 ± 0.012	0.137 ± 0.075	0.194 ± 0.243
Bacilli	0.013 ± 0.061	0.125 ± 0.038	0.043 ± 0.086	0.025 ± 0.179	0.029 ± 0.065	0.028 ± 0.084
Betaproteobacteria	0.031 ± 0.055	0.103 ± 0.054	0.111 ± 0.137	0.112 ± 0.111	0.176 ± 0.086	0.091 ± 0.143
Clostridia	0.039 ± 0.013	0.053 ± 0.047	0.04 ± 0.082	0.012 ± 0.046	0.034 ± 0.014	0.01 ± 0.041
delta/epsilon subdivisions	0 ± 0.569	0 ± 0	0 ± 0.055	0.273 ± 0	0.019 ± 0.08	0.022 ± 0
Gammaprotebacteria	**0.022 ± 0.109** b	**0.342 ± 0.044** a	**0.156 ± 0.272** b	0.168 ± 0.324	0.25 ± 0.16	0.162 ± 0.196
Methanococci	**0.012 ± 0.021** b	**0.087 ± 0.042** a *	**0 ± 0.004** b	0.007 ± 0.081	0.001 ± 0.01	0.003 ± 0
Methanomicrobia	0 ± 0	0 ± 0	0.059 ± 0.012	0 ± 0	0.003 ± 0.18	0.037 ± 0.2
Nostocales	0.037 ± 0.069	0.098 ± 0.132	**0.003 ± 0.134** *	0.197 ± 0.067	0.268 ± 0.203	0.372 ± 0.007
Spirochaetia	0.016 ± 0.003	0.071 ± 0.054	0.029 ± 0.13	0.001 ± 0.08	0.033 ± 0.066	0.013 ± 0.074
Stigonematales	0 ± 0.044	0 ± 0	0 ± 0	0.023 ± 0	0 ± 0	0 ± 0

Table 3.3: Table of OTUs identified by *indicspecies* as associated with N-fixation rates of > 10 µg N per g rhizosphere per day

	cluster	kingdom	phylum	class	order	family	genus
OTU497	cluster I	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	Unidentified
OTU6505	cluster I	Bacteria	Cyanobacteria	Nostocales	Nostocaceae	Anabaena	Anabaena
OTU159	cluster I	Bacteria	Cyanobacteria	Nostocales	Nostocaceae	Nostoc	Nostoc
OTU304	cluster I	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Zoogloea
OTU285	cluster I	Bacteria	Cyanobacteria	Nostocales	Nostocaceae	Anabaena	Anabaena
OTU92	cluster I	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium

Table 3.4: Table of OTUs whose presence appears to be associated with increased N-fixation rates

	cluster	kingdom	phylum	class	order	family	genus
OTU29	cluster I	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	Unidentified
OTU60	cluster I	Bacteria	Cyanobacteria	Nostocales	Nostocaceae	Nostoc	Nostoc
OTU846	cluster I	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	Unidentified
OTU991	cluster I	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	Unidentified

3.5 DISCUSSION

This work aimed to improve our understanding of FLNF and its potential to contribute plant available N to switchgrass cropping systems. In particular, we aimed to evaluate the impact of legacy and short-term N additions on FLNF through measurement of FLNF rates and characterization of the diazotroph community associated with the switchgrass rhizosphere. We found that diazotroph community structure of field soil was significantly different across sites, but was not influenced by legacy N additions. These differences in community structure were no longer evident in

rhizosphere communities at plant harvest. FLNF rates were extremely variable with no significant trends related to N additions or site, or whole diazotroph community structure.

3.5.1 Diazotroph community composition in response to N availability

Perhaps the most intriguing finding of this study is the strong selective pressure which the switchgrass rhizosphere exerted on the diazotroph community. Despite soils being collected from different field sites, and displaying different initial diazotroph communities (Fig. 3.4A), rhizosphere samples collected at the end of the greenhouse experiment showed little to no evidence of this site history. Although site was a significant predictor of field soil diazotroph community composition, neither site nor N treatments were factors in driving overall rhizosphere diazotroph communities in our greenhouse pots. However, we did find that the abundance of some specific community members varied with legacy or short-term N (Fig. 3.6). This contradicts our first and second hypotheses that site, N treatment, and their interactions would drive diazotroph community composition and that short-term N addition would have a greater impact than legacy N. These results are also contrary to other work which found long-term N fertilization to alter diazotroph community composition (Wang et al 2016; Feng et al 2018; Roley et al. 2019). Rather, it seems that rhizosphere effects outweighed the N treatment effects.

Previous studies have observed a similar selective pressure of the rhizosphere on community composition where rhizosphere and root-associated communities were distinct from bulk soil communities (Costa et al. 2006; Singh et al. 2007; Roley et al. 2019). However, the role of plant species is not clear with some studies finding different

plant species have distinct rhizosphere communities (Costa et al. 2006; Garbeva et al. 2008) and others finding plant species is a small or non-significant driver of rhizosphere community composition (Singh et al. 2007; Jesus et al. 2010). Switchgrass has specifically been observed to exert selective pressure on soil communities, cultivating a rhizosphere community distinct from bulk soil and other plant species (Chaudhary et al. 2012) and even other switchgrass cultivars (Rodrigues et al. 2016). Although our results do not allow us to state that the observed rhizosphere diazotroph community is specific to and/or selected by switchgrass, it is clear that the rhizosphere shapes diazotroph community composition.

In addition to switchgrass and root exudate selective pressures, we must also acknowledge the possibility that some of the selection effect on rhizosphere diazotroph communities may be due to growth conditions during the study. In particular, the sandy texture of our growth media may have selected for community members common in sandy soils, like those found at LC. Soil texture at LC was distinct from LUX and ESC, being predominately sand (~84%), while soils at LUX and ESC had a more even distribution of sand, silt, and clay (Kasmerchak and Schaetzl 2018). We observed that diazotroph communities in ESC and LUX field soil, when under rhizosphere conditions, tended to shift towards a composition similar to that observed in LC soils (Fig. 3.5; Supp. Fig. 3.2). We also found LC field soils and LC rhizosphere communities to be indistinguishable, while soil communities clustered separately from rhizosphere communities for LUX and ESC field soils (Supp. Fig. 3.2). Thus, the sandy composition of our growth media may have mimicked conditions most similar to those at LC. The role of soil texture has been observed in other work which found that soil type was a

major driver of bacterial and fungal communities (Singh et al. 2007; Garbeva et al. 2008; Jesus et al. 2010).

In addition to soil texture, greenhouse conditions could have played some role in the homogenization of the rhizosphere diazotroph community. All plants were grown in the greenhouse under controlled conditions of ideal moisture, light, and temperature, which could have exerted selective pressure on the diazotroph community. Collectively, our results suggest that the switchgrass rhizosphere has the potential to select a specific and consistent diazotroph community and that this selection may be moderated by soil texture, but is not moderated by legacy or short-term N additions.

3.5.2 FLNF in response to N availability across sites

We found FLNF rates to be highly variable across all sites and treatments, ranging from below detection to nearly 30 μg N fixed g^{-1} rhizosphere day^{-1}, which is 10 to 30x greater than rates of FLNF typically reported for switchgrass roots and associated soils (Roley et al. 2019; Smercina et al. 2019). These rates even exceed those typically observed in other grassland systems. FLNF of temperate grasslands is estimated at an average of 4.7 kg N ha^{-1} yr^{-1} with an upper limit of 20 kg N ha^{-1} yr^{-1} (Reed et al. 2011). Our highest measured rates would be roughly equivalent to 30.6 kg N ha^{-1} yr^{-1} (assuming 700 g rhizosphere m^{-2} to 15 cm depth; Roley et al. 2019), well above even the upper estimate for temperate grasslands. Surprisingly, none of our N addition treatments or plant aboveground biomass explained FLNF rates. Though, other studies measuring FLNF in the switchgrass rhizosphere have similarly observed no response of FLNF to fertilizer N additions (Roley et al. 2018). Additionally, no drivers significantly influenced the differences in sample variability (average CV = 299.7%) of

FLNF rates. Sporadic and episodic data has been observed in other measurements of switchgrass-associated FLNF rates (Roley et al. 2018) and nifH transcripts (Bahulikar et al. 2014), suggesting that controls on FLNF may be also be variable or sporadic.

We hypothesize that the high variability in FLNF rates observed in this study may relate to heterogeneity in the distribution of diazotrophs, N availability, and/or C availability. We know that unexpectedly high microbial process rates can link to spatial and temporal heterogeneity in microbes and nutrients – so called “hot spots” and “hot moments” (Groffman et al. 2009; Kuzyakov and Blagodatskaya 2015 and references therein). Roots used in our study to measure FLNF rates were chosen at random and therefore were likely heterogeneous in size/volume, diazotroph colonization, and mycorrhizal associations. Additionally, root samples may have included dead and decaying roots. Differences in root size and volume, presence of dead/decaying roots, and abundance of mycorrhizal associates could influence the availability of C in the rhizosphere (Jones et al. 2009; Nie et al. 2013). Diazotrophs are dependent on readily available C to support FLNF activity, thus C is likely a major control on FLNF (Smercina et al. 2019a). Rhizosphere colonization is also likely heterogenous, the controls of which are not well understood, thus it is likely that capture of an active diazotroph community on root surfaces would occur randomly.

Our results of FLNF counter our first two hypotheses that site and N availability would drive FLNF rates and that short-term N would impact FLNF to a greater extent than legacy N. Further, we find no evidence that differences in timing of N availability, legacy versus short-term, impact measured FLNF. Rather, our results seem to suggest that N availability, as measured at the plant to ecosystem-scale, may not accurately

predict N availability at the diazotroph scale. Alternatively, these results may indicate that N availability is not a major driver of FLNF, as expected from past work. This leaves large questions about whether N availability drives FLNF and if/how we can predict FLNF from environmental conditions.

3.5.3 Linking diazotroph community composition and FLNF rates

In contrast to our third hypothesis, we did not find direct evidence for an association between diazotroph community composition and FLNF rates. However, the presence of some diazotrophs may be associated with greater FLNF. Several organisms were identified as indicator species of samples where FLNF rates were greater than 10 μg N fixed g^{-1} rhizosphere day^{-1}. We also identified four organisms whose presence appears to be associated with increased FLNF (i.e. when these organisms were present, FLNF rates tended to be greater than when they were absent). These four organisms were consistently present in the ten samples with the highest overall FLNF rates with eight of these ten samples containing at least one of the identified OTUs and six of these ten samples containing at least three of these OTUs. While this is not direct evidence of these organisms driving FLNF rates, it does suggest that these organisms may warrant further study.

Groups associated with greater FLNF included photosynthetic diazotrophs including *Nostoc* and *Anabaena* species, and diazotrophs typical of symbiotic N-fixation including members of the *Frankiaceae* and *Bradyrhizobiaceae* families. We were surprised to find that OTUs associated with greater FLNF including photosynthetic organisms, which are unlikely to rely on root exuded C from switchgrass to support N-fixation. However, these cyanobacteria may still contribute significantly to plant available

N as *Nostoc* and *Anabeana* species are estimated to release 5 to 70% of fixed N to their surroundings (Belnap 2001). FLNF by cyanobacteria is well-studied in the context of desert biological soil crusts (BSCs), where cyanobacteria interact closely with soil particles to form a cohesive structure (Belnap 2003). These BSCs are vital to maintaining the fertility of desert soil systems by increasing nutrient availability and promoting water retention (Belnap 2003), thus it is plausible these organisms may play similar roles in marginal land systems. Indeed, application of cyanobacteria to marginal lands for improvement of soil fertility has been proposed (D'Acqui 2016; Rossi et al 2017) and tested on African soils with success (D'Acqui 2016). This suggests that cyanobacteria may play an important, yet unexplored, role in supporting bioenergy crop productivity on marginal lands.

We were also surprised to find that known symbiotic N-fixers were associated with greater FLNF in our system. *Bradyrhizobium* species are among the most well-studied N-fixing symbionts, but their contribution to FLNF is not well understood. Many *Bradyrhizobium* ecotypes are found in soils as non-symbiotic or free-living organisms (VanInsberghe et al 2015) and have been shown to associate with many non-legume plants as root endophytes (Schneijderberg et al 2018). They have also been previously identified in the switchgrass rhizosphere (Bahulikar et al. 2014; Roley et al. 2019), including as root endophytes (Bahulikar et al 2019), and are abundant in natural grassland systems (Delmont et al 2012). It has been suggested that these free-living ecotypes lack or are diminished in their N-fixation capacity (VanInsberghe et al 2015; Schneijderberg et al 2018), and may even cheat plant-associates (Sachs et al 2010). However, the association with *Bradyrhizobium* and FLNF rates in our study would

suggest they are actively fixing N in the switchgrass rhizosphere. Indeed, a study of FLNF associated with energy sorghum (*Sorghum bicolor*) cultivars found that those cultivars with highest root N-fixation harbored a high abundance of *Bradyrhizobium* species (Hara et al 2019). This highlights a potentially important role of diazotrophs, typically studied for their symbiotic N-fixing capacity, to contribute N to plants when living associatively rather than symbiotically in the rhizosphere.

3.6 CONCLUSIONS

The results of this study were surprising. FLNF rates were not explained by site or N treatments and overall were highly variable. We posit that these results, coupled with our sampling method, may indicate that spatial heterogeneity of the rhizosphere is a major driver of FLNF. We also found that overall rhizosphere diazotroph community composition was not explained by field site or N treatments. However, we did identify a clear rhizosphere effect on the diazotroph community whereby effects of site on field soil diazotroph communities were completely masked in the rhizosphere.

As with many previous studies, we were unable to establish a direct link between functional community structure and measured process rates. Shifts in relative abundance or physiology of diazotrophs and other functional guilds are difficult to assess, but are likely to be where the link between community and function or process rates resides (Jansson and Hofmockel 2018; Jansson and Hofmockel 2019 and references therein). Other studies have similarly struggled to link *nifH* diversity or *nifH* based community structure and N-fixation function (Fürnkranz et al 2008; Knief et al. 2012). Though no direct link between community composition and function was established in this study, we were able to find evidence for diazotrophs potentially

important for predicting FLNF in the rhizosphere that warrant further investigation. Overall, we need to examine more closely functional groups such as diazotrophs whose role in supporting N demands of bioenergy crops, like switchgrass, has been relatively unexplored.

APPENDIX

SUPPLEMENTAL MATERIAL

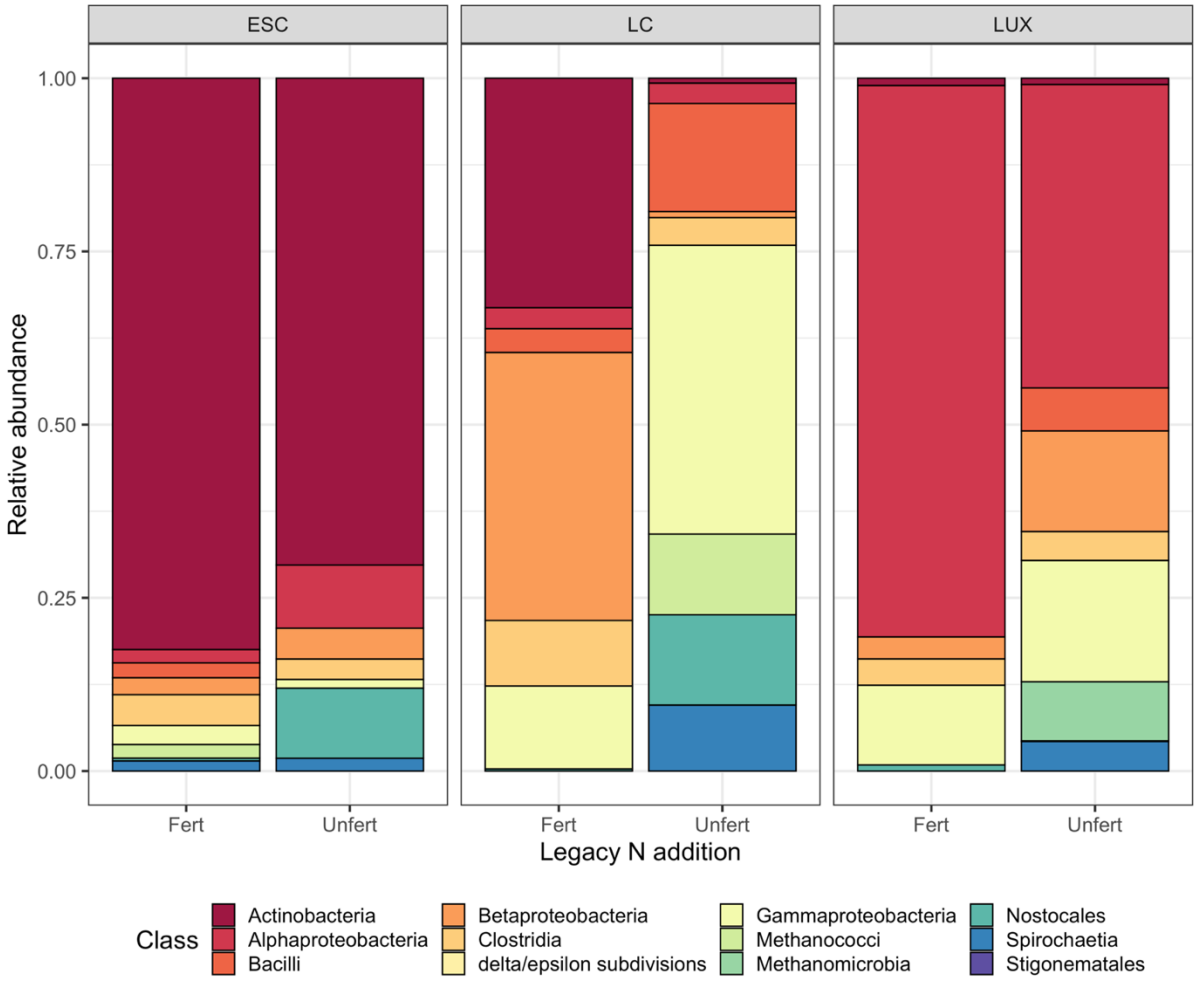

Figure S3.1: Average relative abundance of diazotroph classes by legacy N addition and site for soil samples. No significant differences were found for diazotroph classes by legacy N additions within sites. Bars for ESC and LUX represent the average of 4 samples. LC bars are represented by 1 sample for Fert and 2 samples for Unfert

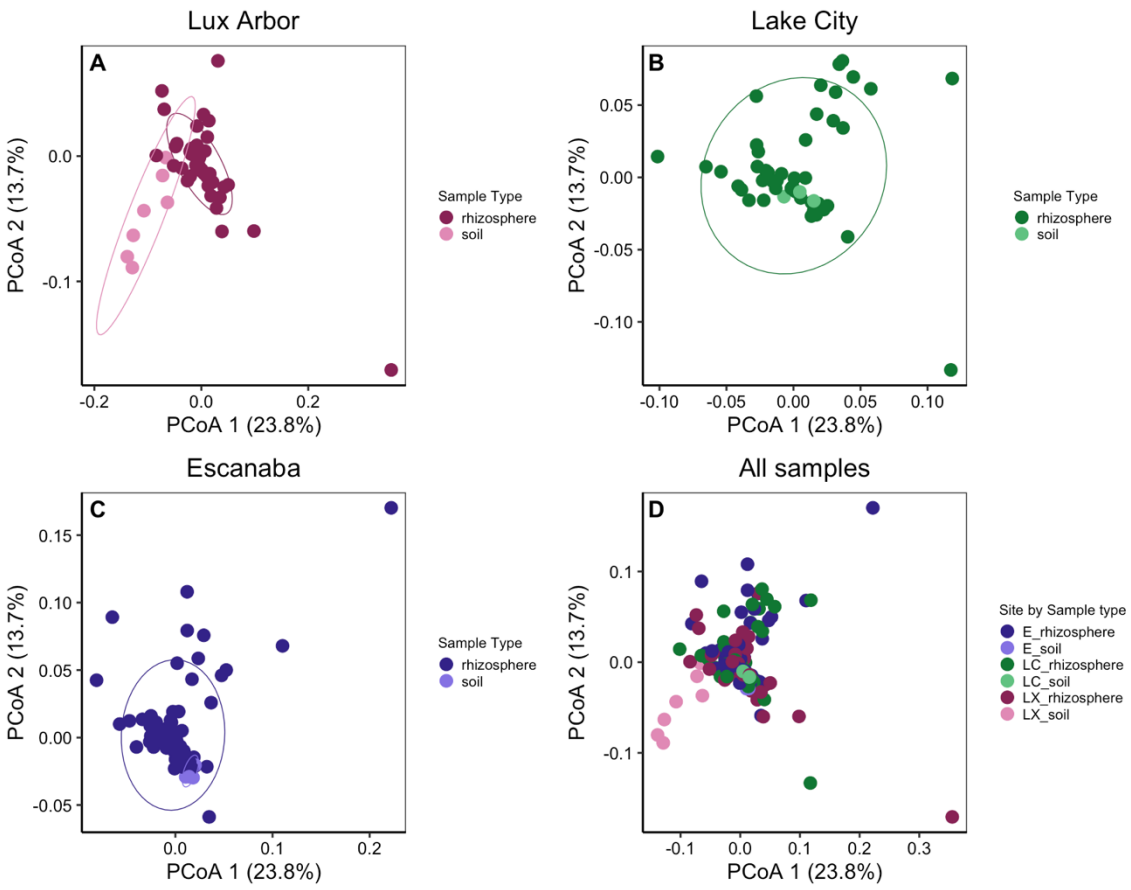

Figure S3.2: Principal coordinates analysis of field soil samples and rhizosphere samples for (A) Lux Arbor, (B) Lake City, (C) Escanaba and (D) All samples. Points are based on a weighted Unifrac distance matrix of the relative abundance of rarefied OTU counts. Points are colored by A-C: sample type (soil or rhizosphere) and D: Site by sample type. Each point represents one sample. Ellipses represent 95% confidence ellipse of the centroid for each sample type. Note that LC soil samples do not have an ellipse because only 3 soil samples (of the 8 total) amplified for *nif*H and were sequenced.

REFERENCES

REFERENCES

Angel, R., Nepel, M., Panhölzl, C., Schmidt, H., Herbold, C. W., Eichorst, S. A., & Woebken, D. (2018). Evaluation of Primers Targeting the Diazotroph Functional Gene and Development of NifMAP–A Bioinformatics Pipeline for Analyzing nifH Amplicon Data. *Frontiers in Microbiology*, *9*, 703.

Bahulikar, R. A., Torres-Jerez, I., Worley, E., Craven, K., & Udvardi, M. K. (2014). Diversity of nitrogen-fixing bacteria associated with switchgrass in the native tallgrass prairie of Northern Oklahoma. Appl. Environ. Microbiol., 80(18), 5636-5643.

Bahulikar, R. A., Chaluvadi, S. R., Torres-Jerez, I., Mosali, J., Bennetzen, J. L., & Udvardi, M. (2019). Nitrogen fertilization reduces nitrogen fixation activity of diverse diazotrophs in switchgrass roots. Phytobiomes Journal, (ja).

Belnap, J. (2001). Factors influencing nitrogen fixation and nitrogen release in biological soil crusts. In *Biological soil crusts: structure, function, and management* (pp. 241-261). Springer, Berlin, Heidelberg.

Belnap, J. (2003). The world at your feet: desert biological soil crusts. *Frontiers in Ecology and the Environment*, *1*(4), 181-189.

Bloch, S. E., Ryu, M. H., Ozaydin, B., & Broglie, R. (2020). Harnessing atmospheric nitrogen for cereal crop production. *Current Opinion in Biotechnology*, *62*, 181-188.

Chaudhary, D. R., Saxena, J., Lorenz, N., Dick, L. K., & Dick, R. P. (2012). Microbial profiles of rhizosphere and bulk soil microbial communities of biofuel crops switchgrass (Panicum virgatum L.) and jatropha (Jatropha curcas L.). *Applied and Environmental Soil Science*, *2012*.

Costa, R., Götz, M., Mrotzek, N., Lottmann, J., Berg, G., & Smalla, K. (2006). Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *FEMS microbiology ecology*, *56*(2), 236-249.

D'Acqui, L. P. (2016). Use of indigenous cyanobacteria for sustainable improvement of biogeochemical and physical fertility of marginal soils in semiarid tropics. In Bioformulations: for Sustainable Agriculture (pp. 213-232). Springer, New Delhi.

Delmont, T. O., Prestat, E., Keegan, K. P., Faubladier, M., Robe, P., Clark, I. M., ... & Le Paslier, D. (2012). Structure, fluctuation and magnitude of a natural grassland soil metagenome. The ISME journal, 6(9), 1677-1687.

Dixon R, Kahn D. 2004. Genetic regulation of biological nitrogen fixation. Nature Reviews Microbiology 2: 621-631.

Fan, K., Delgado-Baquerizo, M., Guo, X., Wang, D., Wu, Y., Zhu, M., ... & Chu, H. (2019). Suppressed N fixation and diazotrophs after four decades of fertilization. *Microbiome*, *7*(1), 1-10.

Feng, M., Adams, J. M., Fan, K., Shi, Y., Sun, R., Wang, D., ... & Chu, H. (2018). Long-term fertilization influences community assembly processes of soil diazotrophs. *Soil Biology and Biochemistry*, *126*, 151-158.

Fürnkranz, M., Wanek, W., Richter, A., Abell, G., Rasche, F., & Sessitsch, A. (2008). Nitrogen fixation by phyllosphere bacteria associated with higher plants and their colonizing epiphytes of a tropical lowland rainforest of Costa Rica. *The ISME journal*, *2*(5), 561-570.

Gaby, J. C., & Buckley, D. H. (2014). A comprehensive aligned nifH gene database: a multipurpose tool for studies of nitrogen-fixing bacteria. *Database*, *2014*.

Gaby, J. C., Rishishwar, L., Valderrama-Aguirre, L. C., Green, S. J., Valderrama-Aguirre, A., Jordan, I. K., & Kostka, J. E. (2018). Diazotroph community characterization via a high-throughput nifH amplicon sequencing and analysis pipeline. *Appl. Environ. Microbiol.*, *84*(4), e01512-17.

Garbeva, P., Van Elsas, J. D., & Van Veen, J. A. (2008). Rhizosphere microbial community and its response to plant species and soil history. *Plant and soil*, *302*(1-2), 19-32.

Gelfand, I., Sahajpal, R., Zhang, X., Izaurralde, R. C., Gross, K. L., & Robertson, G. P. (2013). Sustainable bioenergy production from marginal lands in the US Midwest. *Nature*, *493*(7433), 514-517.

Groffman, P. M., Butterbach-Bahl, K., Fulweiler, R. W., Gold, A. J., Morse, J. L., Stander, E. K., ... & Vidon, P. (2009). Challenges to incorporating spatially and temporally explicit phenomena (hotspots and hot moments) in denitrification models. *Biogeochemistry*, *93*(1-2), 49-77.

Hara, S., Morikawa, T., Wasai, S., Kasahara, Y., Koshiba, T., Yamazaki, K., ... & Minamisawa, K. (2020). Identification of Nitrogen-Fixing Bradyrhizobium Associated With Roots of Field-Grown Sorghum by Metagenome and Proteome. Metabolic Adjustments and Gene Expression Reprogramming for Symbiotic Nitrogen Fixation in Legume Nodules.

Harter, J., Krause, H. M., Schuettler, S., Ruser, R., Fromme, M., Scholten, T., ... & Behrens, S. (2014). Linking N 2 O emissions from biochar-amended soil to the structure and function of the N-cycling microbial community. The ISME journal, 8(3), 660-674.

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(1), 176-190.

Jansson, J. K., & Hofmockel, K. S. (2018). The soil microbiome—from metagenomics to metaphenomics. *Current opinion in microbiology*, *43*, 162-168.

Jansson, J. K., & Hofmockel, K. S. (2019). Soil microbiomes and climate change. *Nature Reviews Microbiology*, 1-12.

Jones, D. L., Nguyen, C., & Finlay, R. D. (2009). Carbon flow in the rhizosphere: carbon trading at the soil–root interface. *Plant and soil*, *321*(1-2), 5-33.

Jesus, E. D. C., Susilawati, E., Smith, S. L., Wang, Q., Chai, B., Farris, R., ... & Tiedje, J. M. (2010). Bacterial communities in the rhizosphere of biofuel crops grown on marginal lands as evaluated by 16S rRNA gene pyrosequences. *Bioenergy research*, *3*(1), 20-27.

Kasmerchak, C. S. and R. Schaetzl. 2018. Soils of the GLBRC Marginal Land Experiment (MLE) Sites. Kellogg Biological Station Longterm Ecological Research Special Publication. Zenodo. http://doi.org/10.5281/zenodo.2578238

Klinger, C. R., Lau, J. A., & Heath, K. D. (2016). Ecological genomics of mutualism decline in nitrogen-fixing bacteria. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1826), 20152563.

Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Wassmann, R., ... & Vorholt, J. A. (2012). Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *The ISME journal*, *6*(7), 1378-1390.

Kuzyakov, Y., & Blagodatskaya, E. (2015). Microbial hotspots and hot moments in soil: concept & review. *Soil Biology and Biochemistry*, *83*, 184-199.

Mehmood, M. A., Ibrahim, M., Rashid, U., Nawaz, M., Ali, S., Hussain, A., & Gull, M. (2017). Biomass production for bioenergy using marginal lands. *Sustainable Production and Consumption*, *9*, 3-21.

Nie, M., Lu, M., Bell, J., Raut, S., & Pendall, E. (2013). Altered root traits due to elevated CO2: A meta-analysis. *Global Ecology and Biogeography*, *22*(10), 1095-1105.

Norman, J. S., & Friesen, M. L. (2017). Complex N acquisition by soil diazotrophs: how the ability to release exoenzymes affects N fixation by terrestrial free-living diazotrophs. *The ISME journal*, *11*(2), 315-326.

Reed, S. C., Cleveland, C. C., & Townsend, A. R. (2011). Functional ecology of free-living nitrogen fixation: a contemporary perspective. Annual review of ecology, evolution, and systematics, 42, 489-512.

Robertson, G. P., Hamilton, S. K., Barham, B. L., Dale, B. E., Izaurralde, R. C., Jackson, R. D., ... & Tiedje, J. M. (2017). Cellulosic biofuel contributions to a sustainable energy future: Choices and outcomes. *Science*, *356*(6345), eaal2324.

Rocca, J. D., Hall, E. K., Lennon, J. T., Evans, S. E., Waldrop, M. P., Cotner, J. B., ... & Wallenstein, M. D. (2015). Relationships between protein-encoding gene abundance and corresponding process are commonly assumed yet rarely observed. The ISME journal, 9(8), 1693-1699.

Rodrigues, R. R., Moon, J., Zhao, B., & Williams, M. A. (2017). Microbial communities and diazotrophic activity differ in the root-zone of Alamo and Dacotah switchgrass feedstocks. *GCB Bioenergy*, *9*(6), 1057-1070.

Roley, S. S., Duncan, D. S., Di Liang, A. G., Jackson, R. D., Tiedje, J. M., & Robertson, G. P. (2018). Associative nitrogen fixation (ANF) in switchgrass (Panicum virgatum) across a nitrogen input gradient. *PloS one*, *13*(6).

Roley, S. S., Xue, C., Hamilton, S. K., Tiedje, J. M., & Robertson, G. P. (2019). Isotopic evidence for episodic nitrogen fixation in switchgrass (Panicum virgatum L.). *Soil Biology and Biochemistry*, *129*, 90-98.

Rossi, F., Li, H., Liu, Y., & De Philippis, R. (2017). Cyanobacterial inoculation (cyanobacterisation): perspectives for the development of a standardized multifunctional technology for soil fertilization and desertification reversal. Earth-Science Reviews, 171, 28-43.

Sachs, J. L., Ehinger, M. O., & Simms, E. L. (2010). Origins of cheating and loss of symbiosis in wild Bradyrhizobium. Journal of evolutionary biology, 23(5), 1075-1089.

Schmidt, J. E., Weese, D. J., & Lau, J. A. (2017). Long-term agricultural management does not alter the evolution of a soybean–rhizobium mutualism. *Ecological Applications*, *27*(8), 2487-2496.

Singh, B. K., Munro, S., Potts, J. M., & Millard, P. (2007). Influence of grass species and soil type on rhizosphere microbial community structure in grassland soils. *Applied Soil Ecology*, *36*(2-3), 147-155.

Smercina, D. N., Evans, S. E., Friesen, M. L., & Tiemann, L. K. (2019a). Optimization of the $^{15}N_2$ incorporation and acetylene reduction methods for free-living nitrogen fixation. *Plant and Soil*, *445*(1), 595-611.

Smercina, D. N., Evans, S. E., Friesen, M. L., & Tiemann, L. K. (2019b). To fix or not to fix: controls on free-living nitrogen fixation in the rhizosphere. Appl. Environ. Microbiol., 85(6), e02546-18.

VanInsberghe, D., Maas, K. R., Cardenas, E., Strachan, C. R., Hallam, S. J., & Mohn, W. W. (2015). Non-symbiotic Bradyrhizobium ecotypes dominate North American forest soils. The ISME journal, 9(11), 2435-2441.

Wang, Q., Quensen, J. F., Fish, J. A., Lee, T. K., Sun, Y., Tiedje, J. M., et al. (2013). Ecological patterns of nifH genes in four terrestrial climatic zones explored with targeted metagenomics using framebot, a new informatics tool. mBio 4:e00592-13. doi: 10.1128/mBio.00592-13

Wang, J., Zhang, D., Zhang, L., Li, J., Raza, W., Huang, Q., & Shen, Q. (2016). Temporal variation of diazotrophic community abundance and structure in surface and subsoil under four fertilization regimes during a wheat growing season. *Agriculture, Ecosystems & Environment*, *216*, 116-124.

Wang, S., Sanford, G. R., Robertson, G. P., Jackson, R. D., & Thelen, K. D. (2019). Perennial Bioenergy Crop Yield and Quality Response to Nitrogen Fertilization. *BioEnergy Research*, 1-10.

White, R.A., Garoutte, A., Mclachlan, E.E., Tiemann, L.K., Evans, S.E., & Friesen, M.L. Genome-resolved metagenomics of nitrogen transformations in the switchgrass rhizosphere microbiome on marginal lands. *In review – Phytobiomes.*

CHAPTER 4:

TEMPORAL DYNAMICS OF FREE-LIVING NITROGEN FIXATION IN THE SWITCHGRASS RHIZOSPHERE

4.1 ABSTRACT

Free-living nitrogen fixation (FLNF) represents an important terrestrial N source and is gaining interest for its potential to contribute plant available N to bioenergy cropping systems. Switchgrass, a cellulosic bioenergy crop, may be particularly reliant on FLNF when grown on low N systems, like marginal lands. However, the potential contributions of FLNF to switchgrass as well as the controls on this process are not well-understood. In this study, we use a combination of field and molecular data to evaluate the important predictors of FLNF in switchgrass systems. We find that climate variables (including precipitation) and soil N availability are major drivers of FLNF rates in switchgrass systems. Generally, increased moisture availability through precipitation events promoted FLNF rates. Soil N availability controls were complex with soil N form (i.e inorganic vs. organic) driving the direction of this effect on FLNF. As in previous studies, we find limited evidence for direct links between N-fixer communities, based on *nifH* amplicon sequencing, and FLNF rates. This work sheds light on an important N source for terrestrial systems and highlights the need to better understand the complex and dynamic controls on this process.

4.2 INTRODUCTION

Free-living nitrogen fixation (FLNF), here defined as N-fixation occurring in and around roots (rhizosphere) without direct plant symbiosis, is increasingly recognized for its importance as a nitrogen (N) input to both natural, terrestrial systems and managed, agriculture systems (Reed et al 2011; Bloch et al 2020; Davies-Barnard and Friedingstein 2020). Recent evidence suggests that FLNF may be a significant N source for cellulosic bioenergy cropping systems, like miscanthus (*Miscanthus* × *giganteus*; Davis et al. 2010) and switchgrass (*Panicum virgatum;* Roley et al. 2018; Smercina et al. 2019a), providing an alternative to fertilizer N additions and potentially increasingly the sustainability of such cropping systems. Switchgrass is a particularly promising bioenergy crop, producing high biomass yields when grown on marginal lands even with minimal inputs of fertilizer nitrogen (N), potentially due to N contributions from FLNF (Gelfand et al. 2013; Mehmood et al. 2017; Robertson et al. 2017). The anticipated reliance of switchgrass on FLNF to support plant N demands has garnered this process much interest in recent years. However, little is still known about the controls on FLNF and the conditions which promote FLNF in switchgrass systems.

Free-living nitrogen fixation is an energy-intensive process, transforming dinitrogen (N_2) gas into biologically-available ammonia, that occurs readily in the rhizospheres of many grasses where roots exude easily accessible C (Chalk 2016; Roley et al. 2018; Smercina et al. 2019a). FLNF in the rhizosphere is carried out by a diverse community of N-fixing organisms (diazotrophs) under complex and dynamic conditions (Smercina et al 2019a). These complex conditions make understanding and predicting FLNF difficult and, to date, this process has remained poorly understood.

Past work has identified several broad controls on FLNF including carbon (C) availability, macro- and micronutrient availability (e.g. N, phosphorus, and metals), and climate (Reed et al. 2011; Smercina et al. 2019a). Given the high energy, and therefore C demands of FLNF, it is generally accepted that FLNF is constrained to regions of the soil, such as the rhizosphere, where C is readily accessible (Cleveland et al. 1999; Smercina et al. 2019a). Because of these high energy demands, FLNF is not likely to be a competitive N-acquisition strategy when external N is available and thus is downregulated as diazotrophs access external N in favor of fixed N (Reed et al. 2011; Norman and Friesen 2017). Phosphorus and metals availability also influence FLNF with increased availability of these nutrients generally supporting more FLNF, yet these controls are not well studied in the rhizosphere (Smercina et al. 2019a). Lastly, climate controls on FLNF are currently tenuous at best, with limited evidence of any strong interactions between climate variables and FLNF rates (Reed et al. 2011; Davies-Barnard and Friedingstein 2020). While these past studies may speak to potential broad-scale patterns, little mechanistic work has been carried out to untangle the controls on FLNF, particularly in the rhizosphere.

In this study, we used field and molecular data collected with high-temporal frequency to explore biological and environmental controls on FLNF. We examined the impact of various soil characteristics, climate conditions, plant metrics and diazotroph community structure and on FLNF process rates. We hypothesized that over the growing season, climate variables and plant metrics will be major drivers of FLNF. Specifically, increased soil moisture and increased plant productivity would result in increased FLNF rates. We also hypothesized that soil N availability and diazotroph

community composition would be major drivers of FLNF whereby increased soil N availability would reduce FLNF rates and key diazotroph community members would be associated with greater FLNF.

4.3 METHODS

4.3.1 Field Site and Weather Data

Samples were collected from the Lux Arbor (LUX; 42.476365, -85.451887) marginal land site in southern Michigan, maintained as part of the Great Lakes Bioenergy Research Center's marginal land experiment (GLBRC; https://www.glbrc.org/). The site contains four replicate split plots of switchgrass (*Panicum virgatum* L.; cv. Cave-in-Rock) divided into fertilized (+ 56 kg urea-N ha-1 yr-1) and unfertilized (no added N) halves. Samples were collected every two weeks from March 27 (pre-emergence) through October 23 (pre-harvest) during the 2017 field season. Fertilizer was applied on May 8, 2017. Sampling locations within each subplot were randomly selected and each subplot was sampled at three pseudo-replicates per collection date. All weather data are collected as part of the GLBRC marginal land experiment via site-based weather stations.

4.3.2 Soil Collection and Nutrient Analyses

At each sampling date, three soil cores (5 cm wide, 10 cm deep) were collected from each replicate sub-plot for a total of 24 samples. Soils were sieved through 4 mm mesh and stored at 4 ˚C until further analysis. A subset of each sieved soil was frozen at -80 ˚C for extracellular enzyme activities and microbial community analyses (described below). Soil moisture content was determined by drying 5 g of field moist soil

at 60 ˚C for 48 hours. Nutrient analyses were carried out on soil extracts. Extracts were generated by shaking 6 g of field moist soil in 30 ml of 0.5 M potassium sulfate (K_2SO_4) at 200 rpm for one hour and then filtering through Whatman grade 202 filter paper to remove soil. The resulting filtrate was used to measure soil inorganic N (NH_4 and NO_3), dissolved organic C (DOC), and dissolved organic N (DON). Soil NH_4 and NO_3 concentrations were determined via 96-well high-throughput colorimetric analyses following methods described by Rhine et al. (1998) and Campbell et al. (2006), respectively. Soil extracts were analyzed on an Elementar Vario TOC cube (Elementar, Langenselbold, Germany) to determine DOC and DON concentration. Concentrations of inorganic N, DOC, and DON were corrected for extract volume and soil moisture content.

4.3.3 Free-living Nitrogen Fixation

Nitrogen fixation rates were measured on intact cores collected from each split plot pseudoreplicate location as described by Smercina et al. (2019a). Briefly, we added a 4-carbon source cocktail solution containing glucose, sucrose, citrate, and malate at a rate of 4 mg C g^{-1} dry soil. Amount of solution added varied by sample date such that all samples were adjusted to 60% water holding capacity. After carbon addition, vials were sealed and evacuated. Vial headspace was replaced with 1 ml of $^{15}N_2$ (Sigma-Aldrich, Inc., St. Louis, MO, USA), 10% Ultra High Purity (UHP) oxygen, and balanced with UHP Helium. Reference samples received UHP-N_2 in place of $^{15}N_2$. Vials were incubated for three days at room temperature. Vials were then uncapped and samples were dried at 60 ˚C for 48 hours before grinding and weighing for ^{15}N analysis. Samples were analyzed following standard procedures at University of California Davis's Stable

Isotope Facility (Davis, CA). FLNF rates were calculated in μg N fixed g^{-1} dry soil day^{-1} as:

$$\frac{AE_i \times TN_i}{AE_{atm} \times t}$$

where AE_i represents atom percent access of sample against an unenriched reference sample, TN_i represents total nitrogen content in sample, AE_{atm} represents atom percent excess in the vial atmosphere (98 atom% in our case), and t is incubation time in days (Warembourg 1993; Roley et al. 2018).

4.3.4 Plant Available Inorganic Nitrogen

Plant available inorganic N (ammonium and nitrate) was measured via field deployed ion exchange membranes following standard protocols of the Kellogg Biological Station Long-term Ecological Research station (KBS-LTER; lter.kbs.msu.edu). The exchange capacity and ion affinity of these membranes mimics that of plant roots, allowing an approximation of plant available inorganic N. Briefly, 10 cm x 2.5 cm strips of cation and anion exchange membranes (Membrane International, Inc., Ringwood, NJ, USA) were cut and activated with washes of 0.5 M hydrochloric acid and 0.5 M sodium bicarbonate. Membrane strips were rinsed and stored with DI water until field deployment. Cation and anion membranes strip pairs were deployed at each split plot pseudoreplicate location and collected at two-week intervals over the course of the 2017 field season. Harvested membranes were collected, rinsed of any adhering soil in field with DI water, and then stored in DI water until extraction. Ammonium and nitrate collected on membranes was extracted by shaking cation and anion membranes pairs in 1M potassium chloride for 24 hours. Ammonium and nitrate concentrations were then analyzed using high-throughput colorimetric methods as

described above. Plant available ammonium and nitrate are presented as rates of ammonium and nitrate “uptake” per day over the two week field incubation.

4.3.5 Soil Extracellular Enzyme Activities

Activities of ten extracellular enzymes including Alanine aminopeptidase (ALA), Arginine aminopeptidase (ARG), β-1,4-glucosidase (BG), β-D-1,4-cellobiosidase (CBH), Glutamine aminopeptidase (GLU), N-acetyl-β-D-glucosaminidase (NAG), Leucine aminopeptidase (LAP), Acid phosphatase (PHOS), Tyroine aminopeptidase (TYR) and Urease (UREA) were measured via high-throughput microplate assays (Saiya-Cork *et al.* 2002; Weintraub et al. 2007). Assays are carried out under optimal conditions with excess substrate to ensure activities were not limited by substrate availability, therefore activity rates are a measure of enzyme potential and not absolute activity. Soil slurries for each sample were made by homogenizing 1 g of soil in 125 ml of distilled water with a hand blender (Cuisinart®, Stamford, CT, USA) for 30 seconds. Slurries were found to match soil sample pH and did not require buffer. Slurries were stirred constantly while 200 µl were pipetted into 24 replicate wells of a 96-well microplate. For the fluorescent assays (ALA, ARG, BG, CBH, GLU, NAG, LAP, PHOS, TYR), sixteen replicate wells of each sample received 50 µl of fluorogenic substrate associated with the target enzyme. The remaining eight replicate wells were used to determine quench coefficients by adding 50 µl of fluorogenic standard corresponding to the fluorescent molecule attached to the substrate, either 4-methylumbelliferone (MUB) for BG, CBH, NAG, and PHOS or 7-Amino-4-methylcoumarin (MC) for ALA, ARG, GLU, LAP and TYR. For colorimetric assays (UREA), sixteen replicate wells of each samples received 10 µl of urea. The remaining eight replicate were used to assess background absorbance and received 10

µl of DI water. Both fluorescent and colorimetric assays included eight replicates each of blanks (200 µl slurry plus 50 µl DI water) and negative controls (200 µl substrate plus 50 µl DI water). All plates were incubated for 18-24 hours and then read on a BioTek Syngery H1 plate reader (BioTek Instruments, Inc., Winooski, VT, USA,). Fluorometric assays were read at excitation of 370 nm and emission of 455 nm for MUB substrates and excitation of 350 nm and emission of 430 nm for MC substrates. Urea plates were analyzed for ammonium production via the Berthelot method (Rhine et al. 1998) and read at absorbance of 610 nm. 100 µl from all well of the urea plates were transferred to fresh clear plates before reading to reduce interference of soil particles. Enzyme activities were corrected for slurry volume and soil moisture content and presented as nmol activity g^{-1} dry soil $hour^{-1}$.

4.3.6 Plant Metrics

Plant metrics were evaluated on each collection date for three plants per field split-plot. At each sampling, a variety of plant metrics were assessed including plant height, photosynthesis parameters, leaf and root C:N, specific stem density, and specific and top leaf area. Plant height was measured in field to the nearest millimeter a standard meterstick as the distance from the ground to the highest reaching point on the plant. Photosynthesis parameters including Phi2 (e.g. quantum yield of photosystem II) and relative chlorophyll were measured in field using a MultiSpeQ (Photosnyq, Inc. East Lansing, MI, USA) at three locations on the plant (low, mid, and upper canopy). Reported values for photosynthesis parameters are averages across canopy samplings for each plant. Leaf and root C:N were measured on a ECS 4010 elemental analyzer (Costech Analytical, Valencia, CA). Leaf C:N was measured by first drying and grinding

leaf material. Root C:N was measured on roots collected during soil core sieving. Roots were washed of adhering soil and then dried and ground before elemental analysis. Specific stem density was measured on the tallest stem for each sampled plant. The tallest stem was first clipped from each measure plant. A 10 cm subset of stem, with leaves removed, was then weighed, dried at 50˚C for 4-7 days and then weighed again. Specific stem density is calculated as g dry stem per g wet stem. Specific and top leaf area were determined by scanning leaves from the clipped stem at 2400 and 1200 dpi using a flatbed scanner. Surface area was calculated using ImageJ software. Specific leaf area was determined by scanning all leaves on the tallest stem for each sampled plant. Top leaf area represents surface area for just the topmost leaf.

4.3.7 Soil Diazotroph Community Composition

Soil diazotroph communities were characterized via *nifH* functional gene amplicon sequencing of soil DNA. Microbial community DNA was extracted from 0.25 g soil via standard procedures of the Qiagen DNeasy PowerSoil kit (QIAGEN, Hilden, Germany). PCR reactions were carried out in two stages to amplify *nifH* genes and then attach linker sequences. Stage 1 was a 15 µl reaction with 0.9 µl of DNA extract, 1X AmpliTaq Gold 360 Master Mix (Applied Biosciences, Foster City, CA), and 1 µM concentrations of both the forward and reverse primers. We used the IGK3 (GCIWTHTAYGGIAARGGIGGIATHGGIAA) and DVV (ATIGCRAAICCICCRCAIACIACRTC) forward and reverse primer pair, recommended by Gaby and Buckley (2012) as optimal primer sets for capturing the widest diversity of diazotrophs. Stage 1 PCR reactions were carried out as follows: 95 ˚C start for 10 minutes, 34 cycles of denaturation at 95 ˚C for 30 secs, annealing at 54 ˚C for 45 secs,

and extension at 72 ˚C for 40 secs, final extension at 72 ˚C for 7 minutes. Amplification of target gene was confirmed via gel electrophoresis (1.5% gel agar, 90 V, 45 minutes) before proceeding to Stage 2 reactions. Stage 2 was a 20 µl reaction with 1.2 µl of Stage 1 PCR product, 1X AmpliTaq Gold 360 Master Mix (Applied Biosciences, Foster City, CA), and 1 µM concentrations of both the forward and reverse primers. The IGK3/DVV primer pair with linker sequences was used for Stage 2 reactions. Stage 2 was carried out as follows: 95 ˚C start for 10 minutes, 4 cycles of denaturation at 95 ˚C for 30 secs, annealing at 56 ˚C for 45 secs, and extension at 72 ˚C for 40 secs, final extension at 72 ˚C for 7 minutes. Successful amplification and absence of non-target products after Stage 2 was confirmed via gel electrophoresis (1.5% gel agar, 90 V, 45 minutes). Stage 2 PCR products were quantified via Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States) and normalized to a 1– 10 ng μl^{-1} DNA concentration range before library prep. Samples were submitted to the Michigan State University RTSF Genomics Core Facility (East Lansing, MI) for library prep and sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) with MiSeq standard reagent kit v.2 and 2 x 250 bp paired-end reads.

Sequence processing was performed following a modified version of the NifMAP pipeline (Angel et al. 2018). Sequence data were received as demultiplexed fastq files. Forward and reverse reads were merged via USEARCH v. 10.0.240 fastq_mergepairs and then quality and length filtered to maximum expected errors of 1 and minimum length of 300 bp via USEARCH v. 10.0.240 fastq_filter. Sequences were then filtered for non-*nifH* reads using four Hidden-Markov Models (HMM) as described in Angel et al. (2018). Sequences were then frameshift corrected and translated to protein sequences

using Framebot (Wang et al. 2013) and then filtered again for homologs using HMM as described in Angel et al. (2018). Frameshift and homolog filtered sequences were then dereplicated using USEARCH v. 10.0.240 fastx_uniques and clustered using USEARCH v. 10.0.240 cluster_otus. The cluster_otus command also filters chimeras. Sequences were then mapped back to reference OTUs using USEARCH v. 10.0.240 usearch_global at 97% similarity. A phylogenetic tree was constructed using an amino acid reference alignment (Angel et al. 2018). Sequences were aligned to the reference using MAFFT v. 7.305 and then used to generate a tree via FastTree v. 2.1.9. Finally, taxonomy was assigned to sequences using Blast+ v. 2.7.1 blastn command and queried against Gaby and Buckley's (2014) *nifH* sequence database. Taxonomy was assigned according to percent similarity using empirically derived cutoffs (Gaby et al. 2018) of 75% similarity for family, 88.1% for genus, and 91.9% for species. All other taxonomic assignments matching at <75% similarity were only assigned at the order level.

4.3.8 Data Analysis

Data for pseudo-replicates were averaged for each field replicate on each collection date giving one data point per field replicate per collection date for statistical analyses. FLNF rates across the 2017 field season were analyzed using a repeated measures ANOVA with Tukey's adjustment for multiple comparisons using the *lmerTest* R package with collection date, fertilizer N treatment, and their interaction as fixed effects and fertilizer N treatment nested within field replicate as a random effect were considered significant at $p \leq 0.05$. Fertilizer N treatment was not a significant predictor of FLNF rates, therefore data are presented as averages across fertilizer N treatments.

nifH OTU counts were first rarefied to an even sampling depth of 1500 using *rarefy_even_depth* in the R *phyloseq* package. This was chosen based on the rarefaction curves which indicated that most of the diversity was captured within this sample size, while limiting loss of samples due to low OTU counts. To evaluate beta-diversity of samples, we used Bray-Curtis to generate distance matrices using the distance function in R *phyloseq* and then ordinated via principal coordinate analysis (PCoA) using the *ordinate* function in R phyloseq. We used *adonis* in R vegan to conduct PERMANOVA of the Bray-Curtis distance matrices by collection data*N treatment. Differences between collection dates and N treatments were considered significant at $p \leq 0.05$. Lastly, we explored diazotroph community data for OTUs whose abundance is potentially associated with peak FLNF rates on August 28 using the *indicspecies* package in R.

Environmental and biological controls on FLNF were evaluated using a random forest regression algorithm via the *randomForest* package in R using the following parameters: ntree=5000, mtry=11 (default), NA values replaced with median (numeric predictors) or mode (categorical predictors. Regressions were performed using all available data for each collection date (the full list of potential predictors are presented in Supplemental Tables S4.1 and S4.2). Two regression analyses were carried out. Regression #1 was carried out using the data from the full field season, but did not include any diazotroph community data. Regression #2 targeted only those dates for which diazotroph community data was available and included all available data for those sample dates.

4.4 RESULTS

4.4.1 N-fixation rates

N-fixation rates varied over the 2017 growing season and collection date was a significant predictor of N-fixation rates (Fig. 4.1; $F = 30.916$; $p < 0.001$). In particular, we observed a sudden pulse of N-fixation during the August 28 sampling, with average rates on Aug. 28 measured at 4.8x greater than the average N-fixation rates for all other collection dates. We also noted a slight increase in N-fixation rates in late June following a decrease in rates from early in the growing season. Noting these "pulses" of FLNF, we

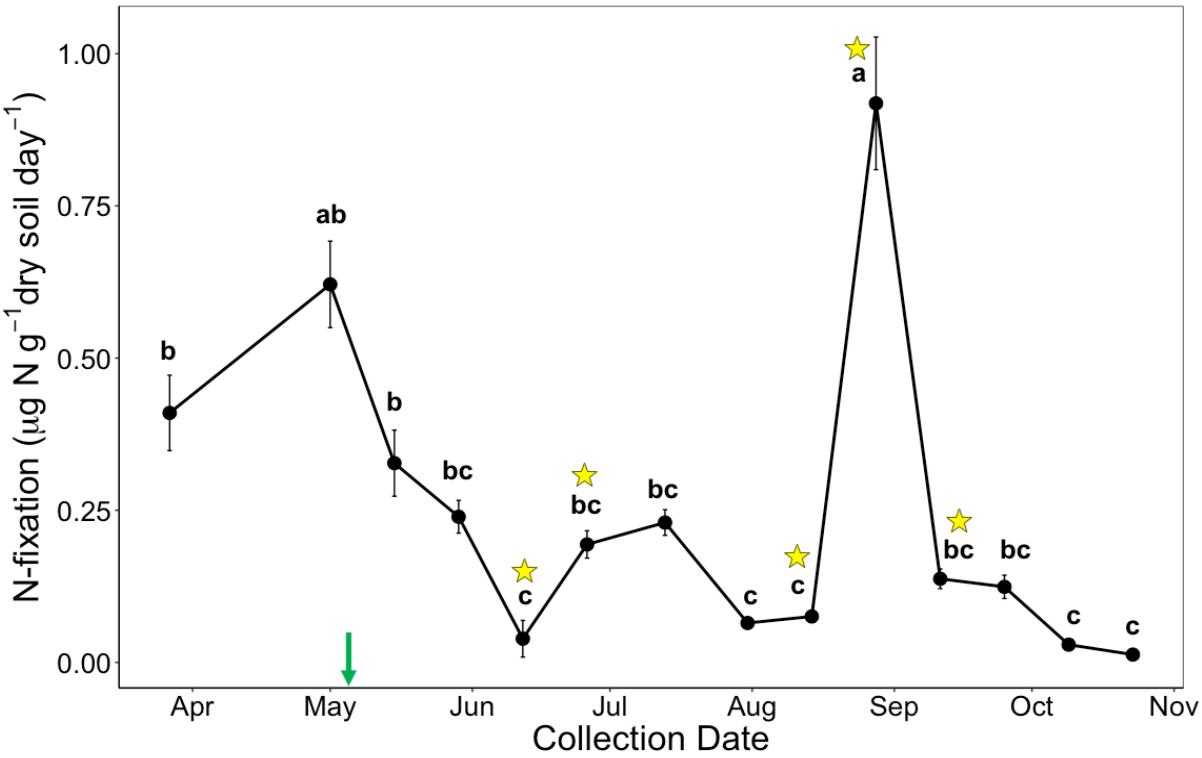

Figure 4.1: Free-living nitrogen fixation rates across the 2017 growing season at the Lux Arbor field site. Samples were collected every two weeks from March 27 to October 23. Each point represents average N-fixation across four replicate field blocks ± standard error (n=8). Significant differences between dates (at $p < 0.05$) are indicated by lowercase letters. Diazotroph community composition was collected on dates marked with stars. The green arrow indicates date of fertilizer application in the field.

targeted these pulse-associated dates to examine diazotroph community composition. N-fixation rates did not differ significantly between field N treatments, so all other analyses of FLNF use averaged results across field N treatments ($F = 0.3406$; $p = 0.5632$).

4.4.2 Diazotroph community composition

Diazotroph community composition was assessed for "pulse-associated" dates (Fig. 4.1). Alpha diversity of diazotroph communities differed by collection date for three evaluated alpha diversity metrics including Chao1, Shannon, and Inverse Simpson (Fig. S4.1). Chao1, a measure of species richness, was lowest on August 28th, while both Shannon and Inverse Simpson results indicate lowest diversity on June 12 with no significant difference between the other sampling dates. Beta diversity of soil diazotroph communities was compared via ordination of Bray-Cutis dissimilarity and PERMANOVA. Spatial distribution of the community dissimilarity was significantly different by field replicate (Fig. 4.2A) with replicate 1 and replicate 4 clustering distinctly from each other and from replicates 2 and 3. Therefore, field replicate was included as a random effect in PERMANOVA analysis of diazotroph community beta diversity by collection date and field replicate. No clear patterns in diazotroph community composition by collection date were observed (Fig. 4.2B) indicating no shifts in community in association with FLNF pulses. Though diazotroph community composition was found to differ significantly by both collection date ($p = 0.001$) and field N treatment ($p = 0.001$), neither of these effects explained much of the spatial distribution observed in the principal coordinates analysis (PCoA; 5.9% and 2.0% variation explained for collection date and field N treatment, respectively). Correlation analyses of Bray-Curtis

dissimilarity for overall diazotroph community composition did significantly correlate with several soil N metrics, suggesting soil N availability may drive diazotroph community structure (Fig. 4.2B). Lastly, we identified seven OTUs indicative of the August 28 sampling date, the collection date with the highest FLNF rates (Table 4.1). All identified OTUs are members of the Proteobacteria phylum and were predominately Alphaproteobacteria.

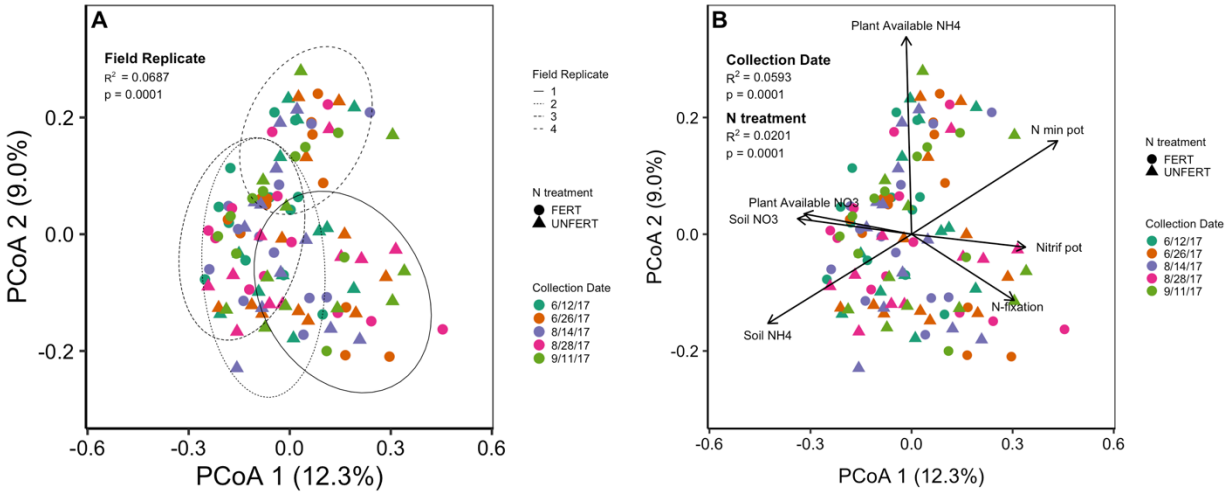

Figure 4.2: Principal coordinate analysis (PCoA) of soil diazotroph communities based on Bray-Curtis dissimilarly of relative abundances for each sample date. (A) PCoA ordination with 95% confidence ellipses of each field replicate. (B) PCoA with overlaid vectors of significantly correlated environmental factors ($p < 0.05$). Diazotroph community composition is based on *nifH* amplicon sequencing. Points are colored by sample dates with shape representing nitrogen treatment. Both collection date and nitrogen treatment were found to be significant, but explained little of the overall pattern in community composition variation across dates. N availability metrics are strong drivers of community composition.

Table 4.1: Table of OTUs associated with the measured pulse in N-fixation on August 28 identified by *indicspecies*.

OTUs	Kingdom	Phylum	Class	Order	Family	Genus	Species
OTU52	Bacteria	Proteobacteria	Alpha-proteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	Unknown
OTU548	Bacteria	Proteobacteria	Gamma-proteobacteria	Enterobacteriales	Enterobacteriaceae	Klebsiella	Unknown
OTU600	Bacteria	Proteobacteria	Alpha-proteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium subterraneum
OTU760	Bacteria	Proteobacteria	Alpha-proteobacteria	Rhizobiales	Bradyrhizobiaceae	Unknown	Unknown
OTU879	Bacteria	Proteobacteria	Alpha-proteobacteria	Rhizobiales	Xanthobacteraceae	Unknown	Unknown
OTU2875	Bacteria	Proteobacteria	Alpha-proteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	Bradyrhizobium japonicum
OTU3509	Bacteria	Proteobacteria	Beta-proteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	Burkholderia sp. JPY629

4.4.3 Random Forest Regression

Important predictors of FLNF varied between full season and pulse-associated dates. Full season results indicate that climate variables (i.e. precipitation metrics and water availability, and temperature) and plant metrics (i.e. top leaf area, plant height, and specific stem density) are the dominant controls on FLNF over the growing season (Fig. 4.3A, $p < 0.05$). Together, these ten variables explained 61.6% of the variation in FLNF rates over the 2017 field season (Fig. 4.3B). We similarly identified precipitation metrics as key predictors of FLNF on pulse-associated dates (Fig. 4.3C). Interestingly, several soil N availability metrics were also identified as key predictors of FLNF for these pulse-associated dates. Together with precipitation and temperature, soil N availability metrics explained 71.4% of the variation in FLNF rates on pulse-associated dates (Fig. 4.3D).

Random forest does not provide directionality, nor does it account for covariance of predictors. Therefore, we explored correlations between FLNF rates and the top ten predictors identified for the full growing season and for pulse-associated dates. Full season FLNF rates were negatively correlated with many of the key predictors (Fig.

4.4). Interestingly, all plant metrics identified as important predictors of FLNF were actually negatively correlated with process rates. We found significant correlation between many of the predictors and FLNF on pulse-associated dates (Fig. 4.5). In particular, several climate variables including precipitation on collection date and days since last rainfall which were significantly, positively correlated with FLNF. This result seems to be specifically driven by the August 28th date. This date was the only pulse-associated date to receive rainfall following the longest "days since last rainfall" window of all pulse-associated dates. August 28th was also the date with the highest FLNF rates during the entire 2017 growing season. We also found that N-fixation on pulse-associated dates was positively correlated with DON, TN, and N mineralization potential, but negatively correlated with the other soil N metrics including NAG activity and soil NH_4^+. There was also a trend towards negative correlations between N-fixation and GLU activity, but this was not significant. Collectively, these results point towards soil water availability metrics (e.g. precipitation, soil moisture) and soil N availability metrics (e.g. organic and inorganic N availability and N-acquiring enzyme activity) as key predictors/drivers of FLNF rates in our marginal land system.

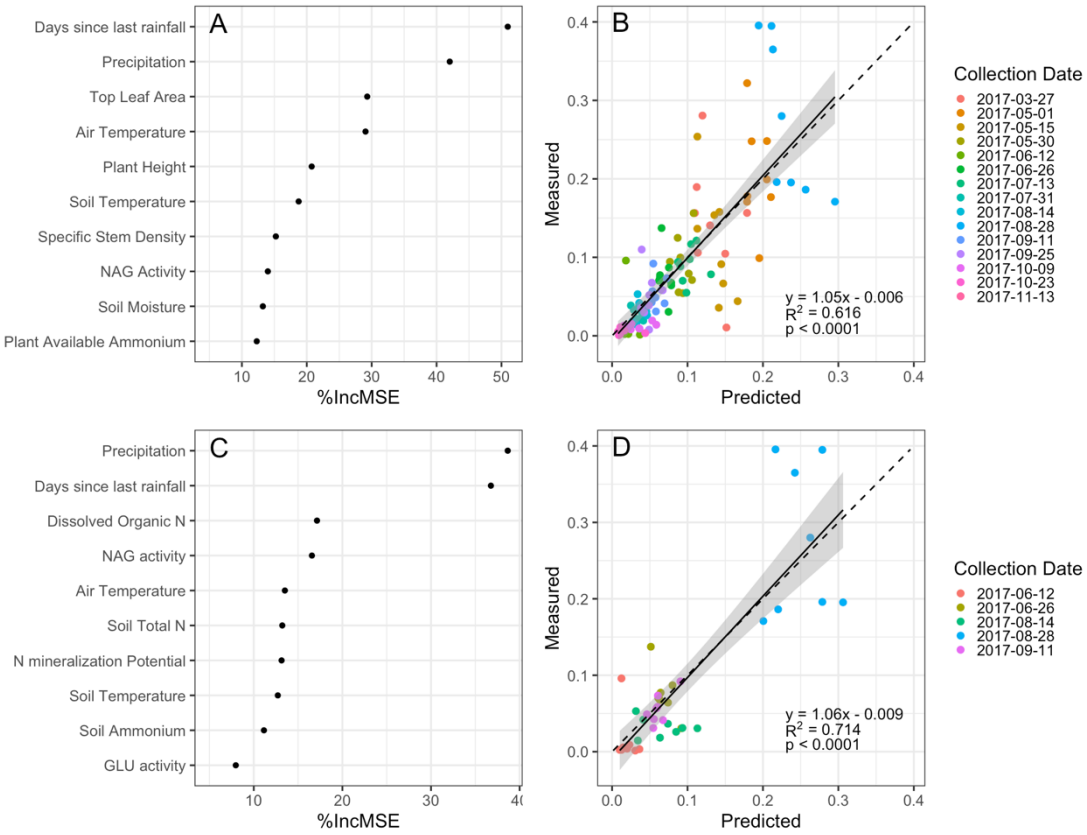

Figure 4.3: Results of random forest regression analysis of free-living nitrogen fixation for full season (A, B) and dates with community data (C, D). (A, C) Top ten important predictors as indicated by associated percent increase in mean square error (%IncMSE). All reported predictors were significant at $p < 0.05$. (B, D) Model predicted vs. measured FLNF rates. Points are colored by collection date. Dashed line represents 1:1 fit line. Solid line shows linear regression of predicted vs. measured line with 95% confidence bands.

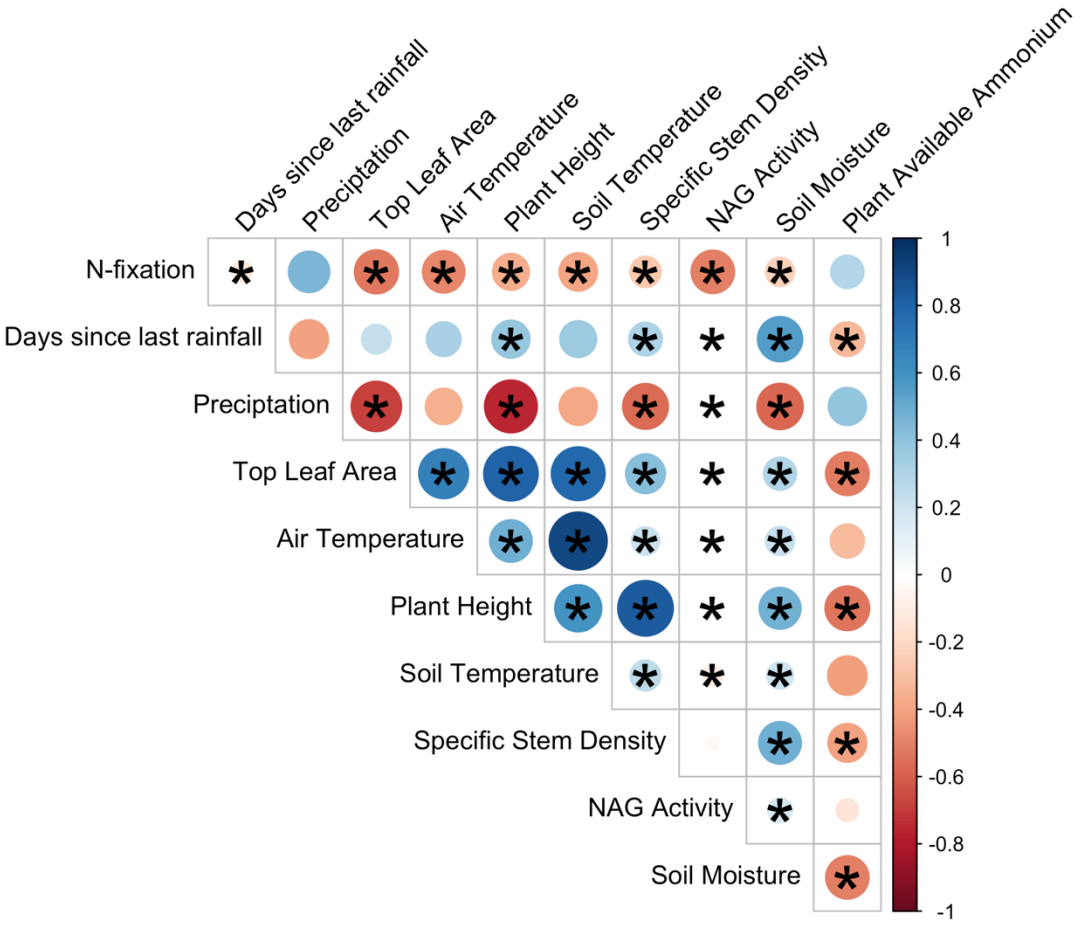

Figure 4.4: Correlations of important full season predictors of N-fixation rates, obtained from random forest analysis of full season data, and N-fixation rates. Point color indicates positive or negative relationship as well as degree of correlation with darker shades indicating stronger relationships. Point size indicates degree of correlation with larger points indicate stronger relationships. Asterisks indicate significant correlation at $p < 0.05$.

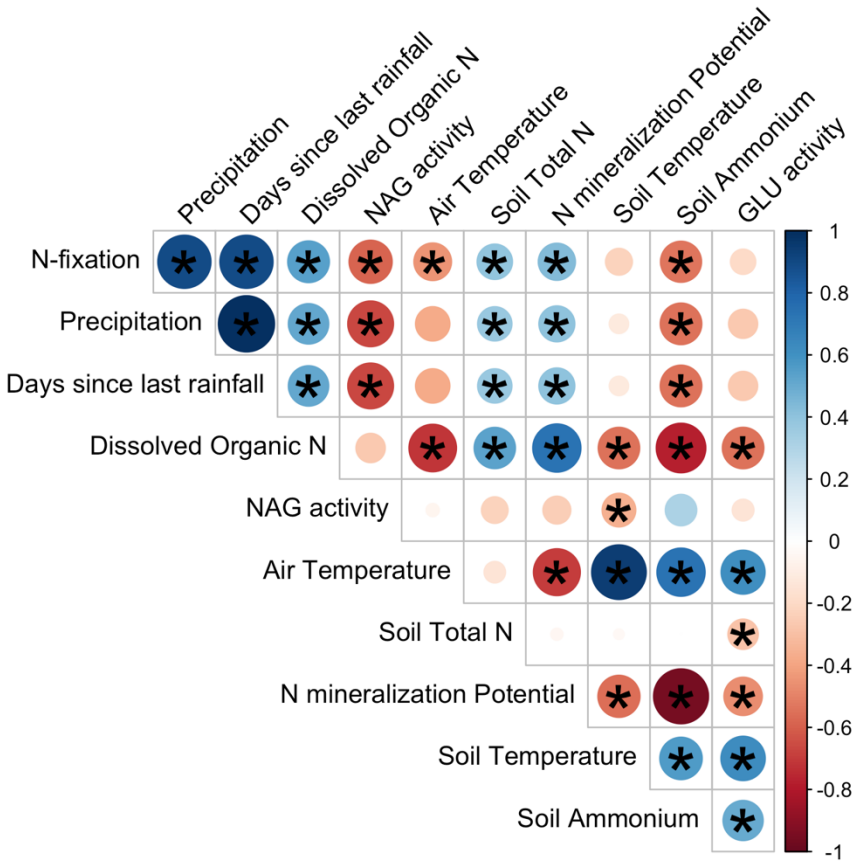

Figure 4.5: Correlations of important pulse-associated predictors of N-fixation rates, obtained from random forest analysis of pulse-associated dates, and N-fixation rates. Point color indicates positive or negative relationship as well as degree of correlation with darker shades indicating stronger relationships. Point size indicates degree of correlation with larger points indicate stronger relationships. Asterisks indicate significant correlation at $p < 0.05$.

4.5 DISCUSSION

In this study, we explored the relationships between FLNF rates and various soil, climate, and biological factors. We identified several key factors that were strongly associated with FLNF rates and may improve our capacity to predict the occurrence of this important N transformation. Overall, we found that factors relating to soil moisture availability, temperature, and soil N availability/acquisition were the strongest drivers of

FLNF across the growing season and on FLNF pulse-associated dates. As with many other studies (Rocca et al. 2015; Jansson and Hofmockel 2018), we found little evidence for a strong link between microbial community structure and function with only weak associations identified between FLNF rates and diazotroph community members based on *nifH* functional gene sequencing.

As hypothesized, climate metrics, and in particular those relating to water availability, were key predictors of FLNF both across the growing season and for pulse-associated dates. The role of water availability was particularly evident for pulse-associated dates, as the August 28th FLNF pulse also corresponded to the first rainfall event in several days preceding this sample date. August 28th was also the only pulse-associated date to have a rainfall event. This seems to indicate that the sudden input of moisture to the system stimulated FLNF activity. Soil water availability is well known to influence microbial function, with greater water availability generally increasing process rates (Wilson and Griffin 1975; Harris et al 2918; Tiemann and Billings 2011; Zhang, S. et al. 2020). FLNF has also been shown to be influenced by water availability as well as temperature, whereby FLNF is typically greater in warmer and wetter environments (Reed et al. 2011). In similar work on FLNF, observed episodic fluxes in FLNF process rates were thought to associated with variation in soil moisture (Roley et al. 2019). Our results add to this body of work and highlight to need to understand water controls on FLNF, particularly in the face of altered precipitation regimes as a result of climate change.

Temperature, including air temperature and soil temperature of the top ten cm of soil, was also a key predictor of FLNF identified by random forest regression. However,

we only found weak, negative correlations between FLNF and either measure of temperature. Our data appeared to identify optimal air and soil temperatures for FLNF, rather than a linear relationship. However, the nature of our data with a single “pulse” date (i.e. August 28) makes it difficult to draw any strong conclusions on this result. Similarly, a recent global meta-analysis found only weak correlations between N-fixation and climate variables, including temperature (Davies-Barnard and Friedlingstein 2020), though previous work identified climate-related variables like evapotranspiration as key predictors of N-fixation (Cleveland et al. 1999). However, global patterns of N-fixation and climate variables do not necessarily predict fine-scale measures of N-fixation, as we observed in this study, (Cleveland et al. 1999) and a more fine-scale view of FLNF may be needed in order to predict this process.

Beyond climate variables, soil N availability was the other dominant control on FLNF rates in the switchgrass rhizosphere with several metrics of soil N availability and acquisition being identified as important predictors for both full season and pulse-associated FLNF rates. This result was most evident for pulse-associated dates where several soil N metrics were identified as key predictors of FLNF for those dates. We expected that increased soil N availability would reduce FLNF rates, but surprisingly, this was not always the case. FLNF rates were found to be positively correlated with soil TN and DON concentrations, but negatively correlated with inorganic N availability including soil ammonium and plant available nitrate. Additionally, the strongest predictor of FLNF was activity of the chitinase enzyme N-acetyl-β-glucosaminidase (NAG), a C and N-acquiring extracellular enzyme, which was negatively correlated with FLNF rates. This inconsistent directionality of relationships between FLNF and soil N metrics

suggests more intricate interactions between soil N and FLNF than previously identified (Reed et al. 2011; Smercina et al. 2019a).

It is generally accepted and has been demonstrated that increased external N availability decreases rates of FLNF (Hobbs and Schimel 1984; Patra et al. 2007; Reed et al 2011; Kox et al. 2016). Diazotrophs are not solely reliant on N-fixation to access N and are known to access both high and low molecular weight N sources, including organic N forms (Norman and Friesen 2017). However, we found that the form of soil N (e.g. organic vs. inorganic) determined the direction of this response. Generally, inorganic N sources were associated with decreased FLNF activity. It was expected that FLNF rates would be negatively correlated with soil ammonium as ammonium is the direct product of N-fixation and its accumulation is well-known to inhibit nitrogenase enzyme function (Steenhoudt and Vanderleyden 2000; Dixon and Kahn 2004)..

In contrast to the results observed for inorganic N availability, soil organic N availability was positively correlated with FLNF and one of the dominant predictors of FLNF. This was surprising as many past studies have found various organic N sources, particularly amino acids, to have a negative or neutral impact on FLNF activity (Dixon and Kahn 2004; Huergo et al. 2012; Klassen et al. 1997). This positive response may be capturing DON concentrations as a product of FLNF activity rather than a control promoting FLNF. DON accumulates in soils as biologically-derived N sources, including proteins and amino acids, are released from dead plant and microbial cells (Robertson and Groffman 2007; Warren 2014). Thus, as FLNF occurs and diazotroph populations grow and die, their internal organic N may accumulate in the soil system increasing DON and TN. This is a particularly important finding for understanding the relationship

between FLNF and plant available N, as organic N sources may represent a significant portion of plant N acquisition (Näsholm et al. 2008; Paungfoo-Lonhienne et al. 2010; Czaban et al. 2016).

Though DON was positively correlated with FLNF, enzyme activity resulting in the release of DON from soil organic matter (e.g. NAG activity) was negatively correlated with FLNF. Previous work has suggested that diazotrophs may invest fixed N from FLNF into production of extracellular enzymes like NAG because they represent a more energy-efficient method of acquiring both N and C (Norman and Friesen 2017). Our results lend some support towards this hypothesis where FLNF and NAG activity are inversely related and NAG activity was also the most important predictor of FLNF. Further, the combination of our DON and NAG results seems to support our hypothesis that FLNF is contributing to the DON pool whereby DON accumulates with FLNF activity which is then replaced by NAG activity as diazotrophs and other soil microorganisms mine the resulting DON pool. Further studies of the relationship between FLNF, DON, and soil extracellular enzymes are needed to elucidate this potentially important trade-off.

The strong relationship between FLNF and NAG activity also highlights a potentially important association and/or interaction between diazotrophs and fungi. Fungi make up a significant portion of soil microbial biomass and, as such, likely represent the dominant source of chitin in soil systems as chitin is a key component of fungal cells (Fernandez and Koide 2012; Wieczoerk et al. 2019). Thus, NAG activity, which is indicative of chitin degradation, could be a proxy for abundance of fungal biomass and or associations between chitinase-producing bacteria and fungal cells. In

the context of our study, the potential trade-off between FLNF and NAG would suggest that diazotrophs are closely associated with fungal cells. This is particularly interesting as recent work has identified associations between fungi and symbiotic N-fixers whereby the biotrophic fungus, *Phomopsis liquidambaris*, assists in the migration of rhizobia from bulk soil to legume host (Zhang et al. 2020). This dispersal of soil bacteria through so-called “fungal highways” has been documented in several other bacterial species, including members of two known diazotrophic genera, *Sphingomonas* and *Burkholderia* (Kohlmeier et al. 2005; Warmink et al. 2011; Simon et al. 2015). Further, interactions between fungi and diazotrophs have previously been observed, whereby direct uptake of diazotroph fixed N was observed in fungi with hypothesized transfer of fungal C to associated diazotrophs (Weißhaupt et al. 2011). The close link we observed between FLNF and NAG adds support to this growing body of literature and suggests that further investigation into fungal-diazotroph associations is warranted.

Lastly, we examined the influence of diazotroph community composition and specific community members on FLNF rates. We found that overall diazotroph community composition did not relate to FLNF rates. In particular, we did not find evidence of a community shift in association with the measured FLNF pulse on August 28. This inability to directly link microbial community structure, even based on functional genes, is a well-documented challenge in soil microbial ecology (Rocca et al. 2015; Jansson and Hofmockel 2018) and for FLNF in particular (Fürnkranz et al 2008; Knief et al. 2012; Smercina et al. *in prep*). Interestingly, differences in overall community composition were correlated with several soil N availability metrics, including the same metrics which were found to significantly impact FLNF rates (Fig. 3B). This suggests

that while large community shifts do not drive differences in FLNF, soil N availability may act on both diazotroph community composition and function to shape FLNF in the switchgrass rhizosphere.

Despite finding no evidence for broad shifts in diazotroph community composition in association with FLNF pulses, we did identify seven potential indicator OTUs associated with the August 28th FLNF pulse. Indicator OTUs were predominately of the order *Rhizobiales*, including several *Bradyrhizobium*. Previous work has identified *Bradyrhizobium* in the switchgrass rhizosphere, including as indicators of greater FLNF rates (Bahulikar et al. 2014; Roley et al. 2019; Smercina et al. *in prep*) and may suggest an important, yet overlooked role for these well-studied symbionts in grassland systems. Our work here further highlights to need to better understand the contributions of symbiotic N-fixers to switchgrass systems.

4.6 CONCLUSIONS

The results of this study, in accordance with past work, find that climate conditions and soil N metrics are the most important predictors of FLNF activity. We find precipitation and precipitation events to be strong predictors of FLNF rates with potential strong explanatory power for observed pulses in FLNF process rates. Our findings also indicate that the relationship between soil N and FLNF may be more complex than previously thought and depends on the form of N (e.g. inorganic vs. organic). In particular, our findings indicate that a potential trade-off between FLNF and enzyme activities surrounding DON concentrations in the soil. Lastly, we find limited evidence of associations between diazotroph community composition, based on *nifH* gene sequencing, and FLNF though some specific members have been identified as

potentially important and warranting further investigation. This work highlights that controls on FLNF are complex and dynamic and that more fine-scale, mechanistic studies are needed to better understand the controls on this ubiquitous and important terrestrial process.

APPENDIX

SUPPLEMENTAL MATERIALS

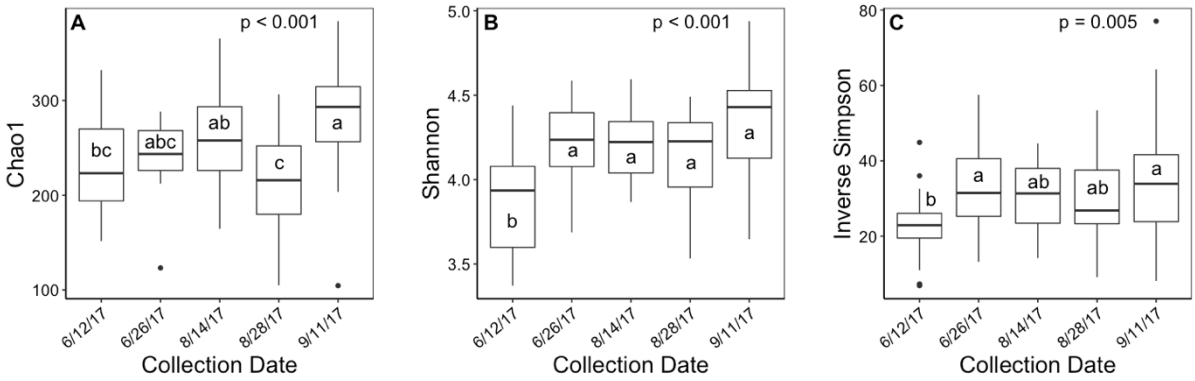

Figure S4.1: Alpha diversity metrics (A) Chao1, (B) Shannon Index, and (C) Inverse Simpson by collection date. All alpha diversity metrics varied significantly by site, but not by field N treatment ($p = 0.803$, $p = 0.385$, and $p = 0.542$ for A, B, and C, respectively). Lowercase letters indicate significant differences between collection dates at $p < 0.05$ based on a repeated measures ANOVA.

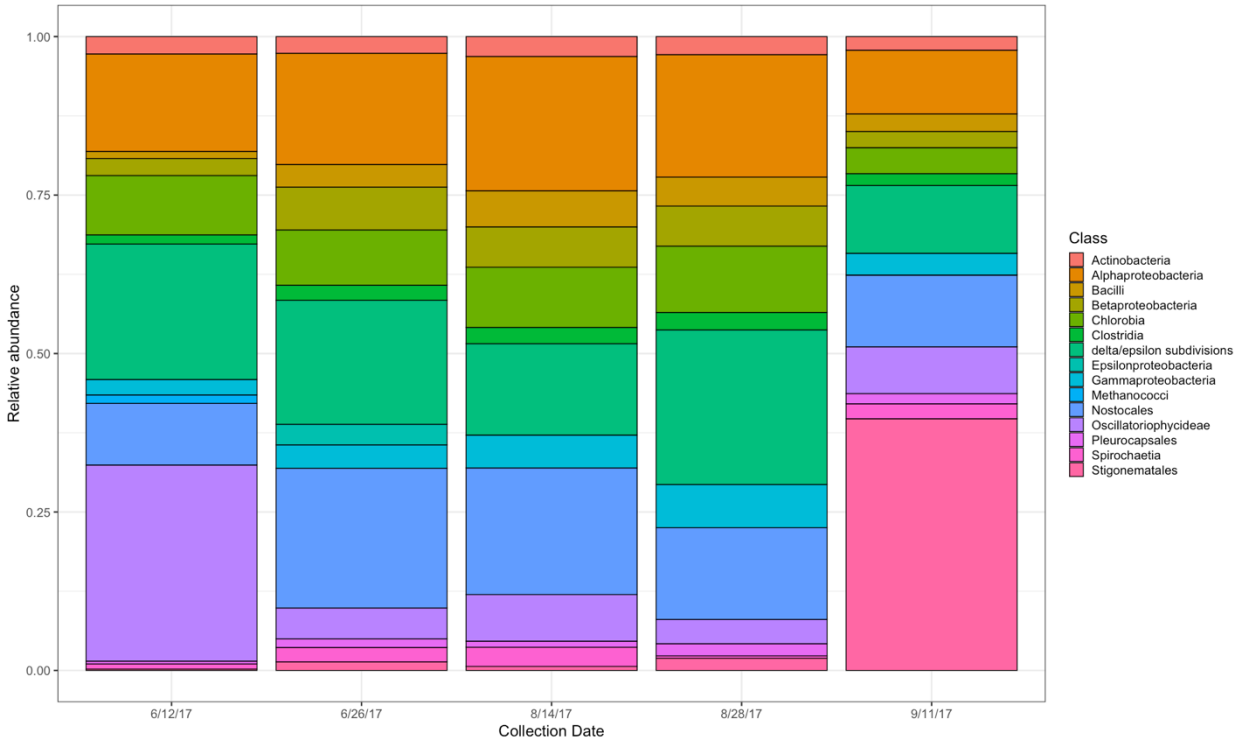

Figure S4.2: **Relative abundance of diazotroph classes, based on *nifH* amplicon sequencing, by sample date**. Colors represent different diazotroph classes. No significant differences were found between fertilized and unfertilized soils. Few significant differences across dates were identified. Notably: Bacilli abundance on June 12 was significantly lower than the Aug. and Sept. dates with no differences between any other dates. Betaproteobacteria and Nostocales abundance was significantly lower on June 12 versus June 26 with no differences between any other dates. Gammaproteobacteria abundance was greater on Aug. and Sept. than June dates. Stigonematales abundance on Sept. 11 was driven by one sample with high Stigonemtales abundance was not actually significant different from any other dates.

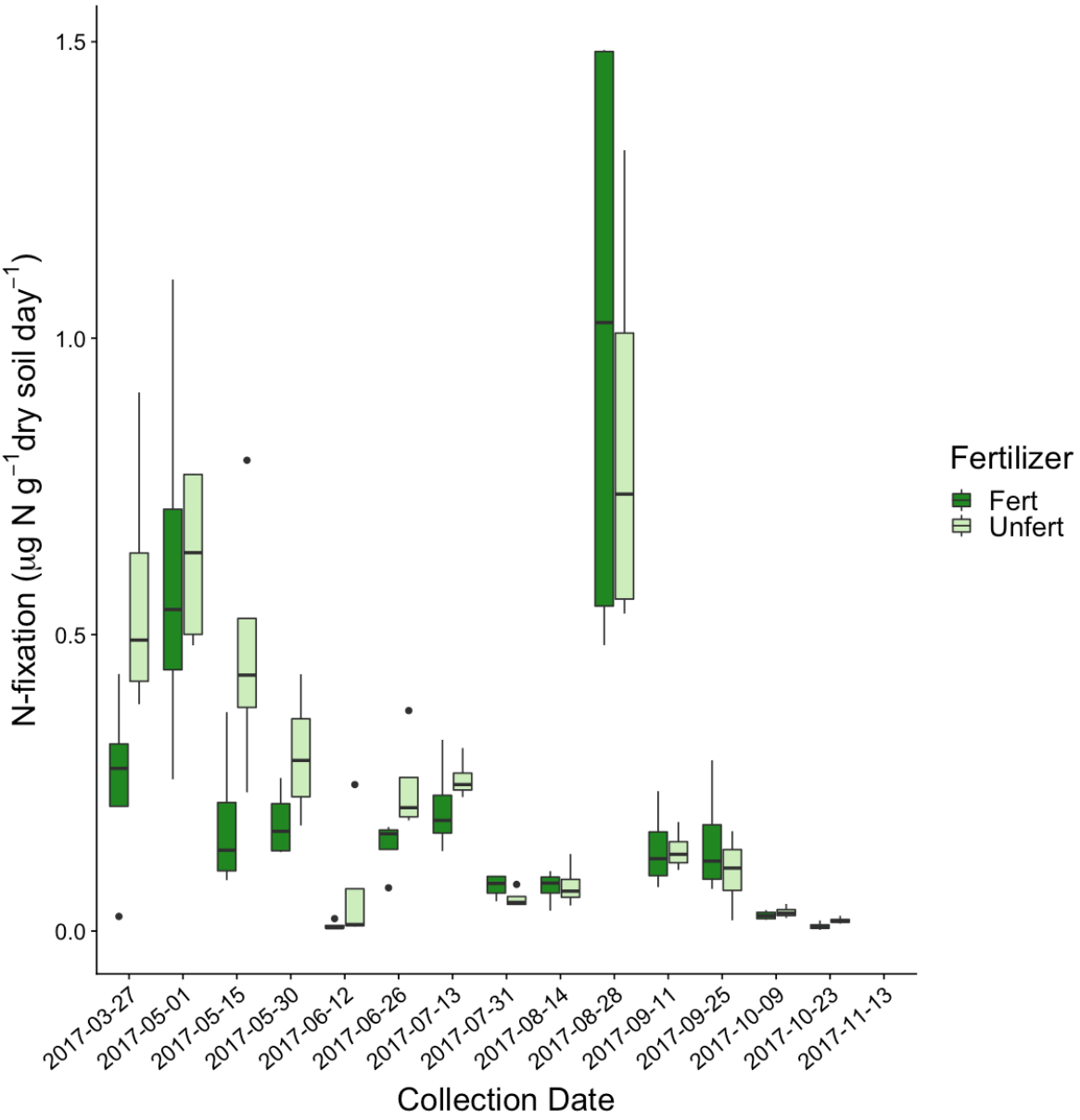

Figure S4.3: Free-living nitrogen fixation rates across the 2017 growing season represented as boxplots for each sample date and field fertilizer treatment (n = 4 per box) where solid, horizontal black lines indicate average FLNF rates.

Table S4.1: Results of randomForest regression analysis for full 2017 field season data. Predictors are sorted by level of importance based on percent increase in mean squared error (%IncMSE).

Predictor	%IncMSE	p-value (%IncMSE)	INP	p-value (INP)
Days since last precipitation event *[a§]	50.96	0.01	0.11	0.01
Precipitation on collection date *[a§]	42.03	0.01	0.11	0.01
Top Leaf Area *[a§]	29.31	0.01	0.08	0.01
Air temperature *[a§]	29.05	0.01	0.04	0.01
Plant height *[§]	20.76	0.02	0.04	0.08
Soil temperature *[§]	18.75	0.02	0.02	0.09
Specific stem density *[§]	15.23	0.02	0.02	0.32
NAG activity *[§]	14.00	0.03	0.01	0.60
Soil moisture *[§]	13.23	0.05	0.02	0.79
Plant available ammonium *[§]	12.29	0.02	0.03	0.41
Soil ammonium	11.81	0.07	0.01	0.97
Leaf C:N	11.75	0.06	0.01	0.47
GLU activity	10.59	0.11	0.02	0.79
Microbial Biomass C:N *	10.58	0.05	0.02	0.54
Urease activity	10.26	0.06	0.01	0.97
N mineralization potential	9.84	0.07	0.01	0.98
TYR activity	8.62	0.13	0.01	0.97
BG activity	8.60	0.20	0.01	0.85
PHOS activity	8.10	0.15	0.01	0.98
Plant available nitrate	7.99	0.18	0.02	0.91
Phi2	7.41	0.05	0.01	0.91
Soil nitrate	7.13	0.27	0.01	1.00
ALA activity	7.09	0.26	0.01	1.00
Specific leaf area	6.97	0.21	0.01	1.00
Dissolved Organic N	6.53	0.13	0.04	0.24
Microbial Biomass N	6.46	0.19	0.03	0.32
Root C:N	6.09	0.24	0.02	0.79
Relative Chlorophyll	5.34	0.13	0.01	0.74
LAP activity	5.09	0.41	0.01	1.00
Microbial Biomass C	4.42	0.31	0.02	0.80
Nitrification potential	4.11	0.39	0.01	1.00
ARG activity	1.43	0.57	0.00	1.00
CBH activity	0.59	0.75	0.01	1.00

***** denotes significant predictors at $p < 0.05$ based on percent increase in mean squared error (%INcMSE).
[a] denotes significant predictors at $p < 0.05$ based on increase in node purity (INP).
[§] denotes top ten most important predictors

Table S4.2: Results of randomForest regression analysis for pulse-associated dates including diazotroph community composition (class-level). Predictors are sorted by level of importance based on percent increase in mean squared error (%IncMSE).

Predictors	%IncMSE	p-value (%IncMSE)	INP	p-value (INP)
Days since last precipitation event *a§	37.94	0.01	0.09	0.01
Precipitation on collection date *a§	37.79	0.01	0.09	0.01
NAG activity *a§	16.09	0.01	0.03	0.04
Air temperature *a§	15.88	0.01	0.03	0.01
Dissolved Organic N *a§	14.25	0.01	0.00	0.04
Soil total N *a§	13.62	0.02	0.03	0.04
Soil temperature *a§	12.95	0.01	0.01	0.02
N mineralization potential *§	12.61	0.02	0.01	0.13
Soil Ammonium *§	10.56	0.05	0.01	0.21
GLU activity *§	10.13	0.04	0.01	0.34
PHOS activity	5.66	0.09	0.00	0.70
Dissolved Organic C	4.85	0.11	0.01	0.53
Phi2	4.31	0.17	0.00	0.98
Gammaproteobacteria Relative Abundance	4.20	0.13	0.00	0.71
Plant available nitrate	4.04	0.20	0.00	0.61
Specific Leaf Area	3.17	0.16	0.00	0.96
TYR activity	2.86	0.14	0.00	0.85
Chlorobia Relative Abundance	2.71	0.15	0.00	1.00
Urease activity	2.60	0.23	0.00	0.90
BG activity	2.11	0.36	0.00	0.70
Microbial Biomass N	1.92	0.17	0.01	0.51
Specific Stem Density	1.74	0.24	0.00	0.88
Root Tissue Density	1.73	0.25	0.01	0.52
Leaf C:N	1.40	0.24	0.00	0.71
Chlorobia Relative Abundance	1.19	0.19	0.00	0.88
Microbial Biomass C:N	0.74	0.24	0.00	0.86
ALA activity	0.61	0.35	0.00	0.99
Alphaproteobacteria Relative Abundance	0.31	0.29	0.00	1.00
Spirochaetia Relative Abundance	0.19	0.25	0.00	1.00
Root C:N	0.17	0.27	0.00	0.65
Spirochaetia Relative Abundance	0.13	0.31	0.00	1.00
Epsilonproteobacteria Relative Abundance	0.00	1.00	0.00	0.95
Plant available ammonium	-0.10	0.36	0.00	0.96
nifH gene copy #	-0.17	0.26	0.00	0.85

Table S4.2 (cont'd):

LAP activity	-0.30	0.52	0.00	1.00
Bacilli Relative Abundance	-0.40	0.39	0.00	0.60
Clostridia Relative Abundance	-0.90	0.44	0.00	1.00
Stigonematales Relative Abundance	-1.06	0.51	0.00	0.39
Pleurocapsales Relative Abundance	-1.06	0.46	0.00	0.95
CBH activity	-1.08	0.63	0.00	1.00
Soil nitrate	-1.16	0.55	0.00	0.77
Soil moisture	-1.70	0.54	0.00	1.00
Nitrification potential	-1.90	0.65	0.00	0.84
Methanococci Relative Abundance	-1.93	0.71	0.00	0.98
Delta/epsilon Relative Abundance	-1.99	0.56	0.00	0.99
Microbial Biomass C	-2.07	0.63	0.00	1.00
ARG activity	-2.60	0.77	0.00	1.00
Plant height	-2.92	0.75	0.00	0.90
Betaproteobacteria Relative Abundance	-3.16	0.70	0.00	0.99
Nostocales Relative Abundance	-3.40	0.81	0.00	0.86
Actinobacteria Relative Abundance	-3.45	0.79	0.00	1.00
Relative Chlorophyll	-3.54	0.62	0.00	0.81
Oscillatoriophycideae Relative Abundance	-4.25	0.92	0.00	1.00

***** denotes significant predictors at $p < 0.05$ based on percent increase in mean squared error (%INcMSE).
[a] denotes significant predictors at $p < 0.05$ based on increase in node purity (INP).
[§] denotes top ten most important predictors

REFERENCES

REFERENCES

Bahulikar, R. A., Torres-Jerez, I., Worley, E., Craven, K., & Udvardi, M. K. (2014). Diversity of nitrogen-fixing bacteria associated with switchgrass in the native tallgrass prairie of Northern Oklahoma. Appl. Environ. Microbiol., 80(18), 5636-5643.

Benhizia, Y., Benhizia, H., Benguedouar, A., Muresu, R., Giacomini, A., & Squartini, A. (2004). Gamma proteobacteria can nodulate legumes of the genus Hedysarum. Systematic and applied microbiology, 27(4), 462-468.

Bloch, S. E., Ryu, M. H., Ozaydin, B., & Broglie, R. (2020). Harnessing atmospheric nitrogen for cereal crop production. *Current Opinion in Biotechnology*, *62*, 181-188.

Campbell, W. H., Song, P., and G. G. Barbier. 2006. Nitrate reductase for nitrate analysis in water. Environmental Chemistry Letters, *4*, 69-73.

Cleveland, C. C., Townsend, A. R., Schimel, D. S., Fisher, H., Howarth, R. W., Hedin, L. O., ... & Wasson, M. F. 1999. Global patterns of terrestrial biological nitrogen (N2) fixation in natural ecosystems. *Global biogeochemical cycles*, *13*(2), 623-645.

Czaban, W., Jämtgård, S., Näsholm, T., Rasmussen, J., Nicolaisen, M., & Fomsgaard, I. S. (2016). Direct acquisition of organic N by white clover even in the presence of inorganic N. Plant and soil, 407(1-2), 91-107.

Davies-Barnard, T., & Friedlingstein, P. 2020. The global distribution of biological nitrogen fixation in terrestrial natural ecosystems. Global Biogeochemical Cycles, 34, e2019GB006387. https://doi-org.proxy2.cl.msu.edu/10.1029/2019GB006387

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.

Dixon R, Kahn D. 2004. Genetic regulation of biological nitrogen fixation. Nature Reviews Microbiology 2: 621-631.

Fernandez, C. W., & Koide, R. T. (2012). The role of chitin in the decomposition of ectomycorrhizal fungal litter. Ecology, 93(1), 24-28.

Gelfand, I., Sahajpal, R., Zhang, X., Izaurralde, R. C., Gross, K. L., & Robertson, G. P. (2013). Sustainable bioenergy production from marginal lands in the US Midwest. *Nature*, *493*(7433), 514-517. 52.

Harris, R. F. (1981). Effect of water potential on microbial growth and activity. *Water potential relations in soil microbiology*, *9*, 23-95.

Hobbs NT, Schimel DS. 1984. Fire effects on nitrogen mineralization and fixation in mountain shrub and grassland communities. Journal of Range Management: 402-405.

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(1), 176-190.

Jansson, J. K., & Hofmockel, K. S. (2018). The soil microbiome—from metagenomics to metaphenomics. *Current opinion in microbiology*, *43*, 162-168.

Kohlmeier, S., Smits, T. H., Ford, R. M., Keel, C., Harms, H., & Wick, L. Y. (2005). Taking the fungal highway: mobilization of pollutant-degrading bacteria by fungi. *Environmental science & technology*, *39*(12), 4640-4646.

Kox MA, Lüke C, Fritz C, van den Elzen E, van Alen T, den Camp HJO, Ettwig KF. 2016. Effects of nitrogen fertilization on diazotrophic activity of microorganisms associated with Sphagnum magellanicum. Plant and Soil 406: 83-100.

Mehmood, M. A., Ibrahim, M., Rashid, U., Nawaz, M., Ali, S., Hussain, A., & Gull, M. (2017). Biomass production for bioenergy using marginal lands. *Sustainable Production and Consumption*, *9*, 3-21.

Näsholm, T., Kielland, K., & Ganeteg, U. (2009). Uptake of organic nitrogen by plants. New phytologist, 182(1), 31-48.

Norman JS, Friesen ML. 2016. Complex N acquisition by soil diazotrophs: how the ability to release exoenzymes affects N fixation by terrestrial free-living diazotrophs. The ISME Journal 11: 315.

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. Journal of Agronomy and Crop Science 193: 153-156.

Paungfoo-Lonhienne, C., Rentsch, D., Robatzek, S., Webb, R. I., Sagulenko, E., Näsholm, T., ... & Lonhienne, T. G. (2010). Turning the table: plants consume microbes as a source of nutrients. PLOS one, 5(7).

Reed, S. C., Cleveland, C. C., & Townsend, A. R. (2011). Functional ecology of free-living nitrogen fixation: a contemporary perspective. Annual review of ecology, evolution, and systematics, 42, 489-512.

Rhine, E. D., Sims, G. K., Mulvaney, R. L., and Pratt, E.J., 1998. Improving the Berthelot reaction for determining ammonium in soil extracts and water. Soil Science Society of America Journal 62: 473-480

Robertson, G. P., & Groffman, P. M. (2007). Nitrogen transformations. *Soil microbiology, ecology, and biochemistry*, *3*, 341-364.

Robertson, G. P., Hamilton, S. K., Barham, B. L., Dale, B. E., Izaurralde, R. C., Jackson, R. D., ... & Tiedje, J. M. (2017). Cellulosic biofuel contributions to a sustainable energy future: Choices and outcomes. *Science*, *356*(6345), eaal2324.

Rocca, J. D., Hall, E. K., Lennon, J. T., Evans, S. E., Waldrop, M. P., Cotner, J. B., ... & Wallenstein, M. D. (2015). Relationships between protein-encoding gene abundance and corresponding process are commonly assumed yet rarely observed. The ISME journal, 9(8), 1693-1699.

Roley, S. S., Duncan, D. S., Di Liang, A. G., Jackson, R. D., Tiedje, J. M., & Robertson, G. P. (2018). Associative nitrogen fixation (ANF) in switchgrass (Panicum virgatum) across a nitrogen input gradient. *PloS one*, *13*(6).

Saiya-Cork, K. R., Sinsabaugh, R. L., & Zak, D. R. (2002). The effects of long-term nitrogen deposition on extracellular enzyme activity in an Acer saccharum forest soil. *Soil Biology and Biochemistry*, *34*(9), 1309-1315.

Shiraishi, A., Matsushita, N., & Hougetsu, T. (2010). Nodulation in black locust by the Gammaproteobacteria Pseudomonas sp. and the Betaproteobacteria Burkholderia sp. Systematic and Applied Microbiology, 33(5), 269-274.

Simon, A., Bindschedler, S., Job, D., Wick, L. Y., Filippidou, S., Kooli, W. M., ... & Junier, P. (2015). Exploiting the fungal highway: development of a novel tool for the in situ isolation of bacteria migrating along fungal mycelium. *FEMS microbiology ecology*, *91*(11), fiv116.

Smercina, D. N., Evans, S. E., Friesen, M. L., & Tiemann, L. K. (2019). To fix or not to fix: controls on free-living nitrogen fixation in the rhizosphere. Appl. Environ. Microbiol., 85(6), e02546-18.

Tiemann, L. K., & Billings, S. A. (2011). Changes in variability of soil moisture alter microbial community C and N resource use. *Soil Biology and Biochemistry*, *43*(9), 1837-1847.

Warmink, J. A., Nazir, R., Corten, B., & Van Elsas, J. D. (2011). Hitchhikers on the fungal highway: the helper effect for bacterial migration via fungal hyphae. *Soil Biology and Biochemistry*, *43*(4), 760-765.

Warren, C. R. (2014). Organic N molecules in the soil solution: what is known, what is unknown and the path forwards. Plant and Soil, 375(1-2), 1-19.

Weißhaupt, P., Pritzkow, W., & Noll, M. (2011). Nitrogen metabolism of wood decomposing basidiomycetes and their interaction with diazotrophs as revealed by IRMS. *International Journal of Mass Spectrometry*, *307*(1-3), 225-231.

Weintraub, M. N., Scott-Denton, L. E., Schmidt, S. K., & Monson, R. K. (2007). The effects of tree rhizodeposition on soil exoenzyme activity, dissolved organic carbon, and nutrient availability in a subalpine forest ecosystem. *Oecologia*, *154*(2), 327-338.

Wieczorek, A. S., Schmidt, O., Chatzinotas, A., von Bergen, M., Gorissen, A., & Kolb, S. (2019). Ecological Functions of Agricultural Soil Bacteria and Microeukaryotes in Chitin Degradation: A Case Study. *Frontiers in microbiology*, *10*.

Wilson, J. M., & Griffin, D. M. (1975). Water potential and the respiration of microorganisms in the soil. *Soil Biology and Biochemistry*, *7*(3), 199-204.

Zhang, S., Yu, Z., Lin, J., & Zhu, B. (2020). Responses of soil carbon decomposition to drying-rewetting cycles: A meta-analysis. *Geoderma*, *361*, 114069.

Zhang, W., Li, X. G., Sun, K., Tang, M. J., Xu, F. J., Zhang, M., & Dai, C. C. (2020). Mycelial network-mediated rhizobial dispersal enhances legume nodulation. *The ISME Journal*, 1-15.