Impacts of Soil Management on Microbial Assemblages Involved in Nitrogen Transformations in Agroecosystems in Tennessee, USA

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Jialin Hu

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ABSTRACT

Nutrient reduction, particularly with respect to nitrogen (N) losses, is an important goal for sustainably managed agroecosystems. Soil N-cycling microbial populations that modulate these processes are affected by agricultural management regimes. This research focused on the controls and dynamics of the major N-cycling microbial populations in high-input cotton field under agricultural management regimes and low-input native C_4 forage grass systems under pasture management practices to determine the effects of management regimes on *in situ* seasonal dynamics of the functional microbes responsible for N fixation, nitrification, and denitrification processes. Molecular microbial ecology methods were combined with soil physicochemical properties and biogeochemical process rates to address research questions.

The results showed that for soils in both cropland and grassland, the variability of the activity of N-cycling microbial populations was consistently and significantly higher than for functional potential. Agricultural season was a key factor affecting the variability of both functional potential and activity of N-cycling microbial populations regardless of agroecosystem types. In cotton fields, leguminous cover crops like hairy vetch promoted functional activity of N-cycling populations through the growing season, equaling or exceeding the effects of inorganic N fertilizer, suggesting the potential of leguminous cover crops for replacing inorganic N fertilization from the perspective of microbial ecology. In native C_4 grasslands, high N fertilization rate changed community composition of both diazotrophs and ammonia-oxidizing bacteria (AOB), inhibited diazotrophs functional activity and diversity of diazotrophs but promoted AOB abundance, activity, and diversity as well as N_2O emission and nitrification potential, indicating the negative influence of excessive N fertilization on soil functional microbial community, N fixation and reservation, and soil and downstream environmental health. Therefore, the significance of reduced N fertilization may be substantial both economically and environmentally. Our findings highlighted the fact that both genetic and transcriptional assessments can provide important information about the microbial populations and functions. Together, our work revealed insights into important relationships between microbial functional capacity and activity and associated nitrogen pools and processes in the field, adding to our

understanding of the effects of soil health management practices on soil N-cycling microbial ecology.

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INTRODUCTION

Microbial transformation of nitrogen (N) is one of the most important processes in soils. Much effort is going into fine-tuning agricultural and land management practices to optimize nitrogen available to plants and minimize excess export. Nutrient reduction strategies aim to maintain crop yields while reducing downstream impacts on the environment (e.g., eutrophication of aquatic systems, production of greenhouse gasses). These strategies include conservation agriculture practices such as reduced tillage and cover crops, and/or switching to more nutrient efficient plant species. Microbes are responsible for the major nitrogen transformations, including fixation of atmospheric N, oxidation of ammonia to nitrate, and denitrification, and ultimately control the fate of N. The composition of the N cycling microbial groups can vary by environment and management. A change in the types of N-cycling microbes can ultimately influence the fate of N and potential for N losses. This dissertation presents research conducted to address some of the knowledge gaps that exist in understanding how agricultural management practices influence N-cycling microbial communities, as well as the roles of soil physicochemical properties in driving the variation of N-cycling populations.

This dissertation is divided into three main sections that include: 1) an introduction encompassing a statement of purpose and a literature review of the current knowledge concerning N-cycling microbial communities in agroecosystems; 2) three main chapters in manuscript form; and 3) a summary that includes conclusions and prospects of this research.

Chapter I is a manuscript entitled “Soil health management enhances microbial nitrogen cycling capacity and activity” published in American Society for Microbiology (ASM) journal *mSphere* in 2021. This paper reported the findings related to biotic mechanisms of N cycling as a part of the USDA-NIFA project “Mitigating nutrient losses from agroecosystems: biotic and abiotic mechanisms of N cycling under conservation agriculture management practices” award to Dr. Sean Schaeffer and Dr. Jennifer DeBruyn. This chapter was a study conducted at a long-term (>30 years) cotton crop field in West Tennessee under various soil health management treatments, allowing us to determine how the N-cycling microbial populations were affected by tillage, cover crops, and N fertilization. The N-cycling microbial population size and functional activity were measured by targeting their marker genes (DNA-level) and gene transcripts (RNA-level)

using quantitative polymerase chain reaction (qPCR) and quantitative reverse-transcription PCR (qRT-PCR).

Chapter II was a study conducted at a native C4 grassland small plot trial with mowing as grass harvesting method in East Tennessee to investigate how the N-cycling microbial communities, especially the diazotrophic and ammonia-oxidizing bacterial communities, were affected by N fertilization rates under different C_4 grass species. The population size and functional activity of N-cycling microbes were quantified by targeting their marker genes and transcripts using qPCR and qRT-PCR. The diazotrophic and ammonia-oxidizing bacterial community structures were determined through high-throughput amplicon sequencing techniques and bioinformatics and statistical analysis, which revealed the structural composition of diazotrophs and ammonia-oxidizing bacteria (AOB) based on their widely used functional marker genes *nifH* and *amoA*, respectively.

Chapter III was a study conducted at a large native pasture with cattle grazing in Middle Tennessee to investigate how the microbial N-cycling capacity was affected by cover crops and N fertilization on a large scale, as well as the difference of microbial N-cycling capacity between Middle and East Tennessee. The microbial N-cycling capacity was quantified by measuring the abundance of N-cycling marker genes through qPCR. Chapter II and chapter III were supported by the USDA-NIFA project "Enhancing agro-grasslands sustainability through innovation and improved soil biodiversity" award to Dr. Patrick Keyser and Dr. Jennifer DeBruyn.

Statement of Purpose

Nutrient reduction, particularly with respect to N losses, is an important goal for sustainably manage agroecosystems. Soil N-cycling microbial populations that modulate these processes are affected by agricultural management. In order to better predict N transformations and potential losses in a given system, we need better information of the quantification, interactions, dynamics and controls on the N-cycling microbial populations in soil.

The objectives of this dissertation research are to assess the controls and dynamics of the major N-cycling microbial populations under high-input (cotton) and low-input (native

forage grass) systems and determine the effects of management (e.g., tilling, fertilizing, cover cropping) on these microbes and their processes. The specific objectives are:

1) Determine seasonal changes to the structure and function of microbial assemblages involved in soil N transformations under long term conservation management practices (reduced tillage, cover cropping, and reduced N fertilization).

2) Determine seasonal changes to the structure and function of microbial assemblages involved in soil N transformations under forage systems with native C_4 grasses and assess the impacts of grass species and management (cover cropping and N fertilization).

3) Determine the roles of environmental factors in driving the variation of the microbial assemblages involved in soil N transformations.

The specific objectives as well as the methodological approaches applied are given in details in subsequent chapters.

Literature Review

The microbial cycling of nutrients impacts many ecological properties of agricultural land including plant growth and productivity, soil carbon (C) sequestration, soil nutrient reservation, and greenhouse gas (GHG) emissions (Levy-Booth et al., 2014). Nitrogen (N) availability is an important limiting factor in the productivity of terrestrial ecosystems (LeBauer & Treseder, 2008). Anthropogenic N inputs to terrestrial ecosystems through fertilization and atmospheric deposition can remove this nutrient limitation, increasing reactive N availability and N losses from soils (Levy-Booth et al., 2014). In recent years, nutrient reduction, particularly with respect to N losses, is an important goal for sustainably managed agroecosystems. Nutrient reduction strategies aim to maintain crop yields while reducing downstream impacts on the environment (e.g., eutrophication of aquatic systems, emission of greenhouse gases). Thus, much effort is going into fine tuning agricultural and land management practices to optimize nitrogen available to plants and minimize excess export, including conservation agriculture practices, such as reduced tillage, reduced

fertilizers application, adoption of winter cover crops, and/or switching to more nutrient efficient plant species.

Microbes are responsible for the major nitrogen transformations in soils, including atmospheric N fixation, nitrification (oxidation of ammonia to nitrate), and denitrification, which ultimately control the fate of N (Ollivier et al., 2011). Many studies have demonstrated that agricultural management practices, such as fertilization and tillage, play major roles as determinants of soil bacterial structure and communities (Fernandez et al., 2016; Nivelle et al., 2016; Verzeaux et al., 2016). The composition of the N cycling microbial groups can vary by environmental factors and management strategies, and a change in the types of N-cycling microbes can ultimately influence the fate of N and the potential for N losses.

In order to understand the functional variation of microbial communities and their resilience to external changes and different agricultural land types, a key issue is to assess the soil community composition, quantify individual microbial population sizes and activity, and study fluctuations thereof. The study of N-cycling microbial assemblages in agroecosystems can provide significant evidence for determining best soil management practices, in turn, an increased understanding of the impacts of management practices on N-cycling microbes could allow modifications in soil management practices to optimize the activity of these organisms. Therefore, research focused on N-cycling microbial assemblages in agroecosystems with long-term impacts of soil management is an important step towards determining sustainable management approaches in agriculture.

Functional microbial assemblages involved in soil N cycling

The biogeochemical cycling of soil N is an important component of soil nutrient cycling. Soil N cycling not only affects soil quality and the productivity and sustainability of agroecosystems, but also has impacts on global environmental change. N cycling is referred to the interconversion processes of N gases, inorganic N, and organic N in nature, including ammonification, nitrification, denitrification, and N fixation, etc. (Figure 1). Among these processes, widespread soil microorganisms play a vital role in soil N cycling. In this literature review, the nitrifiers, denitrifiers, and diazotrophs will be the focus due to

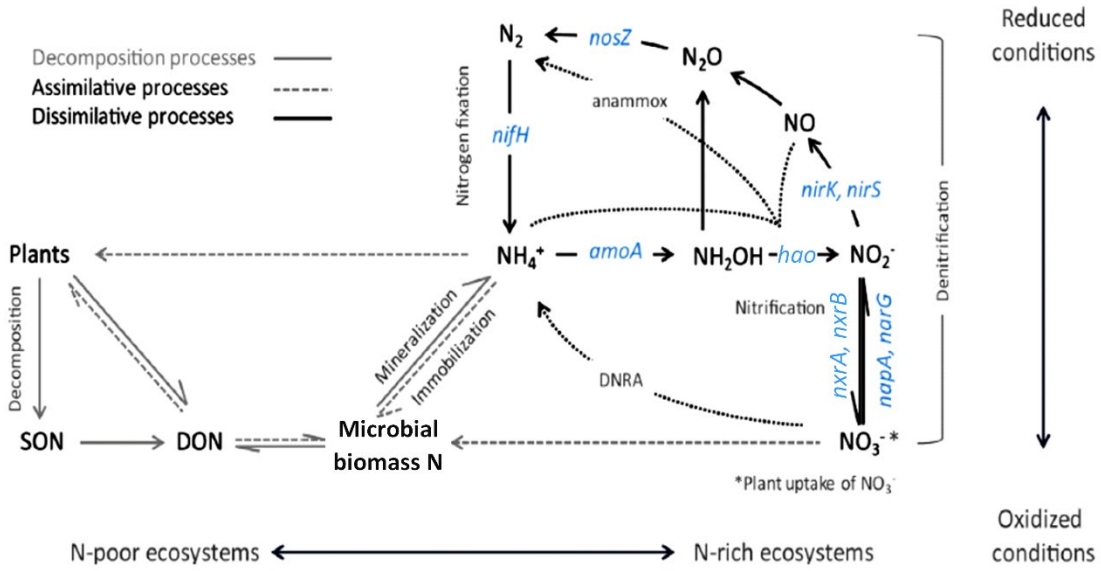

Figure 1. The soil nitrogen cycle process and relevant functional genes.Figure adapted from David J. Levy-Booth et al, 2014.

their important roles in N availability and losses in agricultural soil and their feasibility to be studied by relevant functional marker genes (Levy-Booth et al., 2014).

Nitrification. Nitrification is the biological oxidation of NH_3 to NO_3^-. Microbes that can carry out nitrification are referred to as "nitrifiers". Nitrifiers in agroecosystems can aerobically convert the positively charged ammonium to the negatively charged nitrate that can be easily taken up by plants, but this process can also lead to large N losses because the negatively charged nitrate is not easily absorbed by soil colloids. Consequently, leached nitrate may result in the pollution of streams and groundwater (Cameron et al., 2013). Thus, ammonia-oxidizing and nitrite-oxidizing microorganisms, essential in nitrification, undoubtedly play an important role in the sustainable development of the agricultural ecosystem as well as environmental protection.

Nitrification is a two-step process. The first and rate-limiting step, oxidation of NH_3 to NO_2^-, is carried out by ammonia-oxidizers, which include ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) (Frijlink et al., 1992). AOB convert NH_3 to NO_2^- through the mediation of two enzymes: ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (Araki et al., 2004). The AMO is encoded by the genes *amoA*, *amoB*, and *amoC*, which are co-transcribed as 3.5 kbp mRNA (Sayavedra‐Soto et al., 1998). The nitrifying community is primarily studied by using *amoA* as the marker gene, which encodes the α-subunit (the subunit that carries the active site of the enzyme (Rotthauwe et al., 1997)) of the AMO enzyme (Levy-Booth et al., 2014). The *amoA* gene is well suited for molecular studies of AOA and AOB communities because of its strongly conserved nucleotide sequence and its essential role in energy generating metabolism (Norton et al., 2002). The second step of nitrification, oxidation of NO_2^- to NO_3^-, is mainly carried out by nitrite oxidizing bacteria (NOB). The main genera of NOB are *Nitrospira* and *Nitrobacter*, which carry the key enzyme for nitrite oxidation, nitrite oxidoreductase (NXR) encoded by the genes *nxrA* and *nxrB* (Daims et al., 2015). Both types of nitrifiers use CO_2 as their carbon source for growth. In addition, organisms capable of conducting complete nitrification to convert ammonia to nitrate (termed "Comammox") have been found in most environments. The Comammox bacteria are more competitive than canonical nitrifiers in low-nutrient environments (Daims et al., 2015; van Kessel et

al., 2015) because they had continuing *amoA* gene transcription regardless of the external ammonia content (Xu et al., 2020), but they were 1-2 orders of magnitude less abundant than canonical nitrifiers in agricultural soils (Xu et al., 2020).

According to previous studies, the abundance and community composition of AOB are more responsive than AOA to N source in agricultural soils, indicating that AOB should be the focus when developing strategies to control nitrification in agricultural soils (Ouyang et al., 2016). AOB belong to several genera, including *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovicria* (Levy-Booth et al., 2014). In previous research, the majority of known AOB in soil were *Nitrosomonas,* followed by *Nitrosococcus* and *Nitrosospira* (Purkhold et al., 2000). More recently, different soil types (freshwater marsh, permafrost, garden, agricultural, and desert soils) were investigated using fluorescence microscopy with DAPI staining, revealing that *Nitrosospira*, an important nitrifier in aquatic habitats, also plays an important role in soil nitrification (Bartosch et al., 2002). Due to the difficulties in separation and purification of AOB strains in the lab, the studies of AOB in ecosystems are mainly based on molecular ecology methods targeting the 16S rRNA gene or the *amoA* gene (Kowalchuk et al., 2000; Purkhold et al., 2000).

Denitrification. Denitrification is a process of full or partial dissimilative reduction of NO_3^- conducted by facultative anaerobic microorganisms as a type of respiration to yield dinitrogen gas (N_2) (Colliver & Stephenson, 2000; Kowalchuk & Stephen, 2001). The microbes perform the process are mainly heterotrophic bacteria, such as *Paracoccus denitrificans* and various *Pseudomonas* (Carlson & Ingraham, 1983), but autotrophic denitrifiers also exist, such as *Thiobacillus denitrificans* (Baalsrud & Baalsrud, 1954). Generally, denitrification occurs in anoxic environments where concentrations of O_2, a more energetically favorable electron acceptor is low, so that NO_3^- and NO_2^- can be used as preferred electron acceptors. In these processes, organic matter is the electron donor. Denitrification can regulate NO_3^- leaching by coordinating with nitrification, and is also the primary pathway of nitrous oxide (N_2O) emissions from soil (Shaw et al., 2006). The global warming potential (GWP, a measure of how much energy the emissions of 1 ton of a gas will absorb over a given period of time, relative to the emissions of 1 ton of CO_2) of

N_2O is 296 times that of CO_2 over a 100-year period (IPCC, 2007). It has been pointed out that agricultural activities are the primary source of soil N_2O emissions, accounting for about 60% of total anthropogenic N_2O released into the atmosphere (IPCC, 2013).

The full denitrification process from NO_3^- to N_2 begins with the nitrate reduction to nitrite, which is catalyzed by two different enzymes: one is a membrane-bound reductase (NAR) encoded by the gene *narG*, another is the *napA* gene encoding periplasmic nitrate reductase (NAP). Various microbes contain NAR, including archaea, while the enzyme NAP is only produced by gram-negative bacteria. Nitrate reducing bacteria can carry either one or both of these two genes. Therefore, the *narG* and *napA* genes are utilized most often in studies of the reduction of NO_3^- to nitrite (NO_2^-) (Bru et al., 2010; Kandeler et al., 2009; Tavares et al., 2006). Following nitrate reduction, NO_2^- can be convert to NO or N_2O by Cu-containing nitrite reductase (NIR) encoded by the *nirK* gene or the cytochrome cd_1 NIR encoded by the gene *nirS*. Thus, *nirK* and *nirS* are the marker genes for nitrite reduction (Kandeler et al., 2009). The genes *nirK* and *nirS* evolved convergently, and are generally carried by different organisms due to mutual exclusion of the two reductases in a given strain (Coyne et al., 1989). The gene *nirK* is prevalent in α-proteobacteria and can also be detected in β-proteobacteria, γ-proteobacteria, Firmicutes and Bacteroidetes, while *nirS* is abundant in β-proteobacteria, and also exists in α- and γ-proteobacteira (Heylen et al., 2006). Strains of genus *Thermus* (*T. oshimai* JL-2 and *T. scotoductus* SA-01) are two exceptions to the mutual exclusion, as they contain both *nirS* and *nirK*. As a result, these two strains possess greater denitrification abilities because these isoenzymes are kinetically distinct and regulated differently (Murugapiran et al., 2013).

The emission of N_2O from agricultural soils is a focus of attention in denitrification processes. The nitric oxide produced from nitrite reduction can be very easily converted to N_2O by cytochrome *bc* nitric oxide reductase (NOR) encoded by the gene *norB*. Therefore, nitrite reduction is a crucial step in denitrification for N_2O emission. N_2O can also be further reduced to N_2 by N_2O-reducing bacteria (Sharon, 2008), which is carried out by the nitrous oxide reductase (NOS) enzyme encoded by the gene *nosZ*. It has been observed a co-occurrence pattern of *nirS*, *norB* and *nosZ* genes presented in the genome of *nirS*-type denitrifiers. However, *nirK*-type denitrifiers do not contain nitric oxide or nitrous oxide

reductase genes, suggesting that this group conducts incomplete nitrite reduction to nitrous oxide. Therefore, *nirK*-type denitrifiers may be contributing more to N_2O emissions from soils than *nirS*-type denitrifiers (Helen et al., 2016). N_2O reduction potential is frequently studied using the *nosZ* gene, which encodes the catalytic subunit of multi-Cu NOS (Chan et al., 1997). The populations of denitrifiers in each step of denitrification decrease in abundance (Bru et al., 2010) due to the decreased available free energy liberated along with the sequential reduction step (Koike & Hattori, 1975).

Many studies have demonstrated that denitrifying microbes are widespread in soil, comprising about 0.5 to 5% of the total bacterial population (Bru et al., 2010; Levy-Booth et al., 2014). Denitrifying genes are found in a wide range of both autotrophic and heterotrophic bacteria (Baalsrud & Baalsrud, 1954; Carlson & Ingraham, 1983). The polyphyletic distribution of denitrification genes suggests that they can co-occur with ammonia-oxidation and N-fixation genes in many strains. For example, there are studies proposing that a significant portion of AOB may perform denitrification (Ritchie & Nicholas, 1972; Schmidt et al., 2004). Shaw et al. verified that *Nitrosospira* spp., as a dominant AOB in soil, can produce N_2O via a nitrifier denitrification pathway (Shaw et al., 2006). Nitrifier denitrification had also been demonstrated as a pathway of N_2O production in *Nitrosomonas europaea* (Beaumont et al., 2002).

Bacteria with the ability to reduce N_2O to N_2 can be separated into two types, clade I and clade II, according to the structures of *nos* operon and the features of *nosZ* sequence that they contained (Yoon et al., 2016). The clade I NosZ enzymes and clade II NosZ enzymes perform the same reaction, but the amino acid sequence similarity of the enzymes between clade I and clade II NosZ are less than 50%. Yoon et al. demonstrated that clade I and clade II bacteria exhibit different properties by measuring their growth yields and whole-cell N_2O consumption kinetics (Yoon et al., 2016). Many relevant studies found that clade II *nosZ* is more abundant than clade I *nosZ* in agricultural soils (Jones et al., 2013; Juhanson et al., 2017; Tsiknia et al., 2015), including pastures (Samad et al., 2016), though some studies observed a contradictory pattern (Bowen et al., 2018; Domeignoz-Horta et al., 2015), which may depend on the effects of agricultural management practices, environmental factors, and subsequent niche selection for the bacteria carrying these genes

(Bowen et al., 2018). In general, denitrification is conducted by the combination of different functional microbial groups, with one group only responsible for part of the whole process.

Nitrogen Fixation. Biological nitrogen fixation (BNF), the reduction of atmospheric N_2 gas to biologically available ammonium, is one of the most important microbial processes in plant-soil environment, as it replenishes the pool of N that is lost to the atmosphere via nitrification and denitrification, which makes it a major contributor to the availability of N in agricultural crops (Akter et al., 2014). In this process, rhizobia that symbiotically living with some plants, especially legumes, as well as free-living diazotrophs are the protagonists with nitrogenase enzyme catalyzing this reaction.

Diazotrophs are highly diverse and are widely distributed across bacterial and archaeal taxa (Dixon & Kahn, 2004; Orr et al., 2011). Approximately 80% of BNF is carried out by diazotrophs in symbiosis with legumes (Peoples et al., 1995). But under some certain conditions, free-living diazotrophs can also fix significant amount of N (0 to 60 kg N ha^{-1} $year^{-1}$) (Burgmann et al., 2004). The major genera of diazotrophs include *Achromobacter*, *Azotobacter*, *Azospirillum*, *Beijerinckia*, *Bradyrhizobium*, *Burkholderia*, *Chromotium*, *Chstridium*, *Desulfovibrio*, *Klebsiella*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, *Rhodopseudomonas*, *Rhodosospirillum* and *Thiobacillus*. In addition, some species of *Cyanobacteria* and *Actinobacteria* also have the ability to fix atmospheric N_2 (Levy-Booth et al., 2014). The molecular analysis of diazotrophs is primarily conducted by using the marker gene *nifH*. The *nifH* gene is well suited for use as a marker gene for molecular studies of N-fixing bacteria because it is the most conserved gene in the *nif* operon and encodes the Fe subunit of the nitrogenase enzyme (Orr et al., 2011).

Impacts of soil management on N-cycling microbes in agricultural soil

U.S. land area covers nearly 2.3 billion acres. The proportion of the land base in agricultural uses, which includes crops, pastures, and grazed forestland, has declined from 63% in 1949 to 51% in 2007 (USDA). Cropland can be understood as land with regularly or recently cultivated agricultural, horticultural, and domestic habitats. Grassland or pasture ecosystems are areas covered by grass-dominated vegetation with little or no tree

cover and include meadows, steppes and grasslands grazed with a variable intensity. With the gradual decline in land area used for agriculture, production and crop yields need to be improved to meet demand. Thus, the need for best management practices to improve soil health in agriculture is becoming increasingly important. Conservation agricultural management practices include reduced tillage, increased organic matter inputs, winter cover crops, reduced pesticide use, crop rotations, as well as reduced fertilization. This portion of the literature review focuses on the impacts of tillage, winter cover crops, and N fertilization on the distribution and activity of N-cycling microbes in agricultural soils.

Tillage Effects. Tillage is the agricultural preparation of soil by mechanical agitation of various types, such as digging, stirring, and overturning, which is known as the most important agricultural practice in crop cultivation (Magomedov et al., 2017). It could increase the moisture in deep soil, promote the development of root system of crops (Magomedov et al., 2017), while other studies suggested that it can increase spring soil temperatures and aeration with accompanied decrease in soil water content (McCormick, 2013). However, excessive tillage is harmful to soil health because it can increase oxygen in soil, stimulating microbial activity and lead to the decomposition of organic matter (Carter, 1992; Lupwayi et al., 2001). Tillage can also destroy soil aggregates, exposing organic matter that had been physically protected within aggregates to microbial decomposition (Beare et al., 1994). Therefore, reduced tillage or no tillage is a benetifical management practice to limit disturbance of soil structure and protect soil organic matter from loss via decomposition.

Reduced tillage of agricultural soils has been shown to affect the diversity and abundance of microbes and the microbial biomass. For example, the study by Lupwayi et al. observed higher bacterial diversity under no tillage than under conventional tillage in the soils growing barley in northern British Columbia. They also found that the microbial biomass was greater in large soil aggregates, which are more prevalent under no tillage, than in smaller aggregates due to protection of organic carbon by soil aggregates (Lupwayi et al., 2001). There are studies observed higher population size of soil microbes under no tillage systems (Kladivko, 2001; White & Rice, 2009). Similarly, Mbuthia et al. demonstrated the increases in gram-positive bacteria, *Actinomycetes*, and Mycorrhizae

biomarkers under no tillage compared to tillage in continuous cotton field in West Tennessee (Mbuthia et al., 2015). Furthermore, in a study about long-term impacts of soil management on AOB communities, Liu et al. found that AOB were more diverse in no-till plot than plow-till plot. They speculated that this result may be related to the major characteristics of no-till soil with greater hydraulic conductivity, infiltration rate, and water retention capacity than plow-till plot (Liu et al., 2018).

Tillage also has influence on microbial activity and functionality in agricultural soils. Smith et al. assessed the long-term effects that tillage practice has on N-cycling microbial activities in a large-scale field plots by measuring nitrification and denitrification rates, identifying that tillage is related to higher nitrification rates and N_2O emissions (Smith et al., 2010), which are two primary pathways of N losses. However, Grave et al. found that N_2O emissions were 30 to 200% higher in no tillage soil than in tillage soil, which was explained by the higher soil water-filled pore space under no tillage which favored incomplete denitrification (Grave et al., 2018). Similarly, another study also detected higher N_2O emissions, denitrifying enzyme activity, and total bacteria, and N-cycling genes (such as *amoA* and *nosZ*) abundance in long-term no tillage than those in conventional tillage plots, which was attributed to the stable and favorable conditions of no tillage system for heterotrophic microorganisms (Badagliacca et al., 2018).

Nitrification rates altered by tillage could ultimately affect crop growth and yield. A high nitrification rate can accelerate the conversion of NH_4^+ to NO_3^-, which results in soil nutrient loss by N leaching and by promoting denitrification which release N_2O or N_2, ultimately decreasing the N utilization efficiency by plants and contributing to environmental pollution. Segal et al. reported that the abundance of AOB was 10-fold lower in long-term tillage rainfed continuous maize plots than in no tillage plots while AOA showed no difference (Segal et al., 2017). Similarly, Li et al. also investigated the effects of tillage on soil nitrification in a subtropical rice soil under long-term (22 years) no-tillage and conventional tillage treatments. They found that AOB and AOA *amoA* gene copies showed similar changes and were significantly higher for no tillage than conventional tillage, while nitrification rate showed the contrary trend, which suggested that the practice of no tillage may have the potential to increase soil N retention by keeping N longer in the

NH_4^+ state, thus decreasing the potential for N loss from leaching. Moreover, functional gene expression of *amoA* may not be related to the abundance of nitrifiers (Li et al., 2015), indicating that assessing impacts of tillage on N-cycling microbial activity should take both abundance and functional expression of microbes into consideration. In another long-term soil management study, Liu et al. found that the trends for nitrifier cell density and potential nitrification rate were not consistent. In fact, the abundances of N-cycling microbes measured through marker gene copy numbers and the corresponding N-cycling process rates are usually unrelated, which mainly because of the high dormant proportion of microbes in soils (Che et al., 2018). Therefore, a better way of assessing N-cycling microbial activity is to target the expression of these functional genes at transcriptional level by qRT-PCR. This approach has not been used as frequently as gene-level approach because of challenges with soil RNA extraction and reverse transcription, rendering it more complex and costly than DNA-based studies.

Nitrogen Fertilization Effects. Nitrogen availability is a critical factor for plant growth and the abundance and activity of soil microbes. In order to improve the yield of crops, N is traditionally input by fertilization. However, over-fertilization can result in various environmental problems. For example, NO_3^--N is susceptible to leaching losses because the negative charge of the molecule is not held by cation exchange sites of soil particles. N can also be lost to the atmosphere through conversion into N_2O and NO gases by microorganisms in warm, poorly aerated soil. N in urea-containing fertilizers and manure is susceptible to volatilization losses as ammonia gas when not incorporated into the soil. Therefore, a proper N fertilizer type and fertilization rate can improve N fertilizer use, decrease NO_3^- leaching, and minimize N_2O emissions by affecting the abundance and activity of soil microbes, especially N-cycling microbes.

Compared to inorganic fertilizers, organic fertilizers are better for soil and environmental health by improving soil texture, increasing soil microbial activity, and recycling nitrogen. Many studies have reported that organic N fertilizer types and fertilization rates can differently affect soil N-cycling microbes, especially nitrifiers. For example, Rudisill et al. reported that the potential nitrification activity of ammonia oxidizers was lower in urea treatments compared to animal manure and green manure

despite greater AOB abundance, suggesting that the lower pH and other factors caused by urea may reduce the potential nitrification activity by impacting *amoA* expression (Rudisill et al., 2016). Xiang et al. studied the short-term urea amendment in an alpine grassland and found that short-term urea addition increased the abundance of AOB significantly and resulted in a shift from AOA to AOB dominance, suggesting that AOB were more sensitive to urea fertilizer than AOA (Xiang et al., 2017). Research by Shen et al. also reported that AOB community responded to urea-N substrate while AOA showed no changes in semiarid temperate grassland soil, and N addition rates with 2-4 g N m^{-2} $year^{-1}$ increased AOB abundance and shifted its composition while higher N addition (more than 16 g N m^{-2} $year^{-1}$) decreased AOB diversity significantly (Shen et al., 2011).

Inorganic N fertilizers are more fast acting than organic N fertilizers but may also reduce soil fertility, accelerate soil acidification, and influence the abundance, activity, and diversity of soil microbes, especially functional microbial populations responsible for nitrogen transformations. Ying et al. investigated the effects of different nitrogen forms (NH_4^+-N and NO_3^--N) on ammonia oxidizing microorganisms in a typical grassland ecosystem, showing that AOB, not AOA, had positive responses to various N fertilizer, revealing a dominant role of AOB in nitrification with broad ranges of N availability. However, excessive NH_4^+-N addition (80 mg N kg^{-1} soil) showed a negative effect on AOB (bacterial *amoA* gene abundance), which was considered to be either a direct effect of the high NH_4^+ concentration itself or an indirect affect due to low soil pH caused by the high NH_4^+ concentration. In addition, they also found a negative effect of NO_3^--N on the abundance of AOA, which was speculated to be due to a product inhibition mechanism. The results above may be evidence for limiting contributions of AOA to nitrification in many soils (Ying et al., 2017). Similar results were shown in research by Di et al., which suggested that nitrification is driven by AOB rather than AOA in N-rich grassland soils (Di et al., 2009). Most long-term soil management experiments use NH_4NO_3 as the N fertilizer and focused on the impacts of fertilization rate on soil N-cycling microbes. Many of them reported that increased N fertilization rate can increase AOB diversity, abundance, and activity. For example, Liu et al. found that nitrifier cell densities, diversity, and potential nitrification rate increased with increased N fertilization in a >40 years' continuous maize

field by using both the most probable number method and DGGE combined with PCR method (Liu et al., 2017; Liu et al., 2018). In another research of > 26 year's continuous maize field, N rate showed a moderate correlation with AOB (Segal et al., 2017). However, in grassland soils, AOB diversity was found greater in unfertilized soils than in N fertilized soils (Webster et al., 2002).

N fertilization can also have impact on the denitrification process in agricultural soil, which was considered a main cause of N_2O emissions from soil. Wang et al. collected 353 observations from 74 papers to explore the impacts of N fertilization on the denitrification of agricultural soil (Wang et al., 2018). Their result showed that N fertilization increased soil denitrification by an average of 174%, compared to the no-fertilization control. Besides, within the N fertilization rate of 250 kg N ha^{-1}, soil denitrification increased exponentially with fertilization rate; above this threshold, no further increases were observed. For the abundance and diversity of denitrification bacteria with *nirS* or *nirK* genes, Yang et al. (2017) revealed that in alkaline agricultural soil, the abundance of *nirK*-denitrifiers increased with N fertilization, while *nirS*-denitrifiers decreased. They speculated that the total N concentration and the soil pH change caused by N fertilization could be the dominant factors controlling the communities of denitrification bacteria (Yang et al., 2017). This result was similar to the study by Hallin et al., which pointed out that the high rate (80 kg N ha^{-1} $year^{-1}$) of $(NH_4)_2SO_4$ resulted in lower soil pH and significantly reduced the abundance of denitrification genes (*narG*, *nirK*, *nirS*, and *nosZ*), while organic fertilizers with near-neutral pH can increase *narG*, *nirK*, and *nosZ* abundances (Hallin et al., 2009).

However, research by Soares et al. pointed out that AOB, rather than denitrifiers, via the nitrification pathway, were the main contributors to N_2O emissions in a tropical agricultural soil, because 1) N_2O emissions were significantly correlated with the abundance of AOB *amoA* rather than denitrification gene abundances; 2) nitrogen applied as urea with nitrification inhibitors reduced 95% N_2O emissions than without nitrification inhibitors (Soares et al., 2016). In another study, Kastl et al. found that AOB *amoA* increased with increasing fertilization while AOA *amoA* copy numbers showed no change (Kastl et al., 2014). Interestingly, they also observed that the highest fertilization rate induced an inhibition of denitrifiers, which is similar to the research by Wallenstein et al.,

who suggested that denitrifiers are sensitive to high amounts of nitrate (Wallenstein et al., 2006).

Ouyang et al. conducted a meta-analysis to assess the effect of N fertilization on the abundance of nitrogen cycling genes in agricultural soils. The meta-analysis, comprising 47 field studies in agricultural ecosystems, including both croplands and grasslands, showed that N fertilization had no effect on *nifH* abundance, but significantly increased the abundance of *amoA* of AOA and AOB, *nirK*, *nirS* and *nosZ* genes, respectively. Moreover, organic fertilizers often had a much stronger effect than inorganic fertilizers (Ouyang et al., 2018). In addition to croplands, soil denitrification was also responsive to N fertilization in grasslands. Estavillo et al. (1994) reported immediate N losses through denitrification along with inorganic N fertilization but chronic N losses after organic N fertilization in a natural grassland (Estavillo et al., 1994). Other research conducted by Hartmann et al. investigated the effects of organic (cattle urine) and inorganic (NH_4NO_3) N fertilizers on N cycling in two different grassland soils and found that cattle urine can stimulate nitrification and denitrification but NH_4NO_3 decreased the nitrification and denitrification. These different responses were attributed to a soil pH effect: acidification caused by application of NH_4NO_3, and hydrolysis of urea in urine resulting in ammonia which can combine with H^+ and keep soil pH neutral. However, application of NH_4NO_3 significantly increased the *amoA* gene abundance for both bacteria and archaea but decreased the abundance of denitrification functional genes (*narG* and *nirS*), while urine application did not affect the abundance of N-cycling functional genes (Hartmann et al., 2013).

Therefore, N fertilization mainly have impacts on nitrifiers and denitrifiers in agricultural ecosystems. Over-fertilization can promote nitrification and denitrification in agricultural soil, and consequently result in NO_3^- leaching and N loss by N_2O emissions.

Cover Crop Effects. Cover crops are an important soil management practice, contributing numerous benefits to soil health. They keep the soil covered during winter and other periods of time when crops are not growing, which can reduce the risk of soil erosion, retain NO_3^- and other nutrients and reduce N leaching. After termination, the biomass produced by cover crops can be returned to soil and increase soil organic matter. Legume cover crops in particular can increase the nitrogen in soil by N fixation. Mbuthia et al.

pointed that the continuous period of growing of legume cover crop (vetch) may promote N-cycling in a long-term continuous cotton field by measuring the activities of enzyme β-glucosaminidase which is an index of N mineralization in soils. In some cases, application of N-fixing cover crop could replace N fertilization to improve both crop yields and soil health (Mbuthia et al., 2015).

However, legume cover crops can also release mineral N rapidly and increase denitrification and N_2O emissions relative to other cover crops or no cover crop due to a relatively low C: N ratio (Abdalla et al., 2012; Bowen et al., 2018). In one study, a combination of reduced tillage and mustard cover crop lead to more N_2O emissions than conventional treatment due to the increased soil C and N fixed by cover crops, consequently resulted in higher substrate availability for nitrification and denitrification (Abdalla et al., 2012). Another study combined a perennial grass-forage legume intercropping strategies with different N fertilization rates, concluding that the legume-based forage systems with low N input can replace the high N input conventional systems. However, higher N_2O emissions with the treatment of grass-white clovers intercropping were also detected in this study, which verified that the application of legume can lead to more N_2O emissions than conventional treatment (Hauggaard-Nielsen et al., 2016).

While there are studies revealed that the application of cover crop and cover crop types can affect soil N cycling, including N fixation and N loss from N_2O emissions, less is known about how the genes involved are influenced by cover crops. Therefore, the abundance and activity of N-cycle functional genes based on qPCR and qRT-PCR methods should be considered in future research to reveal the variation of N-cycle genes and gene expressions due to the impacts of winter cover crops.

Summary

The need for best management practices in agriculture to improve soil health and reduce environmental pollution is becoming increasingly important due to the aim of sustainable agriculture. This literature review introduced the main N cycling processes that occur in agricultural soils as well as the relevant functional genes responsible for these processes, summarized the impacts of different management practices on the variation of

abundance and activity of N-cycling microbes revealed by various studies. Collectively these studies have shown that tillage and N fertilization mainly affect microbes responsible for nitrification and denitrification, and cover cropping mainly influences diazotrophs, indicating that reduced tillage, low N fertilization rate, as well as cover crop application are three main management practices to improve N use efficiency and crop yield, reduce N loss by nitrate leaching and nitrous oxide emissions. One major limitation of the existing body of knowledge is that most of studies assessed impacts on N-cycling populations by only measuring the variation of gene abundance (i.e., functional potential) without gene expression (i.e., functional activity). An increasing gene abundance does not necessarily indicate higher activity, therefore the variation of gene expression at transcriptional level due different soil management practices is a research gap that needs to be further studied.

References

Abdalla, M., Rueangritsarakul, K., Jones, M., Osborne, B., Helmy, M., Roth, B., Burke, J., Nolan, P., Smith, P., & Williams, M. (2012). How effective is reduced tillage-cover crop management in reducing N_2O fluxes from arable crop soils? *Water, Air, & Soil Pollution*, *223*(8), 5155-5174. https://doi.org/10.1007/s11270-012-1268-4

Akter, Z., Pageni, B. B., Lupwayi, N. Z., & Balasubramanian, P. M. (2014). Biological nitrogen fixation and *nifH* gene expression in dry beans (*Phaseolus vulgaris* L.). *Canadian Journal of Plant Science*, *94*(2), 203-212. https://doi.org/10.4141/cjps2013-200

Araki, N., Yamaguchi, T., Yamazaki, S., & Harada, H. (2004). Quantification of *amoA* gene abundance and their *amoA* mRNA levels in activated sludge by real-time PCR. *Water Science and Technology*, *50*(8), 1-8. https://doi.org/10.2166/wst.2004.0474

Baalsrud, K., & Baalsrud, K. (1954). Studies on *Thiobacillus denitrificans*. *Archiv für Mikrobiologie*, *20*(1), 34-62. https://doi.org/10.1007/BF00412265

Badagliacca, G., Benitez, E., Amato, G., Badalucco, L., Giambalvo, D., Laudicina, V. A., & Ruisi, P. (2018). Long-term no-tillage application increases soil organic carbon, nitrous oxide emissions and faba bean (*Vicia faba* L.) yields under rain-fed Mediterranean conditions. *Science of the Total Environment*, *639*, 350-359. https://doi.org/10.1016/j.scitotenv.2018.05.157

Bartosch, S., Hartwig, C., Spieck, E., & Bock, E. (2002). Immunological detection of *Nitrospira*-like bacteria in various soils. *Microbial Ecology*, *43*(1), 26-33. https://doi.org/10.1007/s00248-001-0037-5

Beare, M. H., Hendrix, P. F., & Coleman, D. C. (1994). Water-stable aggregates and organic matter fractions in conventional- and no-tillage soils. *Soil Science Society of America Journal*, *58*(3), 777-786. https://doi.org/10.2136/sssaj1994.03615995005800030020x

Beaumont, H. J. E., Hommes, N. G., Sayavedra-Soto, L. A., Arp, D. J., Arciero, D. M., Hooper, A. B., Westerhoff, H. V., & van Spanning, R. J. M. (2002). Nitrite reductase of *Nitrosomonas europaea* is not essential for production of gaseous nitrogen oxides and confers tolerance to nitrite. *The Journal of Bacteriology*, *184*(9), 2557. https://doi.org/10.1128/JB.184.9.2557-2560.2002

Bowen, H., Hanna, P., Jude, E. M., Michel, C., Stephanie, Y., & Steven, M. (2018). Spatial patterns of microbial denitrification genes change in response to poultry litter placement and cover crop species in an agricultural soil. *Biology and fertility of soils*, *54*(6), 769-781. https://doi.org/10.1007/s00374-018-1301-x

Bru, D., Ramette, A., Saby, N. P. A., Dequiedt, S., Ranjard, L., Jolivet, C., Arrouays, D., & Philippot, L. (2010). Determinants of the distribution of nitrogen-cycling microbial communities at the landscape scale. *The ISME Journal*, *5*(3), 532. https://doi.org/10.1038/ismej.2010.130

Burgmann, H., Widmer, F., Von Sigler, W., & Zeyer, J. (2004). New molecular screening tools for analysis of free-living diazotrophs in soil. *Applied and Environmental Microbiology*, *70*(1), 240-247. https://doi.org/10.1128/aem.70.1.240-247.2004

Cameron, K. C., Di, H. J., & Moir, J. L. (2013). Nitrogen losses from the soil/plant system: a review. *Annals of Applied Biology*, *162*(2), 145-173. https://doi.org/10.1111/aab.12014

Carlson, C. A., & Ingraham, J. L. (1983). Comparison of denitrification by *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, and *Paracoccus denitrificans*. *Applied and Environmental Microbiology*, *45*(4), 1247.

Carter, M. R. (1992). Influence of reduced tillage systems on organic matter, microbial biomass, macro-aggregate distribution and structural stability of the surface soil in a humid climate. *Soil and Tillage Research*, *23*(4), 361-372. https://doi.org/10.1016/0167-1987(92)90081-L

Chan, Y. K., McCormick, W. A., & Watson, R. J. (1997). A new *nos* gene downstream from *nosDFY* is essential for dissimilatory reduction of nitrous oxide by *Rhizobium* (*Sinorhizobium*) *meliloti*. *Microbiology*, *143*, 2817-2824. https://doi.org/10.1099/00221287-143-8-2817

Che, R., Qin, J., Tahmasbian, I., Wang, F., Zhou, S., Xu, Z., & Cui, X. (2018). Litter amendment rather than phosphorus can dramatically change inorganic nitrogen pools in a degraded grassland soil by affecting nitrogen-cycling microbes. *Soil Biology and Biochemistry*, *120*, 145-152. https://doi.org/10.1016/j.soilbio.2018.02.006

Colliver, B. B., & Stephenson, T. (2000). Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. *Biotechnology Advances*, *18*(3), 219-232. https://doi.org/10.1016/S0734-9750(00)00035-5

Coyne, M. S., Arunakumari, A., Averill, B. A., & Tiedje, J. M. (1989). Immunological identification and distribution of dissimilatory heme cd_1 and nonheme copper nitrite reductases in denitrifying bacteria. *Applied and Environmental Microbiology*, *55*(11), 2924-2931. https://www.ncbi.nlm.nih.gov/pubmed/2624465

Daims, H., Lebedeva, E. V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R. H., von Bergen, M., Rattei, T., Bendinger, B., Nielsen, P. H., & Wagner, M. (2015). Complete nitrification by *Nitrospira* bacteria. *Nature*, *528*(7583), 504-509. https://doi.org/10.1038/nature16461

Di, H. J., Cameron, K. C., Shen, J. P., Winefield, C. S., O'Callaghan, M., Bowatte, S., & He, J. Z. (2009). Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature Geoscience*, *2*(9), 621-624. https://doi.org/10.1038/ngeo613

Dixon, R., & Kahn, D. (2004). Genetic regulation of biological nitrogen fixation. *Nature Reviews: Microbiology*, *2*(8), 621-631. https://doi.org/10.1038/nrmicro954

Domeignoz-Horta, L. A., Spor, A., Bru, D., Breuil, M. C., Bizouard, F., Leonard, J., & Philippot, L. (2015). The diversity of the N_2O reducers matters for the N_2O:N_2 denitrification end-product ratio across an annual and a perennial cropping system. *Frontiers in Microbiology*, *6*, 971. https://doi.org/10.3389/fmicb.2015.00971

Estavillo, J., Rodriguez, M., Domingo, M., Muñoz-Rueda, A., & Gonzalez-Murua, C.

(1994). Denitrification losses from a natural grassland in the Basque Country under organic and inorganic fertilization. *Plant and Soil*, *162*(1), 19-29. https://doi.org/10.1007/BF01416086

Fernandez, A. L., Sheaffer, C. C., Wyse, D. L., Staley, C., Gould, T. J., & Sadowsky, M. J. (2016). Associations between soil bacterial community structure and nutrient cycling functions in long-term organic farm soils following cover crop and organic fertilizer amendment. *Science of the Total Environment*, *566-567*, 949-959. https://doi.org/10.1016/j.scitotenv.2016.05.073

Frijlink, M., Abee, T., Laanbroek, H., Boer, W., & Konings, W. (1992). The bioenergetics of ammonia and hydroxylamine oxidation in *Nitrosomonas europaea* at acid and alkaline pH. *Archives of Microbiology*, *157*(2), 194-199. https://doi.org/10.1007/BF00245290

Grave, R. A., Nicoloso, R. d. S., Cassol, P. C., da Silva, M. L. B., Mezzari, M. P., Aita, C., & Wuaden, C. R. (2018). Determining the effects of tillage and nitrogen sources on soil N_2O emission. *Soil and Tillage Research*, *175*, 1-12. https://doi.org/10.1016/j.still.2017.08.011

Hallin, S., Jones, C. M., Schloter, M., & Philippot, L. (2009). Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. *The ISME Journal*, *3*(5), 597-605. https://doi.org/10.1038/ismej.2008.128

Hartmann, A. A., Barnard, R. L., Marhan, S., & Niklaus, P. A. (2013). Effects of drought and N-fertilization on N cycling in two grassland soils. *Oecologia*, *171*(3), 705-717. https://doi.org/10.1007/s00442-012-2578-3

Hauggaard-Nielsen, H., Lachouani, P., Knudsen, M. T., Ambus, P., Boelt, B., & Gislum, R. (2016). Productivity and carbon footprint of perennial grass-forage legume intercropping strategies with high or low nitrogen fertilizer input. *Science of the Total Environment*, *541*, 1339-1347. https://doi.org/10.1016/j.scitotenv.2015.10.013

Helen, D., Kim, H., Tytgat, B., & Anne, W. (2016). Highly diverse *nirK* genes comprise two major clades that harbour ammonium-producing denitrifiers. *BMC Genomics*, *17*, 155. https://doi.org/10.1186/s12864-016-2465-0

Heylen, K., Gevers, D., Vanparys, B., Wittebolle, L., Geets, J., Boon, N., & De Vos, P. (2006). The incidence of *nirS* and *nirK* and their genetic heterogeneity in cultivated denitrifiers. *Environmental Microbiology*, *8*(11), 2012-2021. https://doi.org/10.1111/j.1462-2920.2006.01081.x

Jones, C. M., Graf, D. R., Bru, D., Philippot, L., & Hallin, S. (2013). The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *The ISME Journal*, *7*(2), 417-426. https://doi.org/10.1038/ismej.2012.125

Juhanson, J., Hallin, S., Söderström, M., Stenberg, M., & Jones, C. M. (2017). Spatial and phyloecological analyses of *nosZ* genes underscore niche differentiation amongst terrestrial N_2O reducing communities. *Soil Biology and Biochemistry*, *115*, 82-91. https://doi.org/10.1016/j.soilbio.2017.08.013

Kandeler, E., Brune, T., Enowashu, E., Dörr, N., Guggenberger, G., Lamersdorf, N., &

Philippot, L. (2009). Response of total and nitrate-dissimilating bacteria to reduced N deposition in a spruce forest soil profile. *FEMS Microbiology Ecology*, *67*(3), 444-454. https://doi.org/10.1111/j.1574-6941.2008.00632.x

Kastl, E.-M., Schloter-Hai, B., Buegger, F., & Schloter, M. (2014). Impact of fertilization on the abundance of nitrifiers and denitrifiers at the root–soil interface of plants with different uptake strategies for nitrogen. *Biology and Fertility of Soils*, *51*(1), 57-64. https://doi.org/10.1007/s00374-014-0948-1

Kladivko, E. J. (2001). Tillage systems and soil ecology. *Soil and Tillage Research*, *61*(1), 61-76. https://doi.org/10.1016/S0167-1987(01)00179-9

Koike, I., & Hattori, A. (1975). Energy yield of denitrification: an estimate from growth yield in continuous cultures of *Pseudomonas denitrificans* under nitrate-, nitrite- and oxide-limited conditions. *Journal of general microbiology*, *88*(1), 11. https://doi.org/10.1099/00221287-88-1-11

Kowalchuk, G. A., & Stephen, J. R. (2001). Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annual review of microbiology*, *55*, 485.

Kowalchuk, G. A., Stienstra, A. W., Heilig, G. H., Stephen, J. R., & Woldendorp, J. W. (2000). Molecular analysis of ammonia-oxidising bacteria in soil of successional grasslands of the Drentsche A (The Netherlands). *FEMS Microbiology Ecology*, *31*(3), 207-215. https://www.ncbi.nlm.nih.gov/pubmed/10719201

LeBauer, D. S., & Treseder, K. K. (2008). Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology*, *89*(2), 371-379. https://doi.org/Doi 10.1890/06-2057.1

Levy-Booth, D. J., Prescott, C. E., & Grayston, S. J. (2014). Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. *Soil Biology and Biochemistry*, *75*, 11-25. https://doi.org/10.1016/j.soilbio.2014.03.021

Li, S., Jiang, X., Wang, X., & Wright, A. L. (2015). Tillage effects on soil nitrification and the dynamic changes in nitrifying microorganisms in a subtropical rice-based ecosystem: A long-term field study. *Soil and Tillage Research*, *150*, 132-138. https://doi.org/10.1016/j.still.2015.02.005

Liu, S., Coyne, M. S., & Grove, J. H. (2017). Long-term tillage and nitrogen fertilization: consequences for nitrifier density and activity. *Applied Soil Ecology*, *120*, 121-127. https://doi.org/10.1016/j.apsoil.2017.07.034

Liu, S., Coyne, M. S., Grove, J. H., & Flythe, M. D. (2018). Nitrogen, season, and tillage management influence ammonia oxidizing bacterial communities in long-term maize. *Applied Soil Ecology*, *129*, 98-106. https://doi.org/10.1016/j.apsoil.2018.05.002

Lupwayi, N. Z., Arshad, M. A., Rice, W. A., & Clayton, G. W. (2001). Bacterial diversity in water-stable aggregates of soils under conventional and zero tillage management. *Applied Soil Ecology*, *16*(3), 251-261. https://doi.org/Doi 10.1016/S0929-1393(00)00123-2

Magomedov, N. R., Khalilov, M. I., & Bedoeva, S. V. (2017). Resource-serving soil-management practice for winter wheat in the plane regions of Dagestan. *Russian Agricultural Sciences*, *43*(2), 167-169.

https://doi.org/10.3103/s1068367417020124
Mbuthia, L. W., Acosta-Martínez, V., DeBruyn, J., Schaeffer, S., Tyler, D., Odoi, E., Mpheshea, M., Walker, F., & Eash, N. (2015). Long term tillage, cover crop, and fertilization effects on microbial community structure, activity: implications for soil quality. *Soil Biology and Biochemistry*, *89*, 24-34. https://doi.org/10.1016/j.soilbio.2015.06.016
McCormick, I. (2013). Long-term impacts of tillage, crop rotation and cover crop systems on soil bacteria, archaea and their respective ammonia oxidizing communities in an Ontario agricultural soil. In K. Dunfield (Ed.).
Murugapiran, S. K., Huntemann, M., Wei, C. L., Han, J., Detter, J. C., Han, C., Erkkila, T. H., Teshima, H., Chen, A., Kyrpides, N., Mavrommatis, K., Markowitz, V., Szeto, E., Ivanova, N., Pagani, I., Pati, A., Goodwin, L., Peters, L., Pitluck, S., Lam, J., McDonald, A. I., Dodsworth, J. A., Woyke, T., & Hedlund, B. P. (2013). Thermus oshimai JL-2 and T. thermophilus JL-18 genome analysis illuminates pathways for carbon, nitrogen, and sulfur cycling. *Standards in Genomic Sciences*, *7*(3), 449-468. https://doi.org/10.4056/sigs.3667269
Nivelle, E., Verzeaux, J., Habbib, H., Kuzyakov, Y., Decocq, G., Roger, D., Lacoux, J., Duclercq, J., Spicher, F., Nava-Saucedo, J.-E., Catterou, M., Dubois, F., & Tetu, T. (2016). Functional response of soil microbial communities to tillage, cover crops and nitrogen fertilization. *Applied Soil Ecology*, *108*, 147-155. https://doi.org/10.1016/j.apsoil.2016.08.004
Norton, J. M., Alzerreca, J. J., Suwa, Y., & Klotz, M. G. (2002). Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. *Archives of Microbiology*, *177*(2), 139-149. https://doi.org/10.1007/s00203-001-0369-z
Ollivier, J., Towe, S., Bannert, A., Hai, B., Kastl, E. M., Meyer, A., Su, M. X., Kleineidam, K., & Schloter, M. (2011). Nitrogen turnover in soil and global change. *FEMS Microbiology Ecology*, *78*(1), 3-16. https://doi.org/10.1111/j.1574-6941.2011.01165.x
Orr, C. H., James, A., Leifert, C., Cooper, J. M., & Cummings, S. P. (2011). Diversity and activity of free-living nitrogen-fixing bacteria and total bacteria in organic and conventionally managed soils. *Applied and Environmental Microbiology*, *77*(3), 911-919. https://doi.org/10.1128/AEM.01250-10
Ouyang, Y., Evans, S. E., Friesen, M. L., & Tiemann, L. K. (2018). Effect of nitrogen fertilization on the abundance of nitrogen cycling genes in agricultural soils: a meta-analysis of field studies. *Soil Biology and Biochemistry*, *127*, 71-78. https://doi.org/10.1016/j.soilbio.2018.08.024
Ouyang, Y., Norton, J. M., Stark, J. M., Reeve, J. R., & Habteselassie, M. Y. (2016). Ammonia-oxidizing bacteria are more responsive than archaea to nitrogen source in an agricultural soil. *Soil Biology and Biochemistry*, *96*, 4-15. https://doi.org/10.1016/j.soilbio.2016.01.012
Peoples, M., Herridge, D., & Ladha, J. (1995). Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? *Plant and Soil*, *174*(1), 3-28. https://doi.org/10.1007/BF00032239
Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M. C., Koops, H.-P., &

Wagner, M. (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. *Applied and Environmental Microbiology*, *66*, 5368-5382.

Ritchie, G. A., & Nicholas, D. J. (1972). Identification of the sources of nitrous oxide produced by oxidative and reductive processes in Nitrosomonas europaea. *The Biochemical journal*, *126*(5), 1181. https://doi.org/10.1042/bj1261181

Rotthauwe, J. H., Witzel, K. P., & Liesack, W. (1997). The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology*, *63*(12), 4704.

Rudisill, M. A., Turco, R. F., & Hoagland, L. A. (2016). Fertility practices and rhizosphere effects alter ammonia oxidizer community structure and potential nitrification activity in pepper production soils. *Applied Soil Ecology*, *99*, 70-77. https://doi.org/10.1016/j.apsoil.2015.10.011

Samad, M. S., Biswas, A., Bakken, L. R., Clough, T. J., de Klein, C. A., Richards, K. G., Lanigan, G. J., & Morales, S. E. (2016). Phylogenetic and functional potential links pH and N_2O emissions in pasture soils. *Scientific Reports*, *6*, 35990. https://doi.org/10.1038/srep35990

Sayavedra‐Soto, L. A., Hommes, N. G., Alzerreca, J. J., Arp, D. J., Norton, J. M., & Klotz, M. G. (1998). Transcription of the *amoC*, *amoA* and *amoB* genes in *Nitrosomonas europaea* and *Nitrosospira* sp. NpAV. *FEMS Microbiology Letters*, *167*(1), 81-88. https://doi.org/10.1111/j.1574-6968.1998.tb13211.x

Schmidt, I., van Spanning, R. J. M., & Jetten, M. (2004). Denitrification and ammonia oxidation by *Nitrosomonas europaea* wild-type and NirK- and NorB-deficient mutants. *Microbiology*, *150*, 4107-4114.

Segal, L. M., Miller, D. N., McGhee, R. P., Loecke, T. D., Cook, K. L., Shapiro, C. A., & Drijber, R. A. (2017). Bacterial and archaeal ammonia oxidizers respond differently to long-term tillage and fertilizer management at a continuous maize site. *Soil and Tillage Research*, *168*, 110-117. https://doi.org/10.1016/j.still.2016.12.014

Sharon, A. B. (2008). Biogeochemistry: Nitrous oxide in flux. *Nature*, *456*(7224), 888. https://doi.org/10.1038/456888a

Shaw, L. J., Nicol, G. W., Smith, Z., Fear, J., Prosser, J. I., & Baggs, E. M. (2006). *Nitrosospira* spp. can produce nitrous oxide via a nitrifier denitrification pathway. *Environmental Microbiology*, *8*(2), 214-222. https://doi.org/10.1111/j.1462-2920.2005.00882.x

Shen, X.-Y., Zhang, L.-M., Shen, J.-P., Li, L.-H., Yuan, C.-L., & He, J.-Z. (2011). Nitrogen loading levels affect abundance and composition of soil ammonia oxidizing prokaryotes in semiarid temperate grassland. *Journal of Soils and Sediments*, *11*(7), 1243-1252. https://doi.org/10.1007/s11368-011-0375-y

Smith, J., Wagner-Riddle, C., & Dunfield, K. (2010). Season and management related changes in the diversity of nitrifying and denitrifying bacteria over winter and spring. *Applied Soil Ecology*, *44*(2), 138-146.

https://doi.org/10.1016/j.apsoil.2009.11.004
Soares, J. R., Cassman, N. A., Kielak, A. M., Pijl, A., Carmo, J. B., Lourenco, K. S., Laanbroek, H. J., Cantarella, H., & Kuramae, E. E. (2016). Nitrous oxide emission related to ammonia-oxidizing bacteria and mitigation options from N fertilization in a tropical soil. *Scientific Reports*, *6*, 30349. https://doi.org/10.1038/srep30349
Tavares, P., Pereira, A. S., Moura, J. J. G., & Moura, I. (2006). Metalloenzymes of the denitrification pathway. *Journal of Inorganic Biochemistry*, *100*(12), 2087-2100. https://doi.org/10.1016/j.jinorgbio.2006.09.003
Tsiknia, M., Paranychianakis, N. V., Varouchakis, E. A., Nikolaidis, N. P., & Sessitsch, A. (2015). Environmental drivers of the distribution of nitrogen functional genes at a watershed scale. *FEMS Microbiology Ecology*, *91*(6). https://doi.org/10.1093/femsec/fiv052
van Kessel, M. A., Speth, D. R., Albertsen, M., Nielsen, P. H., Op den Camp, H. J., Kartal, B., Jetten, M. S., & Lucker, S. (2015). Complete nitrification by a single microorganism. *Nature*, *528*(7583), 555-559. https://doi.org/10.1038/nature16459
Verzeaux, J., Alahmad, A., Habbib, H., Nivelle, E., Roger, D., Lacoux, J., Decocq, G., Hirel, B., Catterou, M., Spicher, F., Dubois, F., Duclercq, J., & Tetu, T. (2016). Cover crops prevent the deleterious effect of nitrogen fertilisation on bacterial diversity by maintaining the carbon content of ploughed soil. *Geoderma*, *281*, 49-57. https://doi.org/10.1016/j.geoderma.2016.06.035
Wallenstein, M. D., Myrold, D. D., Firestone, M., & Voytek, M. (2006). Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. *Ecological Applications*, *16*(6), 2143-2152. https://www.ncbi.nlm.nih.gov/pubmed/17205893
Wang, J., Chadwick, D. R., Cheng, Y., & Yan, X. (2018). Global analysis of agricultural soil denitrification in response to fertilizer nitrogen. *Science of the Total Environment*, *616-617*, 908-917. https://doi.org/10.1016/j.scitotenv.2017.10.229
Webster, G., Embley, T. M., & Prosser, J. I. (2002). Grassland management regimens reduce small-scale heterogeneity and species diversity of beta-Proteobacterial ammonia oxidizer populations. *Applied and Environmental Microbiology*, *68*(1), 20. https://doi.org/10.1128/AEM.68.1.20-30.2002
White, P. M., & Rice, C. W. (2009). Tillage effects on microbial and carbon dynamics during plant residue decomposition. *Soil Science Society of America Journal*, *73*(1), 138. https://doi.org/10.2136/sssaj2007.0384
Xiang, X., He, D., He, J.-S., Myrold, D. D., & Chu, H. (2017). Ammonia-oxidizing bacteria rather than archaea respond to short-term urea amendment in an alpine grassland. *Soil Biology and Biochemistry*, *107*, 218-225. https://doi.org/10.1016/j.soilbio.2017.01.012
Xu, S., Wang, B., Li, Y., Jiang, D., Zhou, Y., Ding, A., Zong, Y., Ling, X., Zhang, S., & Lu, H. (2020). Ubiquity, diversity, and activity of comammox Nitrospira in agricultural soils. *Science of the Total Environment*, *706*, 135684. https://doi.org/10.1016/j.scitotenv.2019.135684
Yang, Y., Zhao, J., Jiang, Y., Hu, Y., Zhang, M., & Zeng, Z. (2017). Response of bacteria

harboring *nirS* and *nirK* genes to different N fertilization rates in an alkaline northern Chinese soil. *European Journal of Soil Biology*, *82*, 1-9. https://doi.org/10.1016/j.ejsobi.2017.05.006

Ying, J., Li, X., Wang, N., Lan, Z., He, J., & Bai, Y. (2017). Contrasting effects of nitrogen forms and soil pH on ammonia oxidizing microorganisms and their responses to long-term nitrogen fertilization in a typical steppe ecosystem. *Soil Biology and Biochemistry*, *107*, 10-18. https://doi.org/10.1016/j.soilbio.2016.12.023

Yoon, S., Nissen, S., Park, D., Sanford, R. A., & Loffler, F. E. (2016). Nitrous oxide reduction kinetics distinguish bacteria harboring clade I *nosZ* from those harboring clade II *nosZ*. *Applied and Environmental Microbiology*, *82*(13), 3793-3800. https://doi.org/10.1128/AEM.00409-16

CHAPTER I

SOIL HEALTH MANAGEMENT ENHANCES MICROBIAL NITROGEN CYCLING CAPACITY AND ACTIVITY IN A LONG-TERM CONTINUOUS COTTON FIELD IN WEST TENNESSEE

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Abstract

Soil microbial transformations of nitrogen (N) can be affected by soil health management practices. Here, we report *in situ* seasonal dynamics of the population size (gene copy abundances) and functional activity (transcript copy abundances) of five bacterial genes involved in soil N cycling (ammonia-oxidizing bacteria [AOB] *amoA*, *nifH*, *nirK*, *nirS*, and *nosZ*) in a long-term continuous cotton production system under different management practices (cover crops, tillage, and inorganic N fertilization). Hairy vetch (*Vicia villosa* Roth), a leguminous cover crop, most effectively promoted the expression of N-cycle genes, which persisted after cover crop termination throughout the growing season. Moreover, we observed similarly high or even higher N cycle gene transcript abundances under vetch with no fertilizer as no cover crop with N fertilization throughout the cover crop peak and cotton growing seasons (April, May, and October). Further, both the gene and transcript abundances of *amoA* and *nosZ* were positively correlated to soil nitrous oxide (N_2O) emissions. We also found that the abundances of *amoA* genes and transcripts both positively correlated to field and incubated net nitrification rates. Together, our results revealed relationships between microbial functional capacity and activity and *in situ* soil N transformations under different agricultural seasons and soil management practices.

Introduction

Nitrogen (N) is a major nutrient for plant growth, and its transformation and availability in soil is a key factor in terrestrial ecosystem productivity (LeBauer & Treseder,

2008). Nitrogen fertilizer has been widely used to improve crop yields in agroecosystems, but losses of excess N result in the eutrophication of waterbodies and exacerbate atmospheric concentrations of the greenhouse gas nitrous oxide (N_2O) (Galloway et al., 2008). Conservation agriculture and soil health practices, such as growing cover crops, reducing N fertilization, and minimizing tillage, ensure mineral nutrients can be properly used by crops and do not become surplus or deficient (White et al., 2012).

The C-to-N ratios of plant residues can drive the net balance of soil mineral N by affecting microbial mineralization and immobilization of N (Chapin et al., 2002). Cover crops, in addition to the main crop, can take up soil nitrate (NO_3^-) to reduce N losses via leaching and denitrification (Parkin & Kaspar, 2006). Further, leguminous cover crops can supplement crop N requirements through biological N fixation, thereby reducing exogenous N fertilizer requirements and associated N losses (Carsky et al., 2001). The effects of tillage intensity on N losses through N_2O emissions are cropping system-specific but reduced or no tillage generally mitigates N_2O emissions compared to conventional tillage, especially in long-term (> 10 years) production systems (Feng et al., 2018; van Kessel et al., 2013).

Conservation agriculture practices can change soil properties which are important factors influencing microbial functional groups involved in soil N cycling (Forbes et al., 2009). For example, increased soil organic matter (SOM) and soil water content (SWC) under reduced tillage and winter cover cropping can create nutrient-rich anaerobic microsites that favor microbial denitrification (Cambardella et al., 2012; Linn & Doran, 1984). N limitation caused by reduced N fertilization may decrease the population size of ammonia-oxidizing bacteria (AOB) (Yang et al., 2020) but promote the activity of diazotrophs for N-fixation (Bahulikar et al., 2020).

The abundance and expression of genes encoding key enzymes involved in soil N transformations have been widely used to evaluate the abundances and activity of microbial populations involved in specific N cycling processes (Wang et al., 2018). For example, different microbial assemblages are responsible for N fixation, nitrification, and denitrification (Kuypers et al., 2018). Biological N fixation is the reduction of atmospheric N_2 to biologically available ammonium. Molecular analysis of N-fixing bacteria often

targets *nifH* genes encoding a subunit of nitrogenase reductase which catalyzes the reduction of dinitrogen to ammonia (Akter et al., 2014; Gaby & Buckley, 2014; Orr et al., 2011). Nitrification is the process of converting ammonia to nitrate, and is one of the major processes contributing to N_2O production (Khalil et al., 2004; Liu et al., 2016; Shen et al., 2019; White & Lehnert, 2016) and nitrate leaching (Bárta et al., 2017) in soils. The first and rate-limiting step of nitrification is microbial oxidation of ammonia to hydroxylamine catalyzed by ammonia monooxygenases (AMO). Thus, molecular analysis of ammonia-oxidizing bacteria (AOB) and archaea (AOA) commonly targets *amoA* genes encoding subunit A of AMO (Norton et al., 1996; Ward & Bouskill, 2011). Although AOA may be more numerically abundant than AOB in soil (Leininger et al., 2006), AOB have been shown to be generally more responsive than AOA to soil management practices, such as N fertilization (Ouyang et al., 2017; Ouyang et al., 2016; Ying et al., 2017) and tillage (Segal et al., 2017), and were shown to be functionally dominant for ammonia oxidation in agricultural soils (Jia & Conrad, 2009). Denitrification is the step-wise reduction of nitrate to nitrite, nitric oxide, nitrous oxide, and dinitrogen gas under anaerobic conditions, and is catalyzed by nitrate-, nitrite-, nitric oxide-, and nitrous oxide reductases, respectively (Colliver & Stephenson, 2000). Reduction of nitrite to nitric oxide is catalyzed by two structurally different nitrite reductases (NIR) carried by different taxa: 1) Cu-containing NIR (CuNIR) encoded by a single gene *nirK*, sometimes accompanied by gene *nirV*, and 2) cytochrome cd_1 NIR (cdNIR) encoded by at least ten genes (*nirSECFDLGHJN*) with *nirS* gene encoding the functional subunits of cdNIR (Sanchez & Minamisawa, 2018). The reduction of N_2O to N_2 gas is catalyzed by nitrous oxide reductase (NOS), which is frequently studied by using the marker gene *nosZ* that encodes the multi-Cu NOS catalytic subunit (Chan et al., 1997; Levy-Booth et al., 2014). The variation and the ratio between *amoA*, *nirK*, *nirS* that are involved in N_2O production and *nosZ* that is involved in N_2O reduction has been used to predict system-level N_2O emissions (Pereira et al., 2015; Theodorakopoulos et al., 2017).

The reported effects of conservation soil management practices on important functional groups involved in N cycling are inconsistent. For example, many studies indicate that reduced or no tillage can increase the abundance of both AOA and AOB *amoA*

(Li et al., 2015), *nirK* (Tatti et al., 2015), *nosZ* (Badagliacca et al., 2018; Tatti et al., 2015), and (*nirK* + *nirS*)/*nosZ* ratios (Wang & Zou, 2020). Other studies, however, reported positive effect of no tillage on AOB *amoA* (Segal et al., 2017) but did not find significant effects of tillage on the abundances of AOA *amoA* (Segal et al., 2017), *nirK* (Maul et al., 2019) or *nirS* genes (Tatti et al., 2015). Cover cropping may also affect some populations: *nirK* gene abundances were higher under cereal rye compared to hairy vetch while *nirS* gene abundances were unaffected by cover crop species (Bowen et al., 2018). Nitrogen input can suppress *nifH* abundance when the available N is excessive (Cusack et al., 2009; C. Wang et al., 2017), but it can also improve abundances when available N is limited (Lindsay et al., 2010). These inconsistent and sometime contradictory results may be due to a lack of temporal resolution. For example, field trials may not have been conducted long enough to invoke a significant change in the soil system. Moreover, there is seasonal variability in microbial functional dynamics driving N transformations that are not captured if only a single time point sample is taken. Another critical gap in our current understanding of functional group dynamics is the relationship between functional capacity and activity: Many studies have only focused on N-cycling functional groups at either the genetic level (i.e., using extracted DNA) which misses changes in activity or expression, or at the transcriptional level (i.e., using extracted RNA) which misses changes in population abundances.

The objectives of our study were to: 1) Determine the effects of tillage, cover cropping, and N fertilization on abundance and activity of N-cycling microbes; 2) Investigate the seasonal dynamics of abundance and activity of N-cycling functional groups under long-term conservation soil management; and 3) Identify relationships among the N-cycle functional gene abundances, transcripts, microbial-controlled N transformation rates, and soil physicochemical parameters. The study was conducted at a long-term (36 years) continuous cotton experiment with different tillage, cover cropping and N fertilization regimes. To examine the dynamics of soil N-cycling functional groups, we used quantitative polymerase chain reaction (qPCR) and real-time quantitative reverse-transcription PCR (qRT-PCR) to target the N-cycle genes and transcripts of *nifH*, AOB *amoA*, *nirK*, *nirS*, and *nosZ*. We then explored the seasonal dynamics of soil N cycling

populations under these different conservation agricultural management practices, and their relationships to soil N pools and fluxes.

Materials and Methods

Study site, experimental design, and sampling

This study was conducted at a long-term continuous cotton conservation agriculture field experiment, established 1981, at the University of Tennessee West Tennessee Research and Education Center (WTREC) in Jackson, Tennessee. Soils at this site are classified as well-drained Lexington silt loams (fine-silty, mixed, thermic, Ultic Hapludalfs) with a 0-2% slope. The main crop was rainfed cotton (*Gossypium hirsutum* L.). The field study implemented a randomized complete block design with split-split plot treatment arrangements in four replicates such that N fertilization rate was the main plot treatment, cover crop was the sub-plot treatment, and tillage was the sub-sub-plot treatment; a subset of 12 of the 32 plots were used here (Appendix Figure 9). The 12 cropping system management combinations included 2 types of tillage [conventional tillage (CT), no tillage (NT)]; 3 types of winter cover crops [hairy vetch (*Vicia villosa* Roth; V), winter wheat (*Triticum aestivum* L.; W), no cover crop (NC)]; and 2 levels of NH_4NO_3 fertilization rates [0, 67 kg N ha^{-1} (0N, 67N, respectively)] (Table 1). For tilled plots, tillage was done twice before planting by a standard disc harrow followed by smoothing and breaking up of clods by a triple-K harrow. Field operations for these plots, including cover crop termination, cotton planting, and phosphorus and potassium fertilizer application are described in more detail in previous studies of this site (Mbuthia et al., 2015; Nouri et al., 2019).

Subsamples (2.5 cm diameter by 7.5 cm long cores) of soil were collected from ten random locations in each experimental sub-sub-plot (12 cropping systems × 4 replicates = 48 experimental plots), composited, sieved through a 2 mm mesh, then refrigerated until analysis. Collections were obtained four times corresponding to different stages of crop growth in 2017, including cover crop harvest (April, immediately following cover crop harvest), spring crop transition (May, after cotton planting and before fertilizer application), cotton crop peak (October, before cotton harvest), and fall crop transition (November, after

Table 1. Summary of 12 cropping system treatments used in this study.

Cropping system	Tillage	Winter cover crop	N fertilization
NT-NC-0N	No tillage (NT)	No cover (NC)	No fertilizer (0N)
NT-NC-67N			67 kg N ha^{-1} (67N)
NT-V-0N		Hairy Vetch (V)	No fertilizer (0N)
NT-V-67N			67 kg N ha^{-1} (67N)
NT-W-0N		Winter Wheat (W)	No fertilizer (0N)
NT-W-67N			67 kg N ha^{-1} (67N)
CT-NC-0N	Conventional tillage (CT)	No cover (NC)	No fertilizer (0N)
CT-NC-67N			67 kg N ha^{-1} (67N)
CT-V-0N		Hairy Vetch (V)	No fertilizer (0N)
CT-V-67N			67 kg N ha^{-1} (67N)
CT-W-0N		Winter Wheat (W)	No fertilizer (0N)
CT-W-67N			67 kg N ha^{-1} (67N)

cotton harvest and before winter cover crop planting). Soil properties measured included N_2O flux (N_2O-N), soil water content (SWC), soil pH, NO_3^--N, NH_4^+-N, total extractable N (TEN), total extractable C (TEC), total soil C (TC), total soil N (TN), soil ratio of C to N (C:N ratio), microbial biomass N (MBN), microbial biomass C (MBC), field net nitrification rate (field nitrification), field net N mineralization rate (field N mineralization), incubated net nitrification rate (incubated nitrification), and incubated net N mineralization rate (incubated N mineralization). Detailed methods on the measurement of these soil properties are provided in Appendix method.

Soil DNA and RNA extraction

Soil genomic DNA was extracted from 0.25 g of soil using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentrations were quantified using NanoDrop One spectrophotometry (NanoDrop Technologies, Wilmington, DE). DNA was stored at -20℃.

Soil RNA was extracted using the RNeasy PowerSoil Total RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was eluted in RNase-free water and stored at -80℃. RNA quality was checked by 1% agarose gel electrophoresis and NanoDrop One spectrophotometry (NanoDrop Technologies, Wilmington, DE). Samples were checked for DNA contamination by attempting to amplify the N-cycle functional genes by PCR and subsequent agarose gel electrophoresis. Only samples that gave a negative electrophoresis result (i.e., no DNA contamination) were used for reverse transcription. cDNA was produced from RNA using SuperScript IV Reverse Transcriptase (Invitrogen, Paisley, UK) according to the manufacturer's protocol. Random hexamer primers (Invitrogen) were used at a final concentration of 2.5 μM per reaction. cDNA samples were diluted to a final concentration of 15 ng μl^{-1} and stored at -20℃.

Molecular cloning

Genes *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* were amplified from extracted soil DNA with previously designed primer pairs and reaction conditions (Appendix Table 3). PCR products were purified with QIAquick® PCR Purification Kit (Qiagen), ligated into

pCR™4-TOPO® vectors (Invitrogen), and transformed into Transform One Shot® TOP10 Competent Cells (Invitrogen) following the manufacturer's protocol. After shaking at 225 rpm at 37°C for 1 h, cells were spread on LB solid medium containing 50 μg mL^{-1} ampicillin and cultured overnight. Positive clones were grown in liquid LB media amended with ampicillin media overnight and plasmid DNA was extracted using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer's instructions. Plasmid DNA concentrations were determined on a Hoefer DQ 200 fluorometer (Hoefer Inc, San Francisco, CA), which uses a Hoechst dye specific for double stranded DNA that compares DNA fluorescence with a known standard, and then sequenced with primers M13 Forward (-20)/M13 Reverse to confirm the correct target gene was cloned. The copy numbers of *nifH*, *amoA*, *nirK*, *nirS* and *nosZ* genes were calculated using the concentration of the extracted plasmid DNA, size (base pairs) of the plasmids containing cloned products and the molecular weight of DNA. Ten-fold serial dilutions of the plasmid DNA (10^2 to 10^8 copies μl^{-1}) were used during qPCR assays to generate standard curves for quantifying N-cycle genes and transcripts in soil samples.

Quantitative analysis of N-cycle genes and gene transcripts

For both DNA and cDNA samples, qPCR of *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* were performed on a CFX96 Optical Real-Time Detection System (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The 20-μl qPCR reaction mixture contained 10 μl Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific, USA), 1 μl PCR forward and reverse primer (both 10 μM), 2.5 μl DNA or cDNA template, and 5.5 μl nuclease-free water. Primer sets and reaction parameters used are listed in Appendix Table 3. Following the reactions, a melt curve analysis was performed to confirm the specificity of the PCR product for each real-time PCR amplification. Quantitative PCR of 16S rRNA genes and 16S rRNA was done using Femto™ Bacterial DNA Quantification Kit (Zymo Research Corp., CA, USA) following the manufacturer's protocols.

Both absolute abundances (copies per gram dry weight soil) and relative abundances (normalized to 16S rRNA genes or to 16S rRNA) of functional genes and transcripts were analyzed and compared to ensure the reliability of the result. Absolute abundance helped

better understanding the variation of N-cycling bacteria with seasons and management practices while relative abundance mitigated the impact of DNA and RNA extraction efficiency on data accuracy and revealed the relative changes of N-cycling population within the total soil bacteria. The relative abundance of N-cycle genes and transcripts used in this study were calculated as follows:

$$\text{NGA} = \frac{\text{N-cycle gene copies/µl DNA extract}}{\text{16S rRNA gene copies/ µl DNA extract}}$$

$$\text{NTA} = \frac{\text{N-cycle transcript copies/µl RNA extract}}{\text{16S rRNA copies/ µl RNA extract}}$$

Where: NGA is the relative abundance of the gene; NTA is the relative abundance of the gene transcript.

The transcript-to-gene copy number ratio (transcript:gene ratio) for each gene was also calculated to provide a means to determine whether transcript variation was due to changes in population sizes (i.e., genes) or changes in expression (i.e., transcripts) (Cusick et al., 2015) and was calculated as follows:

$$\text{Transcript:gene ratio} = \frac{\text{transcript copies/g dry weight soil}}{\text{gene copies/g dry weight soil}}$$

Statistical analysis

Statistical analyses to test the effects of agricultural season and management practices on the abundance and expression of N-cycle genes were performed in SAS 9.4 (SAS Inst., Cary, NC, USA). The relative and absolute abundances of functional genes and transcripts as well as their transcript:gene ratio were both log transformed to achieve normal distributions of residuals. These log-transformed data were analyzed using a repeated measure mixed model analysis of variance (ANOVA) within the GLIMMIX procedure. The fixed effects included season, tillage system, cover crop, and N fertilizer rate, as well as all interactions. Random effects included the block, block by nitrogen within cover crop, and the block by tillage by nitrogen within cover crop. Means were compared using a Least Significant Difference (LSD) method. The effects of agriculture practices on N-cycling microbes were visualized using scatter plots made by graphing software Origin Pro 2019b.

Pearson correlation analyses were used to determine correlation among genes, gene

transcripts, transcript to gene ratios, and soil properties by 'rcorr' function of package Hmisc in R 3.6.1 (the R Foundation for Statistical Computing). Correlation coefficient *p*-value were then adjusted with a correction for multiple comparisons using the BH method of Hmisc package. A heatmap based on Pearson correlations was used to visualize the significant positive and negative correlations among measured soil properties, N-cycle gene abundance, gene transcript abundance, and transcript to gene ratios, and was performed by pheatmap package in R 3.6.1.

Non-metric Multi-Dimensional Scaling (NMDS) based on Bray-Curtis distances was performed in R 3.6.1 with packages vegan 2.5-5 and phyloseq to reveal the distribution patterns of N-cycling functional groups and their activity among seasons and management practices. Analysis of similarities (ANOSIM), a rank-based non-parametric statistical test, was also performed by R with the function 'anosim' in package vegan to compare groups and test the null hypothesis that the similarity between groups is greater than or equal to the similarity within the groups. Dispersion indices were calculated in R with the function 'betadisper' in package vegan to assess the multivariate homogeneity of group dispersions. The function 'TukeyHSD.betadisper' in package vegan was used to calculate Tukey's Honest Significant Differences between groups. In this study, statistically significant difference was accepted at p-value < 0.05 unless otherwise noted.

Results

Soil properties and ambient temperatures

All the soil physicochemical parameters and N transformation rates measured in this study showed seasonal dynamics, and most of them were significantly affected by hairy vetch (Appendix Table 4 and Appendix Table 5). In particular, increased nitrification and N mineralization rates in vetch plots were mainly observed in cover crop peak season (April) and spring crop transition (May) (Appendix Table 5). N fertilization significantly increased the concentration of NO_3^--N, TC, TN, N_2O-N, and the rate of incubated nitrification and N mineralization (Appendix Table 5). Compared to conventional tillage (CT), no tillage (NT) significantly increased the concentration of TEC, TC and TN, which

was particularly noticeable in April and October for TEC, and in May and October for TC and TN (Appendix Table 5). Ambient temperatures during the cotton growing seasons (May and October, 16.1 and 17.2 ℃, respectively) were about 2 times higher than during the cover crop growing seasons (November and April, 8.3 and 9.4 ℃, respectively).

N-cycle genes and transcripts

The abundances of N-cycle genes and transcripts were influenced differently by agricultural season and soil management practices. In general, for both relative (normalized to 16S rRNA genes and 16S rRNA) and absolute abundances (per gram dry weight soil), genes were mostly affected by only main effects or two-way interaction effects of agricultural season and soil management practices while transcripts were affected by much more complex interaction effects among season and management practices (Table 2; Appendix Table 6 and Appendix Table 7).

Both relative and absolute abundances of marker genes for N-fixation (*nifH*) were significantly affected by agricultural season and tillage (Table 2), with highest abundances during cover crop harvest (April) and under CT (Figure 2). Moreover, although no significant effect of N fertilization on the relative abundance of *nifH* gene was observed (Figure 3A), absolute abundance of *nifH* was significantly higher under no N fertilization than N fertilization, especially in no cover (NC) and vetch (V) cropping systems (Figure 3B). Expression of *nifH* had different and more complex dynamics. N fertilization (67N) increased *nifH* transcript relative abundance in NT but decreased in CT (Appendix Figure 10). In general, *nifH* transcript abundances were higher during fall crop transition (November) than other seasons (Figure 4, 5). *nifH* transcript abundances were also significantly affected by the interaction of season, cover crop and tillage (Table 2). The relative abundance of *nifH* transcripts was not significantly different regardless of cover crops or tillage in April but was significantly higher under CT than NT in spring crop transition (May) for NC plots, in cotton peak season (October) for NC and V plots, and in fall crop transition (November) for wheat (W) plots (Figure 4A). The absolute abundance of *nifH* transcript was significantly higher under CT than NT in April and May for NC plots, in October for V plots, and in November for W plots (Figure 4B). The interaction of season,

cover crop and N fertilization also significantly affected *nifH* transcripts (Table 2). Fertilizer general had a positive effect on *nifH* transcripts in NC plots, but a negative effect in W plots, especially in October (Figure 5).

Both relative and absolute abundances of ammonia oxidation gene AOB *amoA* were significantly affected by season combined with tillage (Table 2), with the highest relative abundance in October under both CT and NT and lowest in November under CT (Figure 2A) as well as the highest absolute abundance in May under CT and in October under both CT and NT but lowest in November under both CT and NT (Figure 2B).

The *amoA* gene abundances were also significantly affected by the combination of cover crop and N fertilization (Table 2): N fertilization increased *amoA* gene relative abundance under V and NC but decreased both relative and absolute abundances under wheat (Figure 3). Similar to *nifH*, addition of N fertilization increased relative abundance of *amoA* transcripts under NT but decreased under CT (Appendix Figure 10). Their absolute abundance was not affected by the interaction of N fertilization and tillage (Appendix Figure 10). In addition, *amoA* transcript abundances were significantly affected by the combination of season, cover crop and tillage (Table 2). In April and May, *amoA* transcript abundances were highest under V in both NT and CT (Figure 4). After a lag in April and May, NC plots had increased *amoA* transcripts in October under CT and November under NT (Figure 4). The interaction of season, cover crop and N fertilization also significantly affected *amoA* transcripts (Table 2). Addition of fertilizer significantly increased *amoA* transcript relative abundance under W in April, and under NC in May, though not to the same level as V (Figure 5A). In October and November, 67N continued to exhibit increased relative abundance of *amoA* transcripts under NC; in contrast, 67N caused a decrease under W in October and November and V in November (Figure 5A). The absolute abundance of *amoA* transcripts showed similar trends to their relative abundance (Figure 5B).

The abundances of nitrite reduction gene *nirK* varied by agricultural season (Table 2), with lowest relative and absolute abundances in May and April, respectively (Figure 2). CT increased absolute abundance rather than relative abundance of *nirK* genes (Figure 2). N fertilization significantly increased their absolute abundance under V (Figure 3B). The relative abundance of *nirK* transcripts increased with fertilization in NT plots, but

Table 2. Results of mixed model ANOVA (based on GLIMMIX procedure in SAS) testing effects of agricultural season and soil management practices on the normalized (N) and absolute (A) abundances of N-cycle genes (G) and transcripts (T). F-values are reported.

Factor		*nifH*		AOB[a] *amoA*		*nirK*		*nirS*		*nosZ*		16S	
		G	T	G	T	G	T	G	T	G	T	G	T
Season (S)	N	**10.97*****	**71.34*****	**56.80*****	**7.91*****	**12.81*****	**49.64*****	**16.04*****	**62.88*****	**41.16*****	**24.29*****	-	-
	A	**13.44*****	**62.28*****	**57.66*****	**15.97*****	**12.54*****	**30.94*****	**25.48*****	**64.89*****	**31.54*****	**33.34*****	**9.30*****	**15.65*****
Tillage (T)	N	**9.18****	**7.12****	0.04	0.02	0.57	0.03	**6.71***	0.78	0.51	**9.87****	-	-
	A	**49.60*****	**13.20*****	**6.63***	2.08	**17.28*****	2.75	**59.71*****	**4.36***	**18.30*****	**17.20*****	**10.12****	3.81
Cover (C)	N	2.08	**4.18***	**88.83*****	**71.70*****	1.09	**26.28*****	2.68	**14.81*****	**6.29****	**28.50*****	-	-
	A	2.21	**10.97*****	**87.58*****	**52.55*****	0.86	**23.39*****	**3.71***	**18.62*****	**7.74*****	**27.22*****	0.40	**8.55*****
Nitrogen (N)	N	0.00	0.03	0.16	0.12	2.63	0.89	**18.81*****	1.22	**17.69*****	1.42	-	-
	A	**4.10***	0.09	**5.65***	0.17	2.69	0.88	**4.99***	1.25	2.24	1.27	**5.18***	0.05
S×T	N	1.33	**2.91***	**3.72***	**3.74***	0.99	0.42	2.47	1.11	1.77	**3.39***	-	-
	A	1.04	**3.07***	**2.97***	**2.68***	0.86	1.81	1.46	0.96	1.52	**3.68***	0.55	1.93
S×C	N	1.03	**2.17***	1.12	**8.41*****	0.70	**5.12*****	0.51	**4.81*****	0.64	**7.56*****	-	-
	A	0.94	1.27	1.78	**4.59*****	1.82	**3.37****	1.31	**5.53*****	2.10	**4.93*****	0.92	**2.37***
S×N	N	0.73	0.88	1.15	**4.82****	2.46	0.67	1.88	0.38	2.06	1.60	-	-
	A	0.10	1.60	0.83	1.74	1.64	2.35	1.07	0.92	0.74	**3.87***	1.05	1.50
T×C	N	0.43	0.61	2.34	0.15	0.04	0.79	0.34	0.77	0.04	1.89	-	-
	A	0.77	1.29	2.02	1.12	0.11	0.16	0.32	1.05	0.07	0.15	0.07	1.70
T×N	N	0.55	**11.63*****	0.76	**9.99****	0.59	**20.20*****	0.03	2.48	0.05	**11.71*****	-	-
	A	0.02	0.56	0.06	0.43	1.09	3.34	2.31	0.07	1.39	0.29	1.55	**5.97***
C×N	N	0.20	**3.86***	**13.66*****	**16.38*****	1.58	1.59	**4.37***	**6.83****	0.67	**7.98*****	-	-
	A	0.80	2.56	**5.51****	**8.78*****	**4.99****	0.69	**16.37*****	**3.99***	**3.38***	**3.97***	2.30	0.26
T×C×N	N	2.10	0.76	2.52	0.23	0.43	1.08	0.96	2.18	0.94	0.45	-	-
	A	2.03	1.05	1.04	0.93	1.79	1.79	0.13	**3.56***	0.06	1.74	0.38	0.84
S×T×C	N	0.72	**5.26*****	1.11	**6.16*****	0.94	**9.88*****	1.11	**4.82*****	0.65	**4.75*****	-	-
	A	1.10	**3.66****	1.78	**2.33***	0.99	**7.46*****	0.28	**4.39*****	0.29	1.82	1.07	2.05
S×T×N	N	0.23	0.29	0.29	0.38	0.17	1.43	0.89	1.59	0.98	0.44	-	-
	A	0.61	0.08	0.74	0.32	2.57	0.76	2.98	1.04	2.05	0.10	1.52	0.08
S×C×N	N	0.93	**2.39***	0.89	**6.40*****	0.60	**3.33****	0.42	1.28	0.64	**4.18*****	-	-
	A	1.63	**2.26***	1.18	**2.97****	0.59	**2.18***	1.03	1.10	0.76	**2.60***	0.18	0.82
S×T×C×N	N	0.30	0.58	1.67	1.80	0.48	1.21	0.78	**2.46***	0.51	**2.26***	-	-
	A	0.29	1.90	1.27	1.84	0.64	1.82	0.95	**2.94****	0.59	**4.49*****	0.47	1.79

Significance level: * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001.

[a]AOB = Ammonia oxidizing bacteria.

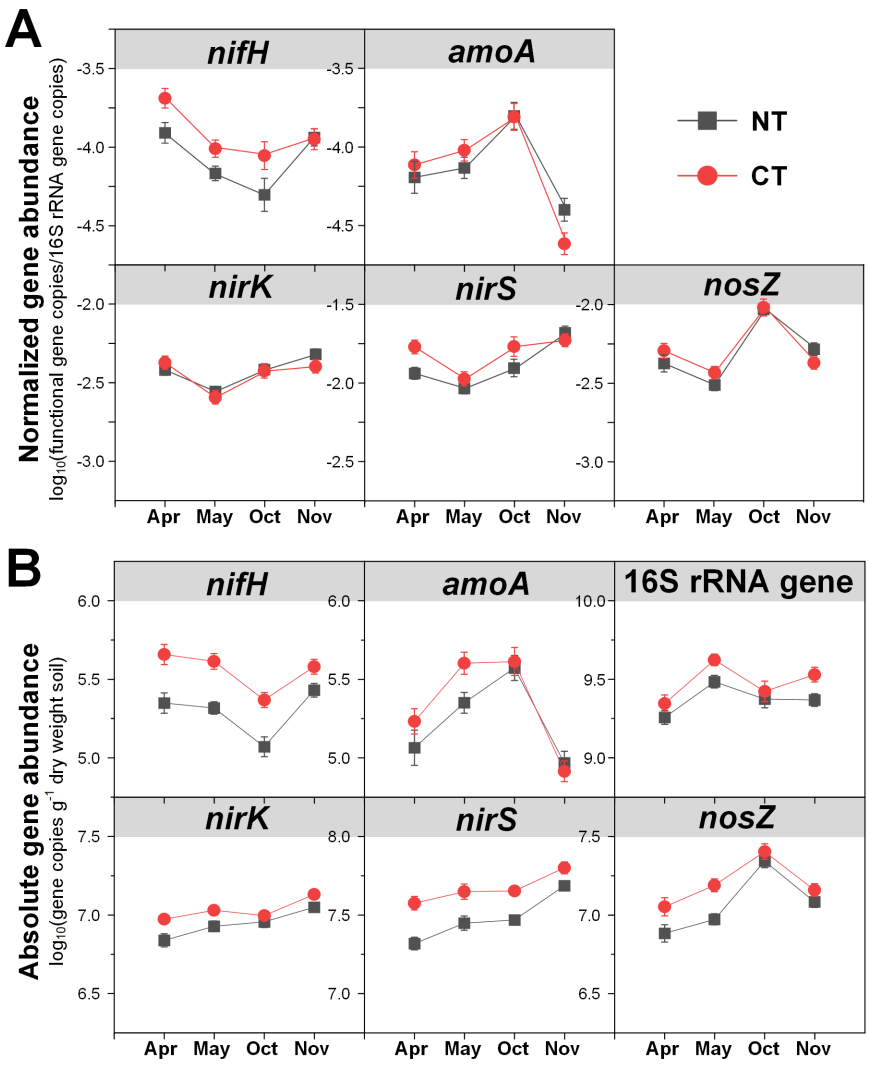

Figure 2. Seasonal dynamics of 16S rRNA gene normalized *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* gene abundances (A) and absolute abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes (B) in relation to tillage. Points represent the mean ± standard error (n = 24). NT = no tillage; CT = conventional tillage.

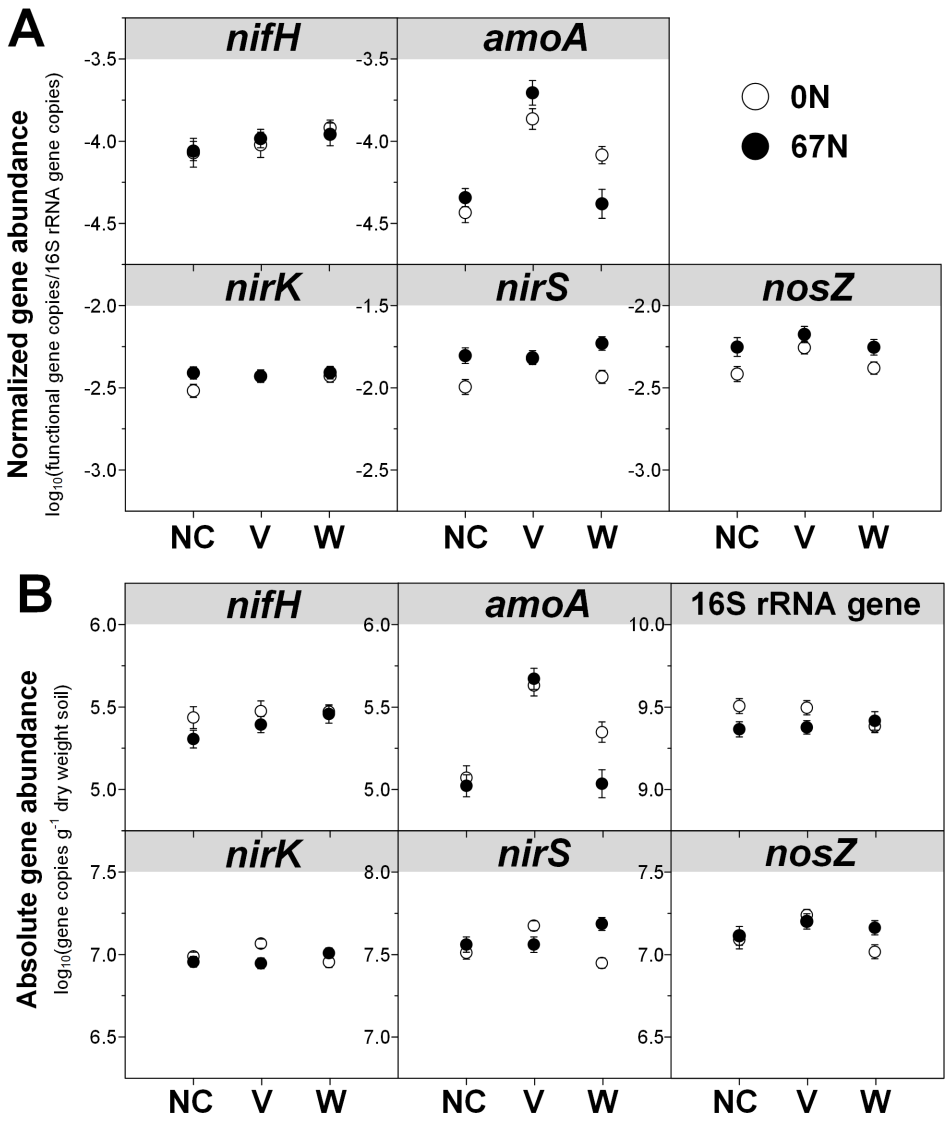

Figure 3. Variation of 16S rRNA gene normalized *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* gene abundances (A) and absolute abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes (B) in relation to N fertilization rate under different cover crop treatments. Points represent the mean ± standard error (n = 32). 0N = no N fertilization; 67N = 67 kg N ha^{-1} fertilization; NC = no cover; V = vetch; W = wheat.

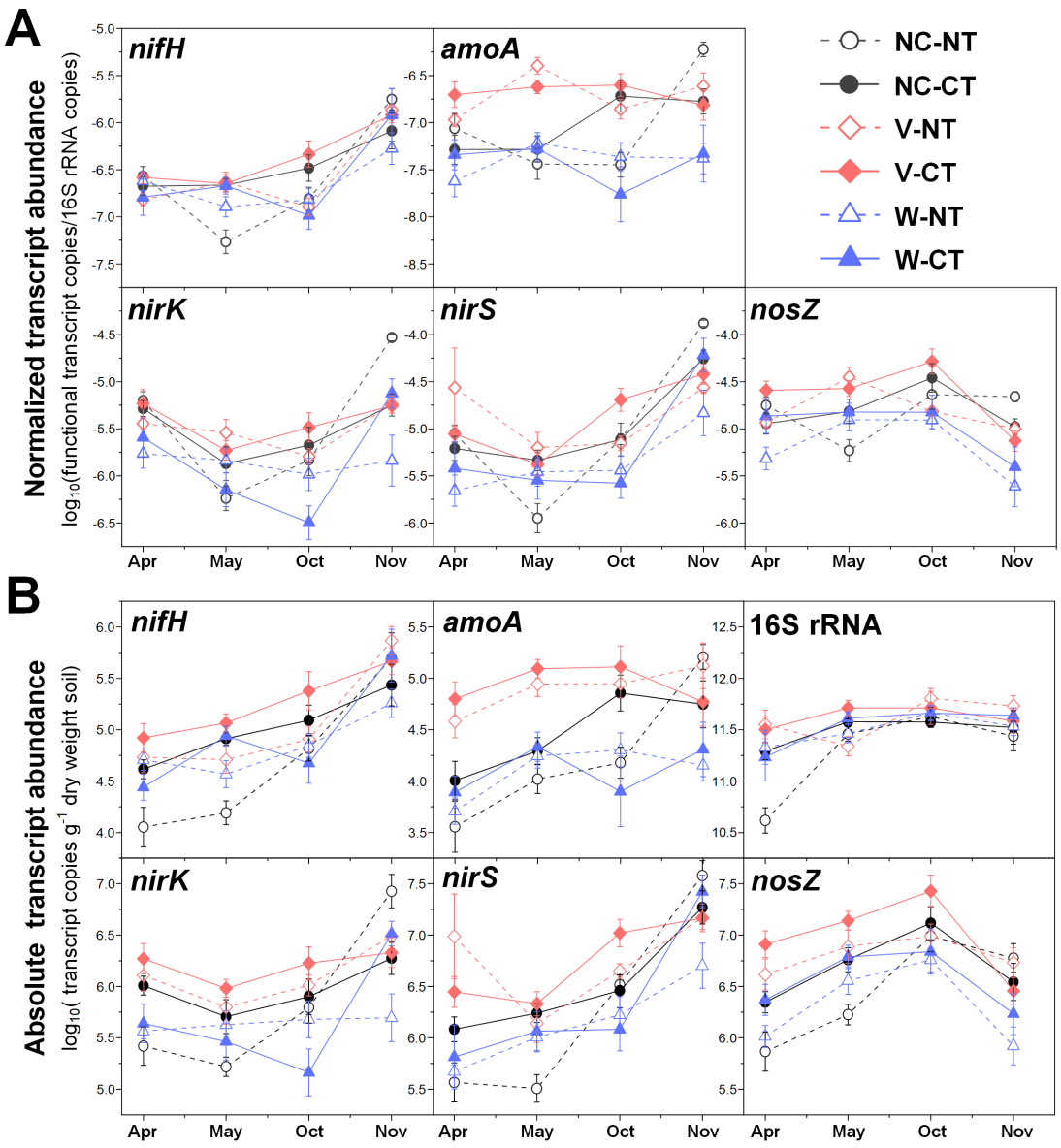

Figure 4. Seasonal dynamics of 16S rRNA normalized transcript abundances of *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* (A) and absolute transcript abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA gene (B) in relation to cover crops and tillage. Points represent the mean ± standard error (n = 8). NC = no cover; V = vetch; W = wheat; NT = no tillage; CT = conventional tillage.

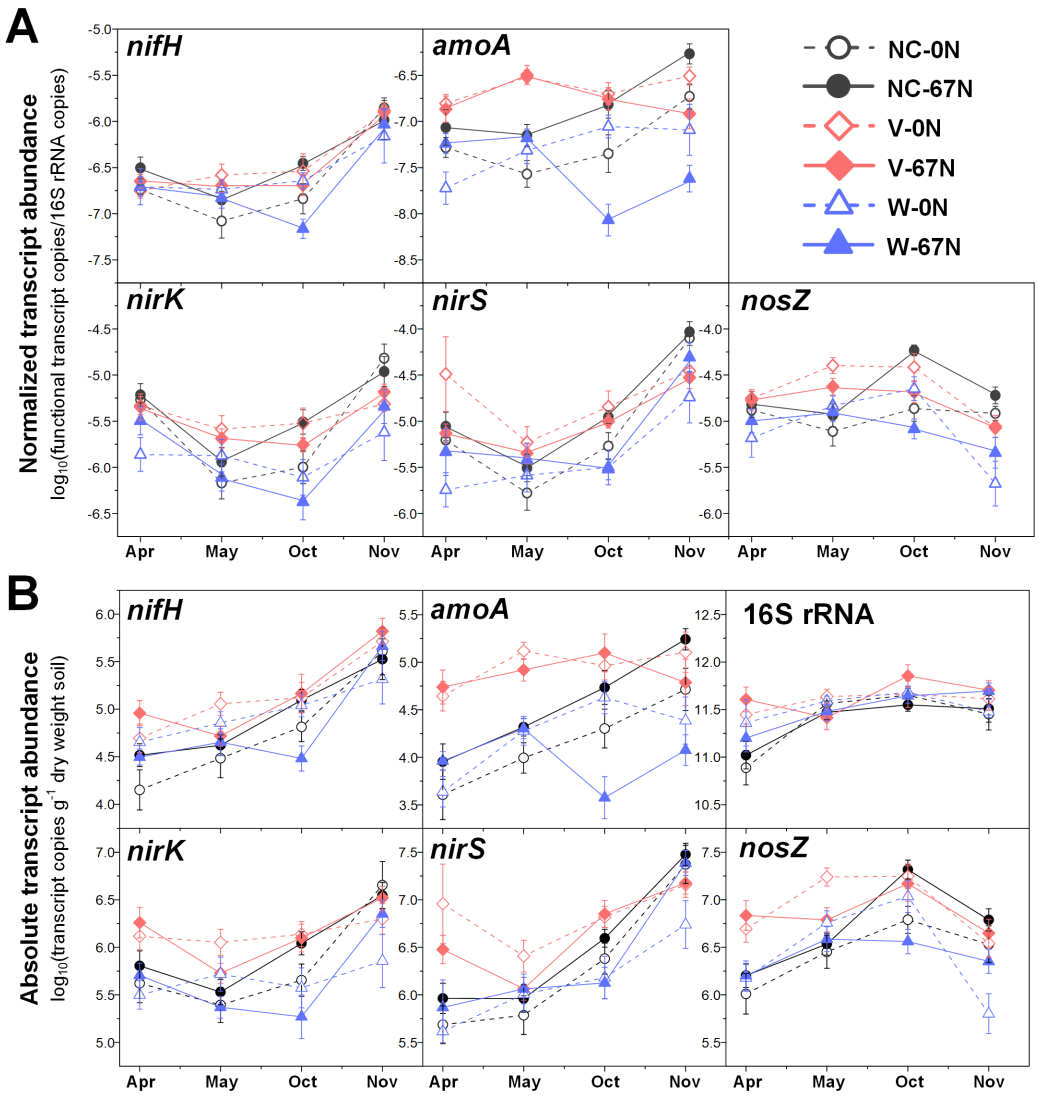

Figure 5. Seasonal dynamics of 16S rRNA normalized transcript abundances of *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* (A) and absolute transcript abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA gene (B) in relation to cover crop treatments and N fertilization rate. Points represent the mean ± standard error (n = 8). NC = no cover; V = vetch; W = wheat; 0N = no N fertilization; 67N = 67 kg N ha^{-1} fertilization.

decreased under CT (Appendix Figure 10). In addition, the *nirK* transcript abundances were significantly affected by the combination of season, cover crop and tillage (Table 2). In April, May and October, *nirK* transcript abundances were generally highest under V, which was more obvious for their absolute abundance (Figure 4). In November, CT plots had similar levels of *nirK* transcripts regardless of cover, while NT plots had much more variable abundances between cover crops (Figure 4). The interaction of season, cover crop and N fertilization rate also significantly affected both relative and absolute abundances of *nirK* transcripts (Table 2). Fertilization generally increased *nirK* transcripts under W in April and November but decreased in May and October (Figure 5).

The abundances of nitrite reduction gene *nirS* were significantly affected by agricultural season and tillage (Table 2), with higher abundances in November and under CT (Figure 2). *nirS* gene abundances were also significantly affected by the combination of cover crop and N fertilization rate (Table 2): N fertilization increased *nirS* gene relative abundance in NC and W plots but had no effect in V plots (Figure 3A); N fertilization increased *nirS* gene absolute abundance in W plots but decreased in V plots (Figure 3B). *nirS* transcript abundances were significantly affected by the interaction between agricultural season and all three soil management practices (Table 2): The highest transcript relative abundance of *nirS* was observed in NT-V-0N, NT-V-67N, CT-V-0N, and NT-NC-67N in April, May, October, and November, respectively, while the highest absolute abundance was observed in NT-V-0N, CT-V-0N, CT-V-67N, and NT-NC-0N in April, May, October, and November, respectively (Appendix Table 6 and Appendix Table 7).

The abundances of nitrous oxide reduction gene *nosZ* were significantly affected by agricultural season (Table 2) with both relative and absolute abundances were highest in October (Figure 2). Their absolute abundance was higher in CT than NT ($p < 0.0001$) (Figure 2B). *nosZ* gene relative abundance was highest under vetch ($p = 0.0024$), and 67N treatments ($p < 0.0001$), while absolute abundance was only increased by 67N under wheat (Figure 3). As with *nirS* transcripts, there was a significant interaction between agricultural season and the three soil management practices on *nosZ* transcript abundances (Table 2). *nosZ* transcript relative abundance was highest in NT-NC-67N in April and November, and CT-V-0N in May and October, while lowest in NT-W-0N in April and November, NT-NC-

0N in May, and CT-W-67N in October (Appendix Table 6). *nosZ* transcript absolute abundance was highest in CT-V-67N in April and October, CT-V-0N in May, and NT-V-67N in November, while lowest in NT-NC-0N in April and May, CT-W-67N in October, and CT-W-0N in November (Appendix Table 7).

16S rRNA genes and 16S rRNA

The abundance of 16S rRNA genes showed significant seasonal dynamics ($p < 0.0001$): highest abundances were observed in May and lowest in April (Appendix Table 7). Compared to NT, CT slightly but significantly promoted 16S rRNA gene abundances, from a mean of 2.35×10^9 copies g^{-1} dry weight soil (gdw^{-1}) in NT to 3.02×10^9 copies gdw^{-1} in CT (Appendix Table 7). 16S rRNA gene copy abundances decreased from 2.92×10^9 gdw^{-1} in 0N to 2.43×10^9 copies gdw^{-1} in 67N (Appendix Table 7). 16S rRNA copy numbers were significantly affected by the combination of season and cover crop ($p < 0.05$), which was lowest in April under NC (9.01×10^{10} copies gdw^{-1}) and highest in October in V (5.74×10^{11} copies gdw^{-1}, Appendix Table 7). Moreover, 67N increased 16S rRNA abundances in CT but decreased in NT (Appendix Figure 10). Unlike N-cycle gene transcripts, the abundance of 16S rRNA was not significantly affected by the interaction of season, tillage, and cover crop/ N fertilization (Table 2; Figure 4, 5).

Cover crop effects on N-cycling microbial activity

In general, V plots tended to exhibit the highest N-cycle gene transcript abundances across seasons. The positive effect of 67N addition on N-cycle gene transcripts was primarily observed in plots with no cover crops (NC), especially during cotton peak season (October) (Figure 5). It was notable that fertilization did not have a positive effect in V plots, with V-0N exhibited similarly high or even higher N-cycle gene transcript abundances as V-67N, especially during the cotton growing season (May and October) (Figure 5). We also observed similarly high or even higher N-cycle gene transcript abundances under vetch with no fertilizer (V-0N) as no cover crop with N fertilization (NC-67N) throughout the cover crop harvest and cotton growing seasons (April, May, October) (Figure 5).

The transcript:gene ratios of N-cycle genes also generally remained higher in V plots under the treatment combination of cover crop with tillage and with N fertilization in April, May, and October (Figure 6; Appendix Table 8), reconfirming the enhancing effect of vetch on N-cycle gene expressions. Moreover, the seasonal dynamics of transcript:gene ratio in relation to cover crop with tillage/N fertilization was generally similar to the trends in transcript absolute abundances (Figure 6), indicating that season and soil management practices had stronger effects at the transcript level compared to the gene abundance level.

Variability of N-cycling microbial functional potential and activity

NMDS analysis showed the variability of the nitrogen cycling bacterial populations in terms of functional potential (i.e., based on the five gene relative abundances) and functional activity (i.e., based on the five transcript relative abundances) (Figure 7). As expected, the statistical dispersion, or variability, of the populations by activity was consistently and significantly higher than for functional potential (dispersion index = 0.3926 for transcripts and 0.2168 for genes, $p < 0.001$). ANOSIM testing showed overall significant differences in functional potential by season (R = 0.1748, $p = 0.001$), tillage (R = 0.0222, $p = 0.020$), and N fertilization (R = 0.0401, $p = 0.003$) but not cover crop. The population distribution by activity was significantly affected by season (R = 0.2656, $p = 0.001$), cover crop (R = 0.0495, $p = 0.001$), and tillage (R = 0.0205, $p = 0.016$) but not fertilization (Appendix Table 9).

Relationships of soil N pools and processes and soil properties with functional gene abundance and expression

Correlation heatmaps showed significant correlations among absolute abundances of N-cycle genes and their transcripts, transcript:gene ratio, and soil properties (Figure 8). We observed significant positive correlations among the five gene transcripts and significant positive correlations between N-cycle genes and their corresponding transcripts for all gene targets except *nifH* (Figure 8). Moreover, the transcript:gene ratios of N-cycle genes were more related to their transcript abundances than gene abundances, reconfirming that transcript variation depended more on changes in expression rather than population sizes.

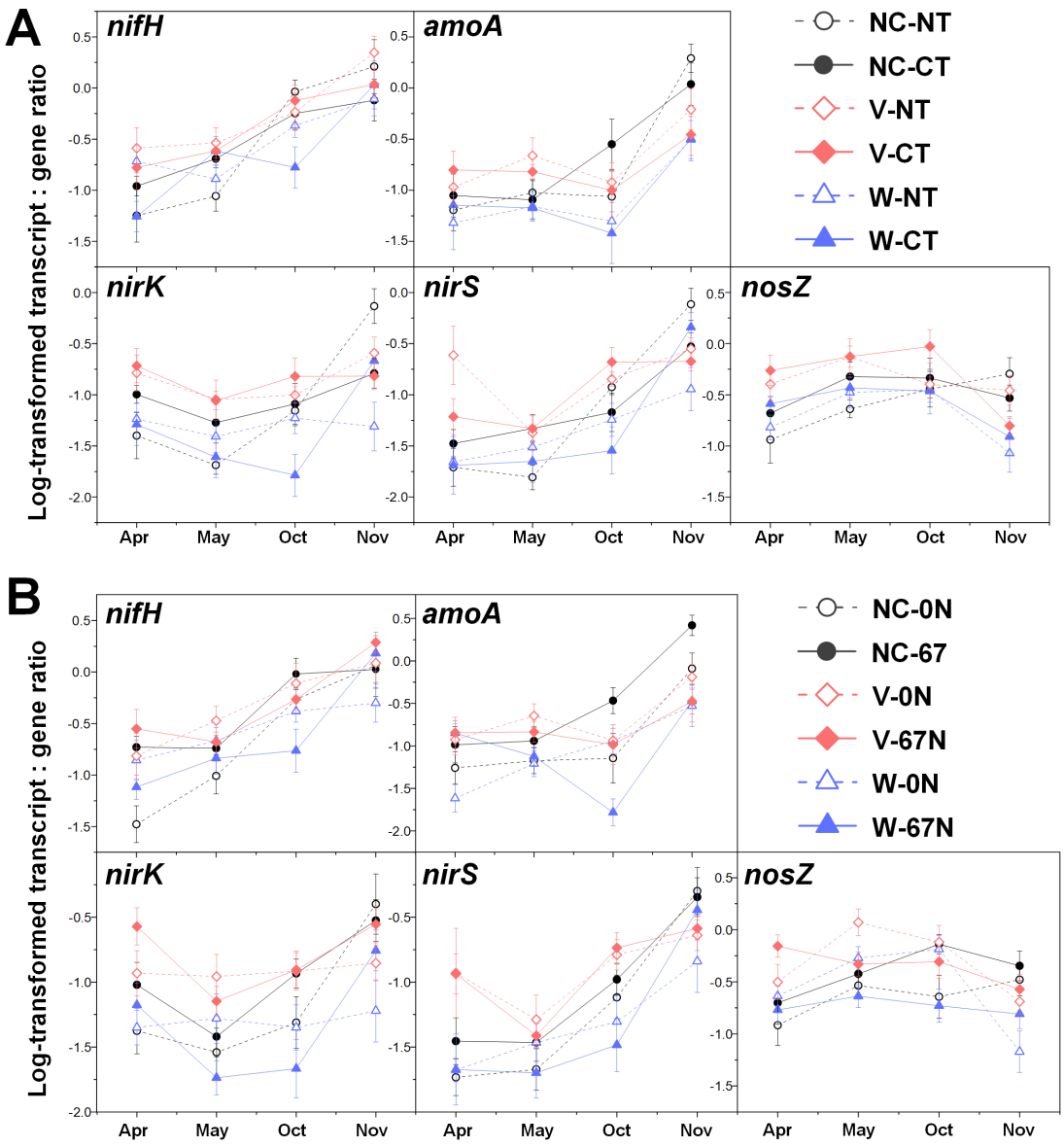

Figure 6. Seasonal dynamics of transcript-to-gene ratio of *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* (B) in relation to cover crops and tillage (A) and in relation to cover crops and N fertilization (B). Points represent the mean ± standard error (n = 8). NC = no cover; V = vetch; W = wheat; NT = no tillage; CT = conventional tillage.

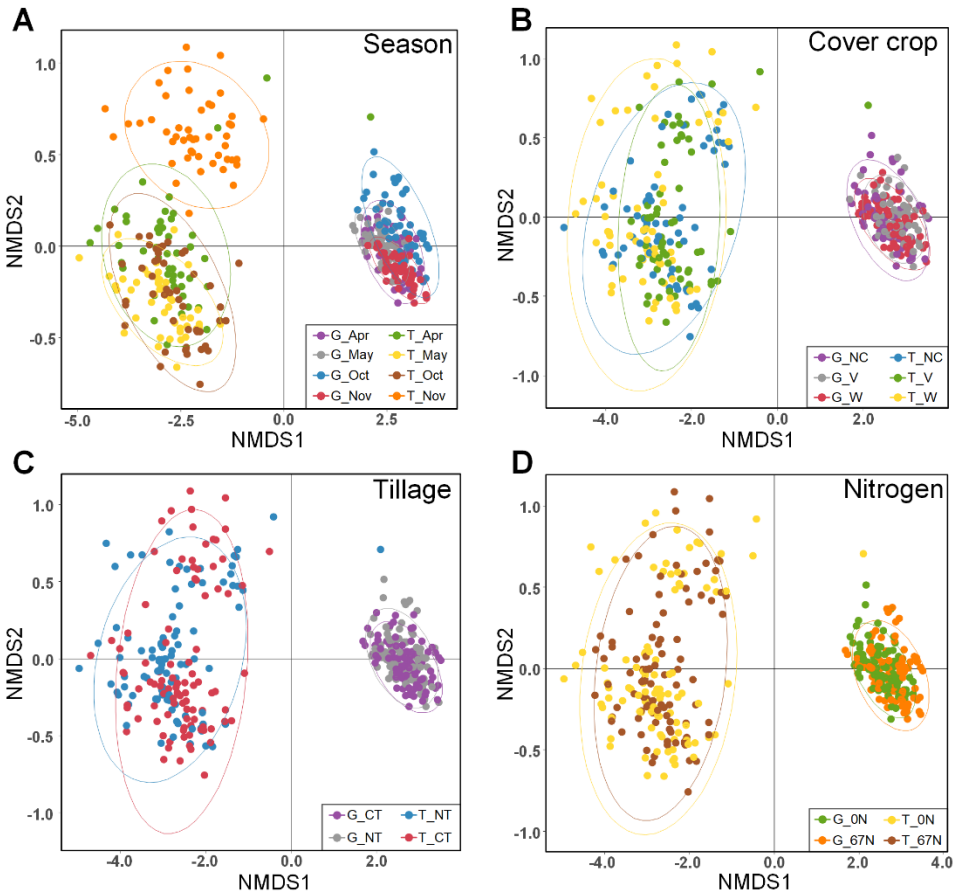

Figure 7. NMDS of Bray-Curtis distances between N-cycling communities, based on abundances of five N-cycling genes (G) and transcripts (T) (Stress = 0.015). Points on the ordination plots are colored by season (A), cover crop treatment (B), tillage (C) and fertilization rate (D). NC = no cover; V = vetch; W = wheat; NT = no tillage; CT = conventional tillage; 0N = no N fertilization; 67N = 67 kg N ha^{-1} fertilization. Ellipses represent 95% confidence interval of each group.

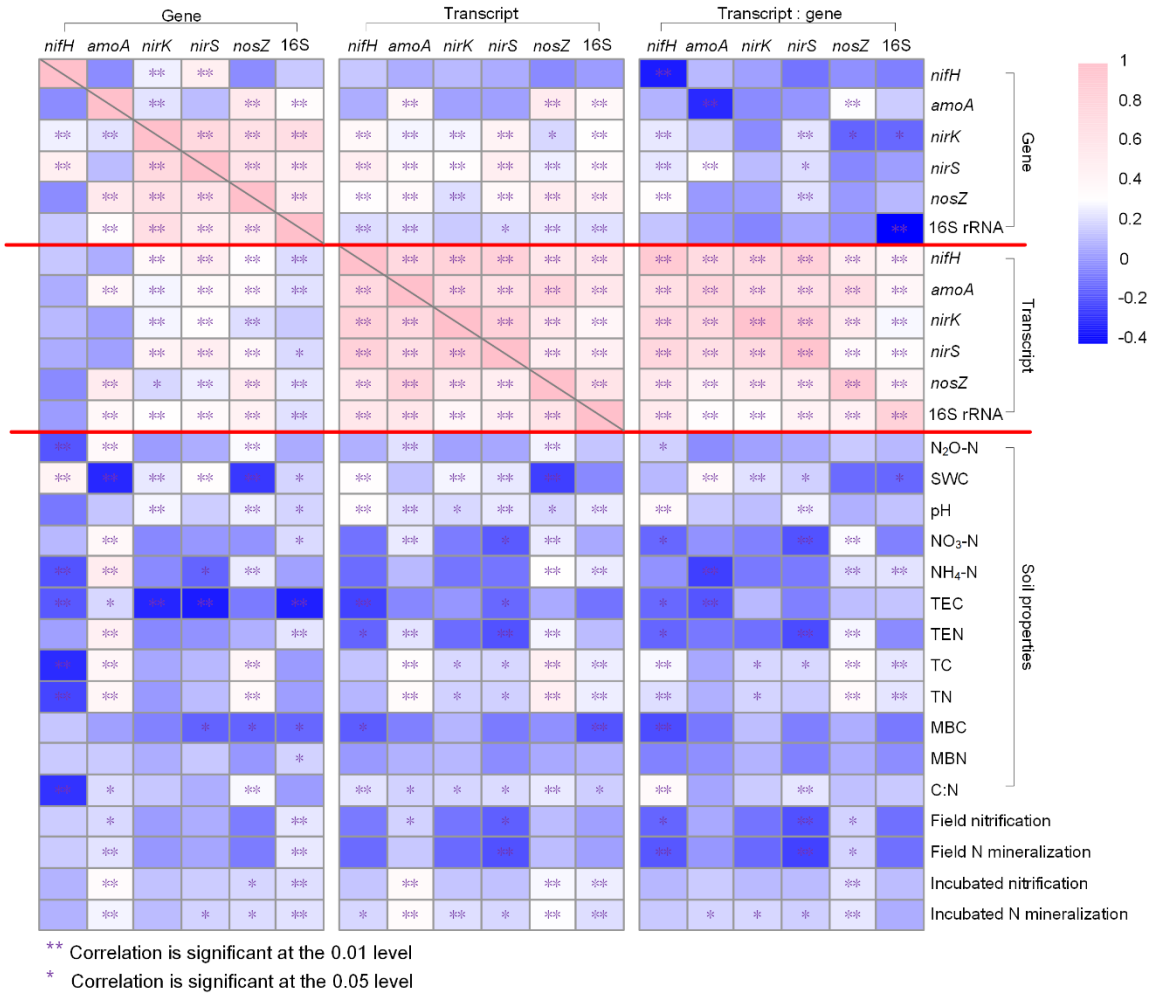

Figure 8. Heatmap showing correlation among genes, gene transcripts, transcript to gene ratios, and soil properties. SWC = soil water content; TC = total carbon; TN = total nitrogen; TEC = total extractable carbon; TEN = total extractable nitrogen; MBC = microbial biomass carbon; MBN = microbial biomass nitrogen.

N-cycle genes and their transcripts, as well as their transcript:gene ratios, showed different patterns of correlations with soil properties (Figure 8). The gene and transcript abundances of *nifH*, *nirK*, and *nirS* were all positively correlated with SWC (Figure 8). *amoA* gene and transcript abundances were both significantly and positively correlated with N_2O-N, NO_3^--N, field nitrification rate, and incubated nitrification rate (Figure 8). Notably, *amoA* gene was positively correlated with NH_4^+-N (R = 0.494) while *amoA* transcript had no relationship with NH_4^+-N, and the transcript:gene ratio of *amoA* was negatively correlated with NH_4^+-N (R = -0.260), indicating that although *amoA* gene abundance was promoted by NH_4^+, its expression was lower with higher NH_4^+-N concentration (Figure 8). In addition, there were significant positive correlations between N_2O-N and the gene and transcript abundances of *amoA* and *nosZ* (Figure 8).

Discussion

Seasonal dynamics in abundance and activity of nitrogen cycling populations

The primary objective of this study was to investigate the impact of soil health management practices on the seasonal population size and activity of soil bacterial communities involved in N transformations by using *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* as indicators. We found that the N-cycling bacterial community activity, as indicated by gene and transcript abundances, respectively, showed seasonal changes.

Without considering soil management practices, the highest functional activity of diazotrophs, as indicated by *nifH* expression, was in November during the fall crop transition, which might be explained by lower N availability at this time. The inhibitory effect of increased N availability (NH_4^+-N and/or NO_3^--N) on *nifH* abundance and expression we observed has been reported in other systems as well (Cusack et al., 2009; Lindsay et al., 2010; Pereira et al., 2013; Reardon et al., 2014). Diazotrophs will preferentially use easily available N (e.g. nitrate and ammonium) as this takes less energy to assimilate compared to fixing dinitrogen gas (Chapin et al., 2002). Soil freeze-thaw cycles, warming, and N addition can stimulate net N mineralization, increasing N availability (Contosta et al., 2011). Compared to November, the warmer temperatures and

N fertilization after cotton planting in May resulted in higher soil N availability during cotton growing seasons, thereby possibly inhibiting the expression of *nifH*. The cotton and winter cover crops take up inorganic and dissolved organic N for growth, leading to increased soil N limitation, especially during the cover crop growing period, which might enhance the competitiveness of diazotrophs and result in their relatively high abundance in April and November. Moreover, N fixation can be affected by soil oxygen content because nitrogenase is an extremely oxygen-sensitive enzyme (Chapin et al., 2002). The high soil water content (SWC) in November may have resulted in lower soil oxygen, contributing to a higher functional activity of diazotrophs. The positive correlation between SWC and *nifH* abundance in our study was also observed in other studies (Chen et al., 2019; Zhang et al., 2020). In addition, the relatively high soil C:N ratio and the decomposition of a large amount of plant residues and roots after cotton harvest season (October) may have led to soil N limitation, stimulating the expression of N-fixation genes.

The *amoA* gene relative and absolute abundances were both highest in October and lowest in November, which corresponded to the highest soil NH_4^+-N concentrations in October and lowest in November. Moreover, a significantly positive correlation between *amoA* gene abundances and NH_4^+-N concentrations was observed in our study, indicating that concentrations of NH_4^+-N, the substrate for nitrification, influence the abundance of nitrifiers (Levy-Booth et al., 2014; Ouyang et al., 2017). The decrease in *amoA* gene abundance in November may due to higher SWC and lower soil oxygen availability because AOB are aerobic bacteria (Levy-Booth et al., 2014). The seasonal dynamics of *amoA* transcript relative and absolute abundances both depended on the interaction of cover crops with either tillage or N fertilization rate. However, contrary to *amoA* gene abundance, relative *amoA* expression (normalized to gene abundance) was higher in November than in October and was negatively correlated to NH_4^+-N concentrations, indicating that while population sizes of AOB were down, their functional activity was increased. One hypothesis to explain this unexpected result was that ammonia oxidizers increased expression of their functional genes under oxygen and/or nutrient stress. We observed a significant positive correlation between expression level of *amoA* and SWC, which was also reported in other studies. For example, Theodorakopoulos et al. (2017) found that >70%

water filled pore space favored transcription of AOB *amoA* gene (Theodorakopoulos et al., 2017). A pure culture experiment by Yu and Chandran (2010) found a low level of dissolved oxygen can promote *amoA* transcription in AOB *Nitrosomas europaea* (Yu & Chandran, 2010). In addition, Bollmann et al. (2005) reported that during short-term (2 weeks) starvation, the *amoA* mRNA of *Nitrosospira briensis* remained present and the ammonia-oxidation functionality maintained at a high level (Bollmann et al., 2005).

The abundances of *nirS* genes and transcripts were greater than those of *nirK* in all agricultural seasons, which suggested that *nirS*-harboring denitrifiers were more predominant at our field site. Both relative and absolute transcript abundances and expressions of *nirK*- and *nirS*-harboring denitrifiers were highest in November, which is likely due to low soil oxygen level resulting from high SWC at that time. The significantly positive correlations of SWC with the transcript abundances and expression levels of *nirK* and *nirS* also support this explanation. Both relative and absolute abundances of *nosZ* genes and transcripts were highest in October. However, the N_2O-N emissions were highest in both May and October. The not fully consistent seasonal trend between *nosZ* and N_2O-N emission suggests that the typical *nosZ* (clade I *nosZ*) we targeted in this study may not independently predict the seasonal dynamics of N_2O emission, and atypical *nosZ* (clade II *nosZ*) may be playing an important role (Orellana et al., 2014; Sanford et al., 2012). In fact, several studies have reported that atypical *nosZ* is more abundant than clade I *nosZ* in agricultural soils (Jones et al., 2013; Juhanson et al., 2017; Tsiknia et al., 2015).

Effects of cover crops on abundance and activity of nitrogen cycling populations

Different responses were observed between the two types of cover crops: In general, compared to winter wheat, hairy vetch had significantly higher transcript abundance and transcript:gene ratios for all N-cycle genes. The elevated activity of N-cycling microbes under vetch was not limited to just the cover crop period (April), but persisted past cover crop termination through the cotton growing season (October). This supports the idea that leguminous cover crops have a lasting stimulation of overall N-cycling microbes in agricultural soil. This is consistent with our previous findings from this site, where we documented significantly increased microbial biomass (both total and microbial biomass

N) and enzyme activities in the vetch treatments (Mbuthia et al., 2015). It may be that the biological N fixation rate stimulated by legumes and the low C:N ratio of legume residues supplied more N to the soil, providing a competitive advantage for N-cycling populations (Carsky et al., 2001; H. Wang et al., 2017). Winter wheat is a non-leguminous cover crop with high C:N residues that takes up additional N from soil for growth, which may decrease soil N availability and inhibit the functional gene expression of N-cycling microbes (except *nifH*). Some studies observed that leguminous cover crops can promote the release of mineral N and increase denitrification and N_2O emissions relative to other cover crops or no cover (Abdalla et al., 2012; Bowen et al., 2018; Hauggaard-Nielsen et al., 2016). The relatively high and generally similar level of N-cycle gene expression under combinations of hairy vetch with no fertilization and no cover crop with N fertilization supports the idea that, in some cases, N-fixing cover crops can replace N fertilization to maintain the N-cycling rate.

Effects of tillage and fertilization on abundance and activity of nitrogen cycling populations

Overall, tillage only affected the relative abundances of genes *nifH* and *nirS*, but it affected the absolute abundances of all five functional genes and 16S rRNA genes, with higher abundances under conventional tillage compared to no tillage. This may be because no-till treatment can decrease surface soil bulk density, increase soil porosity, and result in higher oxygen content in surface soils (López-Vázquez et al., 2019). The aerobic conditions resulting from conventional tillage may suppress the growth of anaerobic N-cycling bacteria, such as N fixers and denitrifiers. Another possible explanation is that conventional tillage makes substrates more available for soil microbes by destroying soil aggregates (Ashman et al., 2003; Elliott, 1986), increasing soil microbial populations, including the N-cycling populations.

Compared to no fertilization, N fertilization with 67 kg N ha^{-1} only significantly increased the relative abundance of genes *nirS* and *nosZ*, but only when no cover crops and wheat were used. This effect was observed only in wheat plots when absolute abundance was taken into consideration. This is partly in accordance with a meta-analysis by Ouyang

et al. (2018), which showed that N fertilization had no effect on *nifH* abundance, but significantly increased the abundance of *amoA*, *nirK*, *nirS* and *nosZ* genes, respectively (Ouyang et al., 2018). Although some studies using NH_4NO_3 as N fertilizer also reported that N fertilization can increase AOB diversity, abundance and activity (Liu et al., 2017; Liu et al., 2018; Segal et al., 2017), we did not observe this in our study, likely because similar concentrations of NH_4^+-N and field nitrification rates were observed under both fertilizer treatments. Increased abundance of denitrification genes *nirS* and *nosZ* with N fertilization corresponded to high NO_3^--N concentration and high N_2O-N emission in fertilized soil.

Linking the nitrogen cycling groups with nitrogen cycling processes

In this study, we assessed the relationships between abundance and expression of functional genes and soil properties. We were particularly interested in determining whether N_2O emissions, a potent greenhouse gas, could be correlated to the population size or activity of bacteria involved in N-cycling processes responsible for N_2O production and transformation. Both the absolute abundances of genes and transcripts of *amoA* and *nosZ* were positively correlated to N_2O emissions. This suggests that soil AOB populations might be a main contributor to N_2O production through nitrification, nitrifier denitrification, or both (Huang et al., 2014; Zhu et al., 2013). Likewise, Soares et al. (2016) also observed that AOB, rather than denitrifiers, were the main contributors to N_2O emissions in tropical agricultural soils (Soares et al., 2016). Moreover, research by Theodorakopoulos et al. (2017) suggested AOB *amoA* transcript abundances, rather than gene abundances, were a good indicator of N_2O emissions, and ammonia oxidation was a major process contributing to N_2O emissions (Theodorakopoulos et al., 2017). However, some studies have found no correlations between N_2O emissions and abundance of related N-cycle genes (AOB *amoA*, *nirK*, *nirS*, and *nosZ*) (Maul et al., 2019; Pereira et al., 2015). Therefore, the contribution of AOB and denitrifiers to N_2O emissions might be site-specific and/or influenced by local soil properties and environmental factors. In addition, although we found significant positive correlation between field and incubated net nitrification rates with absolute abundances of both *amoA* genes and transcripts, a disconnection between AOB *amoA* gene

abundances and nitrification rates has been observed in some studies (Li et al., 2015; Liu et al., 2017). This inconsistent result may be site-specific and might be influenced by the proportion of dormant microbes in soils, substrate limitation, microsite heterogeneity, and/or the fact that measured process rates reflect net, rather than gross, rates.

Conclusions

In summary, our results showed that the distribution patterns, abundances, and expressions of N-cycle functional genes generally changed significantly between seasons and were affected by soil management practices. We demonstrated that leguminous cover crops like hairy vetch can promote functional activity of N-cycling populations through the growing season, equaling or exceeding the effects of inorganic N fertilizer. Our findings highlight the fact that both genetic and transcriptional assessments can provide important information about the microbial populations and functions. Transcriptional-level study has higher resolution than genetic-level in examining the effects of soil management practices on N-cycling communities, with larger variation under different season and management practices were observed. In addition, the interaction effects of season and soil management practices on N-cycle gene expressions based on relative (normalized by 16S rRNA copies) and absolute (copies per gram soil) transcript abundances showed generally similar trend, indicating that active soil N-cycling communities were more responsive than total active soil bacterial populations to agricultural management practices. Together our work revealed insights into important relationships between microbial functional capacity and activity and associated nitrogen pools and processes in the field, adding to our understanding of the effects of soil health management practices on soil microbial ecology.

References

Abdalla, M., Rueangritsarakul, K., Jones, M., Osborne, B., Helmy, M., Roth, B., Burke, J., Nolan, P., Smith, P., & Williams, M. (2012). How effective is reduced tillage-cover crop management in reducing N_2O fluxes from arable crop soils? *Water, Air, & Soil Pollution*, *223*(8), 5155-5174. https://doi.org/10.1007/s11270-012-1268-4

Akter, Z., Pageni, B. B., Lupwayi, N. Z., & Balasubramanian, P. M. (2014). Biological nitrogen fixation and *nifH* gene expression in dry beans (*Phaseolus vulgaris* L.). *Canadian Journal of Plant Science*, *94*(2), 203-212. https://doi.org/10.4141/cjps2013-200

Ashman, M. R., Hallett, P. D., & Brookes, P. C. (2003). Are the links between soil aggregate size class, soil organic matter and respiration rate artefacts of the fractionation procedure? *Soil Biology and Biochemistry*, *35*(3), 435-444. https://doi.org/https://doi.org/10.1016/S0038-0717(02)00295-X

Badagliacca, G., Benitez, E., Amato, G., Badalucco, L., Giambalvo, D., Laudicina, V. A., & Ruisi, P. (2018). Long-term no-tillage application increases soil organic carbon, nitrous oxide emissions and faba bean (*Vicia faba* L.) yields under rain-fed Mediterranean conditions. *Science of the Total Environment*, *639*, 350-359. https://doi.org/10.1016/j.scitotenv.2018.05.157

Bahulikar, R. A., Chaluvadi, S. R., Torres-Jerez, I., Mosali, J., Bennetzen, J. L., & Udvardi, M. (2020). Nitrogen fertilization reduces nitrogen fixation activity of diverse diazotrophs in switchgrass roots. *Phytobiomes Journal*, PBIOMES-09-19-00. https://doi.org/10.1094/pbiomes-09-19-0050-fi

Bárta, J., Tahovská, K., Šantrůčková, H., & Oulehle, F. (2017). Microbial communities with distinct denitrification potential in spruce and beech soils differing in nitrate leaching. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-08554-1

Bollmann, A., Schmidt, I., Saunders, A. M., & Nicolaisen, M. H. (2005). Influence of starvation on potential ammonia-oxidizing activity and *amoA* mRNA levels of *Nitrosospira briensis*. *Applied and Environmental Microbiology*, *71*(3), 1276-1282. https://doi.org/10.1128/AEM.71.3.1276-1282.2005

Bowen, H., Maul, J. E., Poffenbarger, H., Mirsky, S., Cavigelli, M., & Yarwood, S. (2018). Spatial patterns of microbial denitrification genes change in response to poultry litter placement and cover crop species in an agricultural soil. *Biology and Fertility of Soils*, *54*(6), 769-781. https://doi.org/10.1007/s00374-018-1301-x

Cambardella, C. A., Johnson, J. M., & Varvel, G. E. (2012). Soil carbon sequestration in central USA agroecosystems. In M. A. Liebig, A. J. Franzluebbers, & R. F. Follett (Eds.), *Managing Agricultural Greenhouse Gases: Coordinated Agricultural Research through GRACEnet to Address our Changing Climate.* (pp. 41-58). Elsevier Publ. https://doi.org/10.1016/B978-0-12-386897-8.00004-8

Carsky, R. J., Singh, B. B., & Oyewole, B. (2001). Contribution of early season cowpea to late season maize in the savanna zone of West Africa. *Biological Agriculture and Horticulture*, *18*(4), 303-315. https://doi.org/10.1080/01448765.2001.9754894

Chan, Y. K., McCormick, W. A., & Watson, R. J. (1997). A new *nos* gene downstream

from *nosDFY* is essential for dissimilatory reduction of nitrous oxide by *Rhizobium* (*Sinorhizobium*) *meliloti*. *Microbiology*, *143*, 2817-2824. https://doi.org/10.1099/00221287-143-8-2817

Chapin, F. S., Matson, P. A., & Mooney, H. A. (2002). *Principles of terrestrial ecosystem ecology*. Springer, New York, NY. https://doi.org/10.1007/b97397

Chen, J., Shen, W., Xu, H., Li, Y., & Luo, T. (2019). The composition of nitrogen-fixing microorganisms correlates with soil nitrogen content during reforestation: a comparison between legume and non-legume plantations. *Frontiers in Microbiology*, *10*, 508. https://doi.org/10.3389/fmicb.2019.00508

Colliver, B. B., & Stephenson, T. (2000). Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. *Biotechnology Advances*, *18*(3), 219-232. https://www.ncbi.nlm.nih.gov/pubmed/14538109

Contosta, A. R., Frey, S. D., & Cooper, A. B. (2011). Seasonal dynamics of soil respiration and N mineralization in chronically warmed and fertilized soils. *Ecosphere*, *2*(3), art36. https://doi.org/10.1890/es10-00133.1

Cusack, D. F., Silver, W., & McDowell, W. H. (2009). Biological nitrogen fixation in two tropical forests: ecosystem-level patterns and effects of nitrogen fertilization. *Ecosystems*, *12*(8), 1299-1315. https://doi.org/10.1007/s10021-009-9290-0

Cusick, K. D., Fitzgerald, L. A., Cockrell, A. L., & Biffinger, J. C. (2015). Selection and evaluation of reference genes for reverse transcription-quantitative PCR expression studies in a thermophilic bacterium grown under different culture conditions. *PLoS One*, *10*(6), e0131015. https://doi.org/10.1371/journal.pone.0131015

Elliott, E. T. (1986). Aggregate structure and carbon, nitrogen, and phosphorus in native and cultivated soils. *Soil Science Society of America Journal*, *50*(3), 627-633. https://doi.org/10.2136/sssaj1986.03615995005000030017x

Feng, J., Li, F., Zhou, X., Xu, C., Ji, L., Chen, Z., & Fang, F. (2018). Impact of agronomy practices on the effects of reduced tillage systems on CH_4 and N_2O emissions from agricultural fields: a global meta-analysis. *PLoS One*, *13*(5), e0196703. https://doi.org/10.1371/journal.pone.0196703

Forbes, M. S., Broos, K., Baldock, J. A., Gregg, A. L., & Wakelin, S. A. (2009). Environmental and edaphic drivers of bacterial communities involved in soil N-cycling. *Australian Journal of Soil Research*, *47*(4), 380-388. https://doi.org/10.1071/SR08126

Gaby, J. C., & Buckley, D. H. (2014). A comprehensive aligned *nifH* gene database: a multipurpose tool for studies of nitrogen-fixing bacteria. *Database (Oxford)*, *2014*, bau001. https://doi.org/10.1093/database/bau001

Galloway, J. N., Townsend, A. R., Erisman, J. W., Bekunda, M., Cai, Z., Freney, J. R., Martinelli, L. A., Seitzinger, S. P., & Sutton, M. A. (2008). Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science*, *320*(5878), 889-892. https://doi.org/10.1126/science.1136674

Hauggaard-Nielsen, H., Lachouani, P., Knudsen, M. T., Ambus, P., Boelt, B., & Gislum, R. (2016). Productivity and carbon footprint of perennial grass-forage legume intercropping strategies with high or low nitrogen fertilizer input. *Science of the*

Total Environment, *541*, 1339-1347. https://doi.org/10.1016/j.scitotenv.2015.10.013

Huang, T., Gao, B., Hu, X. K., Lu, X., Well, R., Christie, P., Bakken, L. R., & Ju, X. T. (2014). Ammonia-oxidation as an engine to generate nitrous oxide in an intensively managed calcareous fluvo-aquic soil. *Scientific Reports*, *4*, 3950. https://doi.org/10.1038/srep03950

Jia, Z., & Conrad, R. (2009). Bacteria rather than archaea dominate microbial ammonia oxidation in an agricultural soil. *Environmental Microbiology*, *11*(7), 1658-1671. https://doi.org/10.1111/j.1462-2920.2009.01891.x

Jones, C. M., Graf, D. R., Bru, D., Philippot, L., & Hallin, S. (2013). The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *The ISME Journal*, *7*(2), 417-426. https://doi.org/10.1038/ismej.2012.125

Juhanson, J., Hallin, S., Söderström, M., Stenberg, M., & Jones, C. M. (2017). Spatial and phyloecological analyses of *nosZ* genes underscore niche differentiation amongst terrestrial N_2O reducing communities. *Soil Biology and Biochemistry*, *115*, 82-91. https://doi.org/10.1016/j.soilbio.2017.08.013

Khalil, K., Mary, B., & Renault, P. (2004). Nitrous oxide production by nitrification and denitrification in soil aggregates as affected by O2 concentration. *Soil Biology and Biochemistry*, *36*(4), 687-699. https://doi.org/10.1016/j.soilbio.2004.01.004

Kuypers, M. M. M., Marchant, H. K., & Kartal, B. (2018). The microbial nitrogen-cycling network. *Nature Reviews: Microbiology*, *16*(5), 263-276. https://doi.org/10.1038/nrmicro.2018.9

LeBauer, D. S., & Treseder, K. K. (2008). Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology*, *89*(2), 371-379. https://doi.org/Doi 10.1890/06-2057.1

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G. W., Prosser, J. I., Schuster, S. C., & Schleper, C. (2006). Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature*, *442*(7104), 806-809. https://doi.org/10.1038/nature04983

Levy-Booth, D. J., Prescott, C. E., & Grayston, S. J. (2014). Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. *Soil Biology and Biochemistry*, *75*, 11-25. https://doi.org/10.1016/j.soilbio.2014.03.021

Li, S., Jiang, X., Wang, X., & Wright, A. L. (2015). Tillage effects on soil nitrification and the dynamic changes in nitrifying microorganisms in a subtropical rice-based ecosystem: A long-term field study. *Soil and Tillage Research*, *150*, 132-138. https://doi.org/10.1016/j.still.2015.02.005

Lindsay, E. A., Colloff, M. J., Gibb, N. L., & Wakelin, S. A. (2010). The abundance of microbial functional genes in grassy woodlands is influenced more by soil nutrient enrichment than by recent weed invasion or livestock exclusion. *Applied and Environmental Microbiology*, *76*(16), 5547-5555. https://doi.org/10.1128/AEM.03054-09

Linn, D. M., & Doran, J. W. (1984). Aerobic and anaerobic microbial populations in no-

till and plowed soils. *Soil Science Society of America Journal*, *48*(4), 794-799. https://doi.org/10.2136/sssaj1984.03615995004800040019x

Liu, R., Hu, H., Suter, H., Hayden, H. L., He, J., Mele, P., & Chen, D. (2016). Nitrification is a primary driver of nitrous oxide production in laboratory microcosms from different land-use soils. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01373

Liu, S., Coyne, M. S., & Grove, J. H. (2017). Long-term tillage and nitrogen fertilization: consequences for nitrifier density and activity. *Applied Soil Ecology*, *120*, 121-127. https://doi.org/10.1016/j.apsoil.2017.07.034

Liu, S., Coyne, M. S., Grove, J. H., & Flythe, M. D. (2018). Nitrogen, season, and tillage management influence ammonia oxidizing bacterial communities in long-term maize. *Applied Soil Ecology*, *129*, 98-106. https://doi.org/10.1016/j.apsoil.2018.05.002

López-Vázquez, A., Cadena-Zapata, M., Campos-Magaña, S., Zermeño-Gonzalez, A., & Mendez-Dorado, M. (2019). Comparison of energy used and effects on bulk density and yield by tillage systems in a semiarid condition of Mexico. *Agronomy*, *9*(4), 189. https://doi.org/10.3390/agronomy9040189

Maul, J. E., Cavigelli, M. A., Vinyard, B., & Buyer, J. S. (2019). Cropping system history and crop rotation phase drive the abundance of soil denitrification genes *nirK*, *nirS* and *nosZ* in conventional and organic grain agroecosystems. *Agriculture, Ecosystems & Environment*, *273*, 95-106. https://doi.org/10.1016/j.agee.2018.11.022

Mbuthia, L. W., Acosta-Martínez, V., DeBruyn, J., Schaeffer, S., Tyler, D., Odoi, E., Mpheshea, M., Walker, F., & Eash, N. (2015). Long term tillage, cover crop, and fertilization effects on microbial community structure, activity: implications for soil quality. *Soil Biology and Biochemistry*, *89*, 24-34. https://doi.org/10.1016/j.soilbio.2015.06.016

Norton, J. M., Low, J. M., & Klotz, M. G. (1996). The gene encoding ammonia monooxygenase subunit A exists in three nearly identical copies in *Nitrosospira* sp. NpAV. *FEMS Microbiology Letters*, *139*(2-3), 181-188. https://doi.org/10.1016/0378-1097(96)00139-5

Nouri, A., Lee, J., Yin, X., Tyler, D. D., & Saxton, A. M. (2019). Thirty-four years of no-tillage and cover crops improve soil quality and increase cotton yield in Alfisols, Southeastern USA. *Geoderma*, *337*, 998-1008. https://doi.org/10.1016/j.geoderma.2018.10.016

Orellana, L. H., Rodriguez, R. L., Higgins, S., Chee-Sanford, J. C., Sanford, R. A., Ritalahti, K. M., Loffler, F. E., & Konstantinidis, K. T. (2014). Detecting nitrous oxide reductase (NosZ) genes in soil metagenomes: method development and implications for the nitrogen cycle. *mBio*, *5*(3), e01193-01114. https://doi.org/10.1128/mBio.01193-14

Orr, C. H., James, A., Leifert, C., Cooper, J. M., & Cummings, S. P. (2011). Diversity and activity of free-living nitrogen-fixing bacteria and total bacteria in organic and conventionally managed soils. *Applied and Environmental Microbiology*, *77*(3), 911-919. https://doi.org/10.1128/AEM.01250-10

Ouyang, Y., Evans, S. E., Friesen, M. L., & Tiemann, L. K. (2018). Effect of nitrogen fertilization on the abundance of nitrogen cycling genes in agricultural soils: a meta-analysis of field studies. *Soil Biology and Biochemistry*, *127*, 71-78. https://doi.org/10.1016/j.soilbio.2018.08.024

Ouyang, Y., Norton, J. M., & Stark, J. M. (2017). Ammonium availability and temperature control contributions of ammonia oxidizing bacteria and archaea to nitrification in an agricultural soil. *Soil Biology and Biochemistry*, *113*, 161-172. https://doi.org/10.1016/j.soilbio.2017.06.010

Ouyang, Y., Norton, J. M., Stark, J. M., Reeve, J. R., & Habteselassie, M. Y. (2016). Ammonia-oxidizing bacteria are more responsive than archaea to nitrogen source in an agricultural soil. *Soil Biology and Biochemistry*, *96*, 4-15. https://doi.org/10.1016/j.soilbio.2016.01.012

Parkin, T., & Kaspar, T. (2006). Nitrous oxide emissions from corn-soybean systems in the midwest. *Journal of environmental quality*, *35*, 1496-1506. https://doi.org/10.2134/jeq2005.0183

Pereira, E. I. P., Suddick, E. C., Mansour, I., Mukome, F. N. D., Parikh, S. J., Scow, K., & Six, J. (2015). Biochar alters nitrogen transformations but has minimal effects on nitrous oxide emissions in an organically managed lettuce mesocosm. *Biology and Fertility of Soils*, *51*(5), 573-582. https://doi.org/10.1007/s00374-015-1004-5

Pereira, E. S. M. C., Schloter-Hai, B., Schloter, M., van Elsas, J. D., & Salles, J. F. (2013). Temporal dynamics of abundance and composition of nitrogen-fixing communities across agricultural soils. *PLoS One*, *8*(9), e74500. https://doi.org/10.1371/journal.pone.0074500

Reardon, C. L., Gollany, H. T., & Wuest, S. B. (2014). Diazotroph community structure and abundance in wheat-fallow and wheat-pea crop rotations. *Soil Biology and Biochemistry*, *69*, 406-412. https://doi.org/https://doi.org/10.1016/j.soilbio.2013.10.038

Sanchez, C., & Minamisawa, K. (2018). Redundant roles of *Bradyrhizobium oligotrophicum* Cu-type (NirK) and cd_1-type (NirS) nitrite reductase genes under denitrifying conditions. *FEMS Microbiology Letters*, *365*(5), fny015. https://doi.org/10.1093/femsle/fny015

Sanford, R. A., Wagner, D. D., Wu, Q., Chee-Sanford, J. C., Thomas, S. H., Cruz-Garcia, C., Rodriguez, G., Massol-Deya, A., Krishnani, K. K., Ritalahti, K. M., Nissen, S., Konstantinidis, K. T., & Loffler, F. E. (2012). Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proc Natl Acad Sci U S A*, *109*(48), 19709-19714. https://doi.org/10.1073/pnas.1211238109

Segal, L. M., Miller, D. N., McGhee, R. P., Loecke, T. D., Cook, K. L., Shapiro, C. A., & Drijber, R. A. (2017). Bacterial and archaeal ammonia oxidizers respond differently to long-term tillage and fertilizer management at a continuous maize site. *Soil and Tillage Research*, *168*, 110-117. https://doi.org/10.1016/j.still.2016.12.014

Shen, J., Inatomi, M., Hajima, T., & Ito, A. (2019). Fraction of nitrous oxide production in nitrification and its effect on total soil emission: A meta-analysis and global-scale sensitivity analysis using a process-based model. *PLoS One*, *14*(7),

e0219159. https://doi.org/10.1371/journal.pone.0219159
Soares, J. R., Cassman, N. A., Kielak, A. M., Pijl, A., Carmo, J. B., Lourenco, K. S., Laanbroek, H. J., Cantarella, H., & Kuramae, E. E. (2016). Nitrous oxide emission related to ammonia-oxidizing bacteria and mitigation options from N fertilization in a tropical soil. *Scientific Reports*, *6*, 30349. https://doi.org/10.1038/srep30349
Tatti, E., Goyer, C., Burton, D., Wertz, S., Zebarth, B., Chantigny, M., & Filion, M. (2015). Tillage management and seasonal effects on denitrifier community abundance, gene expression and structure over winter. *Microbial Ecology*, *70*(3), 795-808. https://doi.org/10.1007/s00248-015-0591-x
Theodorakopoulos, N., Lognoul, M., Degrune, F., Broux, F., Regaert, D., Muys, C., Heinesch, B., Bodson, B., Aubinet, M., & Vandenbol, M. (2017). Increased expression of bacterial *amoA* during an N_2O emission peak in an agricultural field. *Agriculture, Ecosystems & Environment*, *236*, 212-220. https://doi.org/10.1016/j.agee.2016.12.002
Tsiknia, M., Paranychianakis, N. V., Varouchakis, E. A., Nikolaidis, N. P., & Sessitsch, A. (2015). Environmental drivers of the distribution of nitrogen functional genes at a watershed scale. *FEMS Microbiology Ecology*, *91*(6). https://doi.org/10.1093/femsec/fiv052
van Kessel, C., Venterea, R., Six, J., Adviento-Borbe, M. A., Linquist, B., & van Groenigen, K. J. (2013). Climate, duration, and N placement determine N_2O emissions in reduced tillage systems: a meta-analysis. *Global Change Biology*, *19*(1), 33-44. https://doi.org/10.1111/j.1365-2486.2012.02779.x
Wang, C., Zheng, M., Song, W., Wen, S., Wang, B., Zhu, C., & Shen, R. (2017). Impact of 25 years of inorganic fertilization on diazotrophic abundance and community structure in an acidic soil in southern China. *Soil Biology and Biochemistry*, *113*, 240-249. https://doi.org/10.1016/j.soilbio.2017.06.019
Wang, H., Li, X., Li, X., Li, X., Wang, J., & Zhang, H. (2017). Changes of microbial population and N-cycling function genes with depth in three Chinese paddy soils. *PLoS One*, *12*(12), e0189506. https://doi.org/10.1371/journal.pone.0189506
Wang, J., Chadwick, D. R., Cheng, Y., & Yan, X. (2018). Global analysis of agricultural soil denitrification in response to fertilizer nitrogen. *Science of the Total Environment*, *616-617*, 908-917. https://doi.org/10.1016/j.scitotenv.2017.10.229
Wang, J., & Zou, J. (2020). No-till increases soil denitrification via its positive effects on the activity and abundance of the denitrifying community. *Soil Biology and Biochemistry*, *142*, 107706. https://doi.org/https://doi.org/10.1016/j.soilbio.2020.107706
Ward, B. B., & Bouskill, N. J. (2011). The utility of functional gene arrays for assessing community composition, relative abundance, and distribution of ammonia-oxidizing bacteria and archaea. *Methods in Enzymology*, *496*, 373-396. https://doi.org/10.1016/B978-0-12-386489-5.00015-4
White, C. J., & Lehnert, N. (2016). Is there a pathway for N2O production from hydroxylamine oxidoreductase in ammonia-oxidizing bacteria? *Proceedings of the National Academy of Sciences*, *113*(51), 14474-14476.

https://doi.org/10.1073/pnas.1617953114
White, P. J., Crawford, J. W., Díaz Álvarez, M. C., & García Moreno, R. (2012). Soil management for sustainable agriculture. *Applied and Environmental Soil Science*, *2012*, 1-3. https://doi.org/10.1155/2012/850739
Yang, D., Xiao, X., He, N., Zhu, W., Liu, M., & Xie, G. (2020). Effects of reducing chemical fertilizer combined with organic amendments on ammonia-oxidizing bacteria and archaea communities in a low-fertility red paddy field. *Environmental science and pollution research international*, *27*(23), 29422-29432. https://doi.org/10.1007/s11356-020-09120-5
Ying, J., Li, X., Wang, N., Lan, Z., He, J., & Bai, Y. (2017). Contrasting effects of nitrogen forms and soil pH on ammonia oxidizing microorganisms and their responses to long-term nitrogen fertilization in a typical steppe ecosystem. *Soil Biology and Biochemistry*, *107*, 10-18. https://doi.org/10.1016/j.soilbio.2016.12.023
Yu, R., & Chandran, K. (2010). Strategies of *Nitrosomonas europaea* 19718 to counter low dissolved oxygen and high nitrite concentrations. *BMC Microbiology*, *10*, 70. https://doi.org/10.1186/1471-2180-10-70
Zhang, X., Jia, X., Wu, H., Li, J., Yan, L., Wang, J., Li, Y., & Kang, X. (2020). Depression of soil nitrogen fixation by drying soil in a degraded alpine peatland. *Science of the Total Environment*, *747*, 141084. https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.141084
Zhu, X., Burger, M., Doane, T. A., & Horwath, W. R. (2013). Ammonia oxidation pathways and nitrifier denitrification are significant sources of N_2O and NO under low oxygen availability. *Proceedings of the National Academy of Sciences, USA*, *110*(16), 6328-6333. https://doi.org/10.1073/pnas.1219993110

Appendix

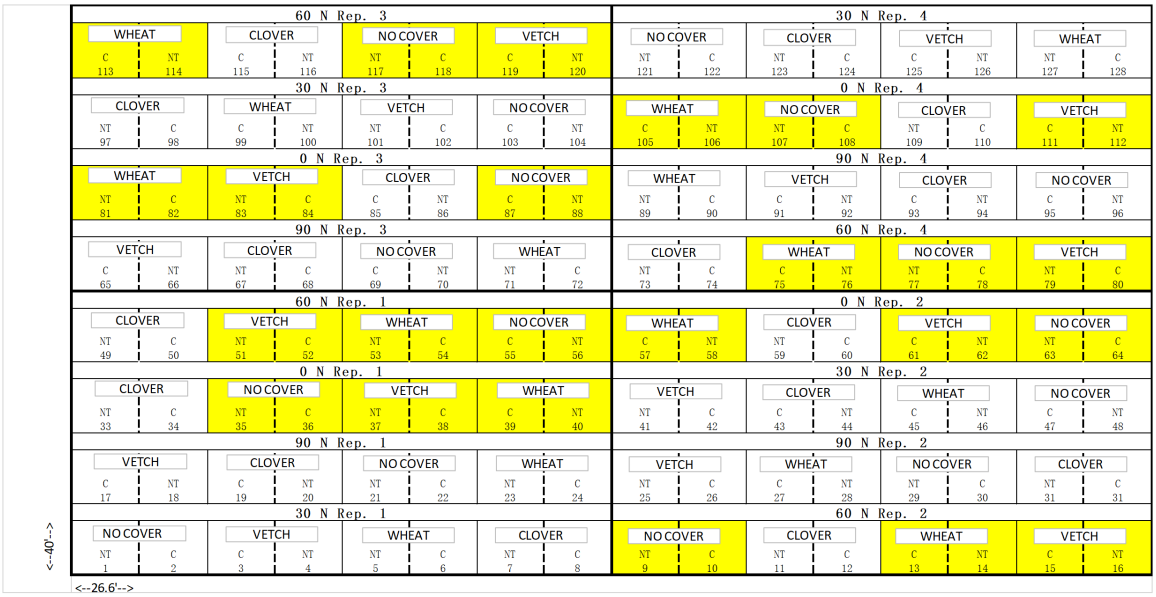

Figure 9. Schematic diagram of the experimental cotton plots located at the University of Tennessee West Tennessee Research and Education Center in Jackson, Tennessee, USA.

Appendix method. Description of methods used for soil properties measurements.

1) Bulk soil total N and C, soil pH, and soil water content

A subsample of 2 mm-sieved fresh soil was oven dried at 65 °C, ground to powder, and analyzed for total N and total C using dry combustion (Stable Isotope Facility; University of California-Davis). A subsample of fresh soil was added to sterile water (1:2 extraction ratio), vortexed, and allowed to settle before measuring for pH (Ultrabasic, Denver Instrument, Bohemia, NY, USA). Another subsample of fresh soil was weighed, then heated at 105 ℃ for 48 hours and weighed again. The difference in weight was considered to be the weight of the water in the samples and the gravimetric soil water content (SWC) was calculated as SWC (%) = [(fresh weight soil – dry weight soil)/dry weight soil] × 100%.

2) Total extractable C, total extractable N, ammonium, and nitrate measurement

For total extractable C (TEC) and total extractable N (TEN) measurement, 10 g field moist soil was extracted in 40 ml of 0.5M K_2SO_4 extract solution by shaking on a reciprocating shaker at 150 rpm for 4 h. The solution was then centrifuged at 3803 rcf for 5 minutes and filtered through a Whatman GF/B filter (0.9 to 1.2 μm pore size) into clean container to collect the filtrate. Filtered extracts were kept under –20℃ until further analysis. Soil microbial biomass N (MBN) and microbial biomass C (MBC) were measured by a chloroform slurry method. For each field sample, one soil subsample was chloroform-exposed and another subsample non-chloroform-exposed, then both were extracted with 0.5 M K_2SO_4 as described above. Filtered extracts were kept under –20℃ until further analysis. Both TEC and TEN concentrations in chloroform-exposed and non-chloroform-exposed soil subsamples were measured using an Organic Carbon analyzer modified with a nitrogen chemiluminescence detector (CLD) for combustion analysis (Aurora 1030W, OI Analytical, College Station, Taxes, USA). Non-chloroform-exposed soil extracts were analyzed for ammonium and nitrate using microplate-based spectrophotometric determinations. Soil extracts were pipetted into 96-well plates. Reagents were added to each well. After reactions complete, the plate was read on a microplate reader (Synergy HT, BioTek, Winooski, VT, USA).

3) Incubation for net N mineralization and nitrification rates

Fresh soils were incubated in mason jars at 25 ℃ for 7 days. The incubated soils were

extracted and analyzed for soil ammonium and nitrate concentrations as described in Method S2 to determine post-incubation soil ammonium and nitrate concentrations ($ammonium_{post\text{-}incubation}$ and $nitrate_{post\text{-}incubation}$), which were then used for N transformation calculations described in Method S4.

4) Soil N and C pool and transformation rate calculations

Soil total N (TN) and total C (TN) was evaluated as concentration (mg N or C g^{-1} soil). Soil microbial biomass N (MBN, μg N g^{-1} soil) concentration was calculated as MBN = $TEN_{chloroform\text{-}exposed} - TEN_{non\text{-}chloroform\text{-}exposed}$. Soil microbial biomass C (MBC, μg C g^{-1} soil) concentration was calculated as MBC = $TEC_{chloroform\text{-}exposed} - TEC_{non\text{-}chloroform\text{-}exposed}$. Potential N mineralization (PNM, μg N g^{-1} soil d^{-1}) rate was calculated as PNM = ($ammonium_{post\text{-}incubation} - ammonium_{pre\text{-}incubation}$)/7 day. Potential N nitrification (PNN, μg N g^{-1} soil d^{-1}) rate was calculated as PNN = $nitrate_{post\text{-}incubation} - nitrate_{pre\text{-}incubation}$)/7 day.

5) Gas sample collection and analysis

Gas emissions were measured and calculated using standardized sampling designs and data processing protocols used by the USDA-ARS Greenhouse Reduction through Agricultural Carbon Enhancement Network (GRACEnet). Gas measurements were taken with static vented chambers using a stratified sampling design in which gases were sampled with syringes and injected into evacuated vials at four evenly spaced time-points over 30 minutes (0, 10, 20, 30 minutes). To account for diurnal variability, gas samples were collected at times of the day closely corresponding to the daily average temperature (midmorning, early evening). Environmental conditions, such as air temperature, soil temperature, and soil moisture, were also measured at the time of collection.

Concentrations of N_2O in air samples from the sampling chamber headspace were measured at the USDA-ARS laboratory in Lincoln, NE using a gas chromatograph equipped with an electron capture detector. Soil gas emission rates were calculated as the change in headspace gas concentration over time within the enclosed chamber volume.

Table 3. Primers and PCR conditions used for qPCR and qRT-PCR.

Gene	Primers	Sequence (5'-3')	Reaction parameters
amoA (AOB†)	*amoA* 1F	GGGGTTTCTACTGGTGGT	95 ℃ for 3 min × 1 cycle; (94 ℃ for 1 min, 56 ℃ for 45 s, 72 ℃ for 1 min) × 40 cycles; 72 ℃ for 10 min × 1 cycle
	amoA 2R	CCCCTCKGSAAAGCCTTCTTC	
nifH	IGK3	GCIWTHTAYGGIAARGGIGGIATHGGIAA	95 ℃ for 10 min × 1 cycle; (95 ℃ for 30 s, 58 ℃ for 1 min, 72 ℃ for 1 min) × 40 cycles; 72 ℃ for 10 min × 1 cycle
	DVV	ATIGCRAAICCICCRCAIACIACRTC	
nirS	cd3aF	GTSAACGTSAAGGARACSGG	95 ℃ for 10 min × 1 cycle; (94 ℃ for 30 s, 57 ℃ for 1 min, 72 ℃ for 1 min) × 40 cycles; 72 ℃ for 10 min × 1 cycle
	R3cd	GASTTCGGRTGSGTCTTGA	
nirK	F1aCu	ATCATGGTSCTGCCGCG	94 ℃ for 2 min × 1 cycle; (94 ℃ for 30 s, 58 ℃ for 1 min, 72 ℃ for 1 min) × 40 cycles; 72 ℃ for 10 min × 1 cycle
	R3Cu	GCCTCGATCAGRTTGTGGTT	
nosZ	*nosZ*-I F	CGCRACGGCAASAAGGTSMSSGT	94 ℃ for 5 min × 1 cycle; (94 ℃ for 40 s, 60 ℃ for 40 s, 72 ℃ for 1 min) × 40 cycles; 72 ℃ for 10 min × 1 cycle
	nosZ-I R	CAKRTGCAKSGCRTGGCAGAA	

†AOB = Ammonia oxidizing bacteria.

Table 4. Results of mixed model ANOVA (based on GLIMMIX procedure in SAS) testing effects of agricultural season and soil management practices on soil parameters. The significance was shown with bold fonts and asterisks. F-values are reported.

Factor	SWC†	pH	NO_3-N	NH_4-N	TEC	TEN	TC	TN	MBC	MBN	C:N	N_2O-N
Season (S)	**401.85****	**17.90****	**552.04****	**34.86****	**107.33****	**265.32****	**34.48****	**31.34****	**60.07****	**13.01****	**11.93****	**15.27****
Tillage (T)	**8.52****	2.55	2.53	3.80	**13.13*****	0.16	**18.94****	**17.71****	1.77	0.01	3.53	0.74
Cover (C)	**5.43****	2.77	**203.02****	**7.45*****	**14.09*****	**96.02*****	**24.98****	**46.25****	**3.73***	**5.05****	1.76	**20.37****
Nitrogen (N)	**12.67*****	0.47	**4.71***	0.24	3.51	0.56	**47.47****	**65.04****	0.90	0.16	1.54	**5.49***
S*T	2.03	1.44	0.43	**8.07*****	**2.85***	1.20	**4.78****	**4.20****	**3.72***	1.84	0.87	0.55
S*C	2.15	0.74	**89.22*****	**8.61*****	1.54	**33.23*****	0.84	0.30	**2.26***	0.74	0.82	**7.01*****
S*N	0.80	0.64	1.02	1.67	0.48	0.42	0.43	0.11	0.07	**3.57***	0.55	1.78
T*C	2.74	2.07	0.13	1.35	1.35	0.24	0.61	0.63	0.48	0.40	0.25	1.28
T*N	0.31	2.90	0.02	2.13	0.05	1.18	1.04	1.03	0.40	0.16	0.09	1.01
C*N	1.22	**7.67*****	0.35	2.32	0.88	0.06	**3.70***	**3.94***	0.42	1.44	1.13	0.22
T*C*N	1.56	0.61	0.62	3.06	0.01	0.51	0.62	0.54	1.76	0.45	0.09	3.06
S*T*C	0.51	0.49	1.00	0.88	0.65	0.19	0.68	0.45	0.41	1.53	0.61	1.18
S*T*N	0.24	0.81	2.05	0.15	0.09	0.36	1.27	0.66	0.16	1.78	0.54	0.88
S*C*N	0.30	0.91	0.27	0.83	0.71	0.50	0.61	0.31	1.08	1.56	0.40	1.66
S*T*C*N	2.22	0.23	0.33	0.46	0.17	0.35	1.49	1.37	1.61	1.40	0.68	0.58

†SWC = soil water content; TEC = total extractable carbon; TEN = total extractable nitrogen; TC = total organic carbon; TN = total nitrogen; MBC = microbial biomass carbon; MBN = microbial biomass nitrogen.

Table 5. Mean values of soil parameters in relation to agricultural season and soil management practices. Only treatments or treatment combinations that significantly affected at least one parameter were shown. Different lowercase letters indicate significant differences between treatment levels within groups. The letters were only shown in groups that have significant effects. Means were compared using Least Significant Difference (LSD) tests ($\alpha = 0.05$).

Treatment†		SWC ‡	pH	NO_3-N	NH_4-N	TEC	TEN	TC	TN	MBC	MBN	C:N	N_2O-N	Nitrification		N Mineralization	
														Field	Incubate	Field	Incubate
Season	Apr	0.17^{c}	5.76^{c}	3.43^{b}	1.40^{c}	128.70^{a}	8.08^{b}	9.97^{c}	1.15^{c}	1139.07^{a}	31.00^{b}	8.62^{c}	0.45^{b}	-0.05^{c}	0.12^{b}	-0.04^{b}	0.14^{b}
	May	0.19^{b}	5.93^{b}	14.53^{a}	1.65^{b}	53.96^{c}	27.17^{a}	11.35^{b}	1.29^{b}	431.33^{b}	39.35^{a}	8.80^{bc}	1.18^{a}	0.27^{a}	0.39^{a}	0.27^{a}	0.32^{a}
	Oct	0.05^{d}	6.27^{a}	2.32^{c}	2.02^{a}	97.46^{b}	9.54^{b}	13.48^{a}	1.40^{a}	508.45^{b}	20.12^{c}	9.54^{a}	1.35^{a}	-0.09^{d}	0.10^{b}	-0.09^{c}	0.12^{b}
	Nov	0.23^{a}	6.23^{a}	1.99^{c}	0.81^{d}	45.74^{c}	5.81^{c}	10.16^{c}	1.12^{c}	426.65^{b}	27.05^{b}	9.07^{b}	0.68^{b}	-0.01^{b}	0.17^{b}	-0.03^{b}	0.19^{b}
Tillage	NT	0.15^{b}	6.00	5.38	1.55	88.26^{a}	12.87	11.84^{a}	1.29^{a}	600.39	29.39	9.12	0.96	0.03	0.19	0.02	0.18
	CT	0.17^{a}	6.09	5.85	1.38	74.68^{b}	12.49	10.64^{b}	1.19^{b}	653.32	29.58	8.90	0.87	0.03	0.21	0.03	0.20
Cover	NC	0.15^{b}	6.07	4.03^{b}	1.30^{b}	74.26^{b}	10.14^{b}	10.49^{b}	1.17^{b}	589.13^{b}	25.74^{b}	8.89	0.68^{b}	0.02^{b}	0.11^{b}	0.02^{b}	0.12^{b}
	V	0.16^{a}	5.96	9.16^{a}	1.70^{a}	95.53^{a}	18.51^{a}	12.61^{a}	1.40^{a}	709.26^{a}	33.33^{a}	8.98	1.40^{a}	0.07^{a}	0.36^{a}	0.07^{a}	0.34^{a}
	W	0.17^{a}	6.12	3.55^{c}	1.41^{b}	74.61^{b}	9.34^{b}	10.62^{b}	1.15^{b}	581.88^{b}	29.20^{b}	9.15	0.66^{b}	0.00^{b}	0.12^{b}	0.00^{b}	0.12^{b}
Nitrogen	0N	0.15^{b}	6.01	5.36^{b}	1.43	77.16	12.38	10.19^{b}	1.13^{b}	644.81	29.00	8.84	0.78^{b}	0.03	0.16^{b}	0.02	0.14^{b}
	67N	0.17^{a}	6.03	5.80^{a}	1.49	84.97	12.84	12.19^{a}	1.34^{a}	602.64	29.65	9.08	1.04^{a}	0.03	0.24^{a}	0.03	0.24^{a}
Season * Tillage	Apr-NT	0.17	5.66	3.10	1.42^{b}	138.25^{a}	7.76	10.32^{cd}	1.19^{de}	983.93^{b}	26.98	8.63	0.58	-0.06	0.10	-0.06	0.13
	Apr-CT	0.17	5.87	3.78	1.38^{b}	119.16^{b}	8.39	9.62^{c}	1.10^{e}	1294.21^{a}	34.84	8.62	0.33	-0.04	0.14	-0.03	0.16
	May-NT	0.18	5.93	14.11	1.57^{b}	56.78^{d}	26.78	11.94^{b}	1.34^{b}	418.48^{c}	42.37	8.93	1.13	0.26	0.34	0.26	0.25
	May-CT	0.20	5.93	14.94	1.72^{b}	51.14^{d}	27.56	10.77^{c}	1.24^{cd}	444.19^{c}	36.33	8.68	1.23	0.27	0.45	0.27	0.39
	Oct-NT	0.04	6.30	2.22	2.47^{a}	111.78^{b}	10.41	14.90^{a}	1.52^{a}	555.48^{c}	21.44	9.79	1.37	-0.09	0.10	-0.08	0.12
	Oct-CT	0.05	6.25	2.43	1.57^{b}	83.14^{c}	8.68	12.05^{b}	1.29^{bc}	463.39^{c}	18.73	9.29	1.34	-0.09	0.10	-0.09	0.12
	Nov-NT	0.23	6.13	1.95	0.75^{c}	46.21^{d}	6.30	10.18^{cd}	1.12^{e}	441.82^{c}	26.13	9.12	0.77	0.00	0.21	-0.04	0.22
	Nov-CT	0.24	6.33	2.03	0.86^{c}	45.27^{d}	5.32	10.14^{cd}	1.13^{e}	411.48^{c}	27.97	9.01	0.58	-0.01	0.14	-0.02	0.15
Season * Cover	Apr-NC	0.16	5.74	2.63^{ef}	0.81^{e}	118.18	7.43^{ef}	9.15	1.08	975.97^{b}	26.87	8.43	0.40^{c}	-0.05^{ef}	-0.09^{g}	-0.05^{ef}	0.01^{fg}
	Apr-V	0.18	5.71	4.78^{d}	2.33^{a}	155.08	10.75^{cd}	10.80	1.28	1390.12^{a}	37.90	8.44	0.55^{c}	-0.03^{de}	0.51^{a}	-0.01^{d}	0.44^{ab}
	Apr-W	0.18	5.84	2.85^{ef}	1.07^{de}	112.85	6.03^{efg}	9.96	1.09	1051.12^{b}	26.96	9.00	0.42^{c}	-0.07^{f}	-0.05^{fg}	-0.07^{f}	-0.03^{g}
	May-NC	0.18	6.02	10.00^{b}	1.77^{bc}	49.68	19.49^{b}	10.80	1.22	439.70^{cd}	35.85	8.83	0.53^{c}	0.18^{b}	0.31^{b}	0.20^{b}	0.22^{cde}
	May-V	0.18	5.69	25.39^{a}	1.39^{cd}	63.81	43.45^{a}	12.60	1.46	398.47^{cd}	41.20	8.67	2.40^{a}	0.49^{a}	0.56^{a}	0.46^{a}	0.49^{a}
	May-W	0.21	6.07	8.19^{c}	1.78^{bc}	48.39	18.57^{b}	10.66	1.19	455.82^{cd}	40.99	8.91	0.61^{c}	0.13^{c}	0.31^{bc}	0.14^{c}	0.25^{cd}
	Oct-NC	0.04	6.28	1.67^{fg}	1.82^{b}	86.30	8.03^{def}	12.62	1.34	482.56^{cd}	15.08	9.33	1.38^{b}	-0.06^{f}	0.05^{efg}	-0.06^{f}	0.14^{defg}
	Oct-V	0.06	6.25	3.62^{de}	2.17^{ab}	110.27	11.67^{c}	15.28	1.58	612.53^{c}	23.25	9.64	1.86^{b}	-0.16^{g}	0.17^{bcde}	-0.15^{g}	0.13^{defg}
	Oct-W	0.05	6.29	1.58^{g}	2.06^{ab}	95.81	8.93^{cde}	12.53	1.29	425.06^{cd}	21.94	9.64	0.82^{c}	-0.05^{ef}	0.07^{def}	-0.05^{def}	0.08^{efg}
	Nov-NC	0.23	6.23	1.82^{fg}	0.80^{e}	42.87	5.45^{fg}	9.40	1.05	458.30^{cd}	24.62	8.97	0.42^{c}	0.00^{d}	0.16^{cde}	-0.02^{de}	0.10^{defg}
	Nov-V	0.24	6.18	2.86^{ef}	0.89^{e}	52.95	8.17^{def}	11.76	1.29	435.91^{cd}	30.96	9.19	0.80^{c}	-0.03^{de}	0.20^{bcd}	-0.03^{def}	0.31^{bc}
	Nov-W	0.23	6.28	1.29^{g}	0.73^{e}	41.40	3.81^{g}	9.32	1.04	385.73^{cd}	25.57	9.05	0.80^{c}	0.00^{d}	0.16^{cde}	-0.03^{def}	0.15^{cdef}

Table 5. Continued.

Treatment[†]		SWC[‡]	pH	NO_3-N	NH_4-N	TEC	TEN	TC	TN	MBC	MBN	C:N	N_2O-N	Nitrification		N Mineralization	
														Field	Incubate	Field	Incubate
Season * Nitrogen	Apr-0N	0.16	5.83	3.57	1.23	121.78	7.64	8.90	1.05	1146.55	25.13^{de}	8.46	0.35	-0.05	0.00^{f}	-0.04	0.02
	Apr-67N	0.18	5.70	3.30	1.58	135.63	8.51	11.04	1.25	1131.59	36.61^{bc}	8.78	0.56	-0.05	0.25^{bc}	-0.04	0.26
	May-0N	0.18	5.89	14.21	1.73	49.96	27.45	10.65	1.21	468.34	44.07^{a}	8.84	1.25	0.26	0.34^{ab}	0.26	0.26
	May-67N	0.20	5.97	14.84	1.56	57.96	26.89	12.06	1.37	394.32	34.63^{b}	8.76	1.12	0.27	0.45^{a}	0.27	0.38
	Oct-0N	0.05	6.32	1.80	2.00	96.45	9.41	12.39	1.31	539.85	21.53^{de}	9.42	1.08	-0.09	0.08^{ef}	-0.09	0.11
	Oct-67N	0.05	6.23	2.80	2.04	98.47	9.68	14.57	1.50	478.36	18.77^{e}	9.66	1.62	-0.09	0.12^{de}	-0.08	0.13
	Nov-0N	0.22	6.23	1.71	0.82	43.64	5.35	9.24	1.03	447.00	25.37^{cde}	9.02	0.48	0.00	0.21^{cd}	-0.03	0.16
	Nov-67N	0.24	6.23	2.27	0.79	47.84	6.28	11.08	1.22	406.30	28.73^{bcd}	9.11	0.87	-0.02	0.14^{cde}	-0.03	0.22
Cover * Nitrogen	NC-0N	0.14	6.04^{bc}	3.78	1.37	68.41	10.04	9.47^{c}	1.08^{d}	638.84	27.72	8.71	0.59	0.02	0.09	0.02	0.08
	NC-67N	0.16	6.10^{ab}	4.28	1.23	80.10	10.24	11.52^{b}	1.26^{c}	539.42	23.69	9.07	0.78	0.02	0.12	0.01	0.15
	V-0N	0.16	5.87^{c}	8.74	1.55	90.92	18.18	12.16^{ab}	1.34^{b}	719.95	31.22	9.01	1.30	0.07	0.32	0.06	0.29
	V-67N	0.17	6.04^{bc}	9.59	1.84	100.14	18.84	13.06^{a}	1.46^{a}	698.57	35.44	8.95	1.51	0.07	0.40	0.07	0.39
	W-0N	0.16	6.30^{a}	3.55	1.43	74.55	9.24	9.26^{c}	1.02^{d}	594.21	28.91	9.09	0.48	0.00	0.06	-0.01	0.04
	W-67N	0.18	5.94^{bc}	3.55	1.39	74.68	9.43	11.97^{b}	1.29^{bc}	569.95	29.45	9.21	0.84	0.01	0.18	0.01	0.19

[†]NC = no cover; V = vetch; W = wheat; NT = no tillage; CT = conventional tillage; 0N = no fertilization; 67N = 67 kg N ha^{-1} fertilization.

[‡]SWC = soil water content; TEC = total extractable carbon; TEN = total extractable nitrogen; TC = total soil carbon; TN = total soil nitrogen.

Note: the unit for SWC is g H_2O g^{-1} dry weight soil (g H_2O gdw^{-1}); unit for NO_3-N, NH_4-N, TEN and MBN is μg N gdw^{-1}; unit for TN is mg N gdw^{-1}; unit for TEC and MBC is μg C gdw^{-1}; unit for TC is mg C gdw^{-1}; unit for nitrification and N mineralization is μg N $gdw^{-1}d^{-1}$; unit for N_2O-N is g N $ha^{-1}d^{-1}$.

Table 6. Mean values of log-transformed relative abundances of N-cycle genes and their transcripts (normalized by 16S rRNA genes and 16S rRNA) in relation to agricultural season and soil management practices.

Treatment[†]		*nifH*		AOB[‡] *amoA*		*nirK*		*nirS*		*nosZ*	
		Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript
Season	April	-3.799^{a}	-6.676^{b}	-4.153^{b}	-7.164^{b}	-2.396^{a}	-5.420^{b}	-1.855^{b}	-5.160^{b}	-2.334^{b}	-4.901^{b}
	May	-4.088^{c}	-6.795^{b}	-4.077^{b}	-7.039^{b}	-2.575^{b}	-5.895^{c}	-2.005^{c}	-5.478^{c}	-2.472^{c}	-4.800^{b}
	October	-4.178^{c}	-6.721^{b}	-3.805^{a}	-7.125^{b}	-2.421^{a}	-5.878^{c}	-1.837^{b}	-5.182^{b}	-2.024^{a}	-4.655^{a}
	November	-3.944^{b}	-5.969^{a}	-4.507^{c}	-6.856^{a}	-2.358^{a}	-5.207^{a}	-1.705^{a}	-4.361^{a}	-2.327^{b}	-5.131^{c}
Cover	NC	-4.065	-6.538^{ab}	-4.389^{c}	-7.030^{b}	-2.464	-5.485^{a}	-1.900	-4.988^{a}	-2.334^{b}	-4.810^{a}
	V	-4.003	-6.461^{a}	-3.785^{a}	-6.696^{a}	-2.431	-5.466^{a}	-1.819	-4.879^{a}	-2.216^{a}	-4.722^{a}
	W	-3.939	-6.622^{b}	-4.233^{b}	-7.411^{c}	-2.418	-5.849^{b}	-1.832	-5.269^{b}	-2.317^{b}	-5.082^{b}
Tillage	NT	-4.079^{b}	-6.601^{b}	-4.132	-7.049	-2.428	-5.604	-1.890^{b}	-5.072	-2.300	-4.935^{b}
	CT	-3.925^{a}	-6.480^{a}	-4.140	-7.042	-2.448	-5.595	-1.811^{a}	-5.019	-2.279	-4.808^{a}
Nitrogen	0N	-4.004	-6.545	-4.128	-7.054	-2.459	-5.623	-1.917^{b}	-5.079	-2.351^{b}	-4.896
	67N	-4.001	-6.537	-4.143	-7.038	-2.416	-5.577	-1.784^{a}	-5.012	-2.227^{a}	-4.847
Tillage * Nitrogen	NT-0N	-4.099	-6.683^{c}	-4.141	-7.135^{b}	-2.459	-5.736^{b}	-1.959	-5.153	-2.365	-5.029^{b}
	NT-67N	-4.059	-6.520^{ab}	-4.123	-6.964^{a}	-2.396	-5.472^{a}	-1.821	-4.991	-2.234	-4.842^{a}
	CT-0N	-3.908	-6.406^{a}	-4.115	-6.974^{a}	-2.459	-5.509^{a}	-1.874	-5.004	-2.337	-4.763^{a}
	CT-67N	-3.943	-6.554^{b}	-4.164	-7.111^{b}	-2.436	-5.682^{b}	-1.747	-5.033	-2.220	-4.853^{a}
Season * Cover	Apr-NC	-3.857	-6.622^{b}	-4.457	-7.175^{cd}	-2.383	-5.242^{b}	-1.875	-5.131^{ef}	-2.378	-4.849^{bc}
	Apr-V	-3.821	-6.701^{bc}	-3.755	-6.836^{b}	-2.391	-5.339^{bc}	-1.826	-4.81^{cd}	-2.237	-4.763^{b}
	Apr-W	-3.718	-6.707^{bc}	-4.248	-7.482^{ef}	-2.415	-5.679^{de}	-1.863	-5.539^{gh}	-2.386	-5.090^{d}
	May-NC	-4.094	-6.965^{d}	-4.306	-7.361^{def}	-2.578	-6.055^{fg}	-2.078	-5.643^{h}	-2.547	-5.024^{cd}
	May-V	-4.056	-6.638^{b}	-3.761	-6.508^{a}	-2.578	-5.636^{de}	-1.932	-5.290^{fg}	-2.376	-4.512^{a}
	May-W	-4.115	-6.783^{bcd}	-4.165	-7.247^{cde}	-2.568	-5.992^{f}	-2.004	-5.502^{gh}	-2.494	-4.865^{bc}
	Oct-NC	-4.358	-6.646^{b}	-4.131	-7.083^{c}	-2.485	-5.753^{e}	-1.918	-5.113^{ef}	-2.011	-4.548^{a}
	Oct-V	-4.187	-6.615^{b}	-3.425	-6.729^{ab}	-2.386	-5.640^{de}	-1.818	-4.924^{de}	-1.994	-4.550^{a}
	Oct-W	-3.990	-6.903^{cd}	-3.860	-7.562^{f}	-2.394	-6.242^{g}	-1.775	-5.510^{gh}	-2.068	-4.866^{bc}
	Nov-NC	-3.953	-5.920^{a}	-4.661	-6.499^{a}	-2.412	-4.890^{a}	-1.729	-4.067^{a}	-2.401	-4.819^{b}
	Nov-V	-3.947	-5.891^{a}	-4.202	-6.713^{ab}	-2.367	-5.249^{bc}	-1.701	-4.492^{b}	-2.256	-5.064^{d}
	Nov-W	-3.931	-6.097^{a}	-4.659	-7.355^{def}	-2.296	-5.482^{cd}	-1.686	-4.524^{bc}	-2.322	-5.509^{e}

Table 6. Continued.

Treatment[†]		*nifH*		AOB[‡] *amoA*		*nirK*		*nirS*		*nosZ*	
		Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript
Season * Tillage	Apr-NT	-3.909	-6.669^{bc}	-4.193^{c}	-7.218^{cd}	-2.419	-5.470	-1.939	-5.091	-2.374	-5.001^{cd}
	Apr-CT	-3.689	-6.684^{bc}	-4.114^{bc}	-7.110^{bcd}	-2.373	-5.370	-1.771	-5.228	-2.294	-4.801^{b}
	May-NT	-4.167	-6.931^{d}	-4.133^{bc}	-7.018^{b}	-2.556	-5.873	-2.035	-5.535	-2.511	-4.863^{bc}
	May-CT	-4.010	-6.660^{b}	-4.021^{b}	-7.059^{bcd}	-2.594	-5.916	-1.974	-5.421	-2.433	-4.737^{b}
	Oct-NT	-4.303	-6.841^{cd}	-3.802^{a}	-7.223^{d}	-2.417	-5.870	-1.905	-5.236	-2.030	-4.787^{b}
	Oct-CT	-4.054	-6.601^{b}	-3.809^{a}	-7.027^{bc}	-2.426	-5.886	-1.769	-5.129	-2.018	-4.523^{a}
	Nov-NT	-3.938	-5.964^{a}	-4.399^{d}	-6.738^{a}	-2.319	-5.204	-1.681	-4.425	-2.283	-5.091^{de}
	Nov-CT	-3.950	-5.975^{a}	-4.615^{e}	-6.973^{b}	-2.398	-5.210	-1.729	-4.297	-2.370	-5.171^{e}
Cover * Nitrogen	NC-0N	-4.071	-6.628^{bc}	-4.435^{d}	-7.233^{c}	-2.519	-5.563^{a}	-1.995^{b}	-5.088^{bc}	-2.416	-4.943^{b}
	NC-67N	-4.060	-6.448^{a}	-4.343^{d}	-6.826^{b}	-2.4109	-5.407^{a}	-1.805^{a}	-4.888^{ab}	-2.252	-4.677^{a}
	V-0N	-4.022	-6.443^{a}	-3.865^{b}	-6.633^{a}	-2.4309	-5.438^{a}	-1.82^{a}	-4.755^{a}	-2.256	-4.660^{a}
	V-67N	-3.98	-6.479^{ab}	-3.706^{a}	-6.760^{ab}	-2.431	-5.494^{a}	-1.817^{a}	-5.003^{bc}	-2.176	-4.785^{a}
	W-0N	-3.919	-6.562^{abc}	-4.085^{c}	-7.296^{c}	-2.429	-5.866^{b}	-1.934^{b}	-5.393^{d}	-2.381	-5.085^{c}
	W-67N	-3.959	-6.682^{c}	-4.381^{d}	-7.527^{d}	-2.407	-5.831^{b}	-1.731^{a}	-5.145^{c}	-2.254	-5.080^{bc}
Season * Cover * Tillage	Apr-NC-NT	-3.915	-6.567^{defg}	-4.596	-7.063^{efg}	-2.394	-5.200^{bc}	-1.937	-5.052^{def}	-2.408	-4.752^{cdefg}
	Apr-NC-CT	-3.799	-6.676^{fgh}	-4.318	-7.287^{fgh}	-2.371	-5.284^{bcde}	-1.814	-5.209^{efgh}	-2.349	-4.946^{gh}
	Apr-V-NT	-3.973	-6.820^{ghi}	-3.743	-6.970^{def}	-2.400	-5.445^{bcdef}	-1.944	-4.564^{bc}	-2.281	-4.935^{fgh}
	Apr-V-CT	-3.669	-6.582^{defg}	-3.766	-6.702^{bcd}	-2.382	-5.233^{bcd}	-1.708	-5.056^{def}	-2.194	-4.592^{bcd}
	Apr-W-NT	-3.839	-6.620^{efgh}	-4.239	-7.622^{ij}	-2.464	-5.765^{fghi}	-1.936	-5.657^{ij}	-2.433	-5.316^{j}
	Apr-W-CT	-3.598	-6.793^{fghi}	-4.256	-7.341^{ghi}	-2.365	-5.593^{efgh}	-1.790	-5.420^{fghi}	-2.338	-4.864^{defgh}
	May-NC-NT	-4.162	-7.266^{j}	-4.368	-7.439^{hij}	-2.504	-6.239^{kl}	-2.095	-5.950^{j}	-2.546	-5.232^{ij}
	May-NC-CT	-4.026	-6.664^{fgh}	-4.244	-7.283^{fgh}	-2.652	-5.870^{hij}	-2.060	-5.335^{fghi}	-2.548	-4.816^{cdefg}
	May-V-NT	-4.200	-6.631^{efgh}	-3.839	-6.396^{ab}	-2.601	-5.542^{defgh}	-1.932	-5.199^{efgh}	-2.425	-4.451^{ab}
	May-V-CT	-3.913	-6.646^{fgh}	-3.682	-6.619^{bc}	-2.555	-5.730^{fghi}	-1.933	-5.381^{fghi}	-2.327	-4.572^{bc}
	May-W-NT	-4.140	-6.895^{hi}	-4.194	-7.218^{fgh}	-2.562	-5.836^{hij}	-2.078	-5.458^{fghi}	-2.563	-4.907^{efgh}
	May-W-CT	-4.090	-6.671^{fgh}	-4.136	-7.276^{fgh}	-2.574	-6.148^{jk}	-1.930	-5.547^{ghij}	-2.425	-4.823^{cdefg}

Table 6. Continued.

Treatment[†]		*nifH*		AOB[‡] *amoA*		*nirK*		*nirS*		*nosZ*	
		Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript
Season * Cover * Tillage	Oct-NC-NT	-4.591	-6.810^{ghi}	-4.204	-7.448^{hij}	-2.494	-5.832^{hij}	-2.003	-5.111^{ef}	-2.009	-4.637^{bcde}
	Oct-NC-CT	-4.125	-6.483^{def}	-4.059	-6.718^{bcd}	-2.476	-5.674^{fghi}	-1.834	-5.115^{ef}	-2.014	-4.459^{ab}
	Oct-V-NT	-4.218	-6.896^{hi}	-3.492	-6.858^{cde}	-2.350	-5.794^{ghi}	-1.865	-5.154^{efg}	-1.975	-4.815^{cdefg}
	Oct-V-CT	-4.156	-6.334^{cde}	-3.357	-6.601^{bc}	-2.422	-5.485^{cdefg}	-1.770	-4.693^{cd}	-2.014	-4.285^{a}
	Oct-W-NT	-4.100	-6.818^{ghi}	-3.709	-7.363^{ghi}	-2.407	-5.985^{ijk}	-1.848	-5.443^{fghi}	-2.107	-4.908^{efgh}
	Oct-W-CT	-3.880	-6.987^{ij}	-4.011	-7.762^{j}	-2.380	-6.498^{l}	-1.703	-5.578^{hij}	-2.028	-4.824^{cdefg}
	Nov-NC-NT	-3.998	-5.752^{a}	-4.569	-6.223^{a}	-2.426	-4.532^{a}	-1.793	-3.880^{a}	-2.415	-4.659^{bcdef}
	Nov-NC-CT	-3.908	-6.088^{bc}	-4.753	-6.775^{cde}	-2.398	-5.249^{bcd}	-1.664	-4.253^{ab}	-2.387	-4.979^{ghi}
	Nov-V-NT	-3.958	-5.865^{ab}	-4.063	-6.611^{bc}	-2.313	-5.242^{bcd}	-1.674	-4.563^{bc}	-2.206	-5.000^{ghi}
	Nov-V-CT	-3.936	-5.918^{ab}	-4.341	-6.814^{cde}	-2.421	-5.256^{bcd}	-1.729	-4.421^{bc}	-2.307	-5.129^{hij}
	Nov-W-NT	-3.857	-6.276^{cd}	-4.565	-7.381^{ghi}	-2.217	-5.839^{hij}	-1.576	-4.832^{cde}	-2.230	-5.613^{k}
	Nov-W-CT	-4.006	-5.918^{ab}	-4.752	-7.330^{ghi}	-2.375	-5.124^{b}	-1.795	-4.216^{ab}	-2.415	-5.405^{jk}
Season * Cover * Nitrogen	Apr-NC-0N	-3.915	-6.739^{cd}	-4.596	-7.283^{hi}	-2.394	-5.268^{bcde}	-1.937	-5.204	-2.408	-4.879^{defghi}
	Apr-NC-67N	-3.973	-6.504^{c}	-3.743	-7.067^{efgh}	-2.400	-5.216^{bcd}	-1.944	-5.058	-2.281	-4.819^{defgh}
	Apr-V-0N	-3.839	-6.752^{cd}	-4.239	-6.804^{bcde}	-2.464	-5.331^{cdef}	-1.936	-4.490	-2.433	-4.754^{defg}
	Apr-V-67N	-3.799	-6.649^{cd}	-4.318	-6.868^{cdef}	-2.371	-5.347^{cdef}	-1.814	-5.130	-2.349	-4.773^{defg}
	Apr-W-0N	-3.669	-6.708^{cd}	-3.766	-7.723^{k}	-2.382	-5.862^{hijk}	-1.708	-5.744	-2.194	-5.183^{jk}
	Apr-W-67N	-3.598	-6.705^{cd}	-4.256	-7.240^{ghi}	-2.365	-5.495^{cdefg}	-1.790	-5.333	-2.338	-4.998^{ghij}
	May-NC-0N	-4.162	-7.080^{ef}	-4.368	-7.570^{ijk}	-2.504	-6.169^{kl}	-2.095	-5.778	-2.546	-5.111^{ijk}
	May-NC-67N	-4.200	-6.850^{de}	-3.839	-7.152^{fgh}	-2.601	-5.941^{ijk}	-1.932	-5.508	-2.425	-4.936^{fghij}
	May-V-0N	-4.140	-6.581^{cd}	-4.194	-6.519^{ab}	-2.562	-5.586^{efgh}	-2.078	-5.230	-2.563	-4.398^{ab}
	May-V-67N	-4.026	-6.696^{cd}	-4.244	-6.496^{ab}	-2.652	-5.687^{ghij}	-2.060	-5.350	-2.548	-4.625^{bcd}
	May-W-0N	-3.913	-6.737^{cd}	-3.682	-7.314^{hij}	-2.555	-5.873^{hijk}	-1.933	-5.586	-2.327	-4.834^{defghi}
	May-W-67N	-4.090	-6.829^{de}	-4.136	-7.179^{fgh}	-2.574	-6.111^{kl}	-1.930	-5.419	-2.425	-4.896^{defghi}
	Oct-NC-0N	-4.591	-6.838^{de}	-4.204	-7.350^{hij}	-2.494	-5.998^{jk}	-2.003	-5.271	-2.009	-4.863^{defghi}
	Oct-NC-67N	-4.218	-6.455^{bc}	-3.492	-6.817^{bcde}	-2.350	-5.509^{cdefg}	-1.865	-4.956	-1.975	-4.233^{a}
	Oct-V-0N	-4.100	-6.540^{cd}	-3.709	-6.701^{bc}	-2.407	-5.523^{defg}	-1.848	-4.844	-2.107	-4.415^{abc}
	Oct-V-67N	-4.125	-6.689^{cd}	-4.059	-6.758^{bcde}	-2.476	-5.756^{ghij}	-1.834	-5.003	-2.014	-4.685^{cdef}
	Oct-W-0N	-4.156	-6.642^{cd}	-3.357	-7.055^{defgh}	-2.422	-6.109^{kl}	-1.770	-5.501	-2.014	-4.647^{bcde}
	Oct-W-67N	-3.880	-7.163^{f}	-4.011	-8.069^{l}	-2.380	-6.374^{l}	-1.703	-5.520	-2.028	-5.084^{hijk}

Table 6. Continued.

Treatment[†]		*nifH*		AOB[‡] *amoA*		*nirK*		*nirS*		*nosZ*	
		Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript
Season * Cover * Nitrogen	Nov-NC-0N	-3.998	-5.856^{a}	-4.569	-6.730^{bcd}	-2.426	-4.818^{a}	-1.793	-4.101	-2.415	-4.917^{efghij}
	Nov-NC-67N	-3.958	-5.984^{a}	-4.063	-6.268^{a}	-2.313	-4.962^{ab}	-1.674	-4.033	-2.206	-4.721^{defg}
	Nov-V-0N	-3.857	-5.899^{a}	-4.565	-6.508^{ab}	-2.217	-5.314^{cdef}	-1.576	-4.457	-2.230	-5.073^{hijk}
	Nov-V-67N	-3.908	-5.883^{a}	-4.753	-6.917^{cdefg}	-2.398	-5.184^{bc}	-1.664	-4.527	-2.387	-5.056^{hij}
	Nov-W-0N	-3.936	-6.162^{ab}	-4.341	-7.092^{efgh}	-2.421	-5.621^{fghi}	-1.729	-4.740	-2.307	-5.676^{l}
	Nov-W-67N	-4.006	-6.032^{a}	-4.752	-7.619^{jk}	-2.375	-5.342^{cdef}	-1.795	-4.308	-2.415	-5.342^{k}
Season * Tillage * Cover * Nitrogen	Apr-NT-NC-0N	-3.738	-6.818	-4.716	-7.380	-2.367	-5.341	-1.995	-5.237	-2.470	-4.956
	Apr-NT-NC-67N	-4.091	-6.316	-4.476	-6.745	-2.422	-5.058	-1.878	-4.868	-2.345	-4.548
	Apr-NT-V-0N	-4.141	-6.886	-3.892	-6.940	-2.364	-5.526	-1.904	-3.889	-2.254	-4.917
	Apr-NT-V-67N	-3.805	-6.753	-3.594	-6.999	-2.435	-5.364	-1.984	-5.239	-2.308	-4.954
	Apr-NT-W-0N	-3.938	-6.638	-3.931	-7.927	-2.536	-6.069	-2.098	-5.922	-2.493	-5.375
	Apr-NT-W-67N	-3.739	-6.602	-4.547	-7.318	-2.391	-5.460	-1.773	-5.393	-2.373	-5.258
	Apr-CT-NC-0N	-3.717	-6.659	-4.266	-7.185	-2.352	-5.196	-1.880	-5.171	-2.388	-4.803
	Apr-CT-NC-67N	-3.881	-6.693	-4.371	-7.389	-2.390	-5.373	-1.748	-5.247	-2.310	-5.089
	Apr-CT-V-0N	-3.620	-6.618	-3.752	-6.668	-2.321	-5.136	-1.684	-5.090	-2.136	-4.591
	Apr-CT-V-67N	-3.719	-6.546	-3.780	-6.737	-2.443	-5.330	-1.732	-5.022	-2.252	-4.592
	Apr-CT-W-0N	-3.632	-6.778	-4.150	-7.520	-2.356	-5.655	-1.906	-5.566	-2.466	-4.991
	Apr-CT-W-67N	-3.563	-6.809	-4.362	-7.162	-2.375	-5.530	-1.675	-5.273	-2.211	-4.738
	May-NT-NC-0N	-4.147	-7.499	-4.522	-7.826	-2.596	-6.389	-2.196	-6.156	-2.616	-5.423
	May-NT-NC-67N	-4.177	-7.033	-4.213	-7.052	-2.412	-6.090	-1.995	-5.744	-2.476	-5.041
	May-NT-V-0N	-4.192	-6.700	-3.920	-6.504	-2.559	-5.581	-1.904	-5.254	-2.472	-4.409
	May-NT-V-67N	-4.208	-6.562	-3.757	-6.288	-2.644	-5.504	-1.960	-5.143	-2.379	-4.493
	May-NT-W-0N	-4.051	-6.807	-4.044	-7.276	-2.547	-5.723	-2.133	-5.528	-2.587	-4.855
	May-NT-W-67N	-4.228	-6.983	-4.343	-7.159	-2.577	-5.950	-2.024	-5.387	-2.538	-4.958
	May-CT-NC-0N	-4.019	-6.662	-4.287	-7.314	-2.683	-5.949	-2.037	-5.399	-2.556	-4.800
	May-CT-NC-67N	-4.032	-6.666	-4.200	-7.253	-2.621	-5.792	-2.082	-5.272	-2.540	-4.832
	May-CT-V-0N	-3.886	-6.461	-3.688	-6.535	-2.560	-5.591	-1.839	-5.205	-2.328	-4.387
	May-CT-V-67N	-3.940	-6.831	-3.677	-6.704	-2.551	-5.870	-2.027	-5.556	-2.326	-4.758
	May-CT-W-0N	-3.992	-6.667	-4.069	-7.352	-2.542	-6.024	-2.011	-5.645	-2.440	-4.812
	May-CT-W-67N	-4.189	-6.675	-4.202	-7.199	-2.605	-6.273	-1.848	-5.450	-2.410	-4.833

Table 6. Continued.

Treatment[†]		*nifH*		AOB[‡] *amoA*		*nirK*		*nirS*		*nosZ*	
		Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript
Season * Tillage * Cover * Nitrogen	Oct-NT-NC-0N	-4.654	-7.111	-4.045	-7.717	-2.584	-6.216	-2.077	-5.239	-2.064	-5.043
	Oct-NT-NC-67N	-4.528	-6.509	-4.362	-7.179	-2.404	-5.448	-1.929	-4.984	-1.953	-4.231
	Oct-NT-V-0N	-4.408	-6.875	-3.773	-6.907	-2.396	-5.881	-1.938	-5.222	-2.095	-4.723
	Oct-NT-V-67N	-4.029	-6.917	-3.212	-6.808	-2.303	-5.707	-1.792	-5.087	-1.855	-4.908
	Oct-NT-W-0N	-4.014	-6.620	-3.676	-7.063	-2.508	-6.064	-1.934	-5.654	-2.186	-4.862
	Oct-NT-W-67N	-4.186	-7.017	-3.741	-7.662	-2.306	-5.906	-1.761	-5.232	-2.028	-4.953
	Oct-CT-NC-0N	-4.343	-6.564	-4.215	-6.982	-2.641	-5.780	-2.082	-5.303	-2.227	-4.683
	Oct-CT-NC-67N	-3.907	-6.402	-3.903	-6.455	-2.310	-5.569	-1.585	-4.927	-1.800	-4.236
	Oct-CT-V-0N	-4.119	-6.206	-3.422	-6.494	-2.486	-5.165	-1.837	-4.466	-2.165	-4.108
	Oct-CT-V-67N	-4.193	-6.462	-3.292	-6.707	-2.357	-5.805	-1.703	-4.919	-1.862	-4.463
	Oct-CT-W-0N	-3.901	-6.665	-3.945	-7.048	-2.404	-6.153	-1.849	-5.348	-2.129	-4.432
	Oct-CT-W-67N	-3.859	-7.309	-4.077	-8.475	-2.356	-6.842	-1.557	-5.809	-1.928	-5.215
	Nov-NT-NC-0N	-4.039	-5.782	-4.641	-6.384	-2.470	-4.482	-1.899	-3.946	-2.517	-4.726
	Nov-NT-NC-67N	-3.958	-5.722	-4.498	-6.062	-2.383	-4.583	-1.687	-3.814	-2.312	-4.591
	Nov-NT-V-0N	-4.050	-5.969	-4.172	-6.451	-2.382	-5.288	-1.809	-4.505	-2.355	-5.072
	Nov-NT-V-67N	-3.866	-5.761	-3.954	-6.770	-2.243	-5.197	-1.538	-4.622	-2.056	-4.928
	Nov-NT-W-0N	-3.820	-6.487	-4.357	-7.240	-2.202	-6.276	-1.624	-5.284	-2.271	-5.988
	Nov-NT-W-67N	-3.893	-6.064	-4.773	-7.522	-2.233	-5.402	-1.528	-4.380	-2.190	-5.237
	Nov-CT-NC-0N	-3.909	-5.930	-4.784	-7.076	-2.457	-5.155	-1.795	-4.256	-2.493	-5.107
	Nov-CT-NC-67N	-3.908	-6.246	-4.722	-6.474	-2.340	-5.342	-1.533	-4.251	-2.282	-4.850
	Nov-CT-V-0N	-3.756	-5.830	-4.300	-6.565	-2.373	-5.340	-1.658	-4.409	-2.247	-5.073
	Nov-CT-V-67N	-4.115	-6.006	-4.382	-7.064	-2.470	-5.171	-1.799	-4.433	-2.368	-5.184
	Nov-CT-W-0N	-4.001	-5.836	-4.504	-6.944	-2.334	-4.967	-1.913	-4.196	-2.474	-5.365
	Nov-CT-W-67N	-4.011	-5.999	-5.001	-7.716	-2.416	-5.282	-1.677	-4.236	-2.355	-5.446

Note: letters that represent the significant differences for the combination effects of season, tillage, cover crop, and nitrogen were not shown due to table space limitation.

[†]NC = no cover; V = vetch; W = wheat; NT = no tillage; CT = conventional tillage; 0N = no fertilization; 67N = 67 kg N ha^{-1} fertilization. [‡]AOB = Ammonia oxidizing bacteria

Table 7. Mean values of log-transformed mean absolute abundances of N-cycle genes and their transcripts in relation to agricultural season and soil management practices.

Treatment†		*nifH*		bacterial *amoA*		*nirK*		*nirS*		*nosZ*		16S	
		Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript
Season	April	5.503^{a}	4.579^{c}	5.149^{c}	4.091^{c}	6.906^{c}	5.835^{b}	7.447^{c}	6.095^{c}	6.968^{c}	6.354^{c}	9.302^{c}	11.255^{c}
	May	5.465^{a}	4.731^{c}	5.477^{b}	4.488^{b}	6.979^{b}	5.632^{c}	7.549^{b}	6.048^{c}	7.081^{b}	6.727^{b}	9.554^{a}	11.527^{b}
	October	5.220^{b}	4.953^{b}	5.593^{a}	4.550^{ab}	6.977^{b}	5.796^{b}	7.561^{b}	6.492^{b}	7.374^{a}	7.020^{a}	9.398^{b}	11.675^{a}
	November	5.505^{a}	5.608^{a}	4.941^{d}	4.718^{a}	7.090^{a}	6.371^{a}	7.743^{a}	7.217^{a}	7.122^{b}	6.444^{c}	9.448^{b}	11.575^{ab}
Tillage	No till (NT)	5.292^{b}	4.863^{b}	5.239^{b}	4.414	6.943^{b}	5.861	7.481^{b}	6.393^{b}	7.071^{b}	6.528^{b}	9.371^{b}	11.463
	Till (CT)	5.555^{a}	5.072^{a}	5.340^{a}	4.510	7.032^{a}	5.957	7.669^{a}	6.533^{a}	7.201^{a}	6.744^{a}	9.480^{a}	11.552
Cover	No cover (NC)	5.371	4.852^{b}	5.047^{c}	4.358^{b}	6.972	5.906^{b}	7.536^{b}	6.403^{b}	7.102^{b}	6.578^{b}	9.436	11.388^{b}
	Vetch (V)	5.434	5.156^{a}	5.651^{a}	4.921^{a}	7.006	6.152^{a}	7.617^{a}	6.739^{a}	7.221^{a}	6.895^{a}	9.437	11.618^{a}
	Wheat (W)	5.465	4.895^{b}	5.171^{b}	4.106^{c}	6.986	5.669^{c}	7.572^{ab}	6.248^{b}	7.086^{b}	6.435^{c}	9.404	11.517^{a}
Nitrogen	0N	5.436^{a}	4.946	5.341^{a}	4.431	7.001	5.867	7.542^{b}	6.429	7.126	6.610	9.466^{a}	11.512
	67N	5.385^{b}	4.976	5.243^{b}	4.475	6.970	5.936	7.602^{a}	6.501	7.159	6.665	9.386^{b}	11.513
Season * Tillage	Apr-NT	5.349	4.498^{d}	5.065^{c}	3.949	6.838	5.697	7.319	6.075	6.884	6.166^{d}	9.258	11.166
	Apr-CT	5.657	4.659^{cd}	5.232^{b}	4.233	6.973	5.973	7.575	6.114	7.052	6.542^{c}	9.346	11.343
	May-NT	5.317	4.490^{d}	5.351^{b}	4.403	6.928	5.547	7.449	5.885	6.973	6.557^{c}	9.484	11.420
	May-CT	5.614	4.973^{b}	5.603^{a}	4.574	7.029	5.717	7.649	6.212	7.190	6.896^{b}	9.623	11.633
	Oct-NT	5.071	4.858^{bc}	5.572^{a}	4.476	6.957	5.829	7.468	6.463	7.343	6.913^{b}	9.374	11.699
	Oct-CT	5.368	5.048^{b}	5.613^{a}	4.623	6.996	5.764	7.653	6.521	7.404	7.127^{a}	9.422	11.650
	Nov-NT	5.430	5.608^{a}	4.969^{c}	4.827	7.049	6.371	7.687	7.150	7.084	6.476^{c}	9.368	11.568
	Nov-CT	5.579	5.608^{a}	4.914^{c}	4.609	7.131	6.372	7.800	7.285	7.159	6.411^{c}	9.529	11.582
Season * Cover	Apr-NC	5.438	4.334	4.837	3.781^{f}	6.912	5.714^{efg}	7.419	5.825^{i}	6.916	6.107^{e}	9.294	10.955^{d}
	Apr-V	5.510	4.827	5.576	4.691^{bc}	6.940	6.189^{bc}	7.505	6.717^{de}	7.094	6.764^{bc}	9.331	11.527^{b}
	Apr-W	5.562	4.575	5.032	3.800^{f}	6.866	5.603^{fgh}	7.417	5.743^{i}	6.894	6.191^{e}	9.280	11.281^{c}
	May-NC	5.427	4.552	5.215	4.156^{e}	6.942	5.462^{gh}	7.443	5.874^{hi}	6.974	6.493^{d}	9.520	11.517^{b}
	May-V	5.463	4.887	5.759	5.018^{a}	6.941	5.890^{de}	7.587	6.236^{fg}	7.143	7.014^{ab}	9.519	11.526^{b}
	May-W	5.506	4.754	5.456	4.290^{de}	7.053	5.545^{gh}	7.617	6.035^{ghi}	7.127	6.672^{cd}	9.621	11.537^{b}
	Oct-NC	5.097	4.955	5.324	4.518^{cd}	6.970	5.848^{def}	7.537	6.488^{ef}	7.444	7.053^{a}	9.455	11.601^{ab}
	Oct-V	5.228	5.144	5.990	5.029^{a}	7.030	6.119^{cd}	7.598	6.835^{cd}	7.421	7.208^{a}	9.415	11.759^{a}
	Oct-W	5.333	4.761	5.463	4.102^{ef}	6.930	5.422^{h}	7.548	6.153^{gh}	7.256	6.798^{bc}	9.323	11.664^{ab}
	Nov-NC	5.521	5.566	4.813	4.977^{ab}	7.062	6.600^{a}	7.746	7.424^{a}	7.073	6.660^{cd}	9.474	11.480^{bc}
	Nov-V	5.534	5.767	5.279	4.946^{ab}	7.114	6.410^{ab}	7.780	7.167^{ab}	7.224	6.594^{cd}	9.481	11.659^{ab}
	Nov-W	5.459	5.490	4.732	4.231^{de}	7.094	6.105^{cd}	7.705	7.062^{bc}	7.068	6.077^{e}	9.390	11.586^{ab}

Table 7. Continued.

Treatment†		*nifH*		bacterial *amoA*		*nirK*		*nirS*		*nosZ*		16S	
		Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript
Season * Nitrogen	Apr-0N	5.550	4.500	5.229	3.963	6.965	5.746	7.436	6.087	6.979	6.294^{f}	9.347	11.232
	Apr-67N	5.457	4.657	5.068	4.218	6.847	5.924	7.458	6.103	6.956	6.414^{ef}	9.256	11.277
	May-0N	5.515	4.799	5.474	4.464	6.981	5.722	7.542	6.067	7.063	6.817^{bc}	9.562	11.598
	May-67N	5.416	4.664	5.479	4.513	6.977	5.542	7.555	6.030	7.100	6.636^{cd}	9.545	11.455
	Oct-0N	5.244	4.992	5.638	4.630	6.981	5.789	7.531	6.460	7.340	7.024^{a}	9.484	11.666
	Oct-67N	5.195	4.914	5.547	4.469	6.972	5.804	7.590	6.524	7.407	7.016^{ab}	9.312	11.683
	Nov-0N	5.535	5.546	5.005	4.735	7.095	6.270	7.681	7.089	7.072	6.292^{f}	9.464	11.514
	Nov-67N	5.474	5.669	4.878	4.701	7.085	6.473	7.805	7.346	7.172	6.596^{de}	9.433	11.636
Tillage * Nitrogen	NT-0N	5.332	4.833	5.291	4.378	6.972	5.781	7.472	6.364	7.066	6.484	9.431	11.514^{ab}
	NT-67N	5.251	4.893	5.188	4.449	6.914	5.941	7.489	6.422	7.076	6.571	9.310	11.413^{b}
	CT-0N	5.590	5.085	5.383	4.518	7.039	5.982	7.624	6.487	7.161	6.729	9.498	11.491^{ab}
	CT-67N	5.519	5.059	5.298	4.501	7.026	5.931	7.715	6.580	7.242	6.759	9.462	11.613^{a}
Cover * Nitrogen	NC-0N	5.436	4.764	5.072^{c}	4.154^{cd}	6.988^{b}	5.831	7.511^{bc}	6.306^{cd}	7.090^{cd}	6.446^{c}	9.506	11.389
	NC-67N	5.305	4.939	5.023^{c}	4.562^{b}	6.955^{b}	5.981	7.561^{b}	6.499^{bc}	7.113^{bcd}	6.710^{b}	9.366	11.388
	V-0N	5.475	5.147	5.631^{a}	4.957^{a}	7.066^{a}	6.152	7.674^{a}	6.835^{a}	7.240^{a}	6.930^{a}	9.496	11.590
	V-67N	5.393	5.166	5.671^{a}	4.885^{a}	6.946^{b}	6.152	7.560^{b}	6.643^{ab}	7.201^{ab}	6.860^{ab}	9.377	11.645
	W-0N	5.471	4.953	5.348^{b}	4.320^{c}	6.954^{b}	5.688	7.448^{c}	6.154^{d}	7.017^{d}	6.457^{c}	9.383	11.535
	W-67N	5.458	4.824	5.035^{c}	3.979^{d}	7.009^{ab}	5.675	7.686^{a}	6.361^{cd}	7.162^{abc}	6.426^{c}	9.416	11.506
Tillage * Cover * Nitrogen	NT-NC-0N	5.327	4.525	4.991	3.991	6.967	5.726	7.430	6.188^{ef}	7.055	6.284	9.472	11.321
	NT-NC-67N	5.118	4.854	4.919	4.489	6.901	5.954	7.434	6.396^{cde}	7.034	6.646	9.306	11.249
	NT-V-0N	5.278	5.069	5.536	4.976	7.050	6.108	7.587	6.959^{a}	7.182	6.896	9.476	11.677
	NT-V-67N	5.295	5.040	5.642	4.821	6.865	6.095	7.453	6.515^{bcd}	7.122	6.717	9.272	11.538
	NT-W-0N	5.391	4.905	5.345	4.167	6.898	5.510	7.399	5.946^{f}	6.962	6.273	9.347	11.543
	NT-W-67N	5.342	4.786	5.002	4.037	6.977	5.773	7.582	6.355^{de}	7.071	6.351	9.353	11.453
	CT-NC-0N	5.545	5.003	5.153	4.317	7.008	5.936	7.593	6.424^{cde}	7.126	6.608	9.541	11.457
	CT-NC-67N	5.493	5.025	5.126	4.634	7.010	6.008	7.688	6.602^{bcd}	7.192	6.775	9.425	11.527
	CT-V-0N	5.671	5.225	5.726	4.938	7.082	6.196	7.762	6.711^{abc}	7.298	6.964	9.517	11.504
	CT-V-67N	5.491	5.292	5.700	4.950	7.027	6.209	7.668	6.770^{ab}	7.281	7.003	9.483	11.753
	CT-W-0N	5.554	5.027	5.268	4.298	7.027	5.814	7.516	6.325^{de}	7.058	6.614	9.436	11.514
	CT-W-67N	5.574	4.861	5.069	3.920	7.041	5.577	7.790	6.366^{de}	7.253	6.500	9.479	11.559

Table 7. Continued.

Treatment†		*nifH*		bacterial *amoA*		*nirK*		*nirS*		*nosZ*		16S	
		Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript
	Apr-NC-NT	5.299	4.052^{j}	4.618	3.556^{i}	6.819	5.419^{ijk}	7.277	5.567^{jk}	6.806	5.867	9.213	10.618
	Apr-NC-CT	5.576	4.616^{gh}	5.05	4.005^{ghi}	7.004	6.008^{defg}	7.561	6.083^{fghi}	7.026	6.346	9.375	11.292
	Apr-V-NT	5.321	4.733^{efgh}	5.551	4.582^{cdef}	6.894	6.107^{cdef}	7.350	6.988^{bc}	7.013	6.617	9.294	11.552
	Apr-V-CT	5.699	4.921^{defg}	5.602	4.800^{abc}	6.986	6.270^{bcde}	7.660	6.447^{defg}	7.174	6.911	9.368	11.50
	Apr-W-NT	5.426	4.709^{efgh}	5.026	3.707^{hi}	6.801	5.565^{hij}	7.330	5.672^{ijk}	6.832	6.013	9.265	11.329
	Apr-W-CT	5.697	4.440^{hij}	5.039	3.892^{ghi}	6.930	5.641^{ghi}	7.505	5.814^{hijk}	6.957	6.369	9.295	11.233
	May-NC-NT	5.249	4.192^{ij}	5.043	4.019^{gh}	6.907	5.218^{jk}	7.315	5.508^{k}	6.865	6.226	9.411	11.458
	May-NC-CT	5.604	4.912^{defg}	5.386	4.293^{efg}	6.978	5.706^{ghi}	7.570	6.241^{efgh}	7.082	6.760	9.630	11.576
	May-V-NT	5.244	4.709^{efgh}	5.605	4.944^{abc}	6.843	5.797^{ghji}	7.512	6.141^{fgh}	7.019	6.888	9.444	11.340
	May-V-CT	5.682	5.066^{cdef}	5.912	5.093^{ab}	7.039	5.982^{defg}	7.662	6.332^{defg}	7.268	7.140	9.595	11.712
Season	May-W-NT	5.457	4.568^{ghi}	5.403	4.245^{fg}	7.035	5.627^{ghi}	7.519	6.005^{ghij}	7.034	6.557	9.597	11.463
*	May-W-CT	5.555	4.940^{defg}	5.510	4.335^{defg}	7.071	5.463^{ijk}	7.715	6.064^{fghi}	7.220	6.788	9.645	11.611
Cover	Oct-NC-NT	4.854	4.817^{efgh}	5.241	4.179^{fg}	6.950	5.795^{fghi}	7.441	6.516^{def}	7.435	6.990	9.444	11.627
*	Oct-NC-CT	5.341	5.092^{cde}	5.407	4.857^{abc}	6.991	5.901^{efgh}	7.633	6.460^{defg}	7.453	7.116	9.466	11.575
Tillage	Oct-V-NT	5.144	4.909^{defg}	5.870	4.947^{abc}	7.013	6.011^{defg}	7.497	6.650^{cde}	7.387	6.989	9.362	11.805
	Oct-V-CT	5.312	5.379^{bc}	6.111	5.112^{ab}	7.047	6.227^{bcde}	7.699	7.020^{bc}	7.455	7.427	9.468	11.712
	Oct-W-NT	5.215	4.847^{efg}	5.606	4.303^{efg}	6.908	5.680^{ghi}	7.467	6.223^{efgh}	7.208	6.758	9.315	11.666
	Oct-W-CT	5.451	4.675^{fgh}	5.320	3.900^{ghi}	6.951	5.164^{k}	7.629	6.084^{fghi}	7.304	6.838	9.332	11.662
	Nov-NC-NT	5.488	5.697^{ab}	4.917	5.208^{a}	7.060	6.927^{a}	7.693	7.578^{a}	7.072	6.776	9.486	11.438
	Nov-NC-CT	5.554	5.435^{bc}	4.710	4.747^{bcde}	7.065	6.274^{bcde}	7.798	7.269^{ab}	7.075	6.544	9.463	11.522
	Nov-V-NT	5.436	5.867^{a}	5.331	5.121^{ab}	7.081	6.490^{bc}	7.720	7.169^{ab}	7.188	6.732	9.394	11.732
	Nov-V-CT	5.632	5.667^{ab}	5.227	4.771^{abcd}	7.146	6.329^{bcd}	7.839	7.164^{ab}	7.260	6.457	9.567	11.585
	Nov-W-NT	5.366	5.258^{cd}	4.658	4.153^{fgh}	7.005	5.695^{ghi}	7.647	6.702^{cd}	6.993	5.921	9.223	11.534
	Nov-W-CT	5.552	5.721^{ab}	4.805	4.309^{efg}	7.183	6.514^{b}	7.763	7.422^{ab}	7.143	6.234	9.558	11.639
	Apr-NC-0N	5.629	4.151^{k}	4.865	3.607^{j}	6.997	5.621^{hij}	7.419	5.686	6.927	6.011^{jk}	9.356	10.889
	Apr-NC-67N	5.246	4.517^{jk}	4.809	3.955^{hij}	6.826	5.806^{defgh}	7.419	5.964	6.905	6.203^{hij}	9.232	11.021
Season	Apr-V-0N	5.510	4.695^{ghij}	5.569	4.643^{cdefg}	7.048	6.117^{cde}	7.597	6.958	7.196	6.694^{cdef}	9.391	11.447
*	Apr-V-67N	5.510	4.958^{defghi}	5.584	4.739^{bcdef}	6.832	6.260^{abc}	7.414	6.477	6.991	6.834^{bcd}	9.271	11.607
	Apr-W-0N	5.509	4.653^{hij}	5.254	3.638^{ij}	6.849	5.499^{hij}	7.293	5.617	6.815	6.178^{ij}	9.295	11.361
Cover	Apr-W-67N	5.614	4.497^{jk}	4.811	3.962^{hij}	6.883	5.707^{fghi}	7.542	5.869	6.973	6.204^{ghij}	9.266	11.202
*	May-NC-0N	5.492	4.484^{jk}	5.170	3.994^{hij}	6.935	5.395^{ij}	7.458	5.786	6.989	6.453^{efghi}	9.575	11.564
Nitrogen	May-NC-67N	5.361	4.620^{ij}	5.259	4.318^{fgh}	6.949	5.530^{hij}	7.427	5.962	6.958	6.534^{defghi}	9.466	11.470
	May-V-0N	5.529	5.057^{defg}	5.764	5.118^{ab}	7.009	6.052^{cdef}	7.696	6.408	7.168	7.240^{a}	9.568	11.638
	May-V-67N	5.397	4.718^{fghij}	5.754	4.918^{abcd}	6.873	5.727^{efghi}	7.478	6.065	7.118	6.789^{cde}	9.471	11.414

Table 7. Continued.

Treatment[†]		*nifH*		bacterial *amoA*		*nirK*		*nirS*		*nosZ*		16S	
		Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript
Season * Cover * Nitrogen	May-W-0N	5.523	4.856^{efghij}	5.488	4.278^{gh}	7.000	5.719^{fghi}	7.473	6.007	7.031	6.759^{cde}	9.544	11.593
	May-W-67N	5.490	4.653^{hij}	5.425	4.302^{fgh}	7.107	5.370^{ij}	7.761	6.063	7.224	6.586^{def}	9.698	11.481
	Oct-NC-0N	5.080	4.814^{efghij}	5.448	4.302^{fgh}	6.965	5.654^{ghij}	7.498	6.381	7.432	6.789^{cde}	9.578	11.652
	Oct-NC-67N	5.115	5.095^{def}	5.200	4.733^{bcdefg}	6.975	6.042^{cdefg}	7.576	6.595	7.456	7.317^{a}	9.333	11.551
	Oct-V-0N	5.232	5.122^{de}	5.898	4.962^{abcd}	7.055	6.139^{bcd}	7.608	6.818	7.366	7.247^{a}	9.496	11.662
	Oct-V-67N	5.224	5.166^{cde}	6.083	5.097^{abc}	7.004	6.099^{cdef}	7.587	6.852	7.476	7.170^{ab}	9.335	11.855
	Oct-W-0N	5.421	5.040^{defgh}	5.568	4.627^{defg}	6.923	5.574^{hij}	7.487	6.182	7.222	7.035^{abc}	9.379	11.683
	Oct-W-67N	5.245	4.482^{jk}	5.358	3.576^{j}	6.936	5.270^{j}	7.608	6.125	7.290	6.560^{defg}	9.268	11.645
	Nov-NC-0N	5.543	5.607^{ab}	4.804	4.714^{bcdefg}	7.054	6.654^{a}	7.670	7.371	7.012	6.532^{defghi}	9.517	11.451
	Nov-NC-67N	5.499	5.525^{abc}	4.822	5.241^{a}	7.071	6.546^{a}	7.822	7.476	7.135	6.788^{cde}	9.432	11.509
	Nov-V-0N	5.626	5.714^{a}	5.294	5.105^{ab}	7.152	6.299^{abc}	7.796	7.156	7.229	6.541^{defgh}	9.530	11.613
	Nov-V-67N	5.441	5.821^{a}	5.264	4.787^{abcde}	7.075	6.520^{ab}	7.763	7.177	7.219	6.648^{def}	9.432	11.704
	Nov-W-0N	5.436	5.316^{bcd}	4.916	4.387^{efgh}	7.078	5.857^{defgh}	7.578	6.738	6.974	5.802^{k}	9.346	11.478
	Nov-W-67N	5.482	5.663^{ab}	4.548	4.075^{hi}	7.110	6.353^{abc}	7.832	7.386	7.162	6.353^{fghij}	9.434	11.695
Season * Tillage * Cover * Nitrogen	Apr-NT-NC-0N	5.580	3.671	4.602	3.109	6.951	5.148	7.323	5.252	6.848	5.533	9.318	10.487
	Apr-NT-NC-67N	5.018	4.432	4.633	4.004	6.687	5.690	7.231	5.881	6.764	6.201	9.109	10.749
	Apr-NT-V-0N	5.326	4.659	5.575	4.606	7.103	6.020	7.563	7.656	7.213	6.629	9.467	11.546
	Apr-NT-V-67N	5.317	4.806	5.527	4.559	6.686	6.195	7.138	6.320	6.814	6.605	9.121	11.558
	Apr-NT-W-0N	5.417	4.903	5.425	3.614	6.819	5.472	7.258	5.619	6.862	6.167	9.356	11.541
	Apr-NT-W-67N	5.435	4.516	4.627	3.800	6.784	5.657	7.401	5.724	6.801	5.859	9.175	11.117
	Apr-CT-NC-0N	5.678	4.631	5.129	4.105	7.043	6.095	7.515	6.119	7.007	6.488	9.395	11.290
	Apr-CT-NC-67N	5.474	4.601	4.984	3.905	6.965	5.921	7.607	6.047	7.045	6.205	9.355	11.294
	Apr-CT-V-0N	5.695	4.731	5.563	4.681	6.993	6.213	7.631	6.259	7.179	6.758	9.315	11.349
	Apr-CT-V-67N	5.703	5.111	5.641	4.919	6.978	6.326	7.690	6.634	7.169	7.064	9.421	11.656
	Apr-CT-W-0N	5.602	4.403	5.083	3.661	6.878	5.525	7.328	5.615	6.768	6.190	9.234	11.181
	Apr-CT-W-67N	5.793	4.478	4.994	4.124	6.981	5.756	7.682	6.013	7.146	6.548	9.357	11.286
	May-NT-NC-0N	5.350	4.009	4.975	3.682	6.901	5.119	7.301	5.352	6.881	6.085	9.497	11.508
	May-NT-NC-67N	5.148	4.374	5.111	4.355	6.913	5.318	7.330	5.664	6.848	6.367	9.324	11.407
	May-NT-V-0N	5.285	4.844	5.557	5.041	6.918	5.964	7.573	6.291	7.005	7.135	9.477	11.545
	May-NT-V-67N	5.203	4.573	5.654	4.846	6.768	5.630	7.452	5.991	7.033	6.641	9.412	11.134
	May-NT-W-0N	5.447	4.740	5.454	4.270	6.950	5.823	7.365	6.018	6.910	6.692	9.498	11.546
	May-NT-W-67N	5.468	4.397	5.353	4.220	7.120	5.430	7.672	5.993	7.159	6.421	9.697	11.380
	May-CT-NC-0N	5.634	4.958	5.365	4.306	6.970	5.671	7.615	6.221	7.097	6.820	9.653	11.620
	May-CT-NC-67N	5.574	4.867	5.407	4.280	6.986	5.741	7.524	6.261	7.067	6.701	9.607	11.533

Table 7. Continued.

Treatment[†]		*nifH*		bacterial *amoA*		*nirK*		*nirS*		*nosZ*		16S	
		Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript	Gene	Transcript
Season * Tillage * Cover * Nitrogen	May-CT-V-0N	5.773	5.269	5.971	5.195	7.099	6.140	7.820	6.525	7.331	7.344	9.659	11.731
	May-CT-V-67N	5.590	4.863	5.854	4.991	6.979	5.824	7.504	6.138	7.204	6.936	9.530	11.694
	May-CT-W-0N	5.600	4.972	5.522	4.287	7.049	5.616	7.580	5.995	7.151	6.827	9.591	11.639
	May-CT-W-67N	5.511	4.909	5.497	4.384	7.094	5.310	7.851	6.133	7.289	6.750	9.699	11.583
	Oct-NT-NC-0N	4.837	4.631	5.446	4.025	6.907	5.527	7.414	6.504	7.426	6.700	9.491	11.742
	Oct-NT-NC-67N	4.870	5.004	5.036	4.333	6.994	6.064	7.469	6.528	7.444	7.281	9.398	11.512
	Oct-NT-V-0N	5.052	5.047	5.687	5.015	7.064	6.041	7.522	6.700	7.365	7.199	9.460	11.922
	Oct-NT-V-67N	5.235	4.771	6.052	4.880	6.961	5.980	7.472	6.601	7.409	6.780	9.264	11.688
	Oct-NT-W-0N	5.358	4.984	5.696	4.541	6.864	5.540	7.438	5.950	7.186	6.742	9.372	11.604
	Oct-NT-W-67N	5.072	4.710	5.517	4.065	6.952	5.821	7.497	6.496	7.230	6.774	9.258	11.727
	Oct-CT-NC-0N	5.322	4.997	5.450	4.579	7.024	5.782	7.583	6.258	7.438	6.878	9.665	11.561
	Oct-CT-NC-67N	5.360	5.187	5.364	5.134	6.957	6.020	7.683	6.662	7.467	7.354	9.267	11.589
	Oct-CT-V-0N	5.412	5.197	6.109	4.909	7.045	6.238	7.695	6.937	7.367	7.295	9.532	11.403
	Oct-CT-V-67N	5.213	5.560	6.113	5.315	7.048	6.217	7.703	7.103	7.543	7.559	9.405	12.022
	Oct-CT-W-0N	5.485	5.096	5.441	4.713	6.982	5.608	7.537	6.414	7.258	7.329	9.387	11.761
	Oct-CT-W-67N	5.417	4.253	5.199	3.087	6.920	4.720	7.720	5.753	7.349	6.347	9.277	11.562
	Nov-NT-NC-0N	5.541	5.790	4.939	5.150	7.110	7.109	7.681	7.643	7.063	6.817	9.580	11.548
	Nov-NT-NC-67N	5.435	5.605	4.895	5.265	7.010	6.745	7.705	7.513	7.080	6.736	9.392	11.327
	Nov-NT-V-0N	5.448	5.725	5.327	5.243	7.116	6.406	7.689	7.189	7.144	6.622	9.499	11.694
	Nov-NT-V-67N	5.424	6.009	5.335	5.000	7.046	6.573	7.751	7.148	7.233	6.842	9.289	11.770
	Nov-NT-W-0N	5.342	4.994	4.805	4.242	6.959	5.205	7.537	6.197	6.891	5.493	9.161	11.481
	Nov-NT-W-67N	5.391	5.523	4.511	4.064	7.051	6.185	7.756	7.207	7.094	6.349	9.284	11.586
	Nov-CT-NC-0N	5.545	5.425	4.669	4.278	6.997	6.199	7.659	7.099	6.961	6.247	9.453	11.354
	Nov-CT-NC-67N	5.564	5.445	4.750	5.216	7.132	6.348	7.938	7.439	7.190	6.840	9.472	11.691
	Nov-CT-V-0N	5.805	5.702	5.261	4.967	7.188	6.192	7.903	7.124	7.314	6.459	9.561	11.532
	Nov-CT-V-67N	5.459	5.632	5.192	4.575	7.104	6.467	7.775	7.205	7.206	6.454	9.574	11.638
	Nov-CT-W-0N	5.530	5.639	5.027	4.532	7.198	6.508	7.618	7.279	7.057	6.111	9.531	11.475
	Nov-CT-W-67N	5.573	5.803	4.584	4.087	7.168	6.521	7.907	7.566	7.229	6.357	9.584	11.803

Note: letters that represent the significant differences for the combination effects of season, tillage, cover crop, and nitrogen were not shown due to table space limitation.

[†]NC = no cover; V = vetch; W = wheat; NT = no tillage; CT = conventional tillage; 0N = no fertilization; 67N = 67 kg N ha^{-1} fertilization.

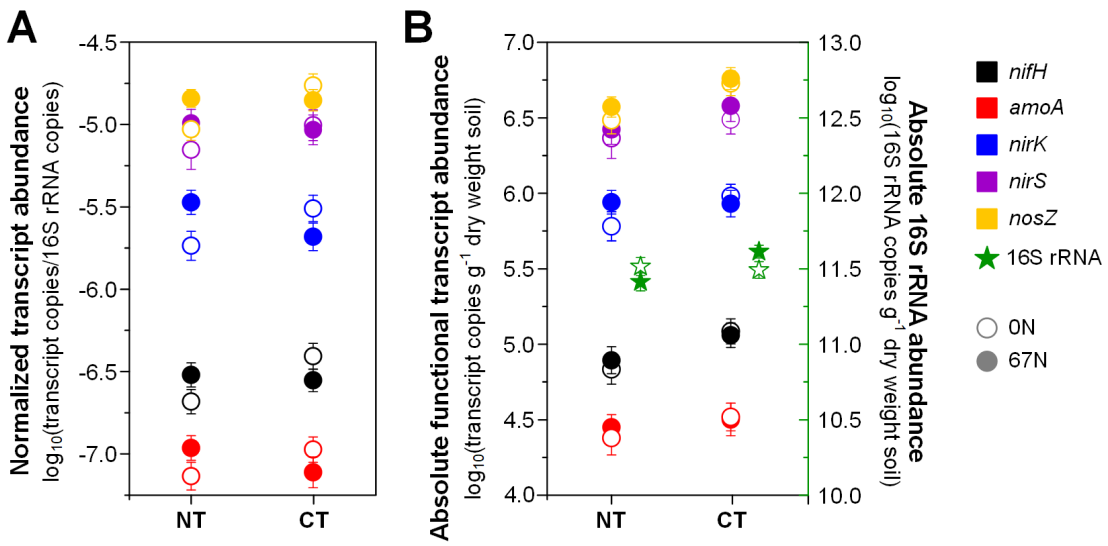

Figure 10. 16S rRNA normalized transcript abundances of *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* (A) and absolute transcript abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA (B) in relation to the combination of tillage and N fertilization (0 or 67 kg N ha^{-1} fertilization). Each value represents the mean ± standard error (n = 48).

Table 8. Mean values of log-transformed transcript-to-gene ratio of N-cycle genes in relation to agricultural season and soil management practices.

Treatment[†]		*nifH*	AOB[‡] *amoA*	*nirK*	*nirS*	*nosZ*
Season	April	-0.925^{d}	-1.080^{b}	-1.071^{b}	-1.411^{c}	-0.614^{b}
	May	-0.734^{c}	-0.988^{b}	-1.347^{c}	-1.500^{c}	-0.355^{a}
	October	-0.266^{b}	-1.043^{b}	-1.180^{bc}	-1.069^{b}	-0.354^{a}
	November	0.103^{a}	-0.223^{a}	-0.719^{a}	-0.526^{a}	-0.678^{b}
Cover	NC	-0.519^{b}	-0.706^{a}	-1.066^{b}	-1.133^{b}	-0.523^{b}
	V	-0.277^{a}	-0.730^{a}	-0.854^{a}	-0.915^{a}	-0.325^{a}
	W	-0.570^{b}	-1.065^{b}	-1.317^{c}	-1.323^{c}	-0.652^{b}
Season * Cover	Apr-NC	-1.104	-1.123^{efgh}	-1.198^{def}	-1.594^{ef}	-0.809^{ef}
	Apr-V	-0.683	-0.885^{def}	-0.751^{abc}	-0.934^{bc}	-0.330^{ab}
	Apr-W	-0.987	-1.233^{gh}	-1.263^{efg}	-1.674^{f}	-0.703^{de}
	May-NC	-0.875	-1.059^{defgh}	-1.480^{fg}	-1.568^{ef}	-0.480^{bcd}
	May-V	-0.576	-0.740^{cd}	-1.051^{cde}	-1.351^{de}	-0.129^{a}
	May-W	-0.752	-1.166^{fgh}	-1.509^{g}	-1.582^{ef}	-0.455^{bcd}
	Oct-NC	-0.143	-0.806^{cde}	-1.122^{de}	-1.049^{cd}	-0.391^{abc}
	Oct-V	-0.084	-0.961^{defg}	-0.911^{bcd}	-0.763^{bc}	-0.213^{ab}
	Oct-W	-0.572	-1.362^{h}	-1.508^{g}	-1.395^{ef}	-0.458^{bcd}
	Nov-NC	0.045	0.164^{a}	-0.462^{a}	-0.322^{a}	-0.413^{bc}
	Nov-V	0.233	-0.333^{b}	-0.704^{ab}	-0.613^{ab}	-0.630^{cde}
	Nov-W	0.031	-0.501^{bc}	-0.989^{bcde}	-0.643^{b}	-0.990^{f}
Season * Nitrogen	Apr-0N	-1.050	-1.267	-1.219^{de}	-1.469	-0.685^{cd}
	Apr-67N	-0.799	-0.894	-0.923^{bc}	-1.355	-0.543^{bc}
	May-0N	-0.716	-1.010	-1.259^{de}	-1.475	-0.246^{a}
	May-67N	-0.752	-0.967	-1.434^{e}	-1.525	-0.464^{abc}
	Oct-0N	-0.252	-1.008	-1.192^{de}	-1.071	-0.316^{a}
	Oct-67N	-0.280	-1.078	-1.168^{cd}	-1.067	-0.391^{ab}
	Nov-0N	0.011	-0.270	-0.825^{ab}	-0.593	-0.780^{d}
	Nov-67N	0.195	-0.177	-0.612^{a}	-0.459	-0.576^{bcd}
Cover * Nitrogen	NC-0N	-0.672^{c}	-0.918^{bc}	-1.157	-1.205	-0.644^{c}
	NC-67N	-0.366^{ab}	-0.494^{a}	-0.975	-1.061	-0.403^{ab}
	V-0N	-0.328^{ab}	-0.674^{ab}	-0.914	-0.911	-0.310^{a}
	V-67N	-0.227^{a}	-0.786^{b}	-0.795	-0.918	-0.341^{a}
	W-0N	-0.506^{bc}	-1.074^{c}	-1.300	-1.322	-0.567^{bc}
	W-67N	-0.634^{c}	-1.057^{c}	-1.334	-1.325	-0.737^{c}
Season * Cover * Tillage	Apr-NC-NT	-1.247^{k}	-1.194	-1.400^{fghi}	-1.710^{ij}	-0.939
	Apr-NC-CT	-0.960^{ijk}	-1.051	-0.996^{bcdef}	-1.478^{fghij}	-0.680
	Apr-V-NT	-0.589^{fghi}	-0.969	-0.787^{bcd}	-0.614^{bc}	-0.396
	Apr-V-CT	-0.778^{hij}	-0.802	-0.716^{bc}	-1.214^{defg}	-0.263
	Apr-W-NT	-0.717^{hij}	-1.319	-1.237^{efg}	-1.658^{ghij}	-0.819
	Apr-W-CT	-1.257^{k}	-1.147	-1.289^{fgh}	-1.691^{hij}	-0.588
	May-NC-NT	-1.057^{jk}	-1.024	-1.688^{hi}	-1.808^{j}	-0.639
	May-NC-CT	-0.692^{ghij}	-1.093	-1.272^{fgh}	-1.329^{efghi}	-0.322
	May-V-NT	-0.536^{efghi}	-0.662	-1.046^{cdef}	-1.371^{efghij}	-0.131
	May-V-CT	-0.615^{fghij}	-0.819	-1.057^{cdef}	-1.330^{efghi}	-0.128
	May-W-NT	-0.889^{ijk}	-1.158	-1.409^{fghi}	-1.513^{fghij}	-0.478
	May-W-CT	-0.615^{fghij}	-1.174	-1.608^{ghi}	-1.651^{ghij}	-0.432

Table 8. Continued.

Treatment[†]		*nifH*	AOB[‡] *amoA*	*nirK*	*nirS*	*nosZ*
Season * Cover * Tillage	Oct-NC-NT	-0.036^{bcd}	-1.062	-1.155^{def}	-0.925^{cde}	-0.445
	Oct-NC-CT	-0.249^{cdefg}	-0.550	-1.090^{cdef}	-1.172^{def}	-0.337
	Oct-V-NT	-0.234^{bcdef}	-0.922	-1.002^{bcdef}	-0.846^{cd}	-0.397
	Oct-V-CT	-0.124^{abcd}	-0.999	-0.819^{bcde}	-0.679^{bc}	-0.028
	Oct-W-NT	-0.367^{defgh}	-1.303	-1.228^{efg}	-1.244^{defgh}	-0.450
	Oct-W-CT	-0.777^{hij}	-1.420	-1.788^{i}	-1.545^{fghij}	-0.466
	Nov-NC-NT	0.210^{ab}	0.290	-0.133^{a}	-0.115^{a}	-0.295
	Nov-NC-CT	-0.119^{bcde}	0.037	-0.791^{bcd}	-0.529^{abc}	-0.532
	Nov-V-NT	0.347^{a}	-0.210	-0.591^{b}	-0.551^{abc}	-0.456
	Nov-V-CT	0.035^{abcd}	-0.456	-0.817^{bcde}	-0.674^{bc}	-0.804
	Nov-W-NT	-0.108^{bcde}	-0.505	-1.310^{fgh}	-0.945^{cde}	-1.072
	Nov-W-CT	0.030^{abc}	-0.496	-0.668^{bc}	-0.340^{ab}	-0.909
Season * Cover * Nitrogen	Apr-NC-0N	-1.478	-1.259^{gh}	-1.376	-1.733	-0.917^{jk}
	Apr-NC-67N	-0.729	-0.987^{efg}	-1.020	-1.455	-0.702^{ghij}
	Apr-V-0N	-0.815	-0.925^{defg}	-0.932	-0.930	-0.502^{bcdefghi}
	Apr-V-67N	-0.551	-0.845^{defg}	-0.571	-0.937	-0.157^{abc}
	Apr-W-0N	-0.856	-1.616^{hi}	-1.350	-1.676	-0.637^{efghij}
	Apr-W-67N	-1.118	-0.849^{defg}	-1.176	-1.673	-0.770^{ij}
	May-NC-0N	-1.008	-1.176^{gh}	-1.541	-1.672	-0.537^{cdefghij}
	May-NC-67N	-0.741	-0.941^{defg}	-1.420	-1.465	-0.424^{bcdefghi}
	May-V-0N	-0.472	-0.646^{cdef}	-0.957	-1.288	0.071^{a}
	May-V-67N	-0.679	-0.835^{defg}	-1.146	-1.413	-0.330^{bcdefg}
	May-W-0N	-0.667	-1.209^{gh}	-1.280	-1.466	-0.271^{abcde}
	May-W-67N	-0.837	-1.123^{fg}	-1.737	-1.699	-0.638^{efghij}
	Oct-NC-0N	-0.266	-1.145^{gh}	-1.311	-1.117	-0.643^{efghij}
	Oct-NC-67N	-0.020	-0.467^{bcd}	-0.933	-0.981	-0.139^{ab}
	Oct-V-0N	-0.110	-0.936^{defg}	-0.915	-0.790	-0.119^{ab}
	Oct-V-67N	-0.265	-0.985^{efg}	-0.906	-0.735	-0.306^{abcdef}
	Oct-W-0N	-0.381	-0.941^{defg}	-1.349	-1.305	-0.186^{abcd}
	Oct-W-67N	-0.763	-1.782^{i}	-1.666	-1.484	-0.729^{hij}
	Nov-NC-0N	0.064	-0.091^{b}	-0.400	-0.299	-0.480^{bcdefghi}
	Nov-NC-67N	0.026	0.418^{a}	-0.525	-0.346	-0.347^{bcdefgh}
	Nov-V-0N	0.087	-0.189^{bc}	-0.853	-0.640	-0.688^{fghij}
	Nov-V-67N	0.288	-0.476^{bcd}	-0.555	-0.586	-0.572^{defghij}
	Nov-W-0N	-0.300	-0.529^{bcde}	-1.222	-0.840	-1.172^{k}
	Nov-W-67N	0.181	-0.472^{bcd}	-0.757	-0.445	-0.809^{ijk}

[†]NC = no cover; V = vetch; W = wheat; NT = no tillage; CT = conventional tillage; 0N = no fertilization; 67N = 67 kg N ha^{-1} fertilization.

[‡]AOB = Ammonia oxidizing bacteria.

Table 9. ANOSIM analysis of the distribution patterns of N-cycling functional groups in four agricultural seasons and under different soil management practices.

Sample type	Treatment	Group		R	*p*-value
Soil DNA (gene level)	Season	Overall level		0.1748	0.001***
		Within group	April/May	0.1039	0.001***
			April/October	0.1194	0.001***
			April/November	0.0495	0.011*
			May/October	0.3101	0.001***
			May/November	0.2751	0.001***
			October/November	0.1764	0.001***
	Winter cover crop	Overall level		0.0150	0.060
		Within group	NC/V†	0.0361	0.017*
			NC/W	0.0007	0.362
			V/W	0.0085	0.185
	Tillage	NT/CT		0.0222	0.020*
	Fertilization	0N/67N		0.0401	0.003**
Soil RNA (transcription level)	Season	Overall level		0.2656	0.001***
		Within group	April/May	0.0597	0.004**
			April/October	0.0610	0.001***
			April/November	0.3793	0.001***
			May/October	0.0204	0.087
			May/November	0.5814	0.001***
			October/November	0.4872	0.001***
	Winter cover crop	Overall level		0.0495	0.001***
		Within group	NC/V	0.0335	0.009**
			NC/W	0.0254	0.029*
			V/W	0.0953	0.001***
	Tillage	NT/CT		0.0205	0.016*
	Fertilization	0N/67N		-0.0053	0.819

Note: * *p*-value ≤ 0.05, ** *p*-value ≤ 0.01, *** *p*-value ≤ 0.001.

†NC = no cover; V = vetch; W = wheat; NT = no tillage; CT = conventional tillage; 0N = no fertilization; 67N = 67 kg N ha^{-1} fertilization.

CHAPTER II

NITROGEN FERTILIZATION AND GRASS SPECIES ALTER NITROGEN CYCLING MICROBIAL COMMUNITIES IN A NATIVE GRASSLAND IN EAST TENNESSEE

This chapter includes three sub-chapters, formatted as manuscripts to be submitted. Chapter II.B has been published by the journal Frontiers in Microbiology. Chapter II.A and chapter II.C have not been published.

Chapter II.A Nitrogen fertilization and grass species alter microbial nitrogen cycling capacity and activity in a native C_4 grassland

Abstract

Soil microbial transformation of nitrogen (N) in low N required native C_4 grassland can be affected by N fertilization rate and C_4 grass species. Here, we reported *in situ* seasonal dynamics of the population size (gene copy abundances) and activity (transcript copy abundances) of five functional genes involved in soil N cycling (*nifH*, bacterial *amoA*, *nirK*, *nirS*, and *nosZ*) in a field experiment with two C_4 grass species (switchgrass [*Panicum virgatum*] and big bluestem [*Andropogon gerardii*]) under three N fertilization rates (0, 67, and 202 kg N ha^{-1}). Our results showed that diazotroph abundance and activity were not affected by N fertilization rate and grass species. Moderate and excessive N fertilization promoted population size and activity of ammonia oxidizing bacteria (AOB) and nitrification potential. The abundances of nitrite-reducing bacteria were increased by moderate N fertilization under switchgrass but were decreased under big bluestem. In general, excessive N fertilization did not promote the abundance and activity of N-cycling microbes and may have a negative effect on these populations compared to moderate N addition. Compared to big bluestem, the soils planted with switchgrass contained higher population size of ammonia-oxidizers and nitrite reducers. In addition, the significant interaction effects of season, grass species, and N fertilization rate on N-cycling population distribution by functional potential (genetic-level) rather than on activity (transcriptional-level) indicated the relative functional stability of N-cycling populations in native C_4 grass systems. Together, our results revealed relationships between N-cycling microbial functional capacity and activity and soil physicochemical properties under different agricultural seasons C_4 grass species, and N fertilization rates.

Introduction

Grasslands are a major agricultural land types, accounting for about 46.8% of all agricultural lands in the United States (USDA-NASS, 2012). C_4 grasses have become the preferred grass species for regional bioenergy development and forage production due to their exceptional drought-tolerance, high productivity, and low nitrogen (N) requirement. The low N requirement of C_4 grasses is hypothesized to be a result of their effects on the microbial assemblages involved in N cycling in C_4 native grassland.

The major soil N transformations regulated by microbes include N fixation, nitrification, and denitrification. Biological N fixation is the process that N gas in atmosphere is converted to ammonia by symbiotic, associative and free-living diazotrophs (Dixon & Kahn, 2004). Nitrification is the biological oxidation of ammonia to nitrate by ammonia- and nitrite-oxidizers, with ammonia oxidation as the first and rate-limiting step performed by ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Frijlink et al., 1992). Denitrification is a process of full or partial dissimilative reduction of NO_3^- conducted by facultative anaerobic microorganisms as a type of respiration to yield nitrous oxide (N_2O) or dinitrogen gas (N_2) (Colliver & Stephenson, 2000). Grassland mobilizes large pools of N with large potential for N losses through N_2O emissions and nitrate leaching via nitrification and denitrification mediated by the population size and activity of nitrifiers and denitrifiers. The global warming potential of N_2O is 296 times that of CO_2 over a 100-year period (IPCC, 2007). Around 100 to 256 kg N ha^{-1} yr^{-1} may be lost via nitrate leaching from grassland cultivation (Di & Cameron, 2002; Duan et al., 2017; Francis et al., 1995; Hansen et al., 2007). Around 40-100 Tg N yr^{-1} can be replenished by biological N fixation to terrestrial ecosystems (Vitousek et al., 2013) and account for about half of annual N inputs into biosphere (Vitousek et al., 1997; Gaby and Buckley, 2014). Therefore, the abundance and activity of microbes responsible for these N cycling processes play important roles in soil N availability and losses, which have been widely assessed by quantifying the functional marker genes *nifH* (N fixation), *amoA* (ammonia oxidation), *nirK* and *nirS* (nitrite reduction), and *nosZ* (nitrous oxide reduction).

The microbially-driven N-cycling processes in soils can be affected by land management practices and plant communities (Lindsay et al., 2010). N fertilization as a

key agricultural management in agroecosystems, impacts N-cycling microbial communities by altering N availability. Biological N fixation can be inhibited under N-rich environment due to that diazotrophs prefer using easily available exogenous N as this is less energy-consuming than fixing N from atmosphere (Chapin et al., 2002). However, previous studies have reported that N fertilization decreased (Cusack et al., 2009; C. Wang et al., 2017), increased (Lindsay et al., 2010), or had no effect on *nifH* abundance (Ouyang et al., 2018), which may depend on the N availability of sampling sites. Nitrogen addition has long been considered to promote nitrification and denitrification rates because it provides initial substrates for microbes involved in these N transformation processes (F. Wang et al., 2018). A meta-analysis of field studies also reported that N fertilization increased the abundance of genes in nitrification and denitrification in agricultural soils, including both croplands and grasslands. Moreover, N fertilization increased nitrification potential and denitrification enzyme activity by 93.7% and 27.9%, respectively (Ouyang et al., 2018). When N fertilizer was applied at the rate no more than 250 kg N ha^{-1}, soil denitrification increased exponentially with N-rate (J. Wang et al., 2018). It has been observed that the pH variation caused by N fertilization could be a dominant factor affecting the response of N-cycling populations to N fertilization (Hallin et al., 2009; Yang et al., 2017). The form of N fertilizer was also an important factor regulating the variability of N-cycling microbes, with stronger variability was observed under organic fertilizers than inorganic fertilizers (Ouyang et al., 2018).

Perennial grass species can affect net N mineralization due to the differences in tissue N concentrations, belowground lignin concentrations, and belowground biomass of the species (Wedin & Tilman, 1990). Compared to annual grasses, perennial grasses are more conservative with N, with lower nitrification rate and less nitrate leaching via a perennial root system (Yé et al., 2015). C_4 perennial grasses, like switchgrass (*Panicum virgatum*) have the potential to select diazotrophic community and form mutualistic symbioses with diazotrophs to improve N use efficiency (Smercina et al., 2020). Grass species may differently affect denitrification potential because of differences in labile C input (Groffman et al., 1996). However, limited studies have evaluated the responses of N-cycling microbes to N fertilization rates under different C_4 grass systems.

Our main objectives were to: 1) Determine the effects of grass species and N fertilization on abundance and activity of N-cycling microbes; 2) Investigate the seasonal dynamics of abundance and activity of N-cycling functional groups under two C_4 grass systems with different N fertilization rates; and 3) Identify relationships among the N-cycle functional microbial abundance, activity, and soil physicochemical parameters. The study was conducted at a native grassland small plot experiment utilizing two C_4 grass species and three N fertilization rates. To examine the dynamics of soil N-cycling functional groups, we used quantitative polymerase chain reaction (qPCR) and quantitative reverse-transcription PCR (qRT-PCR) to target the gene and transcript abundances of *nifH*, AOB *amoA*, *nirK*, *nirS*, and *nosZ*.

Materials and Methods

Study site, experimental design, and sample collection

This study was conducted at a native grassland plot established in 2013 at the University of Tennessee East Tennessee AgResearch and Education Center (ETREC) in Knoxville, Tennessee (35.53° N, 83.06° W). Soils at this site are classified as sandy loam (fine-loamy, mixed, semiactive, thermic Typic Hapludults). This study implemented a randomized complete block design with split-plot treatment arrangements with two native C_4 grass species (switchgrass [*Panicum virgatum*, SG] and big bluestem [*Andropogon gerardii*, BB]) being the main plot treatment and three N application rates (0, 67, and 202 kg N ha^{-1}; 0N, 67N, 202N, respectively) as the sub-plot treatment. These crossed treatments resulted in 6 cropping system combinations. Each treatment combination had three replicated plots. Sub-plots were 1.8 × 7.6 m in size. Grasses were planted in 2013, and N was applied as urea starting in 2014. Other fertilizers (phosphorus, potassium) and lime were applied at the recommended rates based on annual soil test results from the University of Tennessee Institute of Agriculture (UTIA) Soil, Plant and Pest Center (Nashville, TN). In addition, 2,4-Dichlorophenoxyacetic acid (3.5 L ha^{-1}) and Cimarron (52.5 g ha^{-1}) herbicides were sprayed once per year for weed control. Plots were harvested in June and August using a Carter forage harvester (Carter Manufacturing Company, Inc., Brookston, IN) with a 91.4-cm cutting width at a 20.3-cm cutting height.

Soil samples were collected from each sub-plot at three points during the growing seasons in 2019: grass green up (G, late April, one week before the first N fertilization), initial grass harvest (H1, late June, within one week after harvest and before the second N fertilization), and second grass harvest (H2, Mid-August, within one week before the second harvest). Six soil subsamples (2.5 × 10 cm cores) were collected in a systematic manner from each experimental sub-plot along the center-line of the long-axis to minimize influence from adjacent plots. Soil probes were sterilized with 70% ethanol after each sub-plot sampling. Soil samples from each sub-plot were composited and stored in Ziplock® bags and transported to laboratory on ice immediately after sampling. Then, the composited sample from each sub-plot was sieved through 2-mm mesh. A subsample of 10 g sieved soil per sub-plot was stored in -80℃ freezer for DNA and RNA extraction, and the remainder of the soil sample was used for the analysis of soil physicochemical properties.

Soil physicochemical properties

Soil pH was measured in 1:2 soil:water suspension using pH electrode (Ultrabasic, Denver Instrument, Bohemia, NY, USA). Soil water content (SWC) was determined gravimetrically as, SWC (%) = [(fresh soil weight – dry soil weight$_{(105℃, 48h)}$)/dry soil weight] × 100%. Air-dried soil was pulverized and analyzed for total organic carbon (TC) and total nitrogen (TN) by dry combustion using an Elementar vario MAX cube (Elementar, Langenselbold, Germany). Dissolved organic C (DOC) and N (DON) were extracted in Milli-Q water and analyzed using an Elementar vario TOC cube in liquid mode. Soil NH_4^+-N and NO_3^--N were extracted in 0.5 M K_2SO_4 solution and analyzed by a Berthelot reaction-based spectrophotometric method (Doane & Horwáth, 2003; Rhine et al., 1998) using a microplate reader (Synergy HT, BioTek, Winooski, VT, USA).

Soil nitrification potential was measured using the chlorate block method (Belser & Mays, 1980). Nitrification potential was determined in a chlorate slurry followed by nitrite detection over six hours. The upper limit of nitrification was determined in an ammonium and phosphate-rich medium in the presence of chlorate to prevent complete conversion of ammonium to nitrate. To do this, soil samples (2g) were mixed with 14 mL nitrification potential medium (4 mL 0.2M K_2HPO_4, 0.5 mL 0.2M KH_2PO_4, 2.5 mL 0.2M $(NH_4)_2SO_4$

and 10 mL 1M $NaClO_3$ in 983 mL deionized water) in 50 ml tubes and gently shaken for 4-6 hours (175 rpm, 25°C), with 1 mL slurry subsamples removed at 3 h intervals to measure nitrite accumulation rates. The 1 mL subsamples were transferred to 1.5 mL micro-centrifuge tubes and centrifuged before nitrite determination so that soil particles do not interfere with the colorimetric determination of NO_2^- and to stop the reaction (most of the nitrifiers will be associated with the soil particles). The NO_2^- concentrations in the filtrate were determined by adding 50 µL of NO_2^- color reagent (4 g of sulfanilamide and 0.1 g N-(1-naphthyl)-ethylenediamine hydrochloride and 17 mL 85% phosphoric acid in 87.6 mL deionized water) to 200 µL of centrifuged samples in a 96-well plate. After 15 min color reaction time, the absorbance is measured in a microplate reader spectrophotometrically at $\lambda = 543$ nm. Using the absorbance values for the nitrite standards compute the nitrite concentration for each sample, subtracting the non-soil controls. Subsamples of slurry were assayed at five minutes (T0), 3 hours (T1) and 6 hours (T2) for NO_2^-. At the end of the 6 h assay, the potential nitrification rates were calculated as µg NO_2^--N g^{-1} soil h^{-1}.

Soil N_2O emissions were measured using the static chamber method (Collier et al., 2014). A circular stainless-steel base (15 cm in diameter) was inserted into the soil to a depth of approximately 8 cm at the center of each sub-plot as a chamber. For the sampling, the chamber was closed with a lid with two openings. Each opening fitted with a valve, one for gas sampling and other to equilibrate internal and external pressure. Gases were sampled with plastic syringes (30 mL) at four-time intervals (0, 20, 40, and 60 min) after the chamber was closed. The samples were transferred and stored in pre-evacuated glass vials (12 mL) fitted with rubber stoppers. During each gas sampling, soil temperature was measured using digital thermometer (Thermo Scientific, USA). The gas samples were analyzed in a gas chromatograph with an electron capture detector for N_2O determination. N_2O flux was calculated by linear interpolation of the four sampling times. The N_2O-N emission flux was calculated as follows:

$$F = \rho \times h \times \mathrm{d}c/\mathrm{d}t \times 273/(273+T) \times k$$

Where F is emission flux, the unit of which is µg N m^{-2} h^{-1}; ρ is the N_2O-N density in standard state, which is 1.25 kg m^{-3}; h is the net height of static chamber, which is 0.08 m;

d*c*/d*t* is the rate of gas concentration change, the unit of which is μL L^{-1} min^{-1}; *T* is the average soil temperature in the static chamber during gas sampling, the unit of which is ℃; *k* is the time conversion factor, 60 min h^{-1}. The impact of pressure was not taken into consideration because of the almost constant pressure in static chamber during gas sampling.

Soil nucleic acid extraction and cDNA synthesis and quantitative analysis of N-cycle functional genes and 16S rRNA genes and gene transcripts

Soil genomic DNA was extracted from 0.25 g soil from each sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentrations were quantified using the NanoDrop One spectrophotometer (NanoDrop Technologies, Wilmington, DE). Extracted DNA samples were stored at -20℃ for further qPCR amplification and sequencing.

Soil total RNA was extracted using the RNeasy PowerSoil Total RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was eluted in RNase-free water and stored at -80℃. The quality was checked by 1% agarose gel electrophoresis and NanoDrop One spectrophotometry (NanoDrop Technologies, Wilmington, DE). Samples were checked for DNA contamination by attempting to amplify *nifH* genes by PCR and subsequent agarose gel electrophoresis. Only samples that gave a negative electrophoresis result (i.e., did not have DNA contamination) were used for reverse transcription. cDNA was produced with RNA as the template using SuperScript IV Reverse Transcriptase (Invitrogen, Paisley, UK) according to the manufacturer's protocol. Random hexamer primers (Invitrogen) were used at a final concentration of 2.5 μM per reaction. cDNA samples were stored at -20℃ for further qRT-PCR amplification.

The primer sets, reaction systems, and thermal cycle parameters of qPCR and qRT-PCR of *nifH*, *bacterial amoA*, *nirK*, *nirS*, and *nosZ*, as well as the quantification of 16S rRNA genes and 16S rRNA were described in detail in Materials and Methods in Chapter I.

Statistical analysis

A mixed model ANOVA within the GLIMMIX procedure in SAS 9.4 (SAS Inst., Cary, NC, USA) was performed to test the effects of grass growing season, N fertilization rate, and grass species on population size and activity of N-cycling microbes and total bacteria. The abundances of the N-cycle genes and transcripts were log-transformed to achieve normal distributions. The fixed effects included season, N fertilization rate, and grass species, as well as their interactions. Block was included as a random effect. A post-hoc least significant difference (LSD) method was used to compare the means of groups. Pearson correlation analyses were performed in IBM SPSS Statistics v26 to evaluate the correlation among the abundance and activity of N-cycling microbes and total bacteria and soil physico-chemical parameters. A heatmap based on Pearson correlations was used to visualize the significant positive and negative correlations among measured soil properties, N-cycle gene abundance and gene transcript abundance, and was performed by pheatmap package in R 3.6.1.

Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances was performed in R 3.6.1 with packages vegan 2.5-5 and phyloseq to reveal the distribution patterns of N-cycling functional groups and their activity based on the relative abundance of five N-cycle functional genes (*nifH*, *amoA*, *nirK*, *nirS*, *nosZ*) and their transcripts among sampling seasons, grass species, and N fertilization rates.

Analysis of similarities (ANOSIM) was also performed by R with the function 'anosim' in package vegan to compare groups and test the null hypothesis that the similarity between groups is greater than or equal to the similarity within the groups. Dispersion indices were calculated in R with the function 'betadisper' in package vegan to assess the multivariate homogeneity of group dispersions. The function 'TukeyHSD.betadisper' in package vegan was used to calculate Tukey's Honest Significant Differences between groups. In this study, statistically significant difference was accepted at p-value < 0.05 unless otherwise noted.

Results

Soil physicochemical properties

Soil water content (SWC), dissolved organic C (DOC), and dissolved organic N (DON) showed significant differences by agricultural season ($p < 0.001$ for SWC and DOC; $p = 0.006$ for DON) (Table 10; Appendix Table 15). The SWC was highest (0.30 ± 0.01 g H_2O g^{-1} dry weight soil [gdw^{-1}]) at initial grass harvest and lowest (0.15 ± 0.01 g H_2O gdw^{-1}) at second grass harvest. Both DOC and DON were highest (223.00 ± 5.47 μg gdw^{-1} and 22.44 ± 1.98 μg gdw^{-1}, respectively) after second grass harvest and lowest (163.85 ± 4.07 μg gdw^{-1} and 16.16 ± 0.65 μg gdw^{-1}, respectively) after initial grass harvest (Appendix Table 15).

Nitrogen fertilization rate and grass species significantly affected several soil properties (Table 10). With N fertilization, soil nitrate (NO_3^--N) concentrations increased from 0.39 ± 0.16 μg gdw^{-1} to 2.77 ± 0.39 μg gdw^{-1}, soil pH decreased from 6.36 ± 0.05 to 6.12 ± 0.05, and DOC decreased from 210.18 ± 8.60 to 186.07 ± 5.23 μg gdw^{-1}. Soil pH was significantly higher under switchgrass (6.32 ± 0.05) compared to big bluestem (6.19 ± 0.03). Soil water content was higher under N fertilization (67N, 202N) in switchgrass plots while it decreased with N fertilization in big bluestem plots. C: N ratio was not affected by N fertilization in big bluestem plots but was lower under 67N and 202N (11.63 ± 0.10 and 11.53 ± 0.09, respectively) compared to 0N (12.36 ±0.36) in switchgrass plots (Appendix Table 15).

The effect of N fertilization rate on NH_4^+-N concentration depended on the sampling time (Table 10; Appendix Table 15). At grass green up, NH_4^+-N concentration decreased from 12.29 ± 1.26 to 9.54 ± 0.38 μg gdw^{-1} with increased N-rate whereas at both initial and second grass harvest period, NH_4^+-N increased with N-rate (from 9.36 ± 1.14 to 11.71 ± 0.84 μg gdw^{-1} at initial grass harvest; from 6.46 ± 0.62 to 7.61 ±0.61 μg gdw^{-1} at second grass harvest). The interaction between season and grass species was significant for NO_3^--N concentration (Table 10; Appendix Table 15): Under switchgrass, NO_3^--N concentrations remained constant, while under big bluestem, NO_3^--N was higher at initial grass harvest (2.47 ± 0.78 μg gdw^{-1}) compared to grass green up (1.12 ± 0.40 μg gdw^{-1}) and second grass harvest (0.31 ± 0.15 μg gdw^{-1}).

Table 10. Results of mixed model ANOVA (based on GLIMMIX procedure in SAS) testing effects of agricultural season, nitrogen fertilization and grass species on soil properties. F-values are reported.

Effect	Season (S)	Nitrogen (N)	Grass (G)	N×G	S×N	S×G	S×N×G
pH	3.26	11.70***	11.53**	0.06	0.59	1.99	0.13
SWC†	388.39***	0.31	0.01	3.75*	0.89	0.35	0.57
NH_4^+-N	23.06***	0.01	0.04	1.09	3.40*	3.05	1.35
NO_3^--N	9.00***	31.14***	1.95	0.17	1.41	5.42**	1.61
DOC	57.99***	7.71**	0.2	2.62	1.48	0.98	0.21
DON	6.10**	1.2	1.91	1.06	1.64	0.83	1.13
Total organic C	0.43	0.88	0.11	1.13	1.58	1.22	0.84
Total N	1.11	2.44	0.82	0.08	1.79	1.52	0.53
C: N ratio	0.98	2.15	2.01	4.35*	0.24	0.24	0.26
N_2O-N	12.46***	5.94**	2.25	2.38	5.36**	1.78	3.00*
Nitrification potential	31.13***	21.85***	10.44**	0.28	2.72*	0.13	0.3

Significance level: * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001.

†SWC, soil water content; DOC, dissolved organic C; DON, dissolved organic N.

N_2O emission was significantly affected by the interaction of season, N fertilization rate, and grass species (Table 10; Appendix Table 15). At initial grass harvest, N_2O-N increased with N-rate, from 10.01 ± 2.00 to 23.77 ± 2.08 g N_2O-N ha^{-1} d^{-1} in switchgrass plots and from 8.74 ± 3.92 to 74.98 ± 29.33 g N_2O-N ha^{-1} d^{-1} in big bluestem plots. However, at grass green up and second grass harvest, no effect of N fertilization rate and grass species on N_2O-N was observed.

Nitrification potential (NP) varied significantly among different agricultural seasons, N fertilization rates, and grass species (Table 10; Figure 11). In general, NP was highest at second grass harvest and lowest at grass green up for all the six treatment combinations of N fertilization rate and grass species (Figure 11; Appendix Table 15). NP significantly increased with N fertilization rate, from 0.06 ± 0.01 μg NO_2-N gdw^{-1} hr^{-1} under no N fertilization to 0.17 ± 0.02 μg NO_2-N gdw^{-1} hr^{-1} under 202 N-rate (Figure 11; Appendix Table 15). Specifically, agricultural season and N fertilization rate had significantly interaction effect on NP (Table 10; Appendix Table 15): At grass green up, NP increased from 0.04 ± 0.01 to 0.08 ± 0.01 NO_2-N gdw^{-1} hr^{-1} with increased N-rate, but this increase was not statistically significant. At both initial and second grass harvest period, NP was significantly higher under 202 N-rate than no N fertilization (0 N-rate) and 67 N-rate. In addition, NP was significantly higher in switchgrass plots (0.14 ± 0.02 μg NO_2-N gdw^{-1} hr^{-1}) than in big bluestem plots (0.10 ± 0.02 μg NO_2-N gdw^{-1} hr^{-1}) (Figure 11; Appendix Table 15).

Functional genes and transcripts

The abundance of 16S rRNA genes showed significant seasonal dynamics (Table 11), which was highest in August (average 7.62×10^9 copies g^{-1} dry weight soil) and lowest in June (average 3.65×10^9 copies g^{-1} dry weight soil) (Figure 12A; Appendix Table 16). 16S rRNA abundance remained stable among sampling seasons under different treatment of grass species and N fertilization rates (Table 11).

The abundances of N-cycle genes and transcripts under different treatment combinations are shown in Appendix Table 16 and Appendix Table 17. In general, the relative abundances (normalized to 16S rRNA genes and 16S rRNA) changed consistently

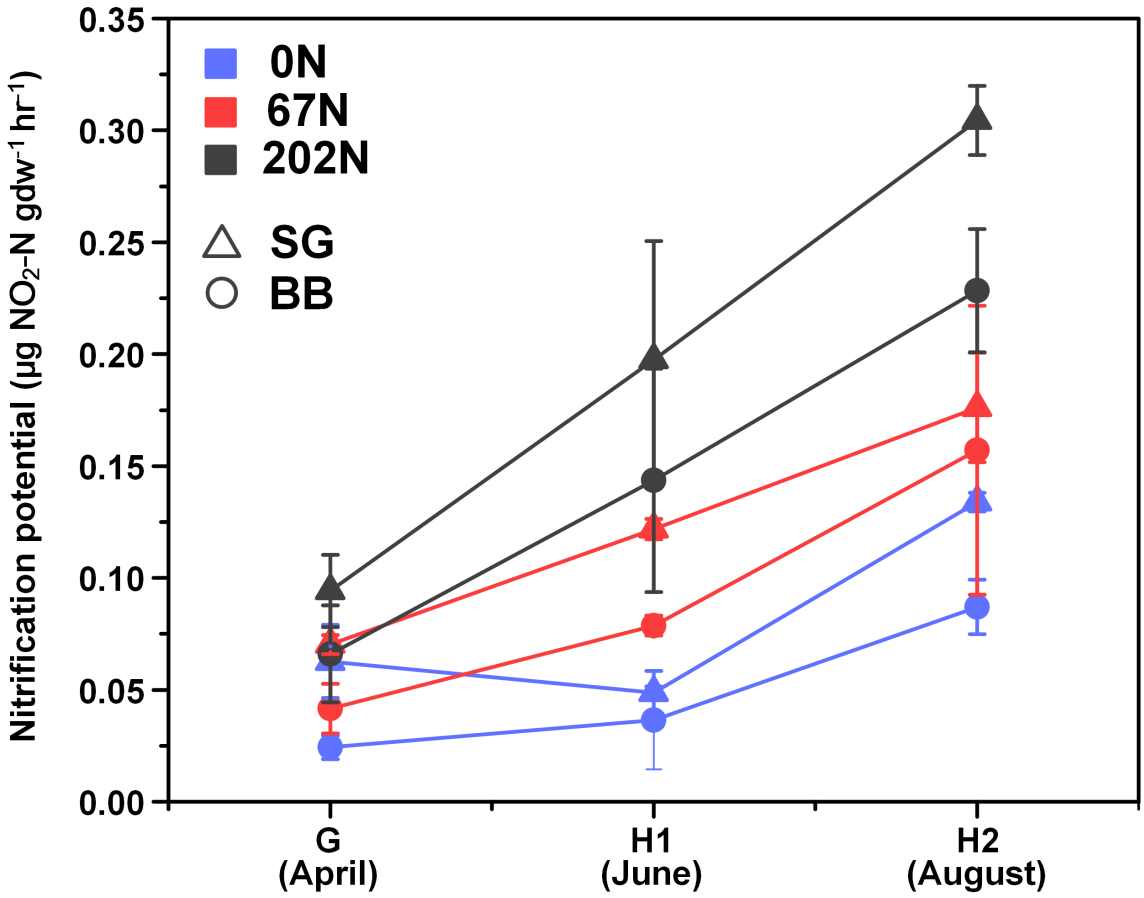

Figure 11. Seasonal dynamics of nitrification potential in relation to nitrogen fertilization rate and grass species. G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

Table 11. Results (F values) of mixed model ANOVA (based on GLIMMIX procedure in SAS) testing effects of sampling season, grass species, and N fertilization rate on the relative (R) and absolute (A) abundances of N-cycle genes (G) and transcripts (T). Significant results are indicated with bold fonts and asterisks.

Factor		*nifH*		AOB[a] *amoA*		*nirK*		*nirS*		*nosZ*		16S	
		G	T	G	T	G	T	G	T	G	T	G	T
Season (S)	R	**32.70*****	**12.87*****	**82.01*****	**9.03*****	**23.59*****	**6.22****	**21.44*****	2.46	**17.64*****	1.82	-	-
	A	**57.42*****	**17.20*****	**91.22*****	**17.20*****	**40.79*****	**13.13*****	**61.48*****	**5.38****	**37.91*****	**20.12*****	**16.63*****	2.23
Grass	R	2.30	0.00	**5.44***	0.33	1.82	1.10	**4.18***	0.16	0.30	0.73	-	-
	A	0.73	0.02	**8.33****	0.67	**5.88***	0.71	**13.31*****	0.24	2.11	1.50	3.78	0.06
Nitrogen (N)	R	0.62	2.26	**24.39*****	**8.97*****	0.77	0.39	0.45	**4.84***	0.68	0.59	-	-
	A	0.43	2.31	**15.72*****	**8.30****	0.04	0.72	0.90	**4.29***	0.00	**4.39***	0.24	0.14
S×Grass	R	0.48	1.75	0.79	1.89	0.17	1.08	0.27	0.63	0.37	1.36	-	-
	A	0.41	1.15	1.56	0.92	0.93	0.44	0.84	0.83	1.95	0.81	1.30	0.59
S×N	R	0.44	2.61	0.86	**4.37****	0.57	**2.84***	0.58	1.53	0.21	1.34	-	-
	A	0.17	1.88	0.34	**3.33***	0.79	1.62	0.34	1.21	0.51	1.00	1.12	0.65
Grass×N	R	0.18	0.76	1.63	2.75	1.03	1.60	2.82	0.27	1.72	1.81	-	-
	A	1.55	0.13	1.13	1.44	3.05	0.07	**7.51****	0.45	3.01	0.23	1.96	1.26
S×Grass×N	R	0.67	2.01	0.26	0.74	2.08	0.74	0.65	1.34	1.44	0.58	-	-
	A	0.44	1.22	0.25	0.48	0.48	0.46	0.52	0.90	0.28	0.57	0.35	0.34

Significance level: * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001.

[a]AOB, Ammonia oxidizing bacteria.

with the absolute abundances (per gram dry weight soil) for both N-cycle genes and transcripts (Figure 12, Figure 13, and Figure 14). Both absolute and relative abundances of N-cycle genes were mostly affected by sampling season, grass species, and N fertilization, as well as the interaction of grass species and N fertilization (Table 11). However, transcript abundances were mostly affected by season and N fertilization rate or the interaction between the two (Table 11).

Both absolute and relative abundances of N fixation gene *nifH* were significantly affected by sampling season (Table 11), with highest abundances at second grass harvest (H2, August) and lowest at initial harvest (H1, June) (Figure 12). No significant effect of grass species and N fertilization on *nifH* gene abundances were observed (Table 11). Both absolute and relative abundances of *nifH* transcript were only significantly affected by sampling season (Table 11), with lowest abundances at H2 (Figure 14; Appendix Table 17).

Both absolute and relative abundances of bacterial ammonia oxidation gene *amoA* were significantly impacted by season, grass species, and N fertilization rate (Table 11). August had the highest *amoA* abundances while June had the lowest abundances. *amoA* abundances were higher under switchgrass than big bluestem. N fertilization increased *amoA* abundances compared to no N addition (Figure 12; Figure 13; Appendix Table 16). Season and N fertilization had significant interaction effect on both absolute and relative abundances of *amoA* transcript (Table 11). N fertilization did not affect *amoA* transcript abundances at grass green up (G) in April. *amoA* transcript abundances were highest under moderate N fertilization (67N) in June but increased with N fertilization rate in August (Figure 14).

Both absolute and relative abundances of nitrite reduction gene *nirK* varied by sampling season (Table 11), with highest abundances in August and lowest in June (Figure 12). Moreover, the absolute abundance of *nirK* was significantly higher under switchgrass than big bluestem (Figure 13A; Appendix Table 16) while no significant effect of grass species on the relative abundance of *nirK* genes was observed (Figure 13B). The absolute abundance of *nirK* transcript varied with sampling season, which was highest in June (Appendix Table 17). *nirK* transcript relative abundance was significantly affected by the interaction of season and N fertilization (Table 11). 67N significantly increased their

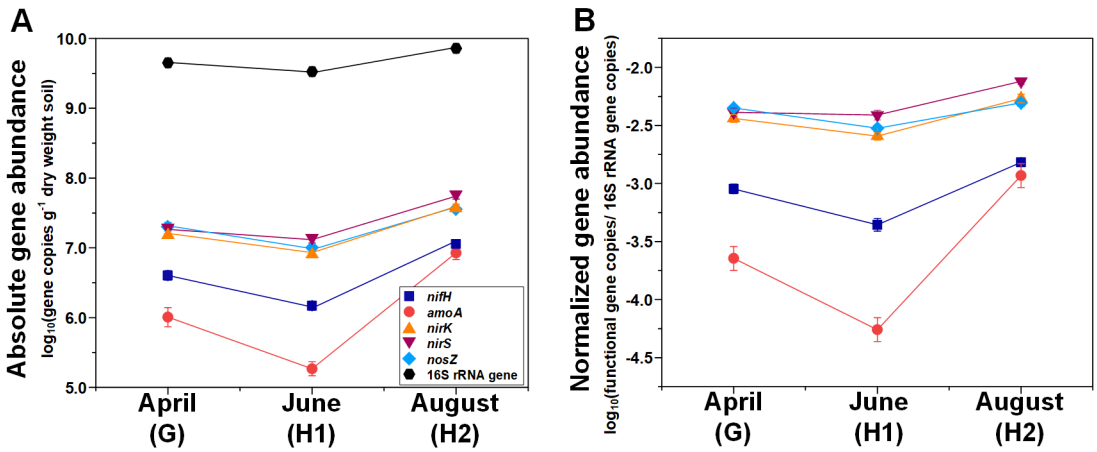

Figure 12. Seasonal dynamics of absolute abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes (A) and 16S rRNA gene normalized *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* gene abundances (B). Points represent the mean ± standard error (n = 18). G, grass green up; H1, initial grass harvest; H2, second grass harvest.

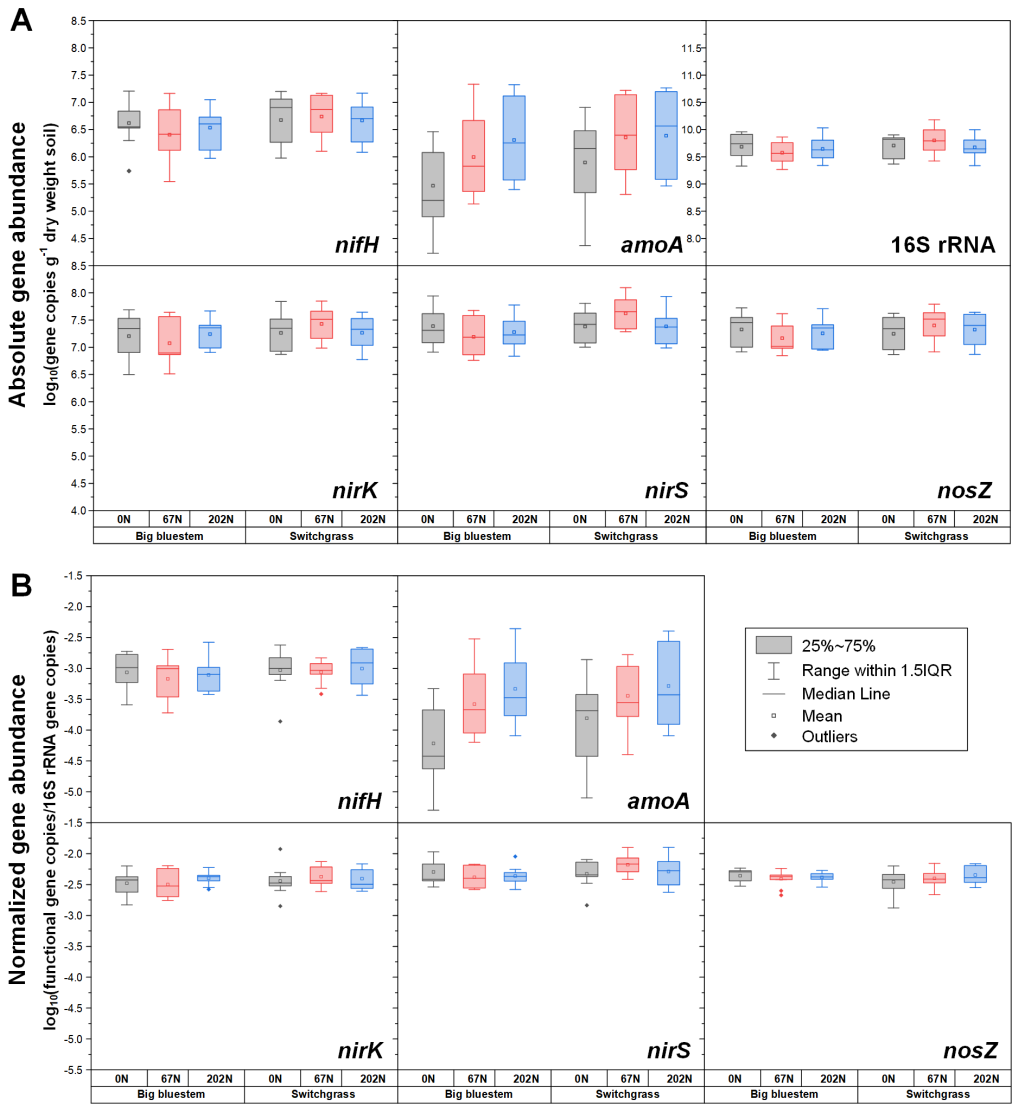

Figure 13. Absolute abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes (A) and abundances normalized to 16S rRNA gene (B) in relation to N fertilization rate under different grass species. 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization.

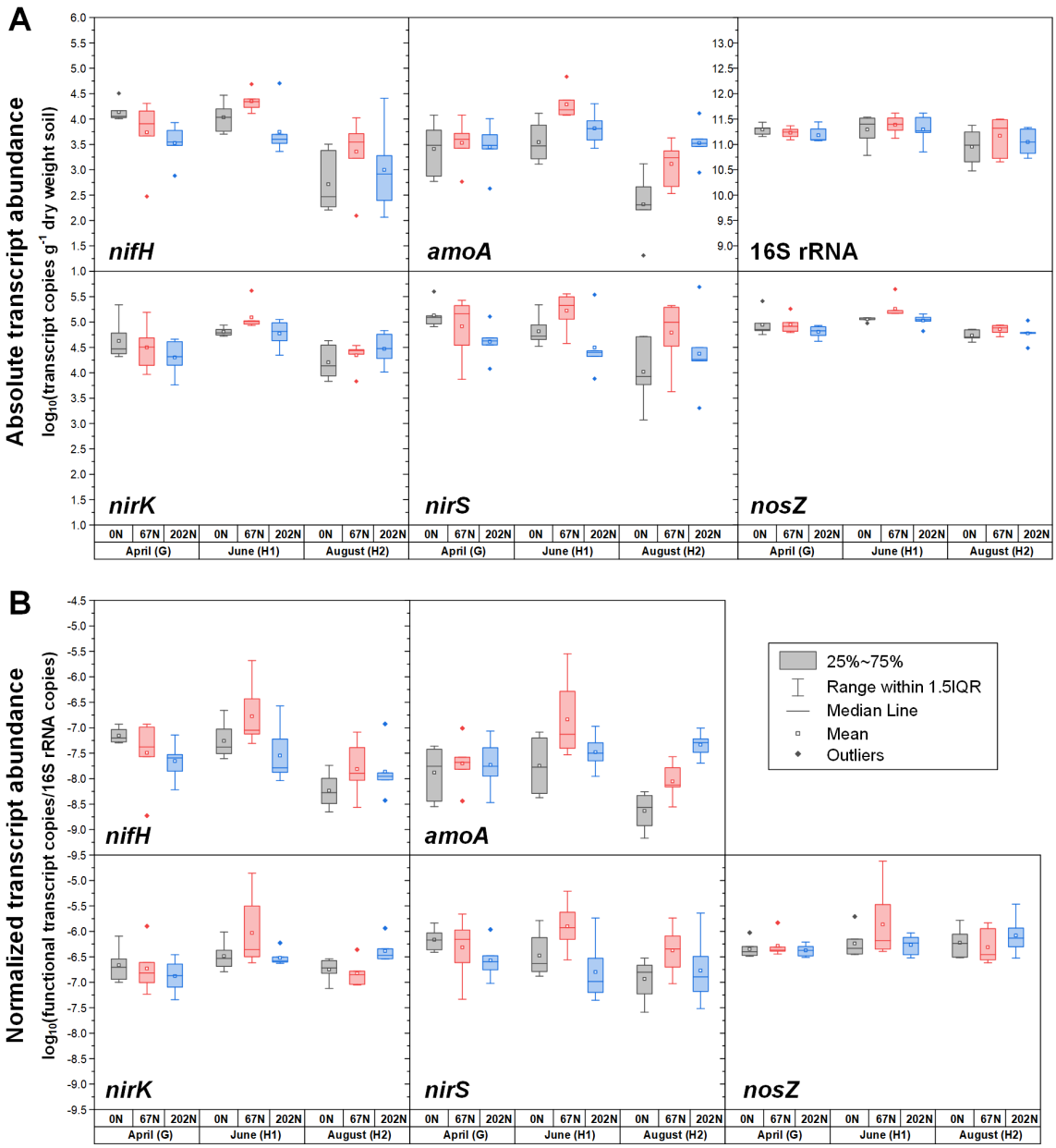

Figure 14. Seasonal dynamics of absolute abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA gene transcripts (A) and transcripts normalized to 16S rRNA transcript abundances (B) in relation to N fertilization rate. 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; G, grass green up; H1, initial grass harvest; H2, second grass harvest.

relative abundance in June but had no effect in April and August (Figure 14B).

The abundances of nitrite reduction gene *nirS* were significantly affected by season (Table 11), with higher abundances in August (Figure 12). *nirS* gene absolute abundance was also significantly affected by the combination of grass species and N fertilization rate (Table 11), which was promoted by 67N under switchgrass but was decreased by 67N under big bluestem (Figure 13A; Appendix Table 16). *nirS* gene relative abundance was higher under switchgrass than under big bluestem (Appendix Table 16). Season only affect absolute abundance of *nirS* transcript, with lower abundance observed in August (Table 11; Appendix Table 16). Both absolute and relative abundance of *nirS* transcript were affected by N fertilization rate (Table 11), with 67N resulted in highest abundances (Appendix Table 17).

The abundances of nitrous oxide reduction gene *nosZ* were only significantly affected by season (Table 11), with both absolute and relative abundances lowest in June (Figure 12; Appendix Table 16). Absolute abundance of *nosZ* transcript was affected by season and N fertilization rate (Table 11), with highest abundance in June and under 67N (Figure 14A; Appendix Table 17). *nosZ* transcript relative abundance was not affected by any treatment (Table 11).

Although the combination effect of grass species and N fertilization rate was not all significant for the abundances of nitrite reduction genes *nirK* and *nirS*, we found that their higher abundances under switchgrass than big bluestem were mainly due to the promotion effect of 67N under switchgrass but inhibition effect under big bluestem (Figure 13; Appendix Table 17).

Variability of N cycling microbial functional potential and activity

As expected, the statistical dispersion, or variability of the populations by activity was significantly higher than by functional potential (dispersion index = 0.307 for transcripts and 0.178 for genes; $P < 0.001$). Principle coordinate analysis (PCoA) showed the variability of the N-cycling microbial populations in terms of functional potential (i.e., based on the five gene relative abundances) and functional activity (i.e., based on the five transcript relative abundances) (Figure 15). Analysis of similarities (ANOSIM) testing

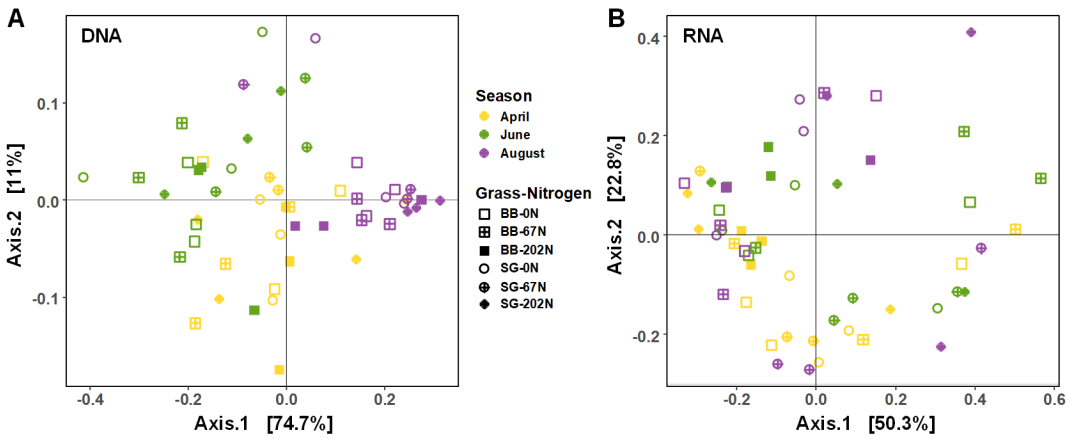

Figure 15. Principle coordinate analysis (PCoA) of Bray-Curtis distances between N-cycling communities based on relative abundances of five N-cycle genes (A) and transcripts (B). BB, big bluestem; SG, switchgrass; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization.

showed that the population distribution by functional potential was significantly affected by the combination of season, grass species, and N fertilization rate ($R = 0.384$; $P = 0.001$) (Table 12; Figure 15A). The population distribution by activity was only significantly affected by season ($R = 0.060$; $P = 0.029$) (Table 12; Figure 15B).

Relationship of soil properties with functional gene and transcript abundance

The correlation heatmap showed significant correlations among absolute abundances of N-cycle genes and their transcripts and soil properties (Figure 16). We observed significant positive correlations among the six marker genes and the six gene transcripts (Figure 16). The genes and their transcripts showed different patterns of correlations with soil properties (Figure 16). In general, the abundances of N-cycle genes and 16S rRNA gene were more closely correlated to soil properties than their transcripts. The 16S rRNA gene abundance was positively correlated to DOC but negatively correlated to SWC and inorganic N (NH_4^+-N and NO_3^--N) where as 16S rRNA abundance was only positively correlated to SWC. The N-cycle genes were both negatively correlated with SWC whereas their transcripts were positively correlated with SWC (Figure 16). Nitrification potential was positively correlated to *amoA* gene abundance but not *amoA* transcript abundances (Figure 16). The abundances of *nirK* and *nirS* genes were both positively correlated to DOC and DON, which was not observed for their transcripts (Figure 16). However, N_2O emission was negatively correlated to both N-cycle genes but had no correlation with gene transcripts (Figure 16).

Discussion

The primary objective of this study was to investigate the impact of grass species and N fertilization rate on the seasonal population size and activity of soil microbial communities involved in N transformations by using *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* as marker genes.

We found that the N-cycling microbial population size showed seasonal changes regardless of grass species and N fertilization rates. The total bacteria, diazotrophs, AOB, and denitrifying bacteria were all most abundant at second grass harvest in August and least

Table 12. Results of ANOSIM examining the importance of the effects of season, N application rate, and grass species on N-cycling microbial communities.

Factor	DNA		RNA	
	R	*p*-value[†]	*R*	*p*-value[†]
Season	**0.475**	**0.001**	**0.060**	**0.029**
Nitrogen	-0.018	0.700	0.017	0.220
Grass	0.021	0.189	0.026	0.106
Season×Grass	**0.425**	**0.001**	0.055	0.053
Season×Nitrogen	**0.342**	**0.001**	0.069	0.068
Nitrogen×Grass	-0.004	0.469	0.039	0.122
Season×Nitrogen×Grass	**0.384**	**0.001**	0.051	0.199

[†]Bold values indicate *p*-value ≤ 0.05.

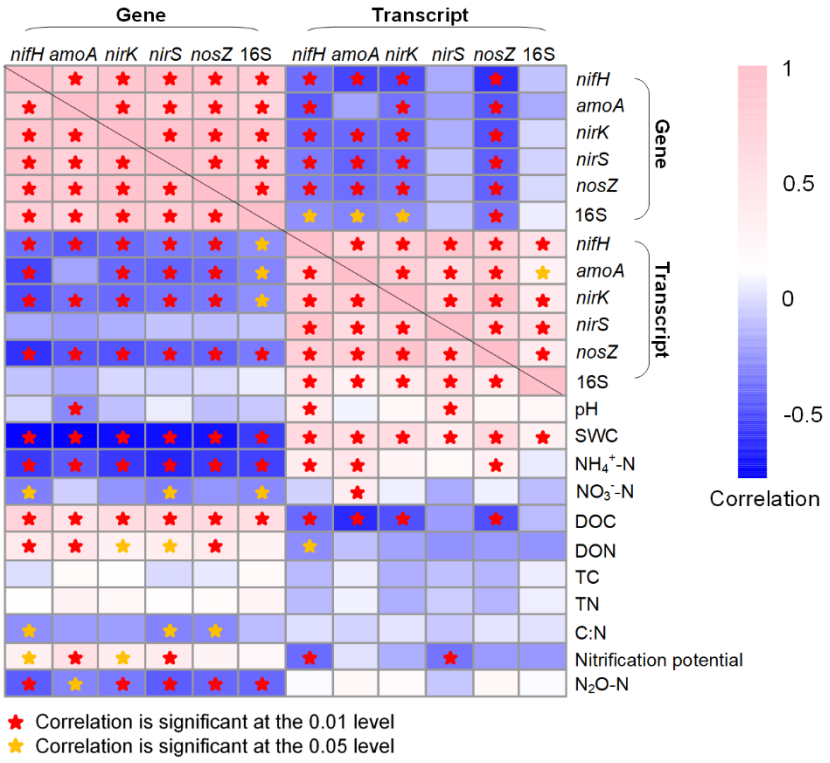

Figure 16. Heatmap showing Pearson correlation coefficients among genes, gene transcripts, and soil properties. SWC, soil water content; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; TC, total organic carbon; TN, total nitrogen.

abundant at initial grass harvest in June, which might be explained by the variability of DOC and DON. We sampled soils after mowing at first grass harvest but before mowing at second grass harvest. Mowing decreased C substrate supply from photosynthesis and grass residues, therefore, decreased decomposition rates and DOC released by soil microbes (Luo et al., 2019). The lower SWC in August may also have reduced the potential of DOC and DON loss through leaching (Poll et al., 2008). In August, the easily accessible C can support the growth of heterotrophic diazotrophs. In addition, denitrifying bacteria are widespread in soils, accounting for about 0.5% to 5% of the total bacterial population and including both autotrophic and heterotrophic bacteria, which were strongly influenced by soil C (Levy-Booth et al., 2014). Consistent with our result, previous studies have demonstrated that the abundance of soil *nirK* and *nosZ* gene quantities were positively correlated to DOC and/or DON (Bárta et al., 2010; Rasche et al., 2011). The positive correlation of AOB abundance with DOC and DON concentrations observed in our study has been observed in some other agroecosystems as well (Hu et al., 2021; Sun et al., 2019), which might be because the C and N mineralization by heterotrophs can provide C and N sources for AOB growth.

The higher soil oxygen content due to lower SWC in August resulted in lower activity of diazotrophs, as indicated by *nifH* transcript abundance, likely because nitrogenase activity is sensitive to oxygen (Dobereiner et al., 1972; Eady & Postgate, 1974). Contrary to our expectation, although ammonia oxidation is an aerobic process, the AOB functional activity was lower in August and was positively correlated to SWC but negatively correlated to DOC. One explanation is that AOB increased the expression of *amoA* genes under oxygen stress (Theodorakopoulos et al., 2017; Yu & Chandran, 2010). Another possible reason is that the exudates containing inhibitory compounds released by some grass roots can inhibit nitrification (Subbarao et al., 2009). Moreover, the weaker competitiveness of autotrophic AOB than heterotrophs for ammonium under labile C-rich conditions is also a possible reason for lower functional activity of AOB in August with high DOC concentrations (Strauss & Lamberti, 2002). The activity of nitrite reducing bacteria and nitrous oxide reducing bacteria were both lowest in August, which is likely because high oxygen content resulted from low SWC suppressed the anaerobic

denitrification processes.

N fertilization had no effect on the abundance of diazotrophs, which was in accordance with the result from a meta-analysis (Ouyang et al., 2018). Compared to no fertilization, moderate urea fertilization with 67 kg N ha^{-1} and excessive fertilization with 202 kg N ha^{-1} similarly increased AOB abundance. The increased AOB abundance by urea addition was also observed in previous studies (Rudisill et al., 2016; Xiang et al., 2017). The promotion effect of N fertilization on AOB activity and nitrification potential was only observed in initial and second harvest seasons after N fertilization, indicating that urea fertilization may only have short-term rather than over-year influence on nitrification process in native C_4 systems. However, a previous study reported that nitrification potential was reduced due to the inhibition of *amoA* expression by lower pH caused by urea fertilization (Rudisill et al., 2016). The inconsistence might be that although pH was reduced by excessive N fertilization in our study (from 6.36 in 0N and 6.29 in 67N to 6.12 in 202N), this decreased pH was not sufficient to suppress AOB activity. N fertilization had higher impact on the functional activity of denitrifying bacteria rather than their population size. The response of *nirS*-type nitrite reducing bacterial abundance to N fertilization rate depended on grass species, with promoted abundance by moderate N addition under switchgrass but decreased abundance by moderate N-rate under big bluestem, which might be related to different N-use efficiency and C-to-N ratio of grass species. Compared to no N addition, moderate N fertilization increased the activity of *nirS*-type nitrite reducing bacteria and nitrous oxide reducing bacteria. Notably, we observed that excessive N addition (202 kg N ha^{-1}) slightly suppressed the activity of denitrifiers, which may be because denitrifiers are sensitive to high amounts of nitrate caused by high N rate (Kastl et al., 2014; Wallenstein et al., 2006).

In addition, our study found that excessive N fertilization significantly increased N_2O emissions, especially in June. Although many previous studies suggested that ammonium oxidizers and/or denitrifiers were main contributors to N_2O emissions in soils (Kastl et al., 2014; Soares et al., 2016). N_2O emission was negatively correlated to N-cycle gene abundances and not correlated to N-cycle gene transcript abundances, which may be attributed to a lot of reasons, such as climatic factors, edaphic properties, and the abundance and activity of fungi. Fungi has been considered to be responsible for a large portion of soil

N_2O emissions due to that they do not contain an ortholog to the enzyme nitrous oxide reductase (NOS) (Kobayashi et al., 1996; Laughlin & Stevens, 2002). It has been reported that fungal denitrification produced up to 89% of N_2O in a grassland soil (Laughlin & Stevens, 2002).

Compared to big bluestem, switchgrass plots had higher absolute and relative abundances of AOB and *nirS*-type nitrite-reducing bacteria as well as the absolute abundance of *nirK*-type nitrite-reducing bacteria, which may be due the higher pH under switchgrass observed in our study. The distinction of soil organic C quality due to the different grass root exudates may also result in the different distribution of N-cycling microbial populations (Strauss & Lamberti, 2002). Although C_4 grass species and N fertilization rate had less impact on the activity than on the population size of N-cycling microbes, the higher statistical dispersion of the functional activity (RNA-level) compared to functional potential (DNA-level) suggested that the dynamics of N-cycling microbial activity may be driven by more complex environmental factors in native C_4 grass systems.

Conclusions

In summary, our results showed that the distribution patterns, abundances, and expression of N-cycle functional genes changed over the growing season and some were affected by N fertilization rate and C_4 grass species. Excessive N fertilization did not promote the abundance and functional activity of N-cycling microbes (except AOB), and instead may have negative effects compared to moderate N addition. Compared to big bluestem, the soils planted with switchgrass contained higher population size of AOB and nitrite reducing bacteria. In addition, the significant interaction effects of season, grass species, and N fertilization rate on N-cycling population distribution by functional potential (genetic-level) rather than by functional activity (transcriptional-level) indicated relative stability in the functional capacity of N-cycling populations in native C_4 grass systems.

Chapter II.B Nitrogen fertilization and C_4 grass species alter abundance, activity and diversity of soil diazotrophic communities

A version of this sub-chapter was originally published by Jialin Hu, Jonathan Richwine, Patrick Keyser, Lidong Li, Fei Yao, Sindhu Jagadamma, and Jennifer DeBruyn:

Jialin Hu, Jonathan Richwine, Patrick Keyser, Lidong Li, Fei Yao, Sindhu Jagadamma, Jennifer DeBruyn "Nitrogen fertilization and native C_4 grass species alter abundance, activity and diversity of soil diazotrophic communities." *Frontiers in Microbiology*; DOI: https://doi.org/10.3389/fmicb.2021.675693.

My role in this work included: experiments operation (DNA and RNA extraction, PCR, qPCR, qRT-PCR, and amplicon sequencing), data analysis, and manuscript preparation.

Abstract

Native C_4 grasses have become the preferred species for native perennial pastures and bioenergy production due to their high productivity under low soil nitrogen (N) status. One reason for their low N requirement is that C_4 grasses may benefit from soil diazotrophs and promote biological N fixation. Our objective was to evaluate the impact of N fertilization rates (0, 67, and 202 kg N ha^{-1}) and grass species (switchgrass [*Panicum virgatum*] and big bluestem [*Andropogon gerardii*]) on the abundance, activity, diversity, and community composition of soil diazotrophs over three agricultural seasons (grass green-up, initial harvest, and second harvest) in a field experiment in East Tennessee, USA. Nitrogen fertilization rate had a stronger influence on diazotroph population size and activity (determined by *nifH* gene and transcript abundances) and community composition (determined by *nifH* gene amplicon sequencing) than agricultural season or grass species. Excessive fertilization (202 kg N ha^{-1}) resulted in fewer *nifH* transcripts compared to moderate fertilization (67 kg N ha^{-1}) and decreased both richness and evenness of diazotrophic community, reflecting an inhibitory effect of high N application rates on soil diazotrophic community. Overall, cluster I and cluster III diazotrophs were dominant in this native C_4 grass system. Diazotroph population size and activity were directly related to soil water content (SWC) based on structural equation modeling. Soil pH, SWC, and C

and N availability were related to the variability of diazotrophic community composition. Our results revealed relationships between soil diazotrophic community and associated soil properties, adding to our understanding of the response of soil diazotrophs to N fertilization and grass species in native C_4 grass systems.

Introduction

Nitrogen (N) is an essential nutrient for plant growth, and its availability limits the productivity of global ecosystems (LeBauer & Treseder, 2008; Vitousek & Howarth, 1991). Biological N fixation (BNF) is the microbial transformation of atmospheric nitrogen (N_2) to ammonia (NH_3), which is carried out by diazotrophs, bacteria and archaea that possess the nitrogenase enzyme complex encoded by the *nif* genes (*nifH*, *nifD*, and *nifK*) (Gaby & Buckley, 2011). BNF is estimated to contribute around 40-100 Tg N input to terrestrial ecosystems per year (Vitousek et al., 2013) and account for about half of annual N inputs into biosphere (Gaby & Buckley, 2014; Vitousek et al., 1997). The distribution of diazotrophs have been investigated in various environments, including seawater (Gilbert et al., 2011; Yang et al., 2019), sediments (Andersson et al., 2014; Burns et al., 2002; Dang et al., 2013), and soils (Feng et al., 2019; Han et al., 2019). Among them, soil harbors the most diverse diazotrophic microbial communities. Diazotrophs belonging to phyla Proteobacteria and Cyanobacteria are typically the most abundant N-fixing bacteria in soil (Gaby & Buckley, 2011). However, soil diazotrophic population size, diversity, and community composition are highly variable across study sites due to different soil characteristics such as pH (Y. Wang et al., 2017), moisture (Srivastava & Ambasht, 1994), organic matter content (Gupta et al., 2014), and temperature (Feng et al., 2019), which are all affected by season (Pereira e Silva et al., 2011; Pereira et al., 2013; Yeager et al., 2012), and plant species (Davis et al., 2011), and N input (Deslippe et al., 2005; C. Wang et al., 2017; Wang et al., 2016).

The highly conserved *nifH* gene has been widely used to identify and quantify N-fixing bacteria and archaea (Gaby & Buckley, 2012; Gaby et al., 2018; Raymond et al., 2004; Yang et al., 2019). *nifH* gene abundances are commonly used to estimate population size, while expression or transcript abundances are used as an indicator of activity. The genetic

divergence of *nifH* does not correlate well to the divergence of 16S rRNA genes in diazotrophs: it has been reported that species having < 3% sequence dissimilarity in their 16S rRNA genes can have up to 23% sequence dissimilarity in *nifH* (Gaby & Buckley, 2014). Based on *nifH* phylogenies, diazotrophs can be classified to five main clusters (clusters I-V). In general, cluster I contains aerobic and facultatively anaerobic diazotrophs belonging to Proteobacteria, Cyanobacteria, Firmicutes, and Actinobacteria. Cluster II contains a relatively small number of diazotrophs belonging to methanogenic archaea. Cluster III are mainly anaerobic bacteria and archaea, including Spirochetes, methanogens, acetogens, sulfate-reducing bacteria, Chloracea, and Clostridia (Gaby & Buckley, 2014; Young, 1992; Zehr et al., 2003). Clusters IV and V are *nifH* paralogs that are not involved in N fixation (Zehr et al., 2003). The dominant clusters are highly variable across study sites. For example, cluster I and II diazotrophs were reported to be dominant in the tundra (Feng et al., 2019). Cluster II and III dominated a mangrove ecosystem in South China (Liu et al., 2020), whereas cluster I and III were more prevalent in tropical mangrove ecosystems in Singapore (Jing et al., 2015), forest soils (Babur et al., 2014; Izquierdo & Klaus, 2015; Yeager et al., 2005), cropland soil (Mårtensson et al., 2009) and pasture soils (Babur et al., 2014; Gupta et al., 2019).

Grasslands account for 46.8% of all agricultural lands in the United States (USDA-NASS, 2012). In the humid Southeastern US, grasslands cover 19.3 million hectares (35.4% of US agricultural land) (USDA-NASS, 2012). In recent years, the emerging bioenergy economy based on dedicated herbaceous energy crops has promoted the widespread planting of native C_4 perennial grasses such as switchgrass (*Panicum virgatum* [SG]) and big bluestem (*Andropogon gerardii* [BB]). These native C_4 grasses are drought-tolerant, highly productive, grow well at low N availability conditions, and ultimately contribute to biodiversity and sustainability of grassland ecosystems (Fike et al., 2006; Parrish & Fike, 2005). One explanation for the low N demands of these native grasses is the high volume of organic matter produced, accumulated, and deposited by the large root systems and/or leaf litter that feeds the growth and activity of diazotrophs in the soil (Gupta et al., 2019; Keuter et al., 2014; Parrish & Fike, 2005). Previous studies have reported that bioenergy grasses promoted diazotrophs (Mao et al., 2013) and root-associated diazotrophic

communities contributed to meeting N demands of switchgrass (Smercina et al., 2020). In addition, different grass species may support different diazotrophic communities due to root systems with varying soil C input capacity and N use efficiency. For example, compared to switchgrass, big bluestem-dominated stands exhibited greater soil C accrual after five growing seasons (Adkins et al., 2019) and showed greater N use efficiency (Friesen & Cattani, 2017).

Soil nitrogen content is a key factor affecting BNF (Patra et al., 2007; Smercina et al., 2019). Many studies have reported that highly available N (urea-N, NH_4^+-N, and NO_3^--N) suppressed *nifH* abundance and expression (Cusack et al., 2009; Lindsay et al., 2010; Pereira et al., 2013; Reardon et al., 2014; C. Wang et al., 2017). Diazotrophs preferentially use easily available exogenous N as this takes less energy to assimilate compared to fixing atmospheric N, which consequently down-regulates the expression of N-fixation genes (Chapin et al., 2002; Smercina et al., 2019). Moreover, enhanced soil acidification caused by excessive N fertilization can also reduce diazotroph abundance and functional activity, and change diazotrophic community composition and diversity (Han et al., 2019; Limmer & Drake, 1996). In contrast, under low N availability, such as bioenergy cropping systems in marginal lands, diazotroph abundance and functional activity may be promoted because BNF offers diazotrophs a competitive advantage (Babur et al., 2014; Lindsay et al., 2010; Smercina et al., 2019; C. Wang et al., 2017). Therefore, N fertilization is likely to affect the diazotrophic microbial community in C_4 grassland ecosystems with low N availability.

In this study, we explored the impact of N fertilization on soil diazotrophic microbial communities under different native C_4 grass systems in infertile, strongly leached Ultisols. There were two main objectives in our study: (i) determine the effects of N fertilization rate and C_4 grass species on diazotrophic microbial communities; (ii) identify soil properties most related to diazotroph abundance, activity, diversity, and community composition. We hypothesized that: 1) increased N availability by N fertilization would suppress the abundance, activity, and diversity of diazotrophs, and excessive N fertilization would alter the community structure of diazotrophs; 2) Because of the higher N use efficiency of big bluestem, the abundance, activity, and diversity of diazotrophs would be higher in big bluestem stands compared to stands dominated by switchgrass, and the

diazotrophic microbial communities would be more responsive to N fertilization under switchgrass; 3) N fertilization would reduce the abundance, activity, and diversity of diazotrophs, and change diazotrophic microbial community due to increased soil N availability (ammonium and nitrate) and decreased soil pH. The study was conducted at a native grassland small plot experiment using two C_4 grass species and three N fertilization rates. To examine the dynamics of soil diazotrophs, we used quantitative polymerase chain reaction (qPCR), quantitative reverse transcription PCR (qRT-PCR), and high-throughput amplicon sequencing to target N fixation gene *nifH* (Figure 1).

Materials and Methods

The study site, experimental design, sample collection, field operations (other fertilizers and lime application, weed control, grass harvest), soil physicochemical properties measurement, soil DNA and RNA extraction and cDNA synthesis were both described in the section Materials and Methods in Chapter II.A.

Quantitative analysis of nifH genes and gene transcripts

Quantitative PCR (qPCR) of *nifH* genes and transcripts were performed on a CFX96 Optical Real-Time Detection System (Bio-Rad, Laboratories Inc., Hercules, California, USA) using the primer pair IGK3 (5'-GCIWTHTAYGGIAARGGIGGIATHGGIAA-3') and DVV (5'-ATIGCRAAICCICCRCAIACIACRTC-3') (Gaby & Buckley, 2012). The 20-µl qPCR reaction mixture contained 10 µl Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific, California, USA), 1 µl of primer IGK3 (final concentration of 10 µM), 1 µl of primer DVV (final concentration of 10 µM), 2.5 µl DNA or cDNA template, and 5.5 µl PCR grade nuclease free water. The thermal conditions were as follows: initial denaturation at 95 ℃ for 10 min, followed by 40 cycles of denaturation at 95 ℃ for 30 s, annealing at 58 ℃ for 1 min and extension at 72 ℃ for 1 min, and final extension at 72 ℃ for 10 min. Ten-fold serial dilutions (10^2 to 10^8 copies μl^{-1}) of the plasmids containing *nifH* genes made through molecular cloning by the TOPO TA Cloning kit (Thermo Scientific, California, USA) were used during qPCR assays to generate standard curves for quantifying *nifH* genes and transcripts in soil samples. Plasmids containing *nifH* genes

amplified from environmental samples, described in our previous study (Hu et al., 2021), were used as standards. Following the reactions, a melt curve analysis was performed to confirm the specificity of the PCR product.

nifH genes amplicon sequencing

Soil DNA samples were diluted to 10 ng μl^{-1} for *nifH* gene amplification. Two-step PCR was used for *nifH* amplicon sequencing library preparation. For the first step, *nifH* genes were amplified from diluted DNA samples with primers IGK3/DVV containing Illumina-compatible adapters (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG for IGK3 and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG for DVV), following manufacturers' recommendations (Klindworth et al., 2013). The amplifications were performed in a 25-μl mixture containing 12.5 μl DreamTaq Green PCR Master Mix (2×) (Thermo Scientific, California, USA), 1 μl of each primer (final concentration of 0.4 μM), 2.5 μl of template DNA, and 8 μl of PCR grade nuclease-free water. The PCR thermal cycling conditions were 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 10 min. Agarose gel electrophoresis (1.5% gel) was applied to confirm the presence of a PCR product. Then, the PCR products were purified using SparQ PureMag Beads (Quantabio, Massachusetts, USA). For the second step, index PCR was performed in a 50-μL reaction which added unique indexes to each sample, containing 25 μl KAPA HiFi HotStart ReadyMix (2×) (Kapa Biosystems, Massachusetts, USA), 5 μl of each forward/reverse NexteraxT index/primer, 5 μl of PCR product from the first step as template DNA, and 10 μl of PCR grade nuclease-free water. The PCR amplification conditions were: denaturation at 95 °C for 3 min, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. SparQ PureMag Beads were used to purify the final indexed PCR products. The final PCR products were quantified by NanoDrop and pooled and diluted to 25 ng μl^{-1} to run on an Agilent Bioanalyzer to ensure quality for sequencing. Finally, 2 × 275-bp paired-ends sequencing was conducted at the University of Tennessee Genomics Core laboratory on an Illumina Miseq system (Illumina, California, USA) with the Miseq reagent kit version 3 (1000 c

ycles) at 4 pM library loading concentration and with a 20% PhiX DNA (Illumina) includ ed in the sequencing run.

Bioinformatics analysis

The generated raw *nifH* gene sequences were processed using Mothur v.1.39.5 (Schloss et al., 2009). Forward and reverse reads were merged using the make.contigs command. Primer sequences were trimmed using trim.seqs. Reads were removed if they contained < 10 bp overlaps, ambiguous bases, homopolymers > 7 bp, or were shorter than 300 bp using screen.seqs. Chimeras were removed by Chimera.uchime using a manually created database containing 7887 *nifH* gene reference sequences from Functional Gene Pipeline and Repository (FunGene; http://fungene.cme.msu.edu/) (Fish et al., 2013). The remaining sequences were compared against a curated *nifH* gene database and its associated taxonomy annotation database (Gaby & Buckley, 2014; Gaby et al., 2018) using BLAST (Altschul et al., 1990) to identify cluster IV/V sequences; all cluster IV/V paralogs were removed by command get.seqs in Mothur. All libraries were subsampled to 5788 reads for downstream analyses. Operational taxonomic units (OTUs) of *nifH* were classified with 95% nucleotide sequence similarity cutoff using vsearch in QIIME2 (Bolyen et al., 2019). Singletons, OTUs appearing only once across all samples, were removed. The representative sequences of *nifH* OTUs were assigned to taxa using the *nifH* database of Gaby and Buckley (Gaby & Buckley, 2014) and GenBank database. An OTU was assigned to either order (BLAST hit, < 75%), family (< 88.1%), genus (< 91.9%), species (≥ 91.9%), or unclassified (no BLAST hit) (Gaby et al., 2018). The raw sequencing data can be accessed from NCBI Sequence Read Archive (SRA) Database, BioProject No. PRJNA687872.

Statistical analysis

In this study, the alpha- and beta-diversity of diazotrophs, *nifH* OTUs, and taxonomy data were derived from *nifH* amplicon sequencing data. The *nifH* OTU abundance refers to relative abundances in the sequence libraries. The *nifH* gene and transcript abundances were absolute abundances measured by qPCR and qRT-PCR, respectively. The alpha- and

beta-diversity indices were calculated with QIIME2-2019.7 based on *nifH* amplicon OTU counts. Chao1 estimates of richness (Chao, 1984), observed number of *nifH* OTUs, Faith's phylogenetic diversity (Faith, 1992), Shannon index, and Pielou's evenness were calculated as metrics of alpha-diversity. Beta-diversity was quantified based on weighted-UniFrac distances (Lozupone et al., 2007). Permutational multivariate analysis of variance (PERMANOVA) based on the weighted-UniFrac distance matrix was used to test the differences among diazotrophic microbial community structures by treatment factors. A neighbor-joining phylogenetic tree was constructed by QIIME using representative sequences of 224 abundant *nifH* OTUs (OTUs with total reads ≥ 100), together with the relative abundance of each OTU in different treatment groups, and was visualized using Interactive Tree of Life (iTOL, v5) (Letunic & Bork, 2011).

Principal coordinates analysis (PCoA) based on weighted-UniFrac distances was performed in open-source software R (version 3.6.1) (Team, 2013) with packages vegan (version 2.5-5) (Oksanen et al., 2020), phyloseq (version 1.28.0) (McMurdie & Holmes, 2013), and ggplot2 (version 3.3.1) (Wickham, 2011) to display the distribution of diazotrophic microbial community structures. Double principal coordinate analysis (DPCoA) (Pavoine et al., 2004) based on beta-diversity and taxonomy information of *nifH* OTUs was performed in R with packages vegan, phyloseq, and ggplot2 to visualize both distribution pattern of samples and *nifH* OTU optima. Redundancy Analysis (RDA) was conducted with vegan to model major factors (experimental treatments, soil physico-chemical properties, alpha-diversity of diazotrophs, and *nifH* gene and transcript abundances) shaping diazotrophic microbial community structures. Indicator species analysis based on the relative abundances of the top 224 abundant *nifH* OTUs was conducted with the multipatt function (number of permutations = 999) implemented in the R package indicspecies (version 1.7.9) (De Caceres et al., 2016) to identify taxon-habitat association patterns. This procedure returns an indicator value index that represent the strength of the association between a *nifH* OTU and a treatment or treatment combinations, with larger value indicating stronger association. The *nifH* OTUs significantly (p-value < 0.05) associated with certain treatments or treatment combinations were identified as representative, or indicator *nifH* OTUs. Association network analysis, which reveals

significant ($p < 0.05$) associations between *nifH* OTUs and the treatment(s) in which they were detected and/or abundant, was conducted using the interactive platform Gephi (WebAtlas, Paris, France) with Force Atlas placement algorithm.

A mixed model ANOVA within the GLIMMIX procedure in SAS 9.4 (SAS Inst., Cary, NC, USA) was performed to test the effects of sampling time, N fertilization rate, and grass species on soil properties, *nifH* gene and transcript abundances, and alpha-diversity of diazotrophic microbial communities. The abundances of *nifH* genes and transcripts were log transformed to achieve normal distributions. The fixed effects included sampling time, N fertilization rate, and grass species, as well as their interactions. Block was included as a random effect. A post-hoc least significant difference (LSD) method was used to compare the means of groups.

Spearman correlation analysis was performed in IBM SPSS Statistics v26 to evaluate the correlation among *nifH* gene and transcript abundances, alpha-diversity of diazotrophic microbial communities, and soil physico-chemical parameters, as well as the correlations between *nifH* gene and transcript abundances and relative abundance of abundant *nifH* OTUs. Correlation coefficient *p* values were adjusted with a multiple comparison correction (BH method) in the stats package (version 4.0.3) within R.

Structural equation modeling (SEM) was performed with the IBM SPSS Amos 27.0 software to characterize the interaction among sampling time, grass species, N fertilization rate, soil physicochemical properties, *nifH* gene and transcript abundances, as well as alpha-diversity of diazotrophs and predominant diazotrophic taxa (orders). We followed the procedures of developing and modifying a structural equation model reported in (Li et al., 2019). Briefly, we proposed a hypothesized model according to existing research and theory, tested if important pathways were left out and if the existing pathways were significant, and then revised the hypothesized model by adding missing pathways and dropping insignificant pathways in consideration of model fit and scientific rationality. Path coefficients were tested by maximum likelihood estimation at $p \leq 0.05$. Multivariate normality was evaluated by Kurtosis value ≤ 7. Chi-square test of model fit, the Chi-square to degree of freedom (Df) ratio (Chi-square/Df), root mean square error of approximation (RMSEA), Tucker-Lewis index (TLI), incremental fit index (IFI), and comparative fit

index (CFI) were used to determine model goodness-of-fit (Li & Schaeffer, 2020). A well fit model should have a Chi-square/Df value in the range of 1.0 to 3.0 with probability level > 0.05, RMSEA value < 0.08, and fit indices (TLI, IFI, and CFI) both > 0.95 (Blunch, 2012; Hooper et al., 2007; Kline, 2015).

Raw data on soil properties and *nifH* gene and transcript abundances are available in the supplemental information.

Results

Soil properties

The soil physicochemical properties involved in this sub-chapter have been reported in the section Results in Chapter II.A and were shown in Table 10 and Appendix Table 15.

Abundances of nifH genes and transcripts

nifH gene and transcript abundances significantly varied among agricultural seasons ($p < 0.0001$ for both) but not among N fertilization rate or grass species; additionally, there were no significant interactions among season, N fertilization rate, and grass species (Table 11 and Figure 17). The mean abundance of *nifH* genes was lower after initial grass harvest (1.8×10^6 copies gdw^{-1}) and higher after second harvest (1.2×10^7 copies gdw^{-1}) (Figure 17A). The mean abundance of *nifH* gene transcripts was higher after initial harvest (1.6×10^4 copies gdw^{-1}) and lower at second grass harvest (3.5×10^3 copies gdw^{-1}) (Figure 17A). Compared to 67N, the abundance of *nifH* transcripts was lower under 202N (Figure 17B). No significant difference of gene or transcript abundance was observed between switchgrass and big bluestem plots (Figure 17C).

Diazotrophic microbial structure and community

A total of 2595 *nifH* OTUs were generated at 95% sequence similarity after subsampling to 5788 reads per sample. N fertilization rate significantly affected diazotrophic alpha-diversity (Appendix Table 18). The richness indices (Chao1, observed OTUs, and phylogenetic diversity) were similarly high at grass green up (G) and second grass harvest (H2) but were lower at initial grass harvest (H1) ($p < 0.05$) (Figure 18A). The

Shannon index reflecting both richness and evenness was similarly low at the initial and second grass harvest and was higher at grass green up ($p < 0.05$) (Figure 18A). Alpha-diversity indices were lower under N fertilization than those under no N fertilization (Figure 18B). The mean values of observed OTUs, phylogenetic diversity, Shannon index, and Pielou's evenness under 202N were lower than that under 0N ($p < 0.05$) (Figure 18B). Grass species did not affect diazotrophic alpha-diversity (Figure 18C). Moreover, no interaction effect of sample time, N fertilization, and grass species on the alpha-diversity of diazotrophic microbial community was observed (Appendix Table 18).

N fertilization rate and grass species had significant effects on the structure of diazotrophic microbial community (Table 13). N fertilization rate had a stronger effect (PERMANOVA $R^2 = 0.210$, $p = 0.001$) than sampling time ($R^2 = 0.148$ $p = 0.001$) and grass species ($R^2 = 0.066$, $p = 0.005$) on diazotrophic microbial community structure (Table 13 and Figure 19). Results of pairwise PERMANOVA showed that diazotrophic community composition in 0N and 67N treatments differed from 202N (Figure 19A; Appendix Table 19). In addition, the response of diazotrophic microbial community composition to N-rate differed between the two grass species: under big bluestem, communities with 67N (67N-BB) differed from 202N (202N-BB); under switchgrass, the communities with 67N (67N-SG) showed no significant difference from 202N (202N-SG) (Figure 19A; Appendix Table 19). Furthermore, the diazotrophic microbial community structure at the two grass harvest times were significantly different from each other (q-value ≤ 0.05), but both showed no significant difference from grass green up (Figure 19B; Appendix Table 19).

Phylogenetic affiliations of diazotrophic communities

The phylum Proteobacteria had the highest representation in the diazotrophic communities, with 1809 *nifH* OTUs and 95.1% of the total reads belonging to Proteobacteria (Appendix Figure 31). Cyanobacteria and Firmicutes accounted for 1.0% and 0.7% of the total reads, respectively (Appendix Figure 31). The *nifH* OUT with highest relative abundance (7.6% of total reads) was classified to family Sphingomonadaceae (class α-Proteobacteria). The next three most abundant *nifH* OTUs all classified as family Bradyrhizobiaceae (order Rhizobiales, class α-Proteobacteria), and accounted for 7.4%,

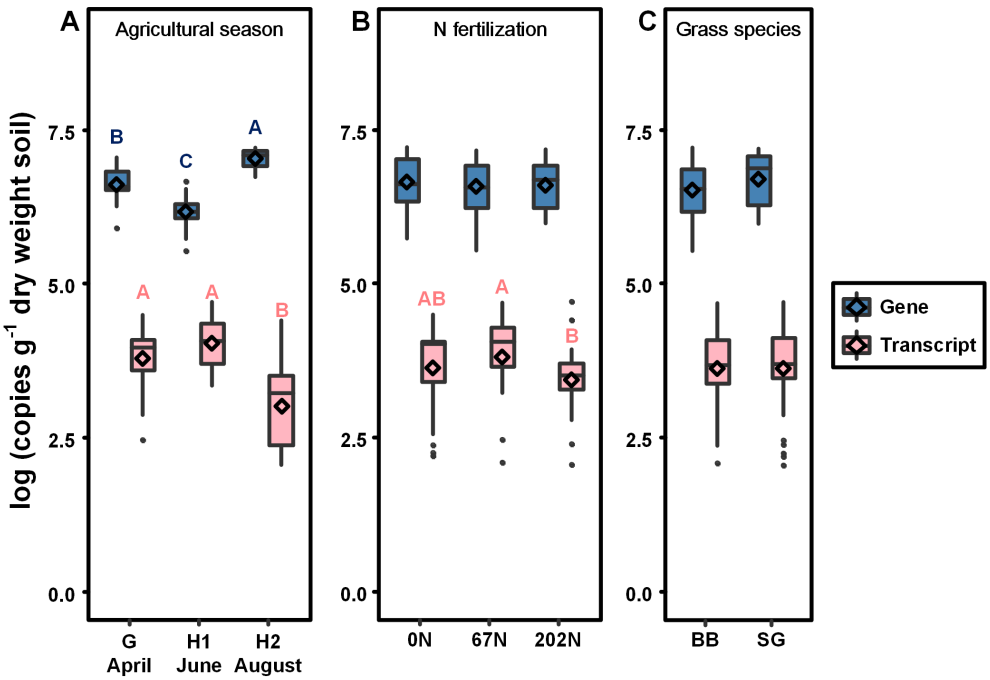

Figure 17. Absolute abundance of *nifH* gene (blue) and transcript (pink) in relation to agricultural seasons (A), nitrogen fertilization rates (B), and grass species (C). Boxes represent the interquartile range (IQR). Lines within boxes represent median values. Whiskers represent the range between minimum and maximum values. Dots represent outliers outside of this range. Diamonds represent mean values. Different letters above boxes indicate significant differences between treatment levels within groups by comparing means using Least Significant Difference (LSD) tests ($\alpha = 0.05$). G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; BB, big bluestem; SG, switchgrass.

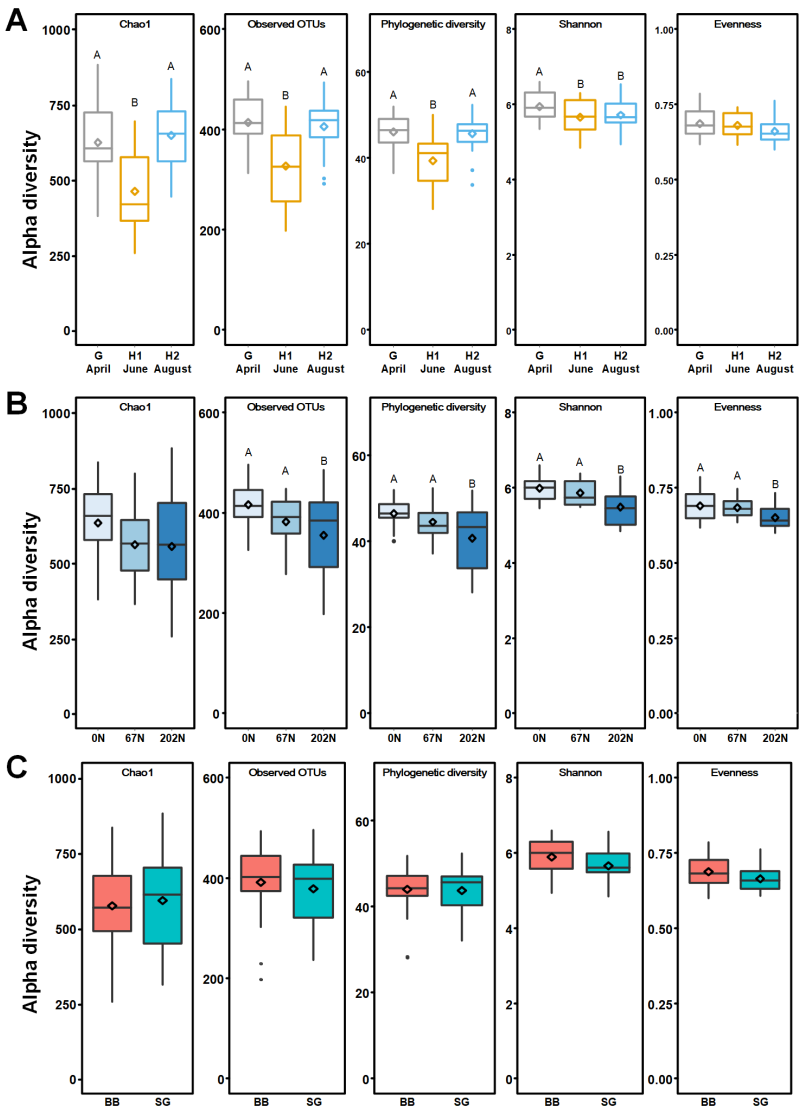

Figure 18. Alpha-diversity of diazotrophic microbial communities in relation to agricultural seasons (A), nitrogen fertilization rates (B), and grass species (C). Boxes represent the interquartile range (IQR). Lines within boxes represent median values. Whiskers represent the range between minimum and maximum values. Dots represent outliers outside of this range. Diamonds represent mean values. Different letters above boxes indicate significant differences between treatment levels within groups by comparing means using Least Significant Difference (LSD) tests ($\alpha = 0.05$). G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; BB, big bluestem; SG, switchgrass.

6.4%, and 6.2% of total reads, respectively.

A phylogenetic tree based on a neighbor-joining method for the 224 most abundant *nifH* OTUs (OTUs with ≥ 100 reads) showed that 149 *nifH* OTUs belong to order Rhizobiales (Figure 20). In our study, the abundant *nifH* OTUs belonged to cluster I or cluster III: 198 of the abundant *nifH* OTUs belonged to phylum Proteobacteria, Cyanobacteria, or Firmicutes and affiliated with cluster I; 24 of the abundant *nifH* OTUs belonged to phylum Proteobacteria, Verrucomicrobia, Spirochaetes, or Chlorobi and affiliated with cluster III (Figure 20). The relative abundances of *nifH* OTUs in cluster I remained unchanged with N fertilization rate (5088 ± 66, 5002 ± 65, and 4961 ± 59 sequences under 0N, 67N, and 202N, respectively), while cluster III OTUs increased in relative abundance with N fertilization rate (266 ± 45, 383 ± 65, and 557 ± 77 sequences under 0N, 67N, and 202N, respectively) (Figure 20).

Indicator species under N fertilization and grass species

We identified 71 *nifH* OTUs significantly associated to one or more treatment combinations of N fertilization rate and grass species (Appendix Table 20), which were represented in an association network (Figure 21). Many *nifH* OTUs were representative of certain N fertilization and/or grass species treatments. For example, one *nifH* OTU (OTU 185, belonging to Rhizobiales) was only detected under 202N (specificity value A = 1.000, fidelity value B = 0.588 $p = 0.003$) whereas OTU 69 (belonging to Nostocales) was only detected under 0N and 67N (A = 1.000, B = 0.697, $p = 0.002$). Moreover, an unclassified diazotroph (OTU 49) appeared in all plots under 0N and 67N (B = 1.000) and was largely (but not completely) restricted to these plots (A = 0.972). Two *nifH* OTUs (OTU 58 and OTU 93, both belonging to Rhizobiales) were significantly associated to big bluestem (BB) ($p = 0.002$ and 0.006, respectively). Some OTUs were significantly associated to only a single treatment combination (e.g., OTU 169 and OTU 207 for 0N combined with switchgrass (0N-SG); OTU 198 for 0N-BB; OTU 215 for 67N-BB).

A double principal coordinate analysis (DPCoA) (Pavoine et al., 2004) showing both distribution pattern of samples and *nifH* OTU optima indicated that OTUs affiliated to Cyanobacteria were mainly found under 0N and 67N treatments rather than 202N, whereas

Table 13. Results of PERMANOVA examining the effects of season, N fertilization rate, and grass species on diazotrophic community composition based on weighted-UniFrac distances of *nifH* OTUs.

Factor	Df	F.Model	R^2	*p*-value[†]
Season	2	5.413	0.148	**0.001**
Nitrogen	2	7.697	0.210	**0.001**
Grass	1	4.804	0.066	**0.005**
Season×Nitrogen	4	1.081	0.059	0.352
Season×Grass	2	0.445	0.012	0.920
Nitrogen×Grass	2	1.667	0.046	0.110
Season×Nitrogen×Grass	4	0.426	0.023	0.982
Residuals	32		0.437	

[†]Bold values indicate *p*-value ≤ 0.05.

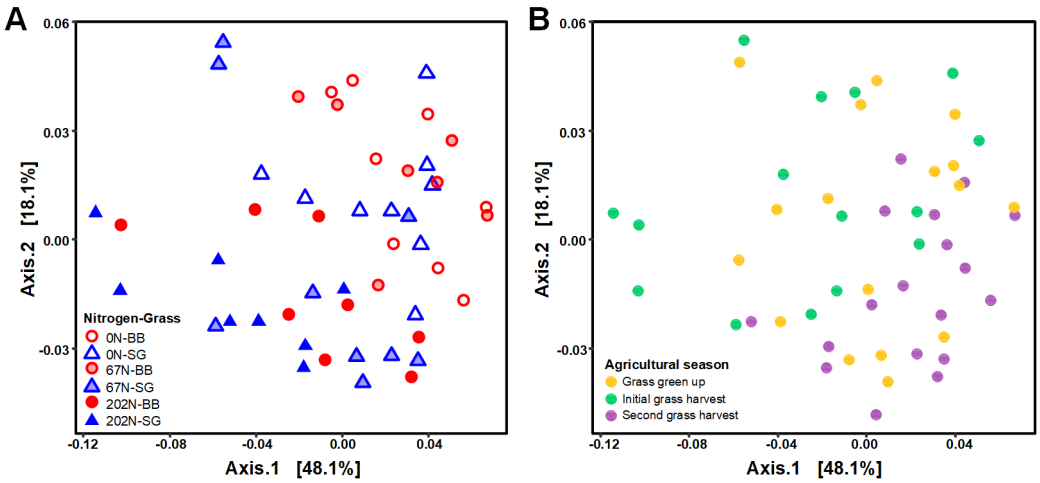

Figure 19. Principal coordinates analysis (PCoA) of weighted-UniFrac distances between diazotrophic microbial communities colored by the combination of N rate and grass species (A) and agricultural season (B). 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

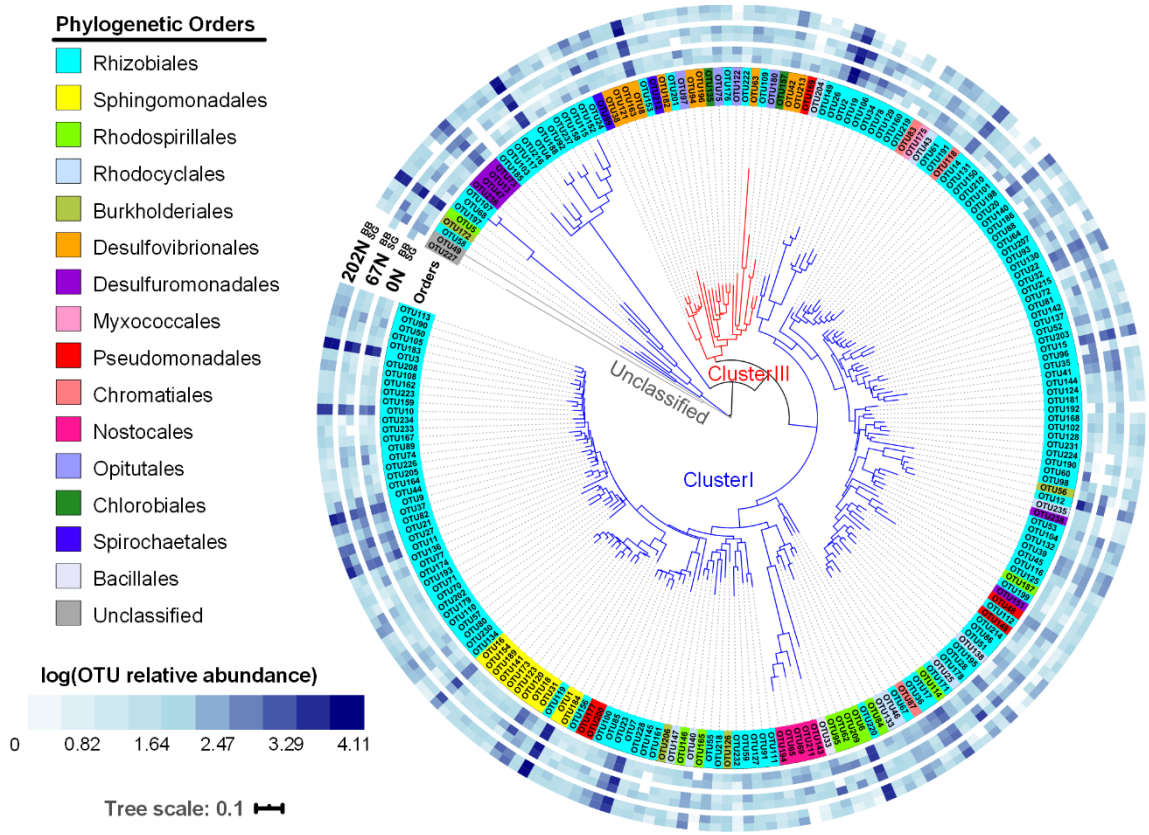

Figure 20. Circular neighbor-joining phylogenetic tree of the top 224 abundant *nifH* OTUs (OTUs with ≥ 100 reads). Tree leaves on the inner circle highlighted by colors show the affiliation to different phylogenetic orders. The outer blue-and-white heatmap circles show the logarithmic value of mean OTU relative abundances in percentages in different N fertilization rates and grass species (all three seasons combined). 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

OTUs affiliated to Firmicutes were mainly found under 67N and 202N (Figure 22). The OTUs affiliated to other phyla did not show a pattern by N fertilization rates. In addition, we observed that the relative abundance of δ-Proteobacteria was higher under 202N than under 0N and 67N regardless of sampling time and grass species, while γ-Proteobacteria was higher under 0N and 67N than under 202N (Appendix Figure 31). The phylum Verrucomicrobia had higher relative abundance in big bluestem plots than in switchgrass plots (Appendix Figure 31).

Relationships among soil properties, diazotrophs abundances, activity, diversity and structure and community

The abundances of *nifH* genes and transcripts were negatively correlated (R = -0.349, $p < 0.05$). The abundance of *nifH* genes was negatively correlated to SWC (R = -0.781, $p < 0.01$) and soil inorganic N (NH_4^+-N: R = -0.510, $p < 0.01$; NO_3^--N: R = -0.387, $p < 0.01$) but positively correlated to DOC (R = 0.728, $p < 0.01$) and DON (R = 0.510, $p < 0.01$). In contrast, the abundance of *nifH* gene transcripts was positively correlated to pH (R = 0.423, $p < 0.01$), SWC (R = 0.590, $p < 0.01$), and NH_4^+-N (R = 0.320, $p < 0.05$) but negatively correlated to DOC (R = -0.280, $p < 0.05$) (Appendix Table 21). Structural equation modeling (SEM) was conducted to identify the impacts of treatments and soil parameters on the abundance, functional activity, diversity, and predominant community composition of diazotrophs (Figure 23; Appendix Table 22). SWC had a direct and strong positive effect on diazotrophs functional activity (*nifH* transcript abundance) (standardized path coefficient = 0.602, *p*-value < 0.001) but had a direct negative effect on diazotrophs abundance (*nifH* gene abundance) (standardized path coefficient = -0.334, *p*-value < 0.001). SWC had a direct and strong positive effect on the major diazotrophic community (standardized path coefficient = 0.677, *p*-value < 0.001). N fertilization rate directly and positively affected soil nitrate concentration (standardized path coefficient = 0.689, p-value < 0.001). The increased soil nitrate by N fertilization negatively affected soil pH (standardized path coefficient = -0.369, *p*-value = 0.001), which negatively affected the relative abundance of Rhodocyclales, one of the most abundant orders within diazotrophic communities (standardized path coefficient = -0.248, *p*-value = 0.011). Moreover,

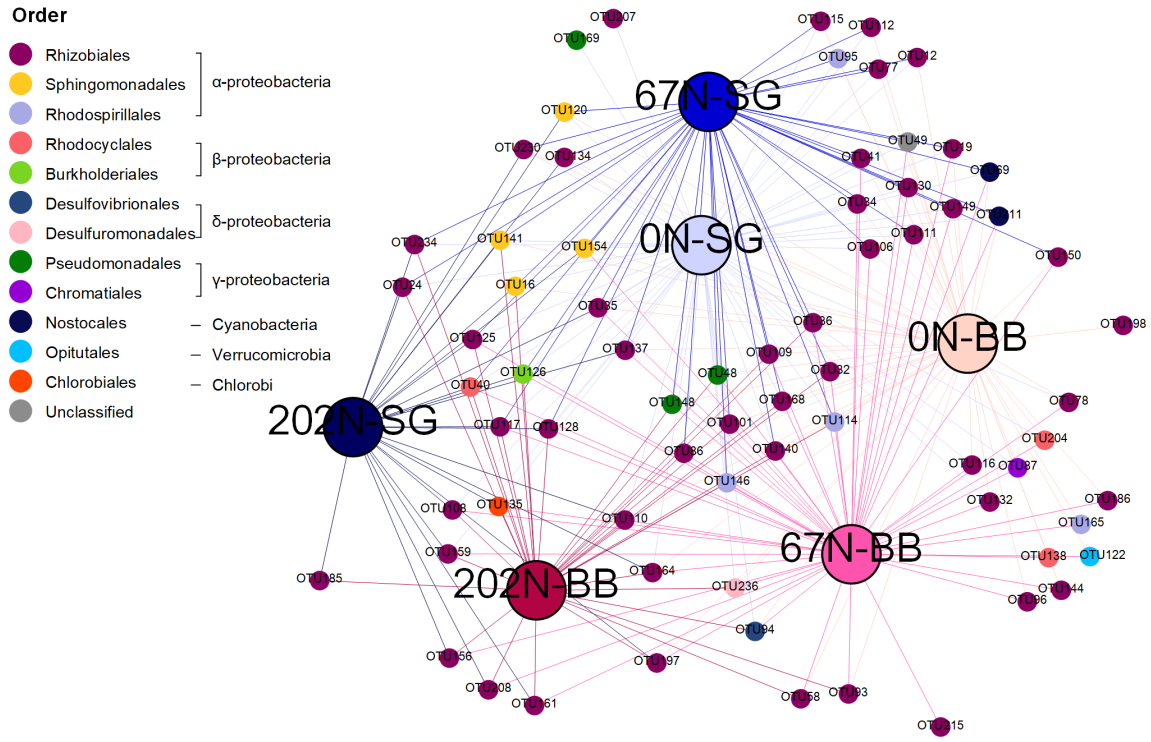

Figure 21. Association network showing significant ($p < 0.05$) positive associations between indicator *nifH* OTUs and specific habitat under two different treatments [specific N fertilization rate (0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization) and grass species (SG, switchgrass; BB, big bluestem)]. Large nodes represent treatments. Small nodes represent *nifH* OTUs. Line colors reflect treatments. *nifH* OTUs were grouped to order level.

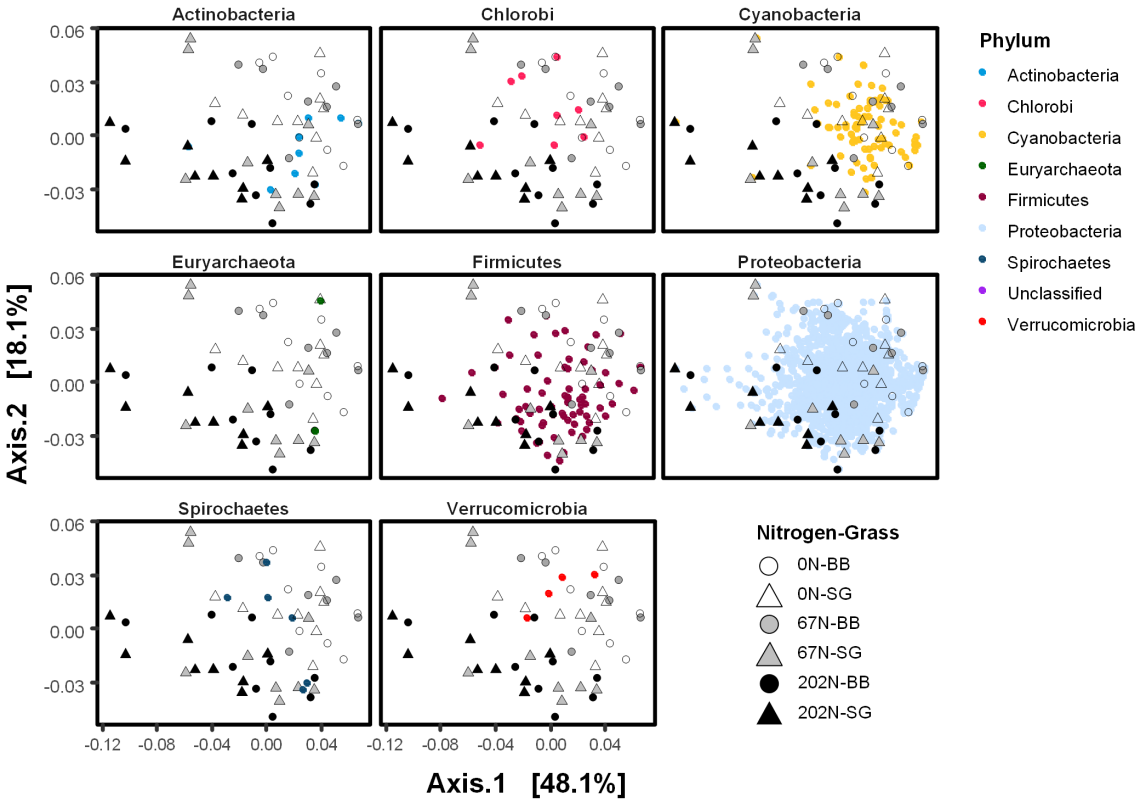

Figure 22. Double principal coordinate analysis (DPCoA) of weighted-UniFrac distance between diazotrophic microbial communities. Both samples and OTU optima were displayed. Small points on the ordination plots represent *nifH* OTUs and are colored by phylum. Large circle and triangle points on the ordination plots represent samples and are shaped by N fertilization rate and grass species. 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

diazotrophs abundance had positive association with observed richness (observed *nifH* OTUs) (standardized path coefficient = 0.427, *p*-value < 0.001). The standardized total effects obtained from structural equation modeling were shown in Appendix Table 23. Compared to switchgrass, big bluestem increased the richness indices of diazotrophs diversity and the relative abundance of Rhizobiales and Rhodocyclales but decreased both diazotrophs abundance and activity as well as the relative abundance of Sphingomonadales and Rhodospirillales. DON had a positive total effect on diazotrophs abundance.

A redundancy analysis (RDA) showed that soil parameters, *nifH* gene and transcript abundances, agricultural season, N fertilization rate, grass species, and five alpha-diversity indices measured in this study explained a total of 68.4% of the variability of diazotrophic community composition, with the first two axes explaining 38.0% of this variability (Figure 24). The RDA showed that soil pH, SWC, NH_4^+-N concentration, NO_3^--N concentration, DOC, *nifH* gene abundance, N fertilization rate, and richness indices of alpha-diversity significantly correlated to the variability of diazotrophic community (p < 0.05) (Figure 24).

Spearman correlation analysis showed that there were positive correlations between the richness indices (Chao1, observed OTUs, and Faith's phylogenetic diversity) of diazotrophic bacteria and *nifH* gene abundance but no correlation between diazotrophic alpha-diversity and *nifH* transcript abundance (Appendix Table 24). These positive correlations were stronger under switchgrass than under big bluestem (Appendix Table 24). In addition, only the relative abundances of four *nifH* OTUs (OTU1, OTU5, OTU6, and OTU18) in the top 20 most abundant *nifH* OTUs were positively correlated to *nifH* gene abundance quantified by qPCR (Appendix Table 25). These four *nifH* OTUs belonged to Sphingomonadales (OTU1 and OTU18) and Rhodospirillales (OTU5 and OTU6), indicating the variation of *nifH* gene abundances measured by qPCR may potentially depend on the variation of these two orders.

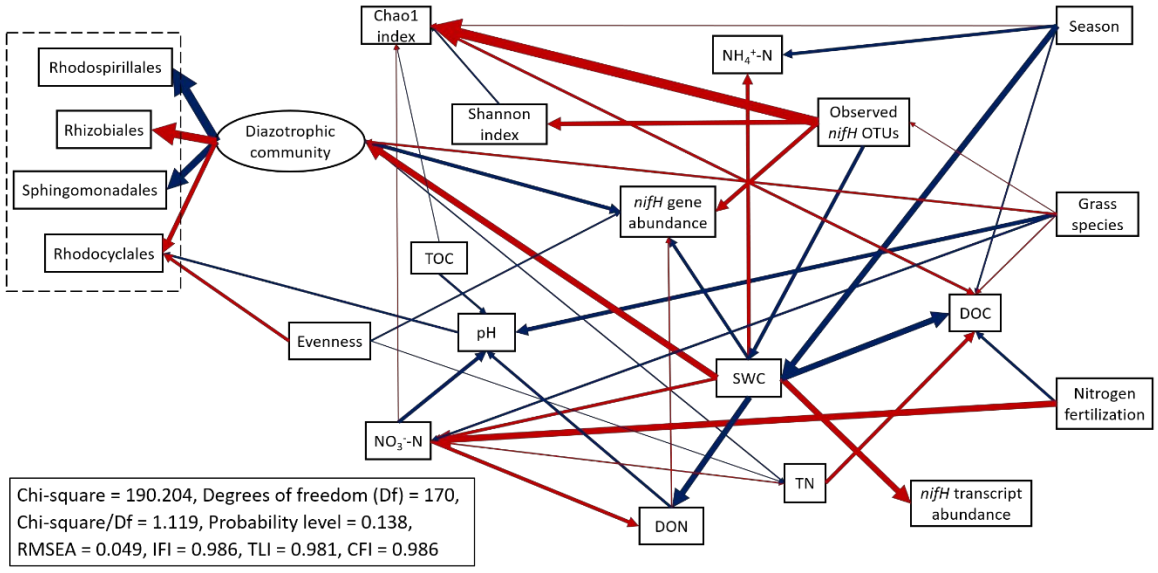

Figure 23. Structural equation modeling (SEM) for diazotrophs abundance, activity, diversity, and major community composition with key soil physico-chemical parameters. Red arrows indicate positive relationships. Blue arrows indicate negative relationships. Single headed arrows represent causal relationship (p-value < 0.05). The direction of arrow indicates the direction of causation. The width of arrow indicates the extent of effects, i.e., the standardized path coefficients proportional to the thickness of arrows. See Appendix Table 22 for specific standardized path coefficients and Appendix Table 23 for standardized total effects. SWC, soil water content; DOC, dissolved organic C; DON, dissolved organic N; TOC, total organic C; TN, total N.

Discussion

The primary objective of this study was to investigate the impacts of N fertilization rate and native grass species on the seasonal population size, activity, and diversity of diazotrophic microbial communities in a native C_4 grass system dominated by infertile, strongly leached Ultisols using qPCR, qRT-PCR, and *nifH* gene amplicon sequencing. The population size, activity, and community composition of diazotrophs varied throughout the season, most likely due to variation of N and C availability (Pereira et al., 2013; Wang et al., 2016), soil temperature (Deslippe et al., 2005), precipitation (Yeager et al., 2012), and other environmental factors. The greatest abundance of diazotrophs, as indicated by *nifH* gene abundance, but lowest activity, as indicated by *nifH* transcript abundance, were observed at the second grass harvest in August, which we attributed to the lower SWC in summer months. In general, SWC is assumed to promote the activity of soil diazotrophs (Feng et al., 2019; Ritchie & Raina, 2016) as higher SWC decreases soil oxygen content (Brusseau et al., 2006) that inhibits nitrogenase activity (Dobereiner et al., 1972; Eady & Postgate, 1974). Here we observed that diazotroph abundances did not decrease in drier soils, perhaps because the most abundant diazotrophs detected in this study were classified as aerobes or facultative anaerobes (e.g., *Sphingomonas*, *Bradyrhizobium*, and *Rhodospirillum*) which would tolerate oxygenated soils. We observed a positive effect of SWC on diazotroph activity (*nifH* transcript abundance) in our structural equation modeling and correlation analysis. This is likely due to the inhibitory effect of oxygen on nitrogenase activity (Eady & Postgate, 1974).

Availability of C is another key factor that may affect the abundance of diazotrophs. We observed a positive correlation between DOC and *nifH* gene copies. Other studies have shown that decreased DOC may limit the growth of heterotrophic diazotrophs (Feng et al., 2019; Yin et al., 2013). One explanation for the lower DOC observed at first grass harvest (June) is that we sampled soils after mowing at first grass harvest but before mowing at second grass harvest. Mowing can decrease soil DOC due to 1) decreased C substrate supplied from photosynthesis and aboveground litter (Gilmullina et al., 2020; Wan & Luo, 2003); 2) increased plant root uptake of easily available C; and 3) decreased plant C flow to soil in the form of root exudates (Bardgett et al., 1999).

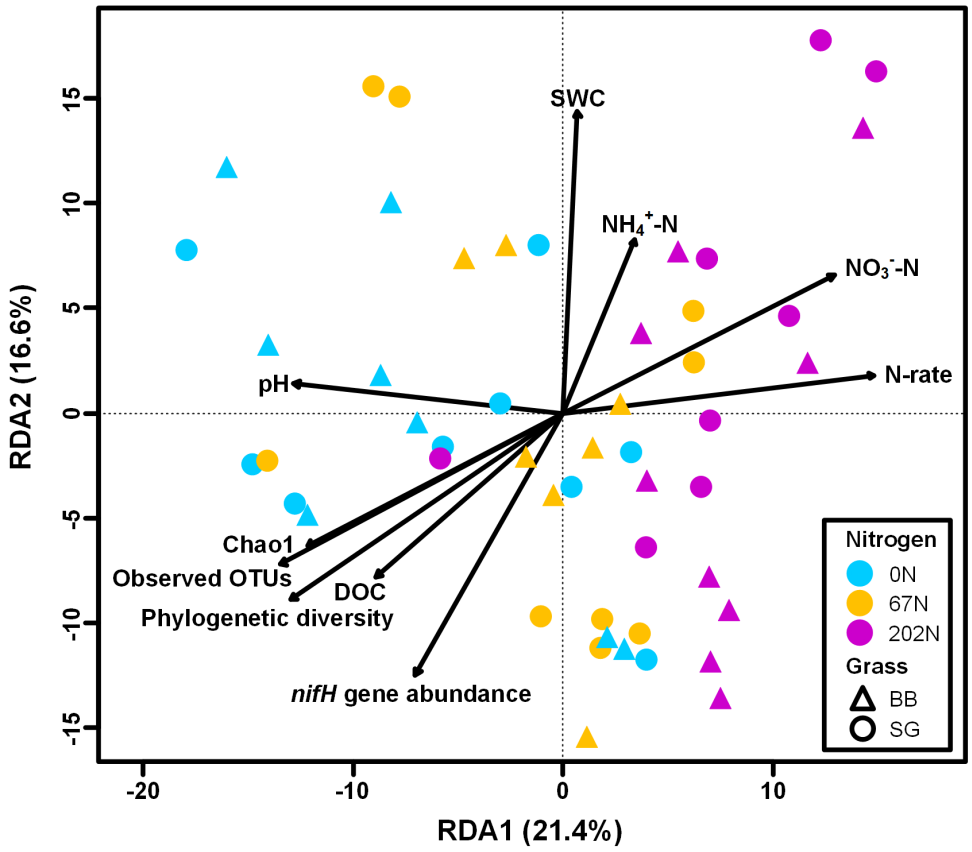

Figure 24. Redundancy Analysis (RDA) of diazotrophic microbial community (circle and triangle symbols) with environmental factors, *nifH* gene and transcript abundances, and community diversity metrics as predictor variables. Significant predictors are indicated with arrows. 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem; SWC, soil water content; DOC, dissolved organic C; N-rate, N fertilization rate.

N fertilization, especially at the highest N rate, resulted in higher soil NH_4^+ and NO_3^- concentrations and lower soil pH and DOC. The lower DOC content at high N rate might be due to the promotion of N immobilization by soil microbes. In contrast to our hypothesis, no effect of N fertilization on the abundance of diazotrophs was observed, which could be explained several ways: 1) diazotrophs were able to get N from soil for growth and reproduction and did not need to rely on atmospheric N_2 fixation; 2) the decreased soil pH by urea fertilization was not enough to suppress the abundance of diazotrophs; and/or 3) the abundant diazotrophs in this system were more nitrate-tolerant. Indeed, some species within the genus *Bradyrhizobium* are denitrifiers and can tolerate higher levels of nitrate (Gao et al., 2020). For instance, OTU 3 detected in our study is an abundant *nifH* OTU affiliated to the legume-root nodulating bacteria *Bradyrhizobium japonicum*. *B. japonicum* has capacity for complete denitrification and nitrate respiration (Bedmar et al., 2005; Vessey & Luit, 2011). High N fertilization rates (202N) may not have affected abundance of diazotrophs, but it did decrease their activity. This is likely because diazotrophs preferentially use easily available N (e.g., nitrate and ammonium) as this takes less energy to assimilate compared to fixing dinitrogen gas (Chapin et al., 2002; Smercina et al., 2019). Another possible reason for the decreased activity of diazotrophs may be the lower pH caused by high N fertilization rate. Diazotrophs typically have a pH optima of 7.0 to 7.5, and reduced activity when pH is lower than 6.7 (Han et al., 2019; Limmer & Drake, 1996; Roper & Gupta, 2016; Roper & Smith, 1991).

N fertilization rate had a strong impact on diazotrophic microbial community composition, with significant changes in diazotrophic microbial community composition, species richness and evenness under high N fertilization. The high N fertilization rate shifted the community composition toward species better adapted to higher nitrate and lower soil pH. We observed that the *nifH* OTUs belonging to Firmicutes were mainly found under high N, where we also observed the lowest pH. The role of pH in shaping diazotrophic community has been observed in other ecosystems, such as alpine meadows (Y. Wang et al., 2017) and wheat fields (Fan et al., 2018). The relative abundance of cluster III *nifH* OTUs, which mainly affiliated with known obligate anaerobes, increased with N-rate. One possible explanation is that the cluster III diazotrophs may better tolerate high

nitrate and/or low pH conditions. Specifically, OTU 8, OTU 38, and OTU 42 within cluster III belonged to Desulfovibrionales and increased in relative abundance with N-rate. This order contains sulfate-reducing bacteria that have been reported to reduce nitrate for growth in the absence of sulfate (Marietou & Boden, 2016; Marietou et al., 2009; Sánchez-Andrea et al., 2015).

According to our RDA analysis and SEM model, N fertilization influenced the variability of diazotrophic microbial community composition by increasing ammonium and/or nitrate concentration and decreasing soil pH. Unmeasured environmental factors, such as climatic factors (Han et al., 2019), and/or other soil nutrients such as phosphorus and potassium (C. Wang et al., 2017; Wang et al., 2020; Zou et al., 2020), may also contribute to the variation of diazotrophic community composition. Many studies have suggested that the metabolic functional potential of a community may be more sensitive to environmental factors than its composition (Louca et al., 2016; Nelson et al., 2016; Raes et al., 2011). We observed this in our SEM, which showed that the soil N concentrations, such as DON and nitrate, elevated by N fertilization, as well as SWC, directly positively affected the population size of diazotrophs. The positive correlation of the diversity with abundance, but not activity, might be due to functional redundancy in these communities. Functional redundancy suggests that in a more diverse community, functionally similar bacteria may be active at different times, depending on conditions (Louca et al., 2016; Louca et al., 2018; Nelson et al., 2016).

Conclusions

We documented the seasonal dynamics of diazotrophic abundance, activity, diversity, and community composition under two C_4 grass species and three N fertilization rates. Our results showed that cluster I and III diazotrophs were dominant in the studied native C_4 grass system. The population size and activity of diazotrophs were related to SWC and C availability over a growing season. Nitrogen fertilization rate had a stronger influence on diazotrophic community composition than grass species. Consistent with our hypotheses, compared to moderate N fertilization, excessive N fertilizer application decreased both alpha-diversity and activity of diazotrophic community, and altered diazotrophic

community composition. This suggests excessive fertilization in native C_4 grass systems may have negative consequences for biological N fixation and soil health. Moreover, soil pH, soil moisture, and C and N availability were related to the variability of diazotrophic community composition. Overall, our work revealed insights into important relationships between diazotrophic community abundance and diversity in the field, adding to our understanding of the response of soil diazotrophs to season, nitrogen fertilization, and grass species in native C_4 grass systems.

Chapter II.C Response of ammonia-oxidizing bacterial community to agricultural season, nitrogen fertilization, and grass species in native C_4 grassland soil

Abstract

Nitrification is a major nitrogen (N) transformation process in soils. Anthropogenic N addition can contribute to N leaching and N_2O emission by affecting microbial populations responsible for nitrification. However, the effects of N fertilization on ammonia oxidizers under different C_4 perennial grasses in the nutrient-poor grassland of humid Southeastern United States have not been well studied. Here, a field experiment was used to assess the effects of N fertilization rate (0, 67, and 202 kg N ha^{-1}) and grass species (switchgrass [*Panicum virgatum*] and big bluestem [*Andropogon gerardii*]) on ammonia-oxidizing bacterial (AOB) communities in a C_4 grassland soil. The AOB abundance and functional activity were quantified using quantitative-PCR and quantitative reverse transcription-PCR of *amoA* genes, respectively. The AOB community was profiled using high-throughput *amoA* amplicon sequencing. Our results showed that bacteria belonging to the genus *Nitrosospira* were dominant AOB in the C_4 grassland soil throughout the growing season. Nitrogen fertilization rate had a stronger influence on AOB community composition than C_4 grass species. Elevated N fertilizer application increased abundance, activity, and alpha-diversity of AOB communities as well as nitrification potential, nitrous oxide (N_2O) emission and soil acidity. Although AOB functional activity (i.e., *amoA* transcript abundance) was similar between the two grass species, the abundance and species richness of AOB was higher under switchgrass compared to big bluestem. Compared to switchgrass, big bluestem system may have less potential for N losses through nitrate leaching and N_2O emission. In addition, soil pH, nitrate, nitrification potential, and N_2O emission significantly related to the variability of ammonia-oxidizing bacterial communities. Together, this study improved our understanding of the response of soil AOB to N addition and C_4 grass species in perennial native grasslands of humid Southeastern United States.

Introduction

Nitrification is the biological oxidation of ammonia (NH_3) to nitrate (NO_3^-), which is one of the major processes in soil nitrogen (N) cycling. Microbes that carry out nitrification are referred to as "nitrifiers", including ammonia- and nitrite-oxidizers. For the two-step nitrification, the ammonia-oxidizers [ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA)] control the first step (oxidation of NH_4^+ to NO_2^-), which is the rate-limiting step of nitrification (Frijlink et al., 1992). Nitrous oxide (N_2O) as a byproduct of ammonia oxidation (Bakken & Frostegård, 2020; Hallin et al., 2018), is a greenhouse gas with a global warming potential 265-298 times that of CO_2 for a 100-year timescale and a major cause of the stratospheric ozone layer destruction (Qin et al., 2014; Ravishankara et al., 2009). It has been reported that about 70% of the atmospheric N_2O is contributed by microbial processes in soils, such as nitrification and denitrification (Conrad, 1996); studies of multiple cropping systems have identified nitrification as the main source of N_2O (Liu et al., 2016). Soil moisture is a main driver of N_2O production due to nitrification, with highest N_2O production in a silt loam arable agricultural soils with 35-60% water-filled pore space (Bateman & Baggs, 2005). In agricultural soils with N fertilization, nitrification can also lead to nitrate leaching (Beeckman et al., 2018), potentially contributing to eutrophication of nearby water systems (Cameron et al., 2013). In addition, major perennial forage crops prefer to take up N in the form of NO_3^- rather than NH_4^+ (Xu et al., 2019), indicating that forage productivity can be affected by the nitrification process conducted by nitrifiers. Thus, nitrifiers play an important role in the sustainability of agricultural ecosystems as well as environmental protection.

Ammonia-oxidizers convert NH_3 to NO_2^- through two enzymes: ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (Araki et al., 2004; Prosser et al., 1986). The analysis of the ammonia-oxidizing microbial community is primarily studied using the marker gene *amoA* as a typical function gene, which encodes the α-subunit, the subunit that carries the active site of the enzyme of the AMO enzyme (Levy-Booth et al., 2014; Rotthauwe et al., 1997). The *amoA* gene is well suited for molecular studies of AOA and AOB communities because of its strongly conserved nucleotide sequence and its essential role in the energy generating metabolism (Norton et

al., 2002). Although AOA appear to be more numerically abundant than AOB in soils (Leininger et al., 2006), the abundance and community composition of AOB are more responsive than AOA to N fertilization, and therefore are functionally dominant for ammonia oxidation in agricultural soils (Di et al., 2009; Jia & Conrad, 2009; Ouyang et al., 2016; Segal et al., 2017; Ying et al., 2017). Moreover, AOB are more abundant and more positively correlated to nitrification rates than AOA in topsoils of agroecosystems (Beeckman et al., 2018; Kong et al., 2010), indicating that AOB should be the focus when developing strategies to control nitrification in agricultural soils. AOB as an important functional group in soil N cycling, including some genera such as *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovicria* (Levy-Booth et al., 2014). In previous research, the majority of known AOB in soil belonged to *Nitrosomonas spp.,* with lesser contributions from *Nitrosococcus* and *Nitrosospira* (Purkhold et al., 2000). More recently, when different terrestrial ecosystems (e.g., freshwater marsh, permafrost, garden, agricultural, and desert soils) were investigated using fluorescence microscopy with DAPI staining, revealing that *Nitrosospira*, an important nitrifier in aquatic habitats, also plays an important role in soil nitrification (Bartosch et al., 2002). Due to the difficulties in separation and purification of AOB strains in the lab, the studies of AOB mostly rely on molecular ecology methods targeting the 16S rRNA gene or the *amoA* gene to analyze the distribution and abundance of AOB in ecosystems (Kowalchuk et al., 2000; Purkhold et al., 2000).

Grasslands accounts for about 46.8% of all agricultural lands in the United States (USDA-NASS, 2012). Grasslands mobilize large pools of N with potential for N losses through N_2O emissions and nitrate leaching via nitrification. Around 100 to 256 kg N ha^{-1} yr^{-1} may be lost via nitrate leaching from grassland cultivation depending on site and management strategies (Di & Cameron, 2002; Duan et al., 2017; Francis et al., 1995; Hansen et al., 2007) with nitrification as a key control. On the contrary, it has been demonstrated that a substantial amount of exudates containing inhibitory compounds released by the roots of specific grass species can block the pathways of enzymes AMO and HAO, resulting in the inhibition of nitrification, which is called biological nitrification inhibition (BNI) (Subbarao et al., 2009). BNI varies significantly among different grass

species. A previous study has shown that C_4 grasses (e.g. brachiaria and sorghum) exhibit greater BNI than C_3 grasses (e.g. ryegrass and fescue) (Subbarao et al., 2006). Perennial native C_4 grasses, such as switchgrass (*Panicum virgatum* [SG]) and big bluestem (*Andropogon gerardii* [BB]), have been widely planted due to their drought tolerance and high productivity under N-poor conditions (Fike et al., 2006; Parrish & Fike, 2005). A previous study reported that the optimum N rate for switchgrass to maximize dry matter yield (50 to 120 kg N ha^{-1}) was higher than that for big bluestem (45 to 90 kg N ha^{-1}) (Brejda, 2000), which may be due to the difference in BNI between these two C_4 grass species and/or the greater N-use efficiency of big bluestem than switchgrass (Friesen & Cattani, 2017). However, the AOB communities associated with these two C_4 grass species have not been well documented.

Many studies have found that AOB abundances and activity increase with ammonium addition, and AOB abundances correlate to nitrification rates (Di et al., 2009; Smits et al., 2010; Wertz et al., 2012). Previous studies also observed that AOB community structure can be altered by N addition (Avrahami et al., 2003; Rooney et al., 2010; Wertz et al., 2012), plant species (Faulwetter et al., 2013), or the interactions between N addition and plant species (Rooney et al., 2010) in soils associated with forest, upland grassland, and wetlands. However, there is a lack of understanding of the impact of N addition on the abundance, activity, diversity, and community structure of AOB under different grass species in native C_4 grasslands.

To address the knowledge gap about impacts of C_4 grass species and N fertilization on soil AOB populations in grasslands, our study had three objectives: (i) investigate the seasonal dynamics of soil AOB communities under native C_4 perennial grasses; (ii) determine the effects of N fertilization rate and grass species on AOB communities; and (iii) identify edaphic properties that mostly related to AOB abundance, activity, diversity, and community composition, with a particular focus on the relationship of AOB with soil nitrification potential and N_2O emissions. We hypothesized that: 1) increased N availability by N fertilization would promote AOB abundance, activity, and diversity as well as nitrification potential and N_2O emission; and a high N fertilization rate would alter AOB community structure; and 2) the abundance, activity, and diversity of AOB would be higher

under switchgrass than big bluestem. This study was conducted at a native grassland small-plot experiment with different C_4 grasses and N fertilization rates over the growing season. Quantitative-PCR (qPCR) of *amoA* genes from extracted DNA was used to estimate AOB abundances, quantitative reverse transcription PCR (qRT-PCR) of *amoA* genes from extracted RNA was used to estimate AOB activity, and high-throughput amplicon sequencing of AOB *amoA* genes was used to examine AOB diversity and community composition. AOB population data was then correlated to a suite of soil physicochemical properties, N_2O emissions, and nitrification potentials.

Materials and Methods

The study site, experimental design, sample collection, field operations (other fertilizers and lime application, weed control, grass harvest), soil physicochemical properties measurement, soil DNA and RNA extraction and cDNA synthesis were both described in the section Materials and Methods in Chapter II.A.

Quantitative analysis of amoA genes and gene transcripts

Quantitative PCR (qPCR) of AOB *amoA* genes and transcripts were performed on a CFX96 Optical Real-Time Detection System (Bio-Rad, Laboratories Inc., Hercules, CA, USA) using the primer pair *amoA*-1F and *amoA*-2R (Aigle et al., 2019). The 20-µl qPCR reaction mixture contained 10 µl Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific, USA), 0.5 µl of primer *amoA*-1F (10 µM), 0.5 µl of primer *amoA*-2R (10 µM), 3 µl DNA or cDNA template, and 6 µl PCR grade nuclease free water. The thermal conditions were as follows: initial denaturation at 95 ℃ for 3 min, followed by 40 cycles of denaturation at 94 ℃ for 1 min, annealing at 56 ℃ for 45 s and extension at 72 ℃ for 1 min, and final extension at 72 ℃ for 10 min. Ten-fold serial dilutions (10^2 to 10^8 copies μl^{-1}) of the plasmids containing *amoA* genes made through molecular cloning by the TOPO TA Cloning kit (Thermo Scientific, USA)were used during qPCR assays to generate standard curves for quantifying *amoA* genes and transcripts in soil samples. Following the reactions, a melt curve analysis was performed to confirm the specificity of the PCR product.

amoA gene amplicon sequencing

Extracted soil DNA samples were diluted to 10 ng μl^{-1} for *amoA* gene amplification. Two-step polymerase chain reaction (PCR) was used for *nifH* amplicon sequencing library preparation. For the first step, *amoA* genes were amplified from diluted DNA samples with primers *amoA*-1F/*amoA*-2R containing Illumina-compatible adapters respectively, following manufacturers' recommendations (Klindworth et al., 2013). The amplifications were performed in a 25-µl mixture containing 12.5 µl KAPA HiFi HotStart ReadyMix (2×) (Kapa Biosystems), 1 µl of each primer (final concentration of 0.4 µM), 2.5 µl of template DNA, and 8 µl of PCR grade nuclease-free water. The PCR thermal cycling conditions were 95 °C for 3 min, followed by 35 cycles of 98 °C for 20 s, 58 °C for 15 s and 72 °C for 15 s, followed by 72 °C for 5 min. Agarose gel electrophoresis (1.5% gel) was applied to confirm the PCR results. Then, the PCR products were purified using SparQ PureMag Beads (Quantabio). For the second step, index PCR was performed in a 50-µL reaction aiming to add unique indexes to each sample, containing 25 µl KAPA HiFi HotStart ReadyMix (2×) (Kapa Biosystems), 5 µl of each forward/ reverse NexteraxT index/primer, 5 µl of PCR product from the first step as template DNA, and 10 µl of PCR grade nuclease-free water. The PCR amplification condition was denaturation at 95 °C for 3 min, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. After that, SparQ PureMag Beads were used again to purify the final indexed PCR products. The final PCR products were quantified by NanoDrop and pooled and run on an Agilent Bioanalyzer to ensure quality for sequencing. Finally, the 2 × 275-bp paired-ends sequencing was run on an Illumina Miseq platform (Illumina, CA, U.S.) in the University of Tennessee Genomics Core laboratory.

Bioinformatics analysis

The generated raw sequences *amoA* genes were processed using Mothur v.1.39.5 (Schloss et al., 2009). Forward and reverse reads were merged using make.contigs command. Primer sequences were trimmed using trim.seqs. The overlaps of the merged reads less than 10 bp, reads of shorter than 400 bp, and ambiguous bases more than 0 were removed using screen.seqs. Chimeras were removed by Chimera.uchime using a manually

created database containing 9682 AOB *amoA* gene reference sequences from Functional Gene Pipeline and Repository (FunGene; http://fungene.cme.msu.edu/) (Fish et al., 2013). All libraries were subsampled to 4307 reads for all downstream analyses. Operational taxonomic units (OTUs) were classified with 97% nucleotide sequence similarity cutoff using vsearch in QIIME2 (Wang et al., 2014; Zheng et al., 2014). Singletons, OTUs appearing only once across all samples, were removed, and representative sequences for each OTU were selected. The abundant OTU representative sequences (OTUs with total reads ≥ 100) were blasted against the FunGene AOB *amoA*-like database using BLASTn. The raw sequencing data can be accessed from NCBI Sequence Read Archive (SRA) Database with BioProject accession number PRJNA733230.

Statistical analysis

The diversity analyses of AOB community were performed with QIIME2-2019.7. Chao1 estimator, observed OTUs, Shannon index, and Pielou's evenness were calculated to determine alpha-diversity. Beta-diversity was quantified based on a weighted-UniFrac method (Lozupone et al., 2007). Permutational multivariate analysis of variance (PERMANOVA) was used to examine the beta-diversity of diazotrophic communities by treatment factors. A pairwise PERMANOVA was conducted to test the significant difference of diazotrophic communities between each treatment pair. A neighbor-joining phylogenetic tree was constructed by QIIME using representative sequences of 28 abundant OTUs (OTUs with total reads ≥ 100) and was visualized using Interactive Tree of Life (iTOL, v5) (Letunic & Bork, 2011).

Principal coordinates analysis (PCoA) based on weighted-UniFrac distances was performed in R 3.6.1 (The R Foundation for Statistical Computing) with packages vegan (2.5-5) and phyloseq to reveal the diazotrophic community structures. Redundancy Analysis (RDA) was calculated with the package vegan to examine the effect of soil physicochemical parameters on the distribution patterns of AOB community.

A mixed model ANOVA within the GLIMMIX procedure in SAS 9.4 (SAS Inst., Cary, NC, USA) was performed to test the effects of grass growing season, N fertilization rate, and grass species on soil properties, abundance and expression of *amoA* genes, and alpha-

diversity of AOB communities. The abundances of *amoA* genes and transcripts were log transformed to achieve normal distributions. The fixed effects included season, N fertilization rate, and grass species, as well as their interactions. Block was included as a random effect. Least significant difference (LSD) method was used to compare the means of each two groups.

Pearson correlation analyses were performed in IBM SPSS Statistics v26 to evaluate the correlation among *amoA* gene and transcript abundances, alpha-diversity of AOB communities, and soil physicochemical parameters.

Structural equation modeling (SEM) was performed with the IBM SPSS Amos 27.0 software to characterize the interaction among agricultural season, grass species, N fertilization rate, soil physico-chemical properties, and AOB abundance, activity, diversity, and community composition. We followed the procedures of developing and modifying a structural equation model in (Li et al., 2019). Briefly, we proposed a hypothesized model according to existing research and theory, tested if important pathways were left out and if the existing pathways were significant, and then revised the hypothesized model by adding missing pathways and dropping insignificant pathways in consideration of model fit and scientific rationality. Path coefficients were tested by maximum likelihood estimation at $p \leq 0.05$. Multivariate normality was evaluated by Kurtosis value ≤ 7. Chi-square test of model fit, the Chi-square to degree of freedom (Df) ratio (Chi-square/Df), root mean square error of approximation (RMSEA), Tucker-Lewis index (TLI), incremental fit index (IFI), and comparative fit index (CFI) were used to determine whether the model was well-fitting. A well-fitting model should have a Chi-square/Df value in the range of 1.0 to 3.0 with *p*-value > 0.05, RMSEA value < 0.08, and three fit indices (TLI, IFI, and CFI) both > 0.95 (Blunch, 2012; Hooper et al., 2007; Kline, 2015).

Results

Soil properties

The soil physicochemical properties involved in this sub-chapter have been reported in the section Results in Chapter II.A and were shown in Table 10 and Appendix Table 15.

Abundances of amoA genes and transcripts

The log-transformed *amoA* gene abundance was significantly varied with agricultural season, N fertilization rate, and grass species (Table 11). In general, *amoA* gene abundances were lowest at initial grass harvest (2.51×10^5 copies g^{-1} dry weight soil [gdw^{-1}]) and highest at second grass harvest (1.14×10^7 copies gdw^{-1}) (Figure 25A). N fertilization significantly increased *amoA* gene abundance (5.03×10^6 and 7.00×10^6 copies gdw^{-1} for 67N and 202N, respectively) compared to no N addition (1.70×10^6 copies gdw^{-1}) (Figure 25B). In addition, *amoA* genes were more abundant in switchgrass plots (5.34×10^6 copies gdw^{-1}) than in big bluestem plots (3.81×10^6 copies gdw^{-1}) ($p = 0.0066$) (Figure 25C).

The *amoA* gene transcript abundance was significantly affected by the interaction effect of season and N fertilization rate (Table 11): at grass green up, *amoA* transcript abundance showed no significant difference under different N-rate; at initial grass harvest, *amoA* transcript abundance was lowest under 0N(5.04×10^3 copies gdw^{-1}) and highest under 67N (2.44×10^4 copies gdw^{-1}); At second harvest, transcript abundances increased with increasing fertilizer (3.93×10^2, 1.83×10^3, and 4.80×10^3 copies gdw^{-1} under 0N, 67N, and 202N, respectively). Grass species did not affect *amoA* transcript abundance (Table 11 and Figure 25C).

Ammonia-oxidizing bacterial structure and community

Using a 97% sequence similarity cutoff, a total of 215943 high-quality bacterial *amoA* gene sequences were clustered into 538 OTUs. Within each sample, the *amoA* OTU numbers ranged from 28 to 134. The main effects of agricultural season, N fertilization rate, and grass species significantly affected the alpha-diversity of AOB community (Appendix Table 26). The AOB species richness (observed OTUs, Chao1) significantly varied with agricultural season (Figure 26A). Observed OTUs was highest at second grass harvest and lowest at initial grass harvest. Chao1 estimator was similarly high at grass green up and second grass harvest and was lowest at initial grass. Shannon index and Pielou's evenness did not vary with agricultural season (Figure 26A). The alpha-diversity indices (except Chao1) increased significantly with increased N fertilization rate (Figure 26B). Compared to no N fertilization (0N), the mean values of observed OTUs, Shannon index, and Pielou's

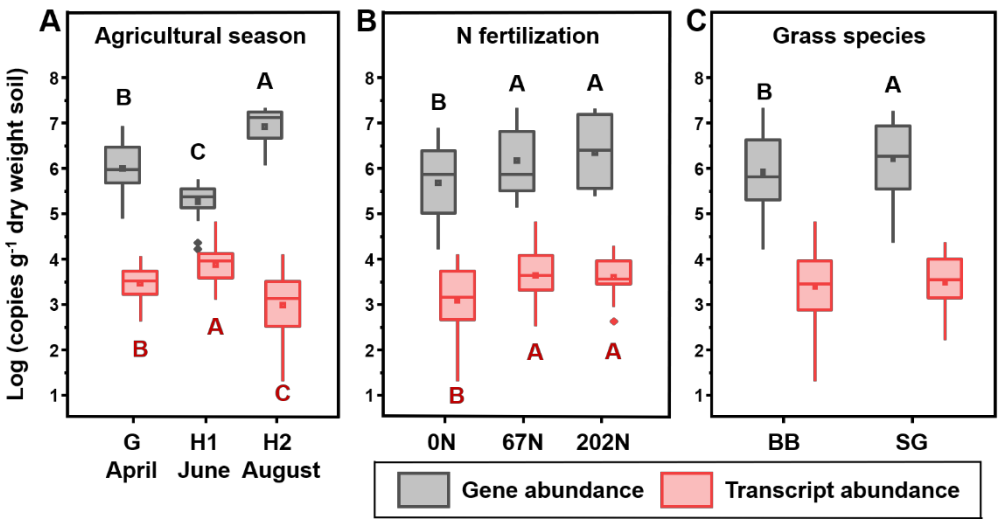

Figure 25. Absolute abundance of *amoA* gene (gray) and transcript (red) in relation to agricultural seasons (A), nitrogen fertilization rates (B), and grass species (C).Boxes represent the interquartile range (IQR). Lines within boxes represent median values. Whiskers represent the range between minimum and maximum values. Diamonds represent outliers outside of this range. Dots within boxes represent mean values. G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

evenness under 67N increased by about 16.1%, 16.3%, and 12.9%, respectively; and those under 202N increased by about 50.5%, 69.6%, and 55.5%, respectively (Figure 26B). Grass species significantly affected the species richness of AOB community, with higher richness observed in switchgrass plots than in big bluestem plots (Figure 26C). Moreover, no two-way or three-way interaction effect of season, N fertilization, and grass species on the alpha-diversity of AOB community was observed (Appendix Table 26).

AOB community structure was not impacted by agricultural season but was significantly affected by N fertilization rate and grass species (Table 14; Figure 27). N fertilization rate was a stronger factor (PERMANOVA $R^2 = 0.504$, $p = 0.001$) than grass species ($R^2 = 0.042$, $p = 0.021$) on influencing AOB structure and community (Table 14), which was confirmed by principal coordinates analysis of beta-diversity (PCoA) (Figure 27). PCoA showed that AOB community composition in 0N and 67N treatments was similar (pairwise PERMANOVA $p = 0.639$), but both obviously different from high N fertilization (202N) (pairwise PERMANOVA $p < 0.001$ for both). Under 0N and 67N fertilization, the communities were much more variable under big bluestem compared to switchgrass (Fig. 27).

Phylogenetic analysis of ammonia-oxidizing bacterial community

There were 28 abundant *amoA* OTUs (OTUs with ≥ 100 reads) which comprised 97.9% of the total reads. A neighbor-joining phylogenetic tree showed that most of the 28 abundant *amoA* OTUs classified as *Nitrosospira*, with 8 OTUs (accounting for 11.8% of total reads) were classified as *Nitrosospira multiformis* (Figure 28).

Many *amoA* OTUs were treatment-specific and can be considered as potential indicator species of plots with certain N fertilization rate and/or grass species treatments. For example, 8 OTUs (OTU4, OTU8, OTU9, OTU10, OTU12, OTU16, OTU19, and OTU22) classified as *Nitrosospira multiformis* and 2 OTUs (OTU11, OTU23) classified as *Nitrosospira* sp. 56-18 were more abundant in high N fertilization rate treatment (202N) compared to moderate (67N) or no N fertilization (0N). Moreover, OTU19 was only detected in big bluestem plots with 202N, whereas OTU3 and OTU6, belonging to

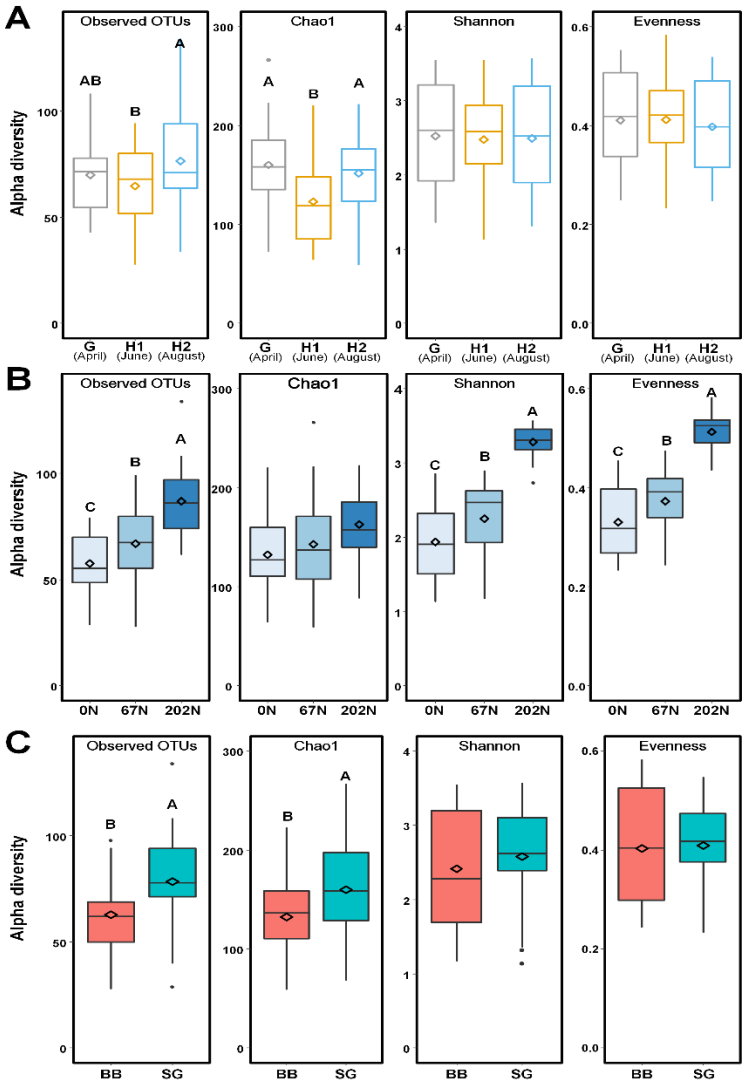

Figure 26. Alpha-diversity of ammonia-oxidizing bacterial communities in relation to agricultural seasons (A), nitrogen fertilization rates (B), and grass species (C). Boxes represent the interquartile range (IQR). Lines within boxes represent median values. Whiskers represent the range between minimum and maximum values. Dots represent outliers outside of this range. Diamonds represent mean values. Different letters above boxes indicate significant differences between treatment levels within groups by comparing means using Least Significant Difference (LSD) tests ($\alpha = 0.05$). G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; BB, big bluestem; SG, switchgrass.

Table 14. Results of PERMANOVA examining the importance of the effects of season, N application rate, and grass species in shaping ammonia-oxidizing bacterial community composition.

Factor	Df	F.Model	R^2	p-value[†]
Season	2	1.184	0.024	0.304
Nitrogen	2	24.706	0.504	**0.001**
Grass	1	4.101	0.042	**0.021**
Season×Nitrogen	4	0.425	0.017	0.931
Season×Grass	2	0.789	0.016	0.534
Nitrogen×Grass	2	1.640	0.033	0.164
Season×Nitrogen×Grass	4	0.661	0.027	0.741
Residuals	33		0.336	

[†]Bold values indicate p-value ≤ 0.05.

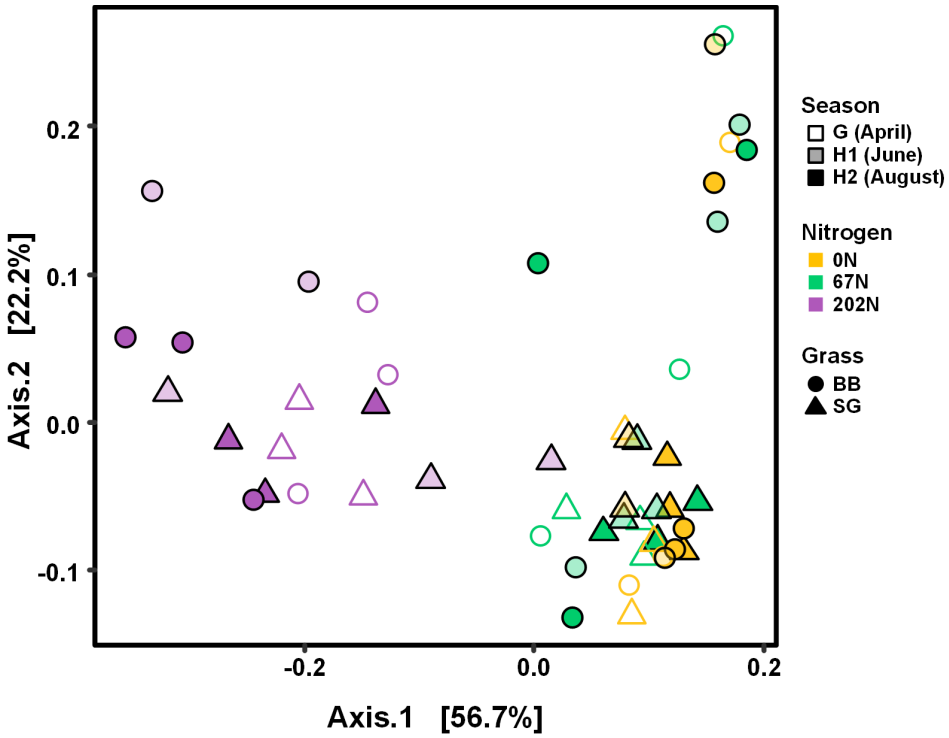

Figure 27. PCoA of weighted-UniFrac distance between ammonia-oxidizing bacterial communities based on agricultural season, N fertilization rate, and grass species. G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

Nitrosospira sp. Nsp14, were more abundant under 0N and 67N in big bluestem plots. OTU2 (belonging to *Nitrosospira* sp. Nsp14) had higher relative abundance under 0N and 67N in both switchgrass and big bluestem plots (Figure 28).

Relationships across soil properties and ammonium oxidizing bacterial populations

The abundance of *amoA* genes and transcripts were not correlated to each other (Appendix Table 27). The *amoA* gene abundance was negatively correlated to soil pH ($R = -0.303$, $p < 0.05$), SWC ($R = -0.792$, $p < 0.01$), NH_4^+-N ($R = -0.471$, $p < 0.01$) but positively correlated to DOC ($R = 0.477$, $p < 0.01$), DON ($R = 0.445$, $p < 0.01$), and nitrification potential ($R = 0.530$, $p < 0.01$). In contrast, the *amoA* gene transcript abundance was positively correlated to SWC ($R = 0.561$, $p < 0.01$), and available inorganic N (NH_4^+-N: $R = 0.448$, $p < 0.01$; NO_3^--N: $R = 0.339$, $p < 0.05$), and negatively correlated to DOC ($R = -0.640$, $p < 0.01$) but not correlated to pH, DOC, DON, and nitrification potential (Appendix Table 27). In addition, the alpha-diversity indices (except Chao1 index) of AOB community were negatively correlated to soil pH and positively correlated to NO_3^--N and nitrification potential (Appendix Table 27). The species richness indices (Chao1 and observed OTUs) and the Shannon index (reflect both richness and evenness) were positively correlated to *amoA* gene abundance but not transcript abundance (Appendix Table 27).

Structural equation modeling (SEM) was conducted to identify the impacts of soil physicochemical parameters on AOB abundance, functional activity, diversity, and major community composition (Figure 29; Appendix Table 28). SWC had a direct negative effect on *amoA* gene abundance (standardized path coefficient = -0.711, $p < 0.001$) but had a direct positive effect on *amoA* transcript abundance (standardized path coefficient = 0.433, $p = 0.010$). This can be interpreted as that 1.000 unit of increase in SWC can cause 0.711 unit of decrease in *amoA* gene abundance but 0.433 unit of increase in *amoA* transcript abundance. The *amoA* transcript abundance was directly and positively affected by ammonium concentration (standardized path coefficient = 0.331, $p = 0.002$) but directly and negatively affected by DOC (standardized path coefficient = -0.493, $p < 0.001$). In addition, *amoA* gene abundance had a direct positive effect on its transcript abundance

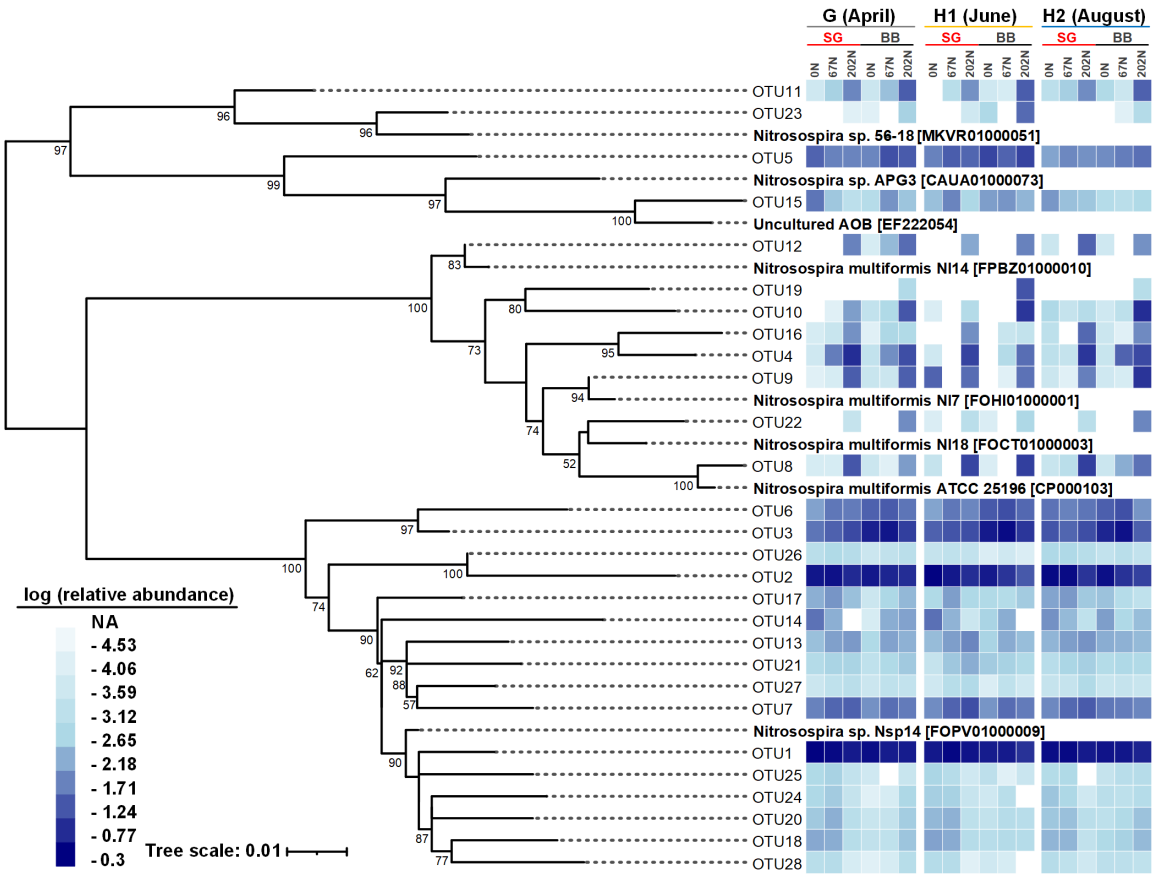

Figure 28. Neighbor-joining phylogenetic tree of the top 28 abundant *amoA* OTUs (OTUs with ≥ 100 reads). Bootstrap values greater than 50% of 1000 resamplings are shown near nodes. The scale bar indicates 0.01 nucleotide substitutions per nucleotide position. The blue-and-white heatmap shows the logarithmic value of OTU relative abundances in percentages in different treatment combinations of N fertilization rate and grass species in different agricultural seasons. GenBank accession numbers are shown for sequences from other studies. G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

(standardized path coefficient = 0.541, $p < 0.001$). The Chao1 index reflecting the richness of AOB community was directly negatively affected by soil nitrate (standardized path coefficient = -0.366, $p < 0.001$), whereas the Pielou's evenness of AOB community was directly and positively affected by ammonium (standardized path coefficient = 0.268, $p < 0.001$). The N_2O emission rate was directly and positively affected by SWC (standardized path coefficient = 0.723, $p < 0.001$) and ammonium (standardized path coefficient = 0.167, $p = 0.022$). The standardized total effects obtained from SEM were shown in Appendix Table 29. The total effects of agricultural season (from cool to warm) on *amoA* gene abundance and nitrification potential were positive but on transcript abundance, alpha-diversity (except observed *amoA* OTUs) and N_2O emission rate were negative. In accordance with our PCoA result shown in Figure 27, agricultural season had no effect on major AOB community composition. The total effects of N fertilization rate on *amoA* gene and transcript abundances, alpha-diversity of AOB community, nitrification potential and N_2O emission rate were both positive. For the most abundant species within AOB community, the total effects of N fertilization rate on *Nitrosospira* sp. 56-18, *Nitrosospira multiformis*, and *Nitrosospira* sp. APG3 were positive but on *Nitrosospira* sp. Nsp14 was negative, which were in accordance with our phylogenetic analysis shown in Figure 28. Compared to big bluestem, switchgrass had higher *amoA* gene and transcript abundances, alpha-diversity of AOB community, nitrification potential, and soil nitrate content, but had no effect on major AOB community composition that was composed of the four species belonging to the genus *Nitrosospira* mentioned above.

A redundancy analysis (RDA) showed that soil physicochemical properties, *amoA* gene and transcript abundances, agricultural season, N fertilization rate, grass species, and alpha-diversity indices measured in this study explained a total of 62.5% of the variability of ammonia-oxidizing bacterial community composition, with the first two axes explaining 50.2% of this variability (Figure 30). Soil pH, NO_3^--N, N_2O emission, nitrification potential, *amoA* transcript abundance, N fertilization rate, grass species, and alpha-diversity significantly correlated to the variability of AOB community structure ($p < 0.05$) (Figure 30). Notably, nitrification potential, NO_3^--N, N_2O emission, N fertilization rate, and Shannon index and Pielou's evenness significantly correlated to the AOB community

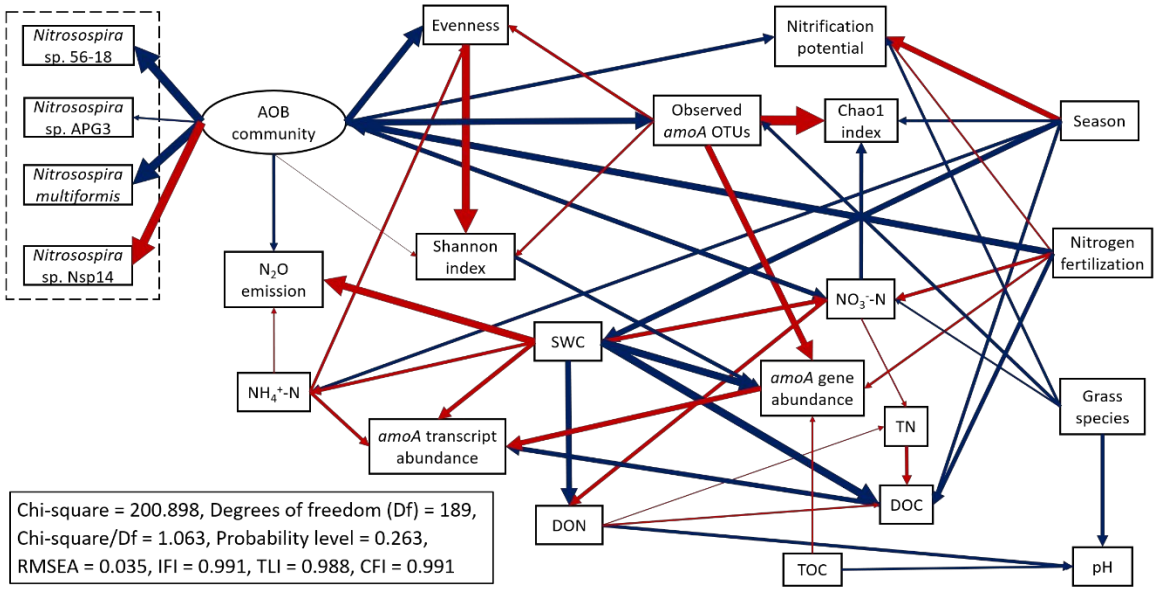

Figure 29. Structural equation modeling (SEM) for ammonia-oxidizing bacterial abundance, functional activity, and major community composition with key soil parameters. Red arrows indicate positive relationships. Blue arrows indicate negative relationships. Single headed arrows represent causal relationship (p-value < 0.05). The direction of arrow indicates the direction of causation. The width of arrow indicates the extent of effects, i.e., the standardized path coefficients proportional to the thickness of arrows. See Appendix Table 28 for specific standardized path coefficients and Appendix Table 29 for standardized total effects. SWC, soil water content; DOC, dissolved organic C; DON, dissolved organic N; TOC, total organic C; TN, total N.

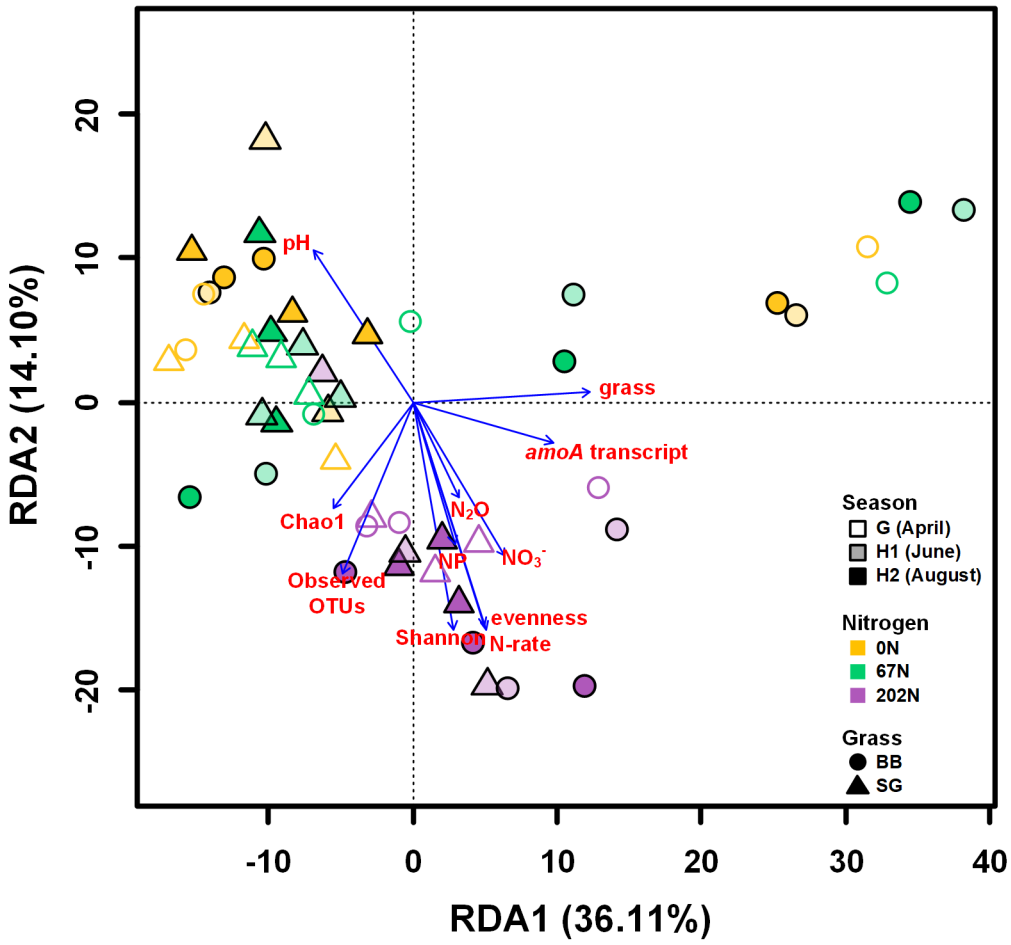

Figure 30. RDA of ammonia-oxidizing bacterial community (circle and triangle symbols) with major environmental factors, *amoA* gene and transcript abundances, and community diversity metrics (arrows). G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem; SWC, soil water content; DOC, dissolved organic C; N-rate, N fertilization rate.

structure under 202N treatment, whereas soil pH was a main factor significantly affecting the AOB community structure under 0N and 67N (Figure 28).

Discussion

The primary objectives of this study were to investigate the impacts of N fertilization rate and native grass species on the seasonal population size, activity, diversity, and community structure of AOB by using qPCR, qRT-PCR, and *amoA* gene amplicon sequencing techniques. One of the main findings of this study was that AOB population size, activity, and species richness varied with sampling season. Although a previous study observed variation of AOB communities with sampling seasons (Liu et al., 2018), our study found that AOB community structure was relatively constant across different sampling seasons, indicating considerable stability of AOB communities in our C_4 grassland soil. The seasonal variation of AOB population size (i.e., *amoA* gene abundance) and activity (i.e., *amoA* transcript abundance) was most likely dominated by the seasonal dynamics of climatic factors, such as precipitation and temperature, as well as soil properties, such as soil moisture and availability of soil nutrients.

The significant negative correlation of *amoA* gene abundance but positive correlation of *amoA* transcript abundance with SWC found in this study was also observed in a cotton field in the similar climatic zone (Hu et al., 2021). One possible explanation was that increasing SWC decreases soil oxygen, resulting in decreased abundances of aerobic AOB. The increased activity we observed could be because AOB increased expression of *amoA genes* under oxygen stress. It has been found that the transcription of bacterial *amoA* genes can be promoted by > 70% water filled pore space (Theodorakopoulos et al., 2017). Another study also reported that low dissolved oxygen content can promote *amoA* gene transcription in *Nitrosomonas europaea* in culture (Yu & Chandran, 2010).

Dissolved organic C (DOC) is another key factor that may have influenced the seasonal variation of AOB abundance and activity, which was highest at second grass harvest, and negatively associated with SWC, indicating possible leaching loss or dilution of DOC under higher SWC conditions (Poll et al., 2008; Zibilske & Makus, 2009) caused by seasonal precipitation. The total precipitation during the initial harvest period (June,

161.29 mm) was higher than during grass green up and second harvest periods (April and August, 94.23 and 120.14 mm, respectively). Our study found that the population size of AOB increased with DOC concentration, which was also observed in some other agroecosystems (Hu et al., 2021; Sun et al., 2019) and in groundwater (van der Wielen et al., 2009). One possible explanation might be that under DOC-rich conditions, mineralization of organic C by heterotrophs provided more CO_2 for the autotrophic AOB. However, there was a negative correlation between DOC and *amoA* transcript abundance in our study, which was also observed in estuarine ecosystems (Happel et al., 2019). Many studies have observed that nitrification rates can be inhibited by increased organic C concentration in terrestrial ecosystems due to 1) biological nitrification inhibition (BNI), that is, active compounds (such as organic acids, brachialactone) released from root exudates of some grass species as well as phenolics, tannins or monoterpenes leached from plant litter that inhibit AMO and HAO enzymes (i.e., biological nitrification inhibition) (Elroy & Sunil, 1973; Janke et al., 2018; Subbarao et al., 2009; White, 1986); 2) phenolics, tannins, and monoterpenes promoting heterotrophic immobilization of ammonium and decreasing substrate availability for nitrifiers (Bremner & McCarty, 1993; Paavolainen et al., 1998); and 3) the increased competition between heterotrophic and nitrifying bacteria for ammonium caused by high quality C (i.e., very labile carbon source) (Strauss & Lamberti, 2002).

Similar to DOC, DON is also an important factor affecting AOB abundance and can be diluted or lost under high SWC conditions. There was a positive correlation between DON and AOB abundance in our study, which may be that the DON, especially low molecular weight DON pool provides initial substrate for nitrification (Jones et al., 2004). However, no correlation between DON and AOB activity was observed, indicating that the expression of *amoA* may be influenced by other climatic factors, such as temperature, soil texture, and other soil characteristics.

Nitrogen fertilization significantly promoted the abundance, activity, and diversity of AOB as well as nitrification potential, as it provided ammonium as initial substrate for nitrification process. Notably, the promotion of AOB functional activity by N addition was only observed in grass growing seasons (June, August) rather than in grass green up (April,

before N fertilization), indicating that urea fertilization may only have short-term influence rather than year-long influence on AOB activity. Moreover, NO_3^--N, a product of nitrification, was closely and positively related to AOB activity and was significantly higher under high N-rate, resulting in lower soil pH.

Our observations that N management can affect AOB community structure is consistent with previous studies (Mendum & Hirsch, 2002; Rooney et al., 2010; Webster et al., 2005). The influence of N fertilization on AOB communities can be partly explained by the significantly lower soil pH with high urea fertilization rate (202 N-rate) (Pommerening-Roser & Koops, 2005; Xi et al., 2017). Although urea can temporarily reduce soil acidity and result in elevated pH conditions, oxidation of ammonium produces H^+ ions that acidify the soil. A decrease in pH could select for a more acidic-tolerant AOB community. Indeed, in this study we observed a very different AOB community structure under high N-rate compared to moderate and no N fertilization. In particular, we found that the OTUs affiliated to *Nitrosospira multiformis* were more abundant under high urea fertilization rate. Norton et al. (2008) reported that *N. multiformis* genome contains genes encoding urease, urea carboxylase, and a putative allophanate (carboxyurea) hydrolase for urea conversion to ammonium and bicarbonate (Norton et al., 2008). The ureolytic capacity of *N. multiformis* makes it well-adapted for soils undergoing urea fertilization and/or acidic pH (Burton & Prosser, 2001; Pommerening-Roser & Koops, 2005). Furthermore, the arginine decarboxylases in *N. multiformis* are known to function in acid-tolerance (Norton et al., 2008).

Grass species did not affect the functional activity of AOB. However, AOB population size and species richness, as well as nitrification potential were significantly higher under switchgrass compared to big bluestem. The AOB community structure under switchgrass and big bluestem was also significantly different. The results we observed indicated that the characteristics of plants may play important roles in shaping AOB communities and selecting distinct AOB populations. As previously mentioned, root exudates of grass contain active compounds which may inhibit nitrification (Subbarao et al., 2009). We speculated that difference in BNI by switchgrass and big bluestem may explain the differences in AOB communities. Moreover, leached compounds from litter (such as

phenolics, tannins or monoterpenes) of these two grass species may also have had different effects on: 1) the inhibition to ammonium monooxygenase (Elroy & Sunil, 1973; White, 1986); 2) the promotion to ammonium immobilization by heterotrophs decreasing ammonium availability for AOB (Bremner & McCarty, 1993); or 3) the quality of soil organic carbon under different grass species (Strauss & Lamberti, 2002). It has been reported previously that big bluestem has a higher cellulose content than switchgrass (Delaquis et al., 2014), which indicates that we should have seen lower nitrification rates and/or AOB population size in the switchgrass treatment. However, our result showed that switchgrass had higher AOB abundance and nitrification potential as well as similar AOB functional activity compared to big bluestem. One possible explanation was that when we sampled soils, the switchgrass had become stemmy and contained more cellulose as it matured (Lemus, 2008), which made its litter more difficult to decompose, and therefore reducing competition for ammonium from heterotrophic decomposers (Strauss & Lamberti, 2002). Lastly, the significant difference of AOB abundance and community structure but similar AOB functional activity between switchgrass and big bluestem reflected functional redundancy within AOB community (Louca et al., 2016; Louca et al., 2018; Short et al., 2013), suggesting that the nitrification processes under switchgrass and big bluestem may be controlled by taxonomically different nitrifiers. It was notable that our SEM result predicted the lower abundance, activity, and alpha-diversity of AOB, as well as nitrification potential and soil nitrate content under big bluestem than under switchgrass, suggesting less potential for N losses through nitrate leaching and N_2O emission in big bluestem systems compared to switchgrass systems.

Nitrification potential was positively correlated to *amoA* gene abundances, confirming the reliability of using nitrification potential to infer the population size of nitrifiers (Belser & Mays, 1980; Bernhard et al., 2010). However, nitrification potential was not correlated to *amoA* transcript abundance, indicating that *in situ* AOB functional activity can be largely influenced by climatic factors, nutrient content, and soil properties (Lew et al., 2019; Nelson et al., 2016). Although many studies have observed a positive correlation between the abundance of ammonia oxidizers and N_2O emissions (Breuillin-Sessoms et al., 2017; Pannu et al., 2019), other studies, including ours, report no correlation of N_2O emissions

with AOB abundance and activity (Lourenco et al., 2018; Pereira et al., 2015). This is probably due to the fact that N_2O emissions are regulated by both nitrification and denitrification processes (Maag & Vinther, 1996). The contribution of nitrifiers and denitrifiers to N_2O emission might be site-specific and can be affected by complex environmental factors, such as soil types, soil moisture, temperatures, and management practices (Maag & Vinther, 1996; Senbayram et al., 2009).

We found that *amoA* transcript abundances significantly and positively associated to its gene abundance according to structural equation modeling, indicating that AOB population size does affect its functional activity. Furthermore, the alpha-diversity of AOB communities was strongly and positively correlated to AOB population size and nitrification potential but had no correlation with AOB functional activity, suggesting that a more diverse AOB community may have more nitrification potential but may not influence *in situ* nitrification rate due to functional redundancy and environmental stress.

Conclusions

We determined the seasonal dynamics of AOB population size, activity, diversity, and community structure under different C_4 grass species and N fertilization rates in a native perennial grassland. The genus *Nitrosospira* was the dominant AOB in this grassland. Our results showed that the abundance, activity, and species richness of AOB varied significantly with sampling season while no shift of AOB community structure with season was observed. Nitrogen fertilization rate had a stronger influence on AOB community composition than C_4 grass species. In accordance with our hypothesis, elevated N fertilizer application changed AOB community composition and increased abundance, activity, and alpha-diversity of AOB community as well as nitrification potential, N_2O emission, soil nitrate content, and soil acidity. This suggests high N addition in native C_4 grass systems may have negative consequences for N retention. The SEM predicted lower abundance, activity, and alpha-diversity of AOB, as well as nitrification potential and soil nitrate content under big bluestem than under switchgrass, suggesting less potential for N losses in big bluestem systems than in switchgrass systems. In addition, soil pH, nitrate, nitrification potential, and N_2O emission significantly related to the variability of AOB

communities. Together, our work revealed insights into important relationships among the abundance, activity, diversity, and community structure of AOB as well as soil properties under different N fertilization rates and C_4 grass species in a field experiment. These results add to our understanding of the response of soil bacterial ammonia-oxidizers to growing-season N fertilization and grass species in perennial native C_4 grass systems.

References

Adkins, J., Jastrow, J. D., Morris, G. P., & de Graaff, M.-A. (2019). Effects of fertilization, plant species, and intra-specific diversity on soil carbon and nitrogen in biofuel cropping systems after five growing seasons. *Biomass and Bioenergy*, *130*, 105393. https://doi.org/10.1016/j.biombioe.2019.105393

Aigle, A., Prosser, J. I., & Gubry-Rangin, C. (2019). The application of high-throughput sequencing technology to analysis of *amoA* phylogeny and environmental niche specialisation of terrestrial bacterial ammonia-oxidisers. *Environmental Microbiome*, *14*(1). https://doi.org/10.1186/s40793-019-0342-6

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403-410. https://doi.org/https://doi.org/10.1016/S0022-2836(05)80360-2

Andersson, B., Sundbäck, K., Hellman, M., Hallin, S., & Alsterberg, C. (2014). Nitrogen fixation in shallow-water sediments: Spatial distribution and controlling factors. *Limnology and Oceanography*, *59*(6), 1932-1944. https://doi.org/10.4319/lo.2014.59.6.1932

Araki, N., Yamaguchi, T., Yamazaki, S., & Harada, H. (2004). Quantification of *amoA* gene abundance and their *amoA* mRNA levels in activated sludge by real-time PCR. *Water Science and Technology*, *50*(8), 1-8. https://doi.org/10.2166/wst.2004.0474

Avrahami, S., Liesack, W., & Conrad, R. (2003). Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers. *Environmental Microbiology*, *5*(8), 691-705. https://doi.org/10.1046/j.1462-2920.2003.00457.x

Babur, S. M., Chotima, P., Klaus, N., Brendan, J. M. B., & Jorge, L. M. R. (2014). Response of free-living nitrogen-fixing microorganisms to land use change in the Amazon rainforest. *Applied and Environmental Microbiology*, *80*(1), 281-288. https://doi.org/10.1128/AEM.02362-13

Bakken, L. R., & Frostegård, Å. (2020). Emerging options for mitigating N_2O emissions from food production by manipulating the soil microbiota. *Current Opinion in Environmental Sustainability*, *47*, 89-94. https://doi.org/10.1016/j.cosust.2020.08.010

Bardgett, Denton, & Cook. (1999). Below-ground herbivory promotes soil nutrient transfer and root growth in grassland. *Ecology Letters*, *2*(6), 357-360. https://doi.org/https://doi.org/10.1046/j.1461-0248.1999.00001.x

Bárta, J., Melichová, T., Vaněk, D., Picek, T., & Šantrůčková, H. (2010). Effect of pH and dissolved organic matter on the abundance of *nirK* and *nirS* denitrifiers in spruce forest soil. *Biogeochemistry*, *101*(1-3), 123-132. https://doi.org/10.1007/s10533-010-9430-9

Bartosch, S., Hartwig, C., Spieck, E., & Bock, E. (2002). Immunological detection of *Nitrospira*-like bacteria in various soils. *Microbial Ecology*, *43*(1), 26-33. https://doi.org/10.1007/s00248-001-0037-5

Bateman, E. J., & Baggs, E. M. (2005). Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space. *Biology and*

Fertility of Soils, *41*(6), 379-388. https://doi.org/10.1007/s00374-005-0858-3
Bedmar, E. J., Robles, E. F., & Delgado, M. J. (2005). The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium *Bradyrhizobium japonicum*. *Biochemical Society Transactions*, *33*(1), 141-144. https://doi.org/10.1042/bst0330141
Beeckman, F., Motte, H., & Beeckman, T. (2018). Nitrification in agricultural soils: impact, actors and mitigation. *Current Opinion in Biotechnology*, *50*, 166-173. https://doi.org/10.1016/j.copbio.2018.01.014
Belser, L. W., & Mays, E. L. (1980). Specific inhibition of nitrite oxidation by chlorate and its use in assessing nitrification in soils and sediments. *Applied and environmental microbiology*, *39*(3), 505-510. https://doi.org/10.1128/AEM.39.3.505-510.1980
Bernhard, A. E., Landry, Z. C., Blevins, A., de la Torre, J. R., Giblin, A. E., & Stahl, D. A. (2010). Abundance of ammonia-oxidizing archaea and bacteria along an estuarine salinity gradient in relation to potential nitrification rates. *Applied and Environmental Microbiology*, *76*(4), 1285-1289. https://doi.org/10.1128/AEM.02018-09
Blunch, N. (2012). *Introduction to structural equation modeling using IBM SPSS statistics and AMOS*. Sage.
Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., Cope, E. K., Da Silva, R., Diener, C., Dorrestein, P. C., Douglas, G. M., Durall, D. M., Duvallet, C., Edwardson, C. F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J. M., Gibbons, S. M., Gibson, D. L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G. A., Janssen, S., Jarmusch, A. K., Jiang, L., Kaehler, B. D., Kang, K. B., Keefe, C. R., Keim, P., Kelley, S. T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M. G. I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B. D., McDonald, D., McIver, L. J., Melnik, A. V., Metcalf, J. L., Morgan, S. C., Morton, J. T., Naimey, A. T., Navas-Molina, J. A., Nothias, L. F., Orchanian, S. B., Pearson, T., Peoples, S. L., Petras, D., Preuss, M. L., Pruesse, E., Rasmussen, L. B., Rivers, A., Robeson, M. S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S. J., Spear, J. R., Swafford, A. D., Thompson, L. R., Torres, P. J., Trinh, P., Tripathi, A., Turnbaugh, P. J., Ul-Hasan, S., van der Hooft, J. J. J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K. C., Williamson, C. H. D., Willis, A. D., Xu, Z. Z., Zaneveld, J. R., Zhang, Y., Zhu, Q., Knight, R., & Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852-857. https://doi.org/10.1038/s41587-019-0209-9
Brejda, J. J. (2000). Fertilization of native warm-season grasses. *Native warm-season grasses: research trends and issues*, 177-200.

https://doi.org/10.2135/cssaspecpub30.c12 (CSSA Special Publications)
Bremner, J., & McCarty, G. (1993). Inhibition of nitrification in soil by allelochemicals derived from plants and plant residues. *Soil biochemistry*, *8*, 181-218.
Breuillin-Sessoms, F., Venterea, R. T., Sadowsky, M. J., Coulter, J. A., Clough, T. J., & Wang, P. (2017). Nitrification gene ratio and free ammonia explain nitrite and nitrous oxide production in urea-amended soils. *Soil Biology and Biochemistry*, *111*, 143-153. https://doi.org/10.1016/j.soilbio.2017.04.007
Brusseau, M. L., Gerba, C. P., & Pepper, I. L. (2006). *Environmental & pollution science* (2nd ed. ed.). Elsevier/Academic Press.
Burns, J. A., Zehr, J. P., & Capone, D. G. (2002). Nitrogen-fixing phylotypes of Chesapeake Bay and Neuse River estuary sediments. *Microbial Ecology*, *44*(4), 336-343. https://doi.org/10.1007/s00248-002-1000-9
Burton, S. A., & Prosser, J. I. (2001). Autotrophic ammonia oxidation at low pH through urea hydrolysis. *Applied and Environmental Microbiology*, *67*(7), 2952-2957. https://doi.org/10.1128/AEM.67.7.2952-2957.2001
Cameron, K. C., Di, H. J., & Moir, J. L. (2013). Nitrogen losses from the soil/plant system: a review. *Annals of Applied Biology*, *162*(2), 145-173. https://doi.org/10.1111/aab.12014
Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics*, *11*(4), 265-270. http://www.jstor.org/stable/4615964
Chapin, F. S., Matson, P. A., & Mooney, H. A. (2002). *Principles of terrestrial ecosystem ecology*. Springer, New York, NY. https://doi.org/10.1007/b97397
Collier, S. M., Ruark, M. D., Oates, L. G., Jokela, W. E., & Dell, C. J. (2014). Measurement of greenhouse gas flux from agricultural soils using static chambers. *Journal of Visualized Experiments*(90). https://doi.org/10.3791/52110
Colliver, B. B., & Stephenson, T. (2000). Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. *Biotechnology Advances*, *18*(3), 219-232. https://www.ncbi.nlm.nih.gov/pubmed/14538109
Conrad, R. (1996). Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). *Microbiological Reviews*, *60*(4), 609-640.
Cusack, D. F., Silver, W., & McDowell, W. H. (2009). Biological nitrogen fixation in two tropical forests: ecosystem-level patterns and effects of nitrogen fertilization. *Ecosystems*, *12*(8), 1299-1315. https://doi.org/10.1007/s10021-009-9290-0
Dang, H., Yang, J., Li, J., Luan, X., Zhang, Y., Gu, G., Xue, R., Zong, M., & Klotz, M. G. (2013). Environment-dependent distribution of the sediment *nifH*-harboring microbiota in the Northern South China Sea. *Applied and Environmental Microbiology*, *79*(1), 121-132. https://doi.org/10.1128/aem.01889-12
Davis, D. A., Gamble, M. D., Bagwell, C. E., Bergholz, P. W., & Lovell, C. R. (2011). Responses of salt marsh plant rhizosphere diazotroph assemblages to changes in marsh elevation, edaphic conditions and plant host species. *Microbial Ecology*, *61*(2), 386-398.
De Caceres, M., Jansen, F., & De Caceres, M. M. (2016). Package 'indicspecies'. *indicators*, *8*, 1.

Delaquis, E., Samson, R., Seguin, P., Mustafa, A., & Martel, H. (2014). Impacts of improved switchgrass and big bluestem selections on yield, morphological characteristics, and biomass quality. *Advances in Agriculture*, *2014*, 192824. https://doi.org/10.1155/2014/192824

Deslippe, J. R., Egger, K. N., & Henry, G. H. R. (2005). Impacts of warming and fertilization on nitrogen-fixing microbial communities in the Canadian High Arctic. *FEMS Microbiology Ecology*, *53*(1), 41-50. https://doi.org/10.1016/j.femsec.2004.12.002

Di, H. J., & Cameron, K. C. (2002). Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. *Nutrient Cycling in Agroecosystems*, *64*(3), 237-256. https://doi.org/10.1023/A:1021471531188

Di, H. J., Cameron, K. C., Shen, J. P., Winefield, C. S., O'Callaghan, M., Bowatte, S., & He, J. Z. (2009). Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature Geoscience*, *2*(9), 621-624. https://doi.org/10.1038/ngeo613

Dixon, R., & Kahn, D. (2004). Genetic regulation of biological nitrogen fixation. *Nature Reviews: Microbiology*, *2*(8), 621-631. https://doi.org/10.1038/nrmicro954

Doane, T. A., & Horwáth, W. R. (2003). Spectrophotometric determination of nitrate with a single reagent. *Analytical Letters*, *36*(12), 2713-2722. https://doi.org/10.1081/AL-120024647

Dobereiner, J., Day, J. M., & Dart, P. J. (1972). Nitrogenase activity and oxygen sensitivity of the *Paspalum notatum-Azotobacter paspali* association. *Journal of general microbiology*, *71*(1), 103-116. https://doi.org/10.1099/00221287-71-1-103

Duan, Y.-F., Kong, X.-W., Schramm, A., Labouriau, R., Eriksen, J., & Petersen, S. O. (2017). Microbial N transformations and N_2O emission after simulated grassland cultivation: effects of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP). *Applied and Environmental Microbiology*, *83*(1), e02019-02016. https://doi.org/10.1128/AEM.02019-16

Eady, R. R., & Postgate, J. R. (1974). Nitrogenase. *Nature*, *249*(5460), 805-810. https://doi.org/10.1038/249805a0

Elroy, L. R., & Sunil, K. P. (1973). Inhibition of nitrification by climax ecosystems. II. additional evidence and possible role of tannins. *American Journal of Botany*, *60*(7), 691-702. https://doi.org/10.1002/j.1537-2197.1973.tb05975.x

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biological Conservation*, *61*(1), 1-10. https://doi.org/https://doi.org/10.1016/0006-3207(92)91201-3

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. https://doi.org/10.1016/j.soilbio.2018.03.017

Faulwetter, J. L., Burr, M. D., Parker, A. E., Stein, O. R., & Camper, A. K. (2013). Influence of season and plant species on the abundance and diversity of sulfate reducing bacteria and ammonia oxidizing bacteria in constructed wetland microcosms. *Microbial Ecology*, *65*(1), 111-127. https://doi.org/10.1007/s00248-

012-0114-y
Feng, J., Penton, C. R., He, Z., Van Nostrand, J. D., Yuan, M. M., Wu, L., Wang, C., Qin, Y., Shi, Z. J., Guo, X., Schuur, E. A. G., Luo, Y., Bracho, R., Konstantinidis, K. T., Cole, J. R., Tiedje, J. M., Yang, Y., & Zhou, J. (2019). Long-term warming in Alaska enlarges the diazotrophic community in deep soils. *mBio*, *10*(1), e02521-02518. https://doi.org/10.1128/mBio.02521-18
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.
Fish, J. A., Chai, B., Wang, Q., Sun, Y., Brown, C. T., Tiedje, J. M., & Cole, J. R. (2013). FunGene: the functional gene pipeline and repository. *Frontiers in Microbiology*, *4*, 291. https://doi.org/10.3389/fmicb.2013.00291
Francis, G. S., Haynes, R. J., Speir, T. W., & Williams, P. H. (1995). The effects of a nitrification inhibitor on leaching losses and recovery of mineralized nitrogen by a wheat crop after ploughing-in temporary leguminous pastures. *Fertilizer Research*, *41*(1), 33-39. https://doi.org/10.1007/BF00749518
Friesen, P. C., & Cattani, D. J. (2017). Nitrogen use efficiency and productivity of first year switchgrass and big bluestem from low to high soil nitrogen. *Biomass and Bioenergy*, *107*, 317-325. https://doi.org/https://doi.org/10.1016/j.biombioe.2017.10.016
Frijlink, M., Abee, T., Laanbroek, H., Boer, W., & Konings, W. (1992). The bioenergetics of ammonia and hydroxylamine oxidation in *Nitrosomonas europaea* at acid and alkaline pH. *Archives of Microbiology*, *157*(2), 194-199. https://doi.org/10.1007/BF00245290
Gaby, J. C., & Buckley, D. H. (2011). A global census of nitrogenase diversity. *Environmental Microbiology*, *13*(7), 1790-1799. https://doi.org/10.1111/j.1462-2920.2011.02488.x
Gaby, J. C., & Buckley, D. H. (2012). A comprehensive evaluation of PCR primers to amplify the *nifH* gene of nitrogenase. *PLoS One*, *7*(7), e42149. https://doi.org/10.1371/journal.pone.0042149
Gaby, J. C., & Buckley, D. H. (2014). A comprehensive aligned *nifH* gene database: a multipurpose tool for studies of nitrogen-fixing bacteria. *Database (Oxford)*, *2014*, bau001. https://doi.org/10.1093/database/bau001
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 amplicon sequencing and analysis pipeline. *Applied and Environmental Microbiology*, *84*(4), e01512-01517. https://doi.org/10.1128/AEM.01512-17
Gao, Y., Mania, D., Mousavi, S. A., Lycus, P., Arntzen, M. O., Lindstrom, K., Shapleigh, J. P., Bakken, L. R., & Frostegard, A. (2020). Competition for electrons favors N_2O reduction in denitrifying *Bradyrhizobium* isolates. *bioRxiv*.
Gilbert, J. A., Fernandez, C., Farías, L., & Ulloa, O. (2011). Nitrogen fixation in denitrified marine waters. *PLoS One*, *6*(6), e20539. https://doi.org/10.1371/journal.pone.0020539

Gilmullina, A., Rumpel, C., Blagodatskaya, E., & Chabbi, A. (2020). Management of grasslands by mowing versus grazing – impacts on soil organic matter quality and microbial functioning. *Applied Soil Ecology*, *156*. https://doi.org/10.1016/j.apsoil.2020.103701

Groffman, P. M., Eagan, P., Sullivan, W. M., & Lemunyon, J. L. (1996). Grass species and soil type effects on microbial biomass and activity. *Plant and Soil*, *183*(1), 61-67. https://doi.org/10.1007/BF02185565

Gupta, V. V. S. R., Kroker, S. J., Hicks, M., Davoren, C. W., 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*(10), 1044. https://doi.org/10.1071/cp14109

Gupta, V. V. S. R., Zhang, B., Penton, C. R., Yu, J., & Tiedje, J. M. (2019). Diazotroph diversity and nitrogen fixation in summer active perennial grasses in a Mediterranean region agricultural soil. *Frontiers in Molecular Biosciences*, *6*. https://doi.org/10.3389/fmolb.2019.00115

Hallin, S., Jones, C. M., Schloter, M., & Philippot, L. (2009). Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. *The ISME Journal*, *3*(5), 597-605. https://doi.org/10.1038/ismej.2008.128

Hallin, S., Philippot, L., Loffler, F. E., Sanford, R. A., & Jones, C. M. (2018). Genomics and ecology of novel N_2O-reducing microorganisms. *Trends Microbiol*, *26*(1), 43-55. https://doi.org/10.1016/j.tim.2017.07.003

Han, L.-L., Wang, Q., Shen, J.-P., Di, H. J., Wang, J.-T., Wei, W.-X., Fang, Y.-T., Zhang, L.-M., & He, J.-Z. (2019). Multiple factors drive the abundance and diversity of the diazotrophic community in typical farmland soils of China. *FEMS Microbiology Ecology*, *95*(8). https://doi.org/10.1093/femsec/fiz113

Hansen, E. M., Eriksen, J., & Vinther, F. P. (2007). Catch crop strategy and nitrate leaching following grazed grass-clover. *Soil use and management*, *23*(4), 348-358. https://doi.org/10.1111/j.1475-2743.2007.00106.x

Happel, E. M., Markussen, T., Teikari, J. E., Huchaiah, V., Alneberg, J., Andersson, A. F., Sivonen, K., Middelboe, M., Kisand, V., & Riemann, L. (2019). Effects of allochthonous dissolved organic matter input on microbial composition and nitrogen-cycling genes at two contrasting estuarine sites. *FEMS Microbiology Ecology*, *95*(9). https://doi.org/10.1093/femsec/fiz123

Hooper, D., Coughlan, J., & Mullen, M. (2007). Structural equation modeling: Guidelines for determining model fit. *The Electronic Journal of Business Research Methods*, *6*.

Hu, J., Jin, V. L., Konkel, J. Y. M., Schaeffer, S. M., Schneider, L. G., & DeBruyn, J. M. (2021). Soil health management enhances microbial nitrogen cycling capacity and activity. *mSphere*, *6*(1), e01237-01220. https://doi.org/10.1128/mSphere.01237-20

Izquierdo, J. A., & Klaus, N. (2015). Variation in diazotrophic community structure in forest soils reflects land use history. *Soil Biology and Biochemistry*, *80*, 1-8. https://doi.org/10.1016/j.soilbio.2014.09.017

Janke, C. K., Wendling, L. A., & Fujinuma, R. (2018). Biological nitrification inhibition

by root exudates of native species, Hibiscus splendens and Solanum echinatum. *PeerJ*, *6*, e4960-e4960. https://doi.org/10.7717/peerj.4960

Jia, Z., & Conrad, R. (2009). Bacteria rather than archaea dominate microbial ammonia oxidation in an agricultural soil. *Environmental Microbiology*, *11*(7), 1658-1671. https://doi.org/10.1111/j.1462-2920.2009.01891.x

Jing, H., Xia, X., Liu, H., Zhou, Z., Wu, C., & Nagarajan, S. (2015). Anthropogenic impact on diazotrophic diversity in the mangrove rhizosphere revealed by *nifH* pyrosequencing. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.01172

Jones, D. L., Shannon, D., V. Murphy, D., & Farrar, J. (2004). Role of dissolved organic nitrogen (DON) in soil N cycling in grassland soils. *Soil Biology and Biochemistry*, *36*(5), 749-756. https://doi.org/10.1016/j.soilbio.2004.01.003

Kastl, E.-M., Schloter-Hai, B., Buegger, F., & Schloter, M. (2014). Impact of fertilization on the abundance of nitrifiers and denitrifiers at the root–soil interface of plants with different uptake strategies for nitrogen. *Biology and Fertility of Soils*, *51*(1), 57-64. https://doi.org/10.1007/s00374-014-0948-1

Keuter, A., Veldkamp, E., & Corre, M. D. (2014). Asymbiotic biological nitrogen fixation in a temperate grassland as affected by management practices. *Soil Biology and Biochemistry*, *70*, 38-46.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glockner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res*, *41*(1), e1. https://doi.org/10.1093/nar/gks808

Kline, R. B. (2015). *Principles and practice of structural equation modeling*. Guilford publications.

Kobayashi, M., Matsuo, Y., Takimoto, A., Suzuki, S., Maruo, F., & Shoun, H. (1996). Denitrification, a novel type of respiratory metabolism in fungal mitochondrion. *The Journal of biological chemistry*, *271*(27), 16263-16267. https://doi.org/10.1074/jbc.271.27.16263

Kong, A. Y., Hristova, K., Scow, K. M., & Six, J. (2010). Impacts of different N management regimes on nitrifier and denitrifier communities and N cycling in soil microenvironments. *Soil Biology and Biochemistry*, *42*(9), 1523-1533. https://doi.org/10.1016/j.soilbio.2010.05.021

Kowalchuk, G. A., Stienstra, A. W., Heilig, G. H., Stephen, J. R., & Woldendorp, J. W. (2000). Molecular analysis of ammonia-oxidising bacteria in soil of successional grasslands of the Drentsche A (The Netherlands). *FEMS Microbiology Ecology*, *31*(3), 207-215. https://www.ncbi.nlm.nih.gov/pubmed/10719201

Laughlin, R. J., & Stevens, R. J. (2002). Evidence for fungal dominance of denitrification and codenitrification in a grassland soil. *Soil Science Society of America Journal*, *66*(5), 1540-1548. https://doi.org/10.2136/sssaj2002.1540

LeBauer, D. S., & Treseder, K. K. (2008). Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology*, *89*(2), 371-379. https://doi.org/Doi 10.1890/06-2057.1

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G. W., Prosser, J. I.,

Schuster, S. C., & Schleper, C. (2006). Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature*, *442*(7104), 806-809. https://doi.org/10.1038/nature04983

Lemus, R. (2008). Establishing and managing switchgrass as a forage. *Forage News. Mississippi State University Extension Service*.

Letunic, I., & Bork, P. (2011). Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res*, *39*(Web Server issue), W475-478. https://doi.org/10.1093/nar/gkr201

Levy-Booth, D. J., Prescott, C. E., & Grayston, S. J. (2014). Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. *Soil Biology and Biochemistry*, *75*, 11-25. https://doi.org/10.1016/j.soilbio.2014.03.021

Lew, S., Glińska-Lewczuk, K., & Lew, M. (2019). The effects of environmental parameters on the microbial activity in peat-bog lakes. *PLoS One*, *14*(10), e0224441-e0224441. https://doi.org/10.1371/journal.pone.0224441

Li, L., & Schaeffer, S. M. (2020). Stabilization mechanisms of isotope-labeled carbon substrates in soil under moisture pulses and conservation agricultural management. *Geoderma*, *380*, 114677. https://doi.org/10.1016/j.geoderma.2020.114677

Li, L., Wilson, C. B., He, H., Zhang, X., Zhou, F., & Schaeffer, S. M. (2019). Physical, biochemical, and microbial controls on amino sugar accumulation in soils under long-term cover cropping and no-tillage farming. *Soil Biology and Biochemistry*, *135*, 369-378. https://doi.org/10.1016/j.soilbio.2019.05.017

Limmer, C., & Drake, H. L. (1996). Non-symbiotic N_2-fixation in acidic and pH-neutral forest soils: Aerobic and anaerobic differentials. *Soil Biology and Biochemistry*, *28*(2), 177-183. https://doi.org/10.1016/0038-0717(95)00118-2

Lindsay, E. A., Colloff, M. J., Gibb, N. L., & Wakelin, S. A. (2010). The abundance of microbial functional genes in grassy woodlands is influenced more by soil nutrient enrichment than by recent weed invasion or livestock exclusion. *Applied and Environmental Microbiology*, *76*(16), 5547-5555. https://doi.org/10.1128/AEM.03054-09

Liu, R., Hu, H., Suter, H., Hayden, H. L., He, J., Mele, P., & Chen, D. (2016). Nitrification is a primary driver of nitrous oxide production in laboratory microcosms from different land-use soils. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01373

Liu, S., Coyne, M. S., Grove, J. H., & Flythe, M. D. (2018). Nitrogen, season, and tillage management influence ammonia oxidizing bacterial communities in long-term maize. *Applied soil ecology : a section of Agriculture, ecosystems & environment*, *129*, 98-106. https://doi.org/10.1016/j.apsoil.2018.05.002

Liu, X., Yang, C., Yu, X., Yu, H., Zhuang, W., Gu, H., Xu, K., Zheng, X., Wang, C., Xiao, F., Wu, B., He, Z., & Yan, Q. (2020). Revealing structure and assembly for rhizophyte-endophyte diazotrophic community in mangrove ecosystem after introduced *Sonneratia apetala* and *Laguncularia racemosa*. *Science of the Total Environment*, *721*, 137807. https://doi.org/10.1016/j.scitotenv.2020.137807

Louca, S., Jacques, S. M. S., Pires, A. P. F., Leal, J. S., Srivastava, D. S., Parfrey, L. W., Farjalla, V. F., & Doebeli, M. (2016). High taxonomic variability despite stable functional structure across microbial communities. *Nature Ecology & Evolution*, *1*(1). https://doi.org/10.1038/s41559-016-0015

Louca, S., Polz, M. F., Mazel, F., Albright, M. B. N., Huber, J. A., O'Connor, M. I., Ackermann, M., Hahn, A. S., Srivastava, D. S., Crowe, S. A., Doebeli, M., & Parfrey, L. W. (2018). Function and functional redundancy in microbial systems. *Nature Ecology & Evolution*, *2*(6), 936-943. https://doi.org/10.1038/s41559-018-0519-1

Lourenco, K. S., Cassman, N. A., Pijl, A. S., van Veen, J. A., Cantarella, H., & Kuramae, E. E. (2018). *Nitrosospira* sp. govern nitrous oxide emissions in a tropical soil amended with residues of bioenergy crop. *Frontiers in Microbiology*, *9*, 674. https://doi.org/10.3389/fmicb.2018.00674

Lozupone, C. A., Hamady, M., Kelley, S. T., & Knight, R. (2007). Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Applied and environmental microbiology*, *73*(5), 1576-1585. https://doi.org/10.1128/AEM.01996-06

Luo, Y., Wang, C., Shen, Y., Sun, W., & Dong, K. (2019). The interactive effects of mowing and N addition did not weaken soil net N mineralization rates in semi-arid grassland of Northern China. *Scientific Reports*, *9*(1), 13457-13411. https://doi.org/10.1038/s41598-019-49787-6

Maag, M., & Vinther, F. P. (1996). Nitrous oxide emission by nitrification and denitrification in different soil types and at different soil moisture contents and temperatures. *Applied Soil Ecology*, *4*(1), 5-14. https://doi.org/https://doi.org/10.1016/0929-1393(96)00106-0

Mao, Y., Yannarell, A. C., Davis, S. C., & Mackie, R. I. (2013). Impact of different bioenergy crops on N-cycling bacterial and archaeal communities in soil. *Environmental Microbiology*, *15*(3), 928-942. https://doi.org/10.1111/j.1462-2920.2012.02844.x

Marietou, A., & Boden, R. (2016). Nitrate reduction in sulfate-reducing bacteria. *FEMS Microbiology Letters*, *363*(15), fnw155. https://doi.org/10.1093/femsle/fnw155

Marietou, A., Griffiths, L., & Cole, J. (2009). Preferential reduction of the thermodynamically less favorable electron acceptor, sulfate, by a nitrate-reducing strain of the sulfate-reducing bacterium *Desulfovibrio desulfuricans* 27774. *Journal of Bacteriology*, *191*(3), 882-889. https://doi.org/10.1128/jb.01171-08

Mårtensson, L., Díez, B., Wartiainen, I., Zheng, W., El-Shehawy, R., & Rasmussen, U. (2009). Diazotrophic diversity, *nifH* gene expression and nitrogenase activity in a rice paddy field in Fujian, China. *Plant and Soil*, *325*(1-2), 207-218. https://doi.org/10.1007/s11104-009-9970-8

McMurdie, P. J., & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, *8*(4), e61217.

Mendum, T. A., & Hirsch, P. R. (2002). Changes in the population structure of β-group autotrophic ammonia oxidising bacteria in arable soils in response to agricultural

practice. *Soil Biology and Biochemistry*, *34*(10), 1479-1485. https://doi.org/10.1016/S0038-0717(02)00092-5

Nelson, M. B., Martiny, A. C., & Martiny, J. B. H. (2016). Global biogeography of microbial nitrogen-cycling traits in soil. *Proceedings of the National Academy of Sciences*, *113*(29), 8033-8040. https://doi.org/10.1073/pnas.1601070113

Norton, J. M., Alzerreca, J. J., Suwa, Y., & Klotz, M. G. (2002). Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. *Archives of Microbiology*, *177*(2), 139-149. https://doi.org/10.1007/s00203-001-0369-z

Norton, J. M., Klotz, M. G., Stein, L. Y., Arp, D. J., Bottomley, P. J., Chain, P. S., Hauser, L. J., Land, M. L., Larimer, F. W., Shin, M. W., & Starkenburg, S. R. (2008). Complete genome sequence of *Nitrosospira multiformis*, an ammonia-oxidizing bacterium from the soil environment. *Applied and Environmental Microbiology*, *74*(11), 3559-3572. https://doi.org/10.1128/AEM.02722-07

Oksanen, J., Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P., O'Hara, R., Simpson, G., & Solymos, P. (2020). vegan: Community Ecology Package. R package version 2.5-5. 2019. In.

Ouyang, Y., Evans, S. E., Friesen, M. L., & Tiemann, L. K. (2018). Effect of nitrogen fertilization on the abundance of nitrogen cycling genes in agricultural soils: a meta-analysis of field studies. *Soil Biology and Biochemistry*, *127*, 71-78. https://doi.org/10.1016/j.soilbio.2018.08.024

Ouyang, Y., Norton, J. M., Stark, J. M., Reeve, J. R., & Habteselassie, M. Y. (2016). Ammonia-oxidizing bacteria are more responsive than archaea to nitrogen source in an agricultural soil. *Soil Biology and Biochemistry*, *96*, 4-15. https://doi.org/10.1016/j.soilbio.2016.01.012

Paavolainen, L., Kitunen, V., & Smolander, A. (1998). Inhibition of nitrification in forest soil by monoterpenes. *Plant and Soil*, *205*(2), 147-154. https://doi.org/10.1023/A:1004335419358

Pannu, M. W., Meinhardt, K. A., Bertagnolli, A., Fransen, S. C., Stahl, D. A., & Strand, S. E. (2019). Nitrous oxide emissions associated with ammonia-oxidizing bacteria abundance in fields of switchgrass with and without intercropped alfalfa [https://doi.org/10.1111/1758-2229.12790]. *Environmental Microbiology Reports*, *11*(5), 727-735. https://doi.org/https://doi.org/10.1111/1758-2229.12790

Parrish, D. J., & Fike, J. H. (2005). The biology and agronomy of switchgrass for biofuels. *BPTS*, *24*(5-6), 423-459.

Patra, A. K., 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*(2), 153-156. https://doi.org/10.1111/j.1439-037X.2006.00247.x

Pavoine, S., Dufour, A.-B., & Chessel, D. (2004). From dissimilarities among species to dissimilarities among communities: a double principal coordinate analysis. *Journal of Theoretical Biology*, *228*(4), 523-537. https://doi.org/10.1016/j.jtbi.2004.02.014

Pereira e Silva, M. C., Semenov, A. V., van Elsas, J. D., & Salles, J. F. (2011). Seasonal

variations in the diversity and abundance of diazotrophic communities across soils. *FEMS Microbiology Ecology*, *77*(1), 57-68. https://doi.org/10.1111/j.1574-6941.2011.01081.x

Pereira, E. I. P., Suddick, E. C., Mansour, I., Mukome, F. N. D., Parikh, S. J., Scow, K., & Six, J. (2015). Biochar alters nitrogen transformations but has minimal effects on nitrous oxide emissions in an organically managed lettuce mesocosm. *Biology and Fertility of Soils*, *51*(5), 573-582. https://doi.org/10.1007/s00374-015-1004-5

Pereira, E. S. M. C., Schloter-Hai, B., Schloter, M., van Elsas, J. D., & Salles, J. F. (2013). Temporal dynamics of abundance and composition of nitrogen-fixing communities across agricultural soils. *PLoS One*, *8*(9), e74500. https://doi.org/10.1371/journal.pone.0074500

Poll, C., Marhan, S., Ingwersen, J., & Kandeler, E. (2008). Dynamics of litter carbon turnover and microbial abundance in a rye detritusphere. *Soil Biology and Biochemistry*, *40*(6), 1306-1321.

Pommerening-Roser, A., & Koops, H. P. (2005). Environmental pH as an important factor for the distribution of urease positive ammonia-oxidizing bacteria. *Microbiological Research*, *160*(1), 27-35. https://doi.org/10.1016/j.micres.2004.09.006

Prosser, J. I., Microbiology, S. f. G., & Group, S. f. G. M. E. (1986). *Nitrification*. Society for General Microbiology. https://books.google.com/books?id=oIIXAQAAIAAJ

Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M. C., Koops, H.-P., & Wagner, M. (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. *Applied and Environmental Microbiology*, *66*, 5368-5382.

Qin, D., Plattner, G., Tignor, M., Allen, S., Boschung, J., Nauels, A., Xia, Y., Bex, V., & Midgley, P. (2014). Climate change 2013: the physical science basis. *Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (eds TF Stocker et al.)*, 5-14.

Raes, J., Letunic, I., Yamada, T., Jensen, L. J., & Bork, P. (2011). Toward molecular trait-based ecology through integration of biogeochemical, geographical and metagenomic data. *Molecular Systems Biology*, *7*, 473. https://doi.org/10.1038/msb.2011.6

Rasche, F., Knapp, D., Kaiser, C., Koranda, M., Kitzler, B., Zechmeister-Boltenstern, S., Richter, A., & Sessitsch, A. (2011). Seasonality and resource availability control bacterial and archaeal communities in soils of a temperate beech forest. *The ISME Journal*, *5*(3), 389-402. https://doi.org/10.1038/ismej.2010.138

Ravishankara, A. R., John, S. D., & Robert, W. P. (2009). Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century. *Science (American Association for the Advancement of Science)*, *326*(5949), 123-125. https://doi.org/10.1126/science.1176985

Raymond, J., Siefert, J. L., Staples, C. R., & Blankenship, R. E. (2004). The natural history of nitrogen fixation. *Molecular Biology and Evolution*, *21*(3), 541-554. https://doi.org/10.1093/molbev/msh047

Reardon, C. L., Gollany, H. T., & Wuest, S. B. (2014). Diazotroph community structure and abundance in wheat-fallow and wheat-pea crop rotations. *Soil Biology and Biochemistry*, *69*, 406-412. https://doi.org/https://doi.org/10.1016/j.soilbio.2013.10.038

Rhine, E. D., Mulvaney, R. L., Pratt, E. J., & Sims, G. K. (1998). Improving the Berthelot reaction for determining ammonium in soil extracts and water. *Soil Science Society of America Journal*, *62*(2), 473-480. https://doi.org/10.2136/sssaj1998.03615995006200020026x

Ritchie, M. E., & Raina, R. (2016). Effects of herbivores on nitrogen fixation by grass endophytes, legume symbionts and free-living soil surface bacteria in the Serengeti. *Pedobiologia*, *59*(5-6), 233-241. https://doi.org/10.1016/j.pedobi.2016.09.001

Rooney, D. C., Kennedy, N. M., Gleeson, D. B., & Clipson, N. J. W. (2010). Responses of ammonia-oxidising bacterial communities to nitrogen, lime, and plant species in upland grassland soil. *Applied and Environmental Soil Science*, *2010*, 1-7. https://doi.org/10.1155/2010/319721

Roper, M., & Gupta, V. (2016). Enhancing non-symbiotic N_2 fixation in agriculture. *The Open Agriculture Journal*, *10*(1).

Roper, M. M., & Smith, N. A. (1991). Straw decomposition and nitrogenase activity (C_2H_2 reduction) by free-living microorganisms from soil: Effects of pH and clay content. *Soil Biology and Biochemistry*, *23*(3), 275-283. https://doi.org/10.1016/0038-0717(91)90064-Q

Rotthauwe, J. H., Witzel, K. P., & Liesack, W. (1997). The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology*, *63*(12), 4704.

Rudisill, M. A., Turco, R. F., & Hoagland, L. A. (2016). Fertility practices and rhizosphere effects alter ammonia oxidizer community structure and potential nitrification activity in pepper production soils. *Applied Soil Ecology*, *99*, 70-77. https://doi.org/10.1016/j.apsoil.2015.10.011

Sánchez-Andrea, I., Stams, A. J. M., Hedrich, S., Ňancucheo, I., & Johnson, D. B. (2015). *Desulfosporosinus acididurans* sp. nov.: an acidophilic sulfate-reducing bacterium isolated from acidic sediments. *Extremophiles*, *19*(1), 39-47. https://doi.org/10.1007/s00792-014-0701-6

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J., & Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*(23), 7537-7541. https://doi.org/10.1128/AEM.01541-09

Segal, L. M., Miller, D. N., McGhee, R. P., Loecke, T. D., Cook, K. L., Shapiro, C. A., & Drijber, R. A. (2017). Bacterial and archaeal ammonia oxidizers respond differently to long-term tillage and fertilizer management at a continuous maize site. *Soil and Tillage Research*, *168*, 110-117.

https://doi.org/10.1016/j.still.2016.12.014
Senbayram, M., Chen, R., Mühling, K. H., & Dittert, K. (2009). Contribution of nitrification and denitrification to nitrous oxide emissions from soils after application of biogas waste and other fertilizers [https://doi.org/10.1002/rcm.4067]. *Rapid Communications in Mass Spectrometry*, *23*(16), 2489-2498. https://doi.org/https://doi.org/10.1002/rcm.4067
Short, M. D., Abell, G. C. J., Bodrossy, L., & van den Akker, B. (2013). Application of a novel functional gene microarray to probe the functional ecology of ammonia oxidation in nitrifying activated sludge. *PLoS One*, *8*(10), e77139-e77139. https://doi.org/10.1371/journal.pone.0077139
Smercina, D. N., Evans, S. E., Friesen, M. L., & Tiemann, L. K. (2020). Impacts of nitrogen addition on switchgrass root-associated diazotrophic community structure and function. *FEMS Microbiology Ecology*, *96*(12). https://doi.org/10.1093/femsec/fiaa208
Smercina, D. N., Evans, S. E., Friesen, M. L., Tiemann, L. K., & Cann, I. (2019). To fix or not to fix: Controls on free-living nitrogen fixation in the rhizosphere. *Applied and Environmental Microbiology*, *85*(6). https://doi.org/10.1128/aem.02546-18
Smits, N. A. C., Hefting, M. M., Kamst-van Agterveld, M. P., Laanbroek, H. J., Paalman, A. J., & Bobbink, R. (2010). Nitrification along a grassland gradient: Inhibition found in matgrass swards. *Soil Biology and Biochemistry*, *42*(4), 635-641. https://doi.org/10.1016/j.soilbio.2010.01.001
Soares, J. R., Cassman, N. A., Kielak, A. M., Pijl, A., Carmo, J. B., Lourenco, K. S., Laanbroek, H. J., Cantarella, H., & Kuramae, E. E. (2016). Nitrous oxide emission related to ammonia-oxidizing bacteria and mitigation options from N fertilization in a tropical soil. *Scientific Reports*, *6*, 30349. https://doi.org/10.1038/srep30349
Srivastava, A. K., & Ambasht, R. S. (1994). Soil moisture control of nitrogen fixation activity in dry tropical *Casuarina* plantation forest. *Journal of Environmental Management*, *42*(1), 49-54. https://doi.org/https://doi.org/10.1006/jema.1994.1059
Strauss, E. A., & Lamberti, G. A. (2002). Effect of dissolved organic carbon quality on microbial decomposition and nitrification rates in stream sediments. *Freshwater Biology*, *47*(1), 65-74. https://doi.org/10.1046/j.1365-2427.2002.00776.x
Subbarao, G. V., Nakahara, K., Hurtado, M. P., Ono, H., Moreta, D. E., Salcedo, A. F., Yoshihashi, A. T., Ishikawa, T., Ishitani, M., Ohnishi-Kameyama, M., Yoshida, M., Rondon, M., Rao, I. M., Lascano, C. E., Berry, W. L., Ito, O., & William, H. S. (2009). Evidence for biological nitrification inhibition in Brachiaria pastures. *Proceedings of the National Academy of Sciences, USA*, *106*(41), 17302-17307. https://doi.org/10.1073/pnas.0903694106
Subbarao, G. V., Rondon, M., Ito, O., Ishikawa, T., Rao, I. M., Nakahara, K., Lascano, C., & Berry, W. L. (2006). Biological nitrification inhibition (BNI)—is it a widespread phenomenon? *Plant and Soil*, *294*(1-2), 5-18. https://doi.org/10.1007/s11104-006-9159-3
Sun, R., Myrold, D. D., Wang, D., Guo, X., & Chu, H. (2019). AOA and AOB communities respond differently to changes of soil pH under long-term

fertilization. *Soil Ecology Letters*, *1*(3-4), 126-135. https://doi.org/10.1007/s42832-019-0016-8
Team, R. C. (2013). R: A language and environment for statistical computing.
Theodorakopoulos, N., Lognoul, M., Degrune, F., Broux, F., Regaert, D., Muys, C., Heinesch, B., Bodson, B., Aubinet, M., & Vandenbol, M. (2017). Increased expression of bacterial *amoA* during an N_2O emission peak in an agricultural field. *Agriculture, Ecosystems & Environment*, *236*, 212-220. https://doi.org/10.1016/j.agee.2016.12.002
van der Wielen, P., Voost, S., & van der Kooij, D. (2009). Ammonia-oxidizing bacteria and archaea in groundwater treatment and drinking water distribution systems. *Applied and environmental microbiology*, *75*, 4687-4695. https://doi.org/10.1128/AEM.00387-09
Vessey, J., & Luit, B. (2011). The nitrate-tolerant symbiosis of *Glycine max* (L.) Merr. *nts*382 and *Bradyrhizobium japonicum* is also tolerant of ammonium. *Canadian Journal of Botany*, *77*, 1432-1438. https://doi.org/10.1139/b99-101
Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens, G. E., Matson, P. A., Schindler, D. W., Schlesinger, W. H., & Tilman, D. G. (1997). Human alteration of the global nitrogen cycle: Sources and consequences. *Ecological Applications*, *7*(3), 737-750.
Vitousek, P. M., & Howarth, R. W. (1991). Nitrogen limitation on land and in the sea: How can it occur? *Biogeochemistry*, *13*(2), 87-115.
Vitousek, P. M., Menge, D. N. L., Reed, S. C., & Cleveland, C. C. (2013). Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1621), 20130119. https://doi.org/10.1098/rstb.2013.0119
Wallenstein, M. D., Myrold, D. D., Firestone, M., & Voytek, M. (2006). Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. *Ecological Applications*, *16*(6), 2143-2152. https://www.ncbi.nlm.nih.gov/pubmed/17205893
Wan, S., & Luo, Y. (2003). Substrate regulation of soil respiration in a tallgrass prairie: Results of a clipping and shading experiment. *Global Biogeochemical Cycles*, *17*(2), n/a-n/a. https://doi.org/10.1029/2002gb001971
Wang, B., Zhao, J., Guo, Z., Ma, J., Xu, H., & Jia, Z. (2014). Differential contributions of ammonia oxidizers and nitrite oxidizers to nitrification in four paddy soils. *The ISME Journal*, *9*(5), 1062-1075. https://doi.org/10.1038/ismej.2014.194
Wang, C., Zheng, M., Song, W., Wen, S., Wang, B., Zhu, C., & Shen, R. (2017). Impact of 25 years of inorganic fertilization on diazotrophic abundance and community structure in an acidic soil in southern China. *Soil Biology and Biochemistry*, *113*, 240-249. https://doi.org/10.1016/j.soilbio.2017.06.019
Wang, C., Zheng, M. M., & Shen, R. F. (2020). Diazotrophic communities are more responsive to maize cultivation than phosphorus fertilization in an acidic soil. *Plant and Soil*, *452*(1), 499-512. https://doi.org/10.1007/s11104-020-04596-z
Wang, F., Chen, S., Wang, Y., Zhang, Y., Hu, C., & Liu, B. (2018). Long-term nitrogen fertilization elevates the activity and abundance of nitrifying and denitrifying

microbial communities in an upland soil: implications for nitrogen loss from intensive agricultural systems. *Frontiers in Microbiology*, *9*, 2424. https://doi.org/10.3389/fmicb.2018.02424

Wang, J., Chadwick, D. R., Cheng, Y., & Yan, X. (2018). Global analysis of agricultural soil denitrification in response to fertilizer nitrogen. *Science of the Total Environment*, *616-617*, 908-917. https://doi.org/10.1016/j.scitotenv.2017.10.229

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. https://doi.org/10.1016/j.agee.2015.09.039

Wang, Y., Li, C., Kou, Y., Wang, J., Tu, B., Li, H., Li, X., Wang, C., & Yao, M. (2017). Soil pH is a major driver of soil diazotrophic community assembly in Qinghai-Tibet alpine meadows. *Soil Biology and Biochemistry*, *115*, 547-555. https://doi.org/10.1016/j.soilbio.2017.09.024

Webster, G., Embley, T. M., Freitag, T. E., Smith, Z., & Prosser, J. I. (2005). Links between ammonia oxidizer species composition, functional diversity and nitrification kinetics in grassland soils. *Environmental Microbiology*, *7*(5), 676-684. https://doi.org/10.1111/j.1462-2920.2005.00740.x

Wedin, D., & Tilman, D. (1990). Species effects on nitrogen cycling: A test with perennial grasses. *Oecologia*, *84*, 433-441. https://doi.org/10.1007/BF00328157

Wertz, S., Leigh, A. K., & Grayston, S. J. (2012). Effects of long-term fertilization of forest soils on potential nitrification and on the abundance and community structure of ammonia oxidizers and nitrite oxidizers. *FEMS Microbiology Ecology*, *79*(1), 142-154. https://doi.org/10.1111/j.1574-6941.2011.01204.x

White, C. S. (1986). Volatile and water-soluble inhibitors of nitrogen mineralization and nitrification in a ponderosa pine ecosystem. *Biology and fertility of soils*, *2*(2), 97-104. https://doi.org/10.1007/BF00257586

Wickham, H. (2011). ggplot2. *Wiley Interdisciplinary Reviews: Computational Statistics*, *3*(2), 180-185.

Xi, R., Long, X. E., Huang, S., & Yao, H. (2017). pH rather than nitrification and urease inhibitors determines the community of ammonia oxidizers in a vegetable soil. *AMB Express*, *7*(1), 129. https://doi.org/10.1186/s13568-017-0426-x

Xiang, X., He, D., He, J.-S., Myrold, D. D., & Chu, H. (2017). Ammonia-oxidizing bacteria rather than archaea respond to short-term urea amendment in an alpine grassland. *Soil Biology and Biochemistry*, *107*, 218-225. https://doi.org/10.1016/j.soilbio.2017.01.012

Xu, L., Cheng, S., Fang, H., Xin, X., Xu, X., & Tang, H. (2019). Soil inorganic nitrogen composition and plant functional type determine forage crops nitrogen uptake preference in the temperate cultivated grassland, Inner Mongolia. *Soil Science and Plant Nutrition*, *65*(5), 501-510. https://doi.org/10.1080/00380768.2019.1671777

Yang, Q.-S., Dong, J.-D., Ahmad, M., Ling, J., Zhou, W.-G., Tan, Y.-H., Zhang, Y.-Z., Shen, D.-D., & Zhang, Y.-Y. (2019). Analysis of *nifH* DNA and RNA reveals a disproportionate contribution to nitrogenase activities by rare plankton-associated

diazotrophs. *BMC Microbiology*, *19*(1). https://doi.org/10.1186/s12866-019-1565-9

Yang, Y., Zhao, J., Jiang, Y., Hu, Y., Zhang, M., & Zeng, Z. (2017). Response of bacteria harboring *nirS* and *nirK* genes to different N fertilization rates in an alkaline northern Chinese soil. *European Journal of Soil Biology*, *82*, 1-9. https://doi.org/10.1016/j.ejsobi.2017.05.006

Yé, L., Abbadie, L., Bardoux, G., Lata, J.-C., Nacro, H. B., Masse, D., de Parseval, H., & Barot, S. (2015). Contrasting impacts of grass species on nitrogen cycling in a grazed Sudanian savanna. *Acta Oecologica*, *63*, 8-15. https://doi.org/10.1016/j.actao.2015.01.002

Yeager, C., Kuske, C., Carney, T., Johnson, S., Ticknor, L., & Belnap, J. (2012). Response of biological soil crust diazotrophs to season, altered summer precipitation, and year-round increased temperature in an arid grassland of the Colorado Plateau, USA [Original Research]. *Frontiers in Microbiology*, *3*(358). https://doi.org/10.3389/fmicb.2012.00358

Yeager, C. M., Northup, D. E., Grow, C. C., Barns, S. M., & Kuske, C. R. (2005). Changes in nitrogen-fixing and ammonia-oxidizing bacterial communities in soil of a mixed conifer forest after wildfire. *Applied and Environmental Microbiology*, *71*(5), 2713-2722. https://doi.org/10.1128/aem.71.5.2713-2722.2005

Yin, H., Li, Y., Xiao, J., Xu, Z., Cheng, X., & Liu, Q. (2013). Enhanced root exudation stimulates soil nitrogen transformations in a subalpine coniferous forest under experimental warming. *Global Change Biology*, *19*(7), 2158-2167. https://doi.org/10.1111/gcb.12161

Ying, J., Li, X., Wang, N., Lan, Z., He, J., & Bai, Y. (2017). Contrasting effects of nitrogen forms and soil pH on ammonia oxidizing microorganisms and their responses to long-term nitrogen fertilization in a typical steppe ecosystem. *Soil Biology and Biochemistry*, *107*, 10-18. https://doi.org/10.1016/j.soilbio.2016.12.023

Young, J. (1992). Phylogenetic classification of nitrogen-fixing organisms. *Biological nitrogen fixation*, *1544*, 43-86.

Yu, R., & Chandran, K. (2010). Strategies of *Nitrosomonas europaea* 19718 to counter low dissolved oxygen and high nitrite concentrations. *BMC Microbiology*, *10*, 70. https://doi.org/10.1186/1471-2180-10-70

Zehr, J. P., Jenkins, B. D., Short, S. M., & Steward, G. F. (2003). Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environmental Microbiology*, *5*(7), 539-554. https://doi.org/10.1046/j.1462-2920.2003.00451.x

Zheng, Y., Huang, R., Wang, B. Z., Bodelier, P. L. E., & Jia, Z. J. (2014). Competitive interactions between methane- and ammonia-oxidizing bacteria modulate carbon and nitrogen cycling in paddy soil. *Biogeosciences*, *11*(12), 3353-3368. https://doi.org/10.5194/bg-11-3353-2014

Zibilske, L. M., & Makus, D. J. (2009). Black oat cover crop management effects on soil temperature and biological properties on a Mollisol in Texas, USA. *Geoderma*, *149*(3-4), 379-385. https://doi.org/10.1016/j.geoderma.2009.01.001

Zou, J., Yao, Q., Liu, J., Li, Y., Song, F., Liu, X., & Wang, G. (2020). Changes of diazotrophic communities in response to cropping systems in a Mollisol of Northeast China. *PeerJ*, *8*, e9550. https://doi.org/10.7717/peerj.9550

Appendix

Table 15. Soil properties in relation to agricultural season, N fertilization rate, and grass species. Different lowercase letters indicate significant differences between treatment levels within groups. The letters were only shown in groups that have significant effects. Means were compared using Least Significant Difference (LSD) tests ($\alpha = 0.05$).

Factor[†]	pH	SWC[‡] (%)	NH_4^+-N	NO_3^--N	DOC	DON	TOC	TN	C: N ratio	N_2O-N ($g\ ha^{-1}\ d^{-1}$)	NP ($\mu g\ NO_2$-N $gdw^{-1}\ hr^{-1}$)
			($\mu g\ gdw^{-1}$)				($mg\ gdw^{-1}$)				
April (G)	6.27±0.05	24.46±0.25^{b*}	10.82±0.49^{a}	1.51±0.30^{a}	203.55±4.20^{b}	18.67±0.56^{ab}	1.82±0.03	21.74±0.43	11.94±0.11	9.71±0.94^{b}	0.06±0.01^{c}
June (H1)	6.30±0.06	30.16±0.46^{a}	10.57±0.60^{a}	2.14±0.46^{a}	163.85±4.07^{c}	16.16±0.65^{b}	1.76±0.06	21.18±0.68	12.05±0.15	24.77±7.44^{a}	0.11±0.02^{b}
August (H2)	6.18±0.03	15.03±0.40^{c}	7.16±0.35^{b}	0.83±0.25^{b}	223.00±5.47^{a}	22.44±1.98^{a}	1.84±0.05	21.66±0.51	11.81±0.12	1.64±0.17^{b}	0.18±0.02^{a}
0N	6.36±0.05^{a}	23.27±1.63	9.37±0.85	0.39±0.16^{c}	210.18±8.60^{a}	17.65±1.10	1.71±0.04	20.73±0.53	12.11±0.16	7.55±1.49^{b}	0.06±0.01^{c}
67N	6.29±0.04^{a}	23.39±1.66	9.53±0.57	1.13±0.19^{b}	196.38±8.02^{b}	19.07±0.95	1.82±0.04	21.74±0.49	11.94±0.12	7.25±1.42^{b}	0.11±0.02^{b}
202N	6.12±0.05^{b}	23.00±1.51	9.62±0.53	2.77±0.39^{a}	186.07±5.23^{b}	20.29±1.83	1.87±0.05	21.97±0.58	11.78±0.09	20.56±7.43^{a}	0.17±0.02^{a}
SG	6.32±0.05^{a}	23.30±1.31	9.52±0.49	1.71±0.24	195.32±6.71	20.28±1.49	1.83±0.04	21.60±0.41	11.79±0.12	9.23±1.57	0.14±0.02^{a}
BB	6.19±0.03^{b}	23.14±1.29	9.51±0.54	1.30±0.33	198.11±5.77	18.03±0.68	1.78±0.04	21.46±0.47	12.06±0.08	14.54±5.11	0.1±0.02^{b}
0N-SG	6.48±0.09	22.23±2.72^{b}	10.03±1.75	0.48±0.17	201.67±13.80	17.34±1.71	1.74±0.06	21.49±0.81	12.36±0.36^{a}	7.28±1.77	0.08±0.02
67N-SG	6.35±0.05	23.77±2.26^{ab}	9.38±0.51	1.39±0.16	194.43±13.99	19.97±1.52	1.83±0.05	21.37±0.54	11.67±0.10^{b}	7.49±2.19	0.12±0.02
202N-SG	6.19±0.08	23.56±2.20^{ab}	9.32±0.54	2.86±0.32	191.98±8.07	22.55±3.48	1.90±0.07	21.90±0.84	11.53±0.09^{b}	12.26±3.29	0.2±0.03
0N-BB	6.27±0.03	23.96±2.13^{a}	8.93±0.87	0.34±0.25	215.85±11.24	17.86±1.51	1.70±0.06	20.22±0.69	11.94±0.12^{ab}	7.74±2.28	0.05±0.01
67N-BB	6.23±0.06	23.02±2.57^{ab}	9.69±1.05	0.88±0.33	198.33±8.76	18.18±1.14	1.81±0.07	22.12±0.82	12.21±0.18^{ab}	7.01±1.94	0.09±0.03
202N-BB	6.06±0.04	22.43±2.20^{b}	9.92±0.95	2.68±0.73	180.16±6.50	18.04±0.93	1.83±0.07	22.04±0.85	12.02±0.11^{ab}	28.87±14.38	0.15±0.03
Apr-0N	6.35±0.10	24.77±0.52	12.29±1.26^{a}	0.45±0.17	220.18±3.73	19.97±0.88	1.79±0.04	21.50±0.48	12.01±0.23	12.00±1.68^{b}	0.04±0.01^{d}
Apr-67N	6.31±0.05	24.22±0.30	10.87±0.51^{ab}	1.20±0.21	204.94±6.82	18.24±0.69	1.77±0.04	21.10±0.71	11.95±0.20	6.99±0.99^{b}	0.06±0.01^{d}
Apr-202N	6.16±0.07	24.44±0.54	9.54±0.38^{bc}	2.70±0.52	188.30±3.30	18.00±1.21	1.90±0.07	22.58±0.89	11.87±0.18	10.52±1.64^{b}	0.08±0.01^{cd}
Jun-0N	6.43±0.09	29.68±0.74	9.36±1.14^{bcd}	0.67±0.44	169.15±6.71	15.21±1.12	1.60±0.06	19.68±1.00	12.31±0.43	9.25±2.30^{b}	0.04±0.01^{d}
Jun-67N	6.38±0.09	31.13±0.76	10.43±1.10^{ab}	1.59±0.39	159.77±9.47	16.23±1.30	1.75±0.07	21.16±1.06	12.05±0.15	13.09±2.54^{b}	0.1±0.01^{cd}
Jun-202N	6.12±0.12	29.60±0.85	11.71±0.84^{a}	3.90±0.76	163.52±4.73	16.88±1.04	1.90±0.11	22.43±1.30	11.82±0.16	49.38±17.44^{a}	0.17±0.03^{b}
Aug-0N	6.30±0.04	15.35±0.74	6.46±0.62^{e}	0.06±0.04	241.21±5.54	17.78±2.79	1.75±0.10	21.01±1.16	12.00±0.18	1.41±0.43^{b}	0.11±0.01^{bc}
Aug-67N	6.19±0.06	14.83±0.87	7.30±0.57^{de}	0.61±0.27	224.43±8.92	22.76±1.58	1.95±0.08	22.96±0.57	11.83±0.28	1.68±0.36^{b}	0.17±0.03^{b}
Aug-202N	6.08±0.03	14.95±0.53	7.61±0.61^{cde}	1.71±0.47	206.38±7.76	26.01±4.66	1.80±0.06	20.89±0.77	11.64±0.14	1.80±0.12^{b}	0.27±0.02^{a}

Table 15. Continued.

Factor†	pH	SWC‡ (%)	NH_4^+-N	NO_3^--N	DOC	DON	TOC	TN	C: N ratio	N_2O-N (g ha^{-1} d^{-1})	NP (µg NO_2-N gdw^{-1} hr^{-1})
			(µg gdw^{-1})				(mg gdw^{-1})				
Apr-SG	6.34±0.08	24.69±0.36	11.30±0.90	1.95±0.42^{ab}	202.78±4.65	19.57±0.72	1.89±0.05	22.14±0.64	11.75±0.15	8.78±1.17	0.08±0.01
Apr-BB	6.20±0.04	24.26±0.36	10.39±0.46	1.12±0.40^{bc}	204.24±7.05	17.86±0.79	1.76±0.03	21.38±0.60	12.11±0.15	10.53±1.45	0.04±0.01
Jun-SG	6.43±0.10	30.00±0.73	9.69±0.48	1.76±0.44^{ab}	158.05±6.59	16.06±0.88	1.72±0.07	20.55±0.87	11.95±0.29	17.08±2.46	0.13±0.03
Jun-BB	6.19±0.07	30.31±0.63	11.35±1.01	2.47±0.78^{a}	169.01±4.65	16.24±1.01	1.79±0.08	21.73±1.03	12.13±0.11	31.60±13.87	0.09±0.02
Aug-SG	6.20±0.05	15.23±0.49	7.57±0.57	1.42±0.42^{b}	225.13±6.71	25.20±3.81	1.89±0.04	22.10±0.53	11.67±0.17	1.82±0.20	0.21±0.03
Aug-BB	6.17±0.04	14.84±0.64	6.80±0.40	0.31±0.15^{c}	221.09±8.77	19.98±1.39	1.79±0.08	21.26±0.85	11.93±0.17	1.49±0.28	0.16±0.03
Apr-0N-SG	6.49±0.20	24.06±0.83	14.44±2.00	0.80±0.14	211.52±1.68	20.59±0.59	1.79±0.07	21.43±0.07	12.00±0.45	9.50±1.58^{bc}	0.06±0.02
Apr-67N-SG	6.36±0.08	24.22±0.49	10.54±0.89	1.54±0.24	204.90±11.62	18.91±1.00	1.80±0.01	20.96±0.38	11.67±0.23	5.74±0.82^{bc}	0.07±0
Apr-202N-SG	6.23±0.15	25.57±0.17	9.98±0.61	3.13±0.62	194.83±2.38	19.55±1.75	2.04±0.08	23.79±1.19	11.65±0.13	11.34±1.68^{bc}	0.09±0.02
Apr-0N-BB	6.25±0.06	25.25±0.53	10.86±0.84	0.21±0.14	225.96±1.84	19.56±1.49	1.79±0.06	21.55±0.87	12.01±0.26	13.66±2.16^{bc}	0.02±0.01
Apr-67N-BB	6.26±0.06	24.22±0.46	11.21±0.62	0.87±0.21	204.99±9.87	17.57±0.97	1.73±0.09	21.23±1.54	12.22±0.28	8.23±1.62^{bc}	0.04±0.01
Apr-202N-BB	6.09±0.03	23.31±0.34	9.10±0.37	2.27±0.88	181.77±2.51	16.45±1.36	1.77±0.04	21.38±1.04	12.09±0.31	9.69±3.17^{bc}	0.07±0.02
Jun-0N-SG	6.58±0.17	28.52±0.57	9.49±1.55	0.52±0.34	160.41±4.48	15.63±0.08	1.58±0.04	20.19±2.00	12.74±0.94	10.01±2.00^{bc}	0.05±0
Jun-67N-SG	6.50±0.05	31.21±1.14	9.51±0.82	1.49±0.35	150.67±17.47	16.25±2.58	1.71±0.08	20.12±0.86	11.80±0.07	15.09±3.04^{bc}	0.12±0
Jun-202N-SG	6.25±0.21	29.76±1.40	10.00±0.43	2.87±0.69	163.85±6.69	16.18±0.65	1.83±0.17	21.23±1.97	11.59±0.24	23.77±2.08^{b}	0.2±0.05
Jun-0N-BB	6.33±0.05	30.45±0.96	9.28±1.77	0.77±0.75	174.97±9.88	14.93±2.03	1.61±0.10	19.34±1.08	12.03±0.25	8.74±3.92^{bc}	0.04±0.02
Jun-67N-BB	6.25±0.14	31.05±1.25	11.35±2.13	1.70±0.78	168.87±7.78	16.21±1.34	1.80±0.14	22.21±1.94	12.31±0.20	11.09±4.35^{bc}	0.08±0
Jun-202N-BB	5.99±0.11	29.44±1.27	13.43±0.62	4.93±1.16	163.18±8.19	17.58±2.13	1.96±0.16	23.64±1.77	12.05±0.13	74.98±29.33^{a}	0.14±0.05
Aug-0N-SG	6.39±0.04	14.10±0.16	6.15±0.97	0.11±0.08	233.07±4.94	15.80±4.28	1.86±0.05	22.87±0.40	12.33±0.14	2.32±0.44^{bc}	0.13±0
Aug-67N-SG	6.19±0.05	15.87±0.79	8.09±0.41	1.14±0.27	227.73±18.46	24.75±1.08	1.99±0.06	23.02±0.66	11.55±0.20	1.64±0.35^{c}	0.18±0.02
Aug-202N-SG	6.09±0.06	15.35±0.96	7.99±1.25	2.56±0.53	217.25±3.50	31.92±8.56	1.82±0.06	20.67±0.68	11.36±0.09	1.66±0.17^{c}	0.3±0.02
Aug-0N-BB	6.24±0.03	16.19±0.98	6.66±0.87	0.02±0.00	246.63±7.31	19.10±3.83	1.68±0.16	19.78±1.57	11.78±0.18	0.80±0.24^{c}	0.09±0.01
Aug-67N-BB	6.18±0.12	13.79±1.44	6.51±0.92	0.07±0.05	221.13±6.81	20.76±2.72	1.91±0.16	22.91±1.09	12.10±0.52	1.73±0.71^{c}	0.16±0.06
Aug-202N-BB	6.08±0.04	14.55±0.58	7.22±0.41	0.85±0.25	195.52±13.07	20.10±0.65	1.77±0.13	21.10±1.56	11.92±0.12	1.94±0.14^{c}	0.23±0.03

† 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

‡SWC, soil water content; DOC, dissolved organic C; DON, dissolved organic N; TOC, total organic C; TN, total N; NP, nitrification potential.

Table 16. Absolute abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes as well as relative abundances of *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* genes in relation to agricultural season, nitrogen fertilization, grass species, and their combinations. Different lowercase letters indicate significant differences between treatment levels within groups. The letters were only shown in groups that have significant effects. Means were compared using Least Significant Difference (LSD) tests ($\alpha = 0.05$).

Factor†	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*	16S rRNA	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*
	Log(functional gene copies g^{-1} dry weight soil						Log(functional gene copies/16S rRNA gene copies)				
April (G)	6.61±0.07^{b}	6.01±0.14^{b}	7.21±0.07^{b}	7.27±0.06^{b}	7.30±0.06^{b}	9.65±0.05^{b}	-3.05±0.04^{b}	-3.65±0.10^{b}	-2.44±0.03^{b}	-2.39±0.04^{b}	-2.35±0.02^{a}
June (H1)	6.17±0.06^{c}	5.27±0.10^{c}	6.93±0.05^{c}	7.12±0.04^{c}	7.00±0.04^{c}	9.53±0.04^{c}	-3.36±0.05^{c}	-4.26±0.10^{c}	-2.59±0.03^{c}	-2.41±0.04^{b}	-2.52±0.03^{b}
August (H2)	7.04±0.04^{a}	6.93±0.09^{a}	7.59±0.03^{a}	7.74±0.04^{a}	7.55±0.03^{a}	9.86±0.03^{a}	-2.82±0.04^{a}	-2.93±0.10^{a}	-2.27±0.04^{a}	-2.12±0.03^{a}	-2.31±0.03^{a}
0N	6.65±0.10	5.68±0.20^{b}	7.23±0.09	7.38±0.08	7.29±0.07	9.69±0.05	-3.05±0.08	-4.01±0.17^{b}	-2.46±0.05	-2.31±0.05	-2.41±0.04
67N	6.57±0.11	6.17±0.18^{a}	7.25±0.10	7.41±0.09	7.28±0.07	9.69±0.06	-3.12±0.06	-3.51±0.13^{a}	-2.44±0.05	-2.28±0.05	-2.40±0.03
202N	6.60±0.09	6.35±0.18^{a}	7.25±0.07	7.33±0.07	7.29±0.07	9.66±0.05	-3.06±0.07	-3.31±0.15^{a}	-2.40±0.03	-2.33±0.05	-2.37±0.03
SG	6.69±0.08	6.21±0.16^{a}	7.32±0.06^{a}	7.46±0.06^{a}	7.32±0.06	9.73±0.04	-3.03±0.05	-3.51±0.13^{a}	-2.41±0.04	-2.26±0.04^{a}	-2.40±0.03
BB	6.52±0.09	5.92±0.16^{b}	7.17±0.07^{b}	7.29±0.07^{b}	7.25±0.06	9.63±0.04	-3.11±0.06	-3.71±0.14^{b}	-2.46±0.04	-2.35±0.03^{b}	-2.38±0.02
0N-SG	6.67±0.15	5.90±0.29	7.26±0.12	7.38±0.10^{b}	7.25±0.10	9.71±0.07	-3.03±0.12	-3.81±0.25	-2.44±0.08	-2.33±0.08	-2.46±0.07
67N-SG	6.74±0.13	6.35±0.25	7.43±0.10	7.62±0.10^{a}	7.40±0.10	9.80±0.08	-3.06±0.07	-3.45±0.19	-2.37±0.06	-2.18±0.05	-2.40±0.05
202N-SG	6.67±0.13	6.39±0.27	7.26±0.11	7.38±0.10^{b}	7.32±0.10	9.67±0.07	-3.00±0.10	-3.29±0.23	-2.41±0.06	-2.29±0.08	-2.35±0.05
0N-BB	6.62±0.15	5.47±0.27	7.20±0.13	7.39±0.12^{b}	7.33±0.10	9.68±0.08	-3.07±0.10	-4.22±0.22	-2.48±0.07	-2.30±0.07	-2.36±0.04
67N-BB	6.40±0.17	5.99±0.25	7.07±0.14	7.19±0.12^{c}	7.17±0.10	9.57±0.07	-3.17±0.11	-3.58±0.19	-2.50±0.08	-2.38±0.06	-2.41±0.05
202N-BB	6.53±0.13	6.31±0.27	7.24±0.09	7.28±0.10^{bc}	7.25±0.09	9.64±0.07	-3.11±0.09	-3.33±0.21	-2.40±0.04	-2.36±0.05	-2.39±0.03
Apr-0N	6.66±0.12	5.72±0.26	7.19±0.11	7.26±0.07	7.28±0.11	9.64±0.09	-2.98±0.07	-3.92±0.20	-2.45±0.04	-2.37±0.05	-2.36±0.04
Apr-67N	6.54±0.15	6.02±0.21	7.15±0.16	7.26±0.16	7.26±0.11	9.61±0.09	-3.07±0.08	-3.59±0.15	-2.46±0.08	-2.34±0.08	-2.35±0.04
Apr-202N	6.62±0.09	6.29±0.22	7.30±0.11	7.27±0.08	7.37±0.11	9.71±0.09	-3.09±0.07	-3.43±0.15	-2.41±0.05	-2.44±0.06	-2.34±0.04
Jun-0N	6.21±0.12	4.83±0.18	6.91±0.11	7.12±0.07	7.03±0.09	9.59±0.10	-3.38±0.12	-4.76±0.15	-2.67±0.06	-2.47±0.09	-2.56±0.07
Jun-67N	6.12±0.13	5.45±0.09	6.93±0.10	7.15±0.10	6.99±0.06	9.51±0.06	-3.40±0.08	-4.06±0.09	-2.58±0.06	-2.36±0.08	-2.52±0.04
Jun-202N	6.18±0.10	5.52±0.04	6.95±0.04	7.07±0.07	6.98±0.03	9.48±0.05	-3.29±0.08	-3.96±0.05	-2.52±0.04	-2.41±0.06	-2.50±0.03
Aug-0N	7.07±0.07	6.50±0.13	7.60±0.06	7.76±0.05	7.55±0.06	9.86±0.03	-2.79±0.05	-3.36±0.16	-2.26±0.08	-2.09±0.03	-2.31±0.05
Aug-67N	7.06±0.06	7.05±0.11	7.67±0.05	7.81±0.08	7.6±0.06	9.94±0.06	-2.88±0.05	-2.89±0.09	-2.27±0.07	-2.14±0.06	-2.34±0.07
Aug-202N	7.00±0.06	7.24±0.03	7.51±0.06	7.65±0.07	7.51±0.06	9.78±0.06	-2.78±0.08	-2.54±0.08	-2.27±0.04	-2.13±0.06	-2.26±0.04
Apr-SG	6.72±0.10	6.28±0.16	7.34±0.09	7.39±0.08	7.41±0.09	9.75±0.07	-3.03±0.05	-3.48±0.12	-2.42±0.05	-2.36±0.05	-2.34±0.04
Apr-BB	6.49±0.08	5.74±0.19	7.09±0.09	7.14±0.07	7.19±0.07	9.55±0.05	-3.06±0.07	-3.81±0.16	-2.46±0.05	-2.41±0.05	-2.36±0.02
Jun-SG	6.27±0.07	5.33±0.14	7.00±0.04	7.20±0.05	7.00±0.04	9.55±0.05	-3.28±0.09	-4.22±0.14	-2.55±0.04	-2.35±0.08	-2.55±0.04
Jun-BB	6.07±0.10	5.20±0.15	6.87±0.09	7.03±0.06	7.00±0.06	9.50±0.07	-3.43±0.05	-4.30±0.16	-2.63±0.05	-2.47±0.02	-2.50±0.03
Aug-SG	7.09±0.04	7.03±0.11	7.62±0.06	7.79±0.06	7.55±0.05	9.87±0.06	-2.79±0.04	-2.84±0.12	-2.26±0.07	-2.08±0.04	-2.32±0.06
Aug-BB	7.00±0.06	6.82±0.15	7.56±0.04	7.69±0.05	7.55±0.05	9.85±0.04	-2.85±0.06	-3.02±0.17	-2.28±0.03	-2.16±0.04	-2.29±0.02

Table 16. Continued.

Factor†	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*	16S rRNA	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*
	Log(functional gene copies g^{-1} dry weight soil						Log(functional gene copies/16S rRNA gene copies)				
Apr-0N-SG	6.74±0.24	6.10±0.23	7.27±0.18	7.31±0.14	7.35±0.20	9.71±0.17	-2.96±0.09	-3.60±0.09	-2.44±0.04	-2.40±0.04	-2.36±0.04
Apr-67N-SG	6.79±0.11	6.36±0.28	7.44±0.15	7.57±0.12	7.46±0.13	9.79±0.09	-3.00±0.04	-3.43±0.24	-2.35±0.11	-2.22±0.07	-2.33±0.09
Apr-202N-SG	6.63±0.19	6.36±0.40	7.30±0.21	7.30±0.16	7.43±0.20	9.76±0.15	-3.14±0.12	-3.40±0.30	-2.46±0.09	-2.47±0.10	-2.34±0.09
Apr-0N-BB	6.58±0.05	5.33±0.37	7.11±0.13	7.22±0.07	7.21±0.13	9.57±0.09	-2.99±0.13	-4.24±0.29	-2.46±0.08	-2.35±0.09	-2.36±0.08
Apr-67N-BB	6.28±0.19	5.68±0.18	6.86±0.13	6.95±0.13	7.06±0.04	9.43±0.04	-3.14±0.16	-3.75±0.15	-2.56±0.09	-2.47±0.09	-2.37±0.02
Apr-202N-BB	6.61±0.04	6.21±0.26	7.29±0.14	7.24±0.06	7.32±0.12	9.66±0.11	-3.05±0.09	-3.45±0.15	-2.37±0.03	-2.42±0.08	-2.34±0.02
Jun-0N-SG	6.22±0.14	4.91±0.29	6.92±0.03	7.11±0.08	6.93±0.03	9.58±0.13	-3.36±0.26	-4.67±0.21	-2.66±0.10	-2.47±0.19	-2.65±0.12
Jun-67N-SG	6.28±0.10	5.53±0.13	7.11±0.06	7.33±0.03	7.08±0.09	9.56±0.08	-3.28±0.10	-4.03±0.19	-2.45±0.04	-2.23±0.10	-2.48±0.02
Jun-202N-SG	6.32±0.18	5.56±0.06	6.96±0.09	7.16±0.10	7.01±0.07	9.52±0.09	-3.20±0.15	-3.96±0.07	-2.56±0.03	-2.36±0.11	-2.51±0.03
Jun-0N-BB	6.20±0.24	4.76±0.28	6.90±0.25	7.13±0.14	7.14±0.18	9.59±0.18	-3.40±0.10	-4.84±0.24	-2.69±0.08	-2.46±0.04	-2.46±0.03
Jun-67N-BB	5.96±0.21	5.38±0.15	6.75±0.12	6.98±0.12	6.90±0.04	9.47±0.10	-3.51±0.11	-4.09±0.08	-2.72±0.02	-2.49±0.05	-2.57±0.07
Jun-202N-BB	6.05±0.04	5.47±0.05	6.95±0.02	6.98±0.07	6.96±0.01	9.44±0.05	-3.39±0.02	-3.96±0.10	-2.49±0.07	-2.46±0.02	-2.48±0.05
Aug-0N-SG	7.06±0.09	6.68±0.20	7.59±0.13	7.72±0.05	7.47±0.08	9.83±0.04	-2.77±0.07	-3.15±0.24	-2.24±0.16	-2.11±0.01	-2.36±0.11
Aug-67N-SG	7.15±0.01	7.17±0.02	7.73±0.09	7.97±0.07	7.66±0.08	10.06±0.06	-2.91±0.06	-2.88±0.07	-2.33±0.15	-2.09±0.12	-2.40±0.14
Aug-202N-SG	7.05±0.08	7.24±0.02	7.53±0.10	7.69±0.14	7.54±0.07	9.73±0.07	-2.68±0.01	-2.49±0.05	-2.20±0.03	-2.05±0.07	-2.19±0.01
Aug-0N-BB	7.08±0.12	6.31±0.12	7.60±0.05	7.81±0.10	7.63±0.05	9.89±0.05	-2.81±0.07	-3.57±0.16	-2.28±0.07	-2.07±0.06	-2.26±0.02
Aug-67N-BB	6.97±0.10	6.92±0.21	7.61±0.02	7.65±0.03	7.54±0.07	9.83±0.03	-2.86±0.09	-2.90±0.19	-2.22±0.01	-2.18±0.01	-2.29±0.04
Aug-202N-BB	6.94±0.11	7.24±0.06	7.48±0.09	7.61±0.09	7.49±0.11	9.83±0.12	-2.89±0.16	-2.59±0.17	-2.34±0.06	-2.21±0.09	-2.34±0.04

† G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

Table 17. Absolute transcript abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes as well as relative transcript abundances of *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* in relation to agricultural season, nitrogen fertilization, grass species, and their combinations. Different lowercase letters indicate significant differences between treatment levels within groups. The letters were only shown in groups that have significant effects. Means were compared using Least Significant Difference (LSD) tests ($\alpha = 0.05$).

Factor†	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*	16S rRNA	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*
	Log(functional gene copies g^{-1} dry weight soil						Log(functional gene copies/16S rRNA gene copies)				
April (G)	3.80±0.12^{a}	3.46±0.11^{b}	4.48±0.09^{b}	4.89±0.11^{a}	4.90±0.04^{b}	11.23±0.03	-7.44±0.11^{a}	-7.77±0.11^{b}	-6.76±0.09^{b}	-6.35±0.10	-6.34±0.04
June (H1)	4.04±0.10^{a}	3.88±0.11^{a}	4.89±0.06^{a}	4.85±0.12^{a}	5.11±0.04^{a}	11.33±0.06	-7.19±0.14^{a}	-7.36±0.16^{a}	-6.35±0.11^{a}	-6.39±0.14	-6.12±0.11
August (H2)	3.02±0.17^{b}	2.95±0.16^{c}	4.33±0.07^{b}	4.40±0.17^{b}	4.79±0.03^{c}	11.06±0.08	-7.97±0.12^{b}	-8.04±0.15^{b}	-6.66±0.07^{b}	-6.69±0.13	-6.20±0.08
0N	3.63±0.18	3.09±0.18^{b}	4.55±0.09	4.66±0.15^{ab}	4.91±0.05^{b}	11.18±0.07	-7.55±0.14	-8.09±0.14^{b}	-6.63±0.07	-6.52±0.11^{ab}	-6.27±0.06
67N	3.81±0.16	3.64±0.15^{a}	4.65±0.11	4.98±0.13^{a}	5.02±0.05^{a}	11.26±0.06	-7.36±0.17	-7.53±0.18^{a}	-6.53±0.14	-6.20±0.12^{a}	-6.15±0.11
202N	3.45±0.15	3.60±0.10^{a}	4.51±0.08	4.51±0.13^{b}	4.88±0.04^{b}	11.18±0.06	-7.67±0.11	-7.52±0.09^{a}	-6.60±0.08	-6.71±0.13^{b}	-6.24±0.06
SG	3.63±0.15	3.49±0.11	4.53±0.07	4.78±0.13	4.91±0.03	11.21±0.05	-7.54±0.11	-7.68±0.09	-6.64±0.06	-6.45±0.11	-6.26±0.05
BB	3.63±0.12	3.39±0.14	4.61±0.08	4.65±0.10	4.97±0.05	11.21±0.05	-7.51±0.12	-7.76±0.15	-6.54±0.10	-6.49±0.10	-6.18±0.08
0N-SG	3.59±0.27	3.29±0.22	4.53±0.11	4.61±0.24	4.87±0.05	11.16±0.09	-7.57±0.21	-7.87±0.15	-6.63±0.06	-6.55±0.18	-6.29±0.06
67N-SG	3.85±0.20	3.63±0.17	4.59±0.12	5.11±0.16	4.99±0.05	11.30±0.08	-7.45±0.18	-7.67±0.15	-6.71±0.11	-6.19±0.16	-6.31±0.07
202N-SG	3.46±0.29	3.56±0.18	4.47±0.15	4.63±0.25	4.87±0.07	11.18±0.09	-7.59±0.21	-7.49±0.17	-6.58±0.14	-6.62±0.23	-6.19±0.11
0N-BB	3.67±0.25	2.89±0.27	4.56±0.14	4.70±0.18	4.95±0.08	11.20±0.12	-7.53±0.18	-8.31±0.23	-6.64±0.13	-6.50±0.14	-6.25±0.11
67N-BB	3.78±0.26	3.66±0.25	4.70±0.18	4.84±0.20	5.05±0.10	11.22±0.09	-7.27±0.29	-7.39±0.32	-6.35±0.25	-6.21±0.20	-6.00±0.21
202N-BB	3.43±0.10	3.64±0.07	4.56±0.09	4.38±0.08	4.89±0.04	11.19±0.08	-7.79±0.06	-7.54±0.06	-6.61±0.06	-6.81±0.10	-6.29±0.06
Apr-0N	4.14±0.08	3.41±0.22^{bc}	4.63±0.16	5.13±0.10	4.95±0.10	11.29±0.04	-7.16±0.06	-7.88±0.21^{bc}	-6.67±0.13^{bc}	-6.16±0.09	-6.35±0.07
Apr-67N	3.74±0.27	3.53±0.18^{bc}	4.50±0.18	4.92±0.24	4.95±0.07	11.23±0.04	-7.50±0.27	-7.70±0.19^{bc}	-6.73±0.19^{bc}	-6.31±0.24	-6.28±0.09
Apr-202N	3.53±0.15	3.45±0.19^{bc}	4.30±0.13	4.61±0.13	4.81±0.05	11.18±0.06	-7.65±0.15	-7.73±0.20^{bc}	-6.88±0.13^{c}	-6.57±0.14	-6.37±0.05
Jun-0N	4.03±0.12	3.54±0.17^{bc}	4.81±0.03	4.82±0.12	5.06±0.02	11.29±0.12	-7.26±0.15	-7.75±0.22^{bc}	-6.49±0.12^{bc}	-6.47±0.18	-6.24±0.12
Jun-67N	4.35±0.08	4.29±0.12^{a}	5.09±0.11	5.22±0.15	5.26±0.08	11.39±0.07	-6.77±0.25	-6.84±0.32^{a}	-6.03±0.29^{a}	-5.90±0.19	-5.87±0.28
Jun-202N	3.75±0.20	3.82±0.13^{ab}	4.77±0.11	4.50±0.22	5.03±0.05	11.30±0.11	-7.55±0.23	-7.48±0.14^{b}	-6.52±0.06^{bc}	-6.80±0.24	-6.26±0.08
Aug-0N	2.72±0.23	2.32±0.24^{d}	4.20±0.13	4.02±0.26	4.73±0.04	10.95±0.15	-8.24±0.14	-8.63±0.14^{d}	-6.75±0.09^{bc}	-6.93±0.16	-6.22±0.12
Aug-67N	3.36±0.28	3.11±0.17^{c}	4.34±0.11	4.79±0.26	4.86±0.04	11.17±0.16	-7.81±0.21	-8.05±0.14^{c}	-6.82±0.10^{bc}	-6.37±0.19	-6.31±0.14
Aug-202N	2.99±0.37	3.53±0.17^{bc}	4.45±0.14	4.40±0.35	4.77±0.08	11.05±0.11	-7.86±0.20	-7.33±0.10^{ab}	-6.38±0.09^{ab}	-6.77±0.27	-6.08±0.15
Apr-SG	3.65±0.20	3.43±0.15	4.38±0.11	4.81±0.17	4.85±0.04	11.24±0.04	-7.58±0.19	-7.81±0.15	-6.86±0.10	-6.43±0.16	-6.39±0.03
Apr-BB	3.94±0.12	3.50±0.15	4.57±0.15	4.96±0.14	4.95±0.08	11.23±0.04	-7.29±0.09	-7.73±0.17	-6.66±0.14	-6.27±0.12	-6.28±0.07
Jun-SG	4.12±0.14	3.91±0.13	4.87±0.08	5.01±0.17	5.08±0.04	11.32±0.08	-7.20±0.15	-7.42±0.12	-6.46±0.06	-6.31±0.18	-6.25±0.05
Jun-BB	3.96±0.14	3.86±0.18	4.92±0.10	4.68±0.15	5.15±0.07	11.33±0.09	-7.19±0.24	-7.29±0.30	-6.24±0.22	-6.47±0.23	-6.00±0.21
Aug-SG	3.12±0.30	3.15±0.20	4.35±0.10	4.53±0.31	4.80±0.06	11.07±0.11	-7.83±0.20	-7.80±0.18	-6.60±0.11	-6.61±0.24	-6.15±0.12
Aug-BB	2.91±0.18	2.73±0.25	4.30±0.11	4.27±0.16	4.77±0.02	11.04±0.12	-8.11±0.10	-8.22±0.22	-6.70±0.09	-6.77±0.09	-6.26±0.10

Table 17. Continued.

Factor†	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*	16S rRNA	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*
	Log(functional gene copies g^{-1} dry weight soil						Log(functional gene copies/16S rRNA gene copies)				
Apr-0N-SG	4.07±0.05	3.58±0.17	4.58±0.11	5.07±0.05	4.86±0.06	11.23±0.05	-7.16±0.07	-7.65±0.17	-6.65±0.06	-6.16±0.10	-6.37±0.05
Apr-67N-SG	3.43±0.49	3.32±0.28	4.33±0.18	4.73±0.43	4.88±0.03	11.28±0.05	-7.85±0.45	-7.96±0.24	-6.96±0.14	-6.55±0.39	-6.40±0.02
Apr-202N-SG	3.47±0.31	3.38±0.40	4.24±0.25	4.62±0.30	4.80±0.09	11.20±0.12	-7.74±0.32	-7.83±0.41	-6.96±0.26	-6.58±0.32	-6.40±0.10
Apr-0N-BB	4.20±0.15	3.24±0.42	4.68±0.33	5.19±0.21	5.03±0.19	11.36±0.04	-7.15±0.11	-8.12±0.38	-6.68±0.29	-6.17±0.17	-6.33±0.15
Apr-67N-BB	4.04±0.19	3.74±0.19	4.67±0.30	5.10±0.28	5.01±0.14	11.18±0.06	-7.14±0.18	-7.44±0.22	-6.51±0.32	-6.08±0.28	-6.17±0.17
Apr-202N-BB	3.59±0.09	3.52±0.09	4.36±0.15	4.60±0.03	4.81±0.05	11.16±0.07	-7.57±0.02	-7.64±0.13	-6.79±0.08	-6.55±0.05	-6.35±0.03
Jun-0N-SG	4.07±0.22	3.74±0.27	4.80±0.07	4.88±0.24	5.03±0.03	11.32±0.11	-7.25±0.30	-7.58±0.21	-6.52±0.14	-6.43±0.32	-6.29±0.08
Jun-67N-SG	4.33±0.05	4.19±0.09	5.00±0.02	5.40±0.08	5.17±0.01	11.41±0.07	-7.07±0.02	-7.22±0.16	-6.40±0.05	-6.00±0.14	-6.24±0.07
Jun-202N-SG	3.97±0.37	3.79±0.26	4.79±0.22	4.76±0.39	5.02±0.10	11.25±0.22	-7.28±0.42	-7.45±0.28	-6.45±0.12	-6.49±0.42	-6.22±0.15
Jun-0N-BB	4.00±0.13	3.35±0.18	4.81±0.02	4.75±0.10	5.08±0.01	11.27±0.24	-7.27±0.14	-7.92±0.42	-6.46±0.22	-6.51±0.22	-6.19±0.24
Jun-67N-BB	4.37±0.17	4.39±0.23	5.18±0.22	5.04±0.27	5.35±0.15	11.37±0.14	-6.47±0.47	-6.45±0.58	-5.66±0.51	-5.8±0.40	-5.50±0.51
Jun-202N-BB	3.53±0.10	3.84±0.13	4.75±0.10	4.24±0.18	5.04±0.02	11.34±0.09	-7.82±0.04	-7.51±0.11	-6.59±0.01	-7.11±0.14	-6.31±0.08
Aug-0N-SG	2.62±0.38	2.55±0.28	4.22±0.23	3.88±0.47	4.71±0.08	10.93±0.22	-8.31±0.16	-8.38±0.09	-6.71±0.07	-7.05±0.28	-6.22±0.15
Aug-67N-SG	3.79±0.12	3.38±0.14	4.45±0.05	5.20±0.11	4.92±0.01	11.21±0.24	-7.42±0.20	-7.83±0.16	-6.76±0.21	-6.01±0.14	-6.29±0.23
Aug-202N-SG	2.96±0.73	3.52±0.34	4.38±0.24	4.50±0.69	4.77±0.16	11.08±0.15	-7.75±0.44	-7.18±0.09	-6.33±0.19	-6.78±0.58	-5.94±0.25
Aug-0N-BB	2.81±0.35	2.09±0.40	4.19±0.18	4.16±0.29	4.75±0.05	10.98±0.25	-8.16±0.27	-8.89±0.17	-6.79±0.17	-6.82±0.21	-6.23±0.23
Aug-67N-BB	2.92±0.42	2.84±0.24	4.24±0.20	4.39±0.40	4.79±0.05	11.12±0.24	-8.20±0.18	-8.28±0.14	-6.89±0.08	-6.74±0.16	-6.33±0.20
Aug-202N-BB	3.04±0.20	3.53±0.06	4.57±0.15	4.25±0.01	4.78±0.01	11.01±0.23	-7.98±0.03	-7.48±0.12	-6.44±0.06	-6.76±0.15	-6.23±0.17

† G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

Table 18. Results of mixed model ANOVA (based on GLIMMIX procedure in SAS) testing effects of agricultural season, nitrogen fertilization rate, and grass species on the alpha-diversity metrics of *nifH* genes. F-values are reported.

Alpha diversity	Chao1 estimator	Observed OTUs	Phylogenetic diversity	Shannon index	Pielou's evenness
Season	**13.06*****	**15.39*****	**13.48*****	**3.93***	2.57
Nitrogen	2.80	**8.55****	**10.97*****	**11.77*****	**6.98****
Grass	0.02	0.77	0.21	4.16	2.46
Season×Nitrogen	1.66	2.39	2.52	0.58	0.37
Season×Grass	3.55	2.86	1.67	0.85	0.65
Nitrogen×Grass	0.07	0.10	1.41	1.39	1.52
Season×Nitrogen×Grass	1.76	1.63	2.02	1.08	1.44

Significance: * $0.01 < p\text{-value} \leq 0.05$; ** $0.001 < p\text{-value} \leq 0.01$; *** $p\text{-value} \leq 0.001$.

Table 19. Results of pairwise PERMANOVA of diazotrophic community in three agricultural seasons and under different N fertilization rate and grass species.

Treatment	Group	Pseudo-F	*p*-value	*q*-value
Season	G†/H1	2.285	0.076	0.076
	G/H2	2.315	0.070	0.076
	H1/H2	7.550	0.001***	0.003
Nitrogen	0N/67N	1.224	0.282	0.282
	0N/202N	11.760	0.001***	0.003
	67N/202N	4.738	0.007**	0.011
Grass	SG/BB	3.486	0.016*	0.016
Nitrogen × Grass	0N-BB/0N-SG	1.313	0.248	0.266
	0N-BB/67N-BB	0.898	0.426	0.426
	0N-BB/67N-SG	3.497	0.025*	0.047
	0N-BB/202N-BB	4.874	0.001***	0.004
	0N-BB/202N-SG	11.136	0.001***	0.004
	0N-SG/67N-BB	1.470	0.176	0.203
	0N-SG/67N-SG	2.019	0.120	0.164
	0N-SG/202N-BB	3.720	0.008**	0.020
	0N-SG/202N-SG	9.692	0.001***	0.004
	67N-BB/67N-SG	3.761	0.021*	0.045
	67N-BB/202N-BB	3.848	0.007**	0.020
	67N-BB/202N-SG	9.416	0.001***	0.004
	67N-SG/202N-BB	1.680	0.157	0.196
	67N-SG/202N-SG	3.058	0.048*	0.080
	202N-BB/202N-SG	2.248	0.117	0.164
Season × Nitrogen	G_0N/G_67N	1.199	0.270	0.360
	G_0N/G_202N	4.525	0.012*	0.039
	G_67N/G_202N	1.149	0.326	0.419
	G_0N/H1_0N	0.811	0.559	0.610
	G_0N/H2_0N	1.097	0.361	0.448
	G_67N/H1_67N	0.513	0.741	0.762
	G_67N/H2_67N	2.170	0.058	0.110
	G_202N/H1_202N	3.507	0.064	0.110
	G_202N/H2_202N	0.747	0.548	0.610
	H1_0N/H1_67N	0.941	0.447	0.519
	H1_0N/H1_202N	6.231	0.028*	0.068
	H1_67N/H1_202N	2.340	0.088	0.144
	H1_0N/H2_0N	2.335	0.048*	0.102
	H1_67N/H2_67N	3.687	0.013*	0.039
	H1_202N/H2_202N	5.316	0.020*	0.055
	H2_0N/H2_67N	0.380	0.938	0.938
	H2_0N/H2_202N	4.694	0.006**	0.031
	H2_67N/H2_202N	4.592	0.007**	0.032

Table 19. Continued.

Treatment	Group	Pseudo-F	*p*-value	*q*-value
	G_SG/G_BB	1.577	0.181	0.265
	G_SG/H1_SG	1.598	0.184	0.265
	G_SG/H2_SG	1.181	0.313	0.361
Season	G_BB/H1_BB	0.854	0.503	0.503
×	G_BB/H2_BB	1.508	0.194	0.265
Grass	H1_SG/H1_BB	0.993	0.339	0.363
	H1_SG/H2_SG	4.576	0.019*	0.078
	H1_BB/H2_BB	3.468	0.006**	0.045
	H2_SG/H2_BB	2.055	0.072	0.135

Significance: *0.01< *p*-value ≤ 0.05; **0.001 < *p*-value ≤ 0.01; ***p-value ≤ 0.001.

[†]G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

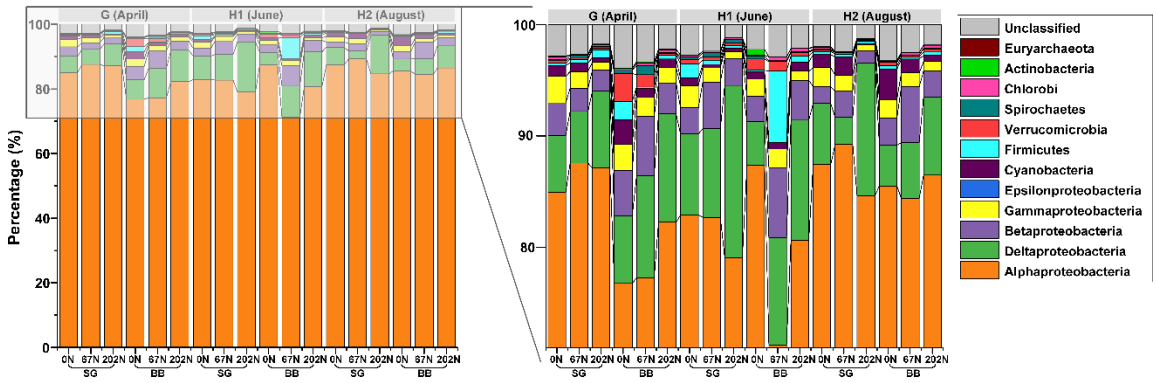

Figure 31. Composition of *nifH* taxa in different treatment combinations in different sampling times. G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

Table 20. Results from indicator species analysis aiming at identifying *nifH* OTUs typical of different combination of N fertilization rate and grass species or groups of the combinations. The classification of OTUs at phylum, class and order level, as well as the specificity value (A-value), fidelity value (B-value), indicator value index (stat-value) and the significance (*p*-value) of the test are reported.

0N-SG[†]							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU169	Proteobacteria	Gammaproteobacteria	Pseudomonadales	0.885	0.667	0.768	0.002**
OTU207	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.702	0.778	0.739	0.009**
0N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU198	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.936	0.375	0.593	0.048*
67N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU215	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.931	0.857	0.893	0.001***
202N-SG & 202N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU185	Proteobacteria	Alphaproteobacteria	Rhizobiales	1	0.588	0.767	0.003**
0N-BB & 67N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU144	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.7561	1	0.87	0.009**
OTU122	Verrucomicrobia	Opitutae	Opitutales	0.840	0.867	0.853	0.001***
OTU165	Proteobacteria	Alphaproteobacteria	Rhodospirillales	0.9285	0.6667	0.787	0.001***
OTU96	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.988	0.533	0.726	0.01**
OTU138	Proteobacteria	Betaproteobacteria	Rhodocyclales	0.951	0.533	0.712	0.019*
OTU186	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.947	0.533	0.711	0.007**

Table 20. Continued.

0N-SG & 67N-SG & 0N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU77	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.883	0.962	0.921	0.001***
OTU112	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.906	0.923	0.914	0.001***
OTU95	Proteobacteria	Alphaproteobacteria	Rhodospirillales	0.918	0.885	0.901	0.001***
OTU12	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.881	0.885	0.883	0.001***
OTU115	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.931	0.808	0.867	0.001***
0N-SG & 0N-BB & 67N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU132	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.800	1	0.894	0.016*
OTU87	Proteobacteria	Gammaproteobacteria	Chromatiales	0.895	0.875	0.885	0.001***
OTU116	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.912	0.833	0.872	0.002**
OTU204	Proteobacteria	Betaproteobacteria	Rhodocyclales	0.9101	0.7083	0.803	0.004**
OTU78	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.943	0.667	0.793	0.004**
67N-SG & 0N-BB & 67N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU150	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.959	0.792	0.871	0.002**
202N-SG & 67N-BB & 202N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU208	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.884	0.958	0.92	0.001***
OTU156	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.885	0.917	0.901	0.001***
OTU161	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.913	0.542	0.703	0.017*
0N-BB & 67N-BB & 202N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU58	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.905	0.917	0.911	0.002**
OTU93	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.855	0.750	0.801	0.006**

Table 20. Continued.

0N-SG & 67N-SG & 202N-SG & 0N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU120	Proteobacteria	Alphaproteobacteria	Sphingomonadales	0.957	0.824	0.888	0.002**
OTU230	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.950	0.794	0.869	0.001***
OTU134	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.946	0.794	0.867	0.002**
0N-SG & 67N-SG & 202N-SG & 202N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU24	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.933	0.914	0.924	0.016*
OTU234	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.956	0.771	0.859	0.002**
0N-SG & 67N-SG & 0N-BB & 67N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU49	Unclassified	Unclassified	Unclassified	0.970	1	0.985	0.001***
OTU111	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.947	0.879	0.912	0.001***
OTU19	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.9824	0.8182	0.897	0.006**
OTU41	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.953	0.818	0.883	0.007**
OTU34	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.968	0.788	0.873	0.013*
OTU69	Cyanobacteria	Cyanophyceae	Nostocales	1	0.697	0.835	0.002**
OTU130	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.969	0.697	0.822	0.002**
OTU106	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.994	0.636	0.795	0.007**
OTU211	Cyanobacteria	Cyanophyceae	Nostocales	0.963	0.576	0.744	0.03*
OTU149	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.963	0.515	0.704	0.041*
0N-SG & 202N-SG & 67N-BB & 202N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU108	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.957	0.939	0.948	0.001***
OTU135	Chlorobi	Chlorobia	Chlorobiales	0.952	0.818	0.882	0.001***
OTU159	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.900	0.758	0.826	0.01**

Table 20. Continued.

0N-SG & 0N-BB & 67N-BB & 202N-BB								
OTU	*Phylum*	*Class*	*Order*	*A*		*B*	*stat*	*p. value*
OTU94	Proteobacteria	Deltaproteobacteria	Desulfovibrionales		0.935	0.909	0.922	0.004**
OTU236	Proteobacteria	Deltaproteobacteria	Desulfuromonadales		0.983	0.667	0.809	0.001***
202N-SG & 0N-BB & 67N-BB & 202N-BB								
OTU	*Phylum*	*Class*	*Order*	*A*		*B*	*stat*	*p. value*
OTU197	Proteobacteria	Alphaproteobacteria	Rhizobiales		0.923	0.594	0.74	0.045*
0N-SG & 67N-SG & 202N-SG & 0N-BB & 67N-BB								
OTU	*Phylum*	*Class*	*Order*	*A*		*B*	*stat*	*p. value*
OTU35	Proteobacteria	Alphaproteobacteria	Rhizobiales		0.976	0.951	0.963	0.01**
OTU137	Proteobacteria	Alphaproteobacteria	Rhizobiales		0.988	0.854	0.918	0.001***
OTU154	Proteobacteria	Alphaproteobacteria	Sphingomonadales		0.964	0.829	0.894	0.014*
0N-SG & 67N-SG & 202N-SG & 0N-BB & 202N-BB								
OTU	*Phylum*	*Class*	*Order*	*A*		*B*	*stat*	*p. value*
OTU16	Proteobacteria	Alphaproteobacteria	Sphingomonadales		0.966	0.977	0.971	0.003**
OTU141	Proteobacteria	Alphaproteobacteria	Sphingomonadales		0.924	0.954	0.938	0.01**
0N-SG & 67N-SG & 202N-SG & 67N-BB & 202N-BB								
OTU	*Phylum*	*Class*	*Order*	*A*		*B*	*stat*	*p. value*
OTU40	Proteobacteria	Betaproteobacteria	Rhodocyclales		0.971	0.976	0.973	0.002**
OTU126	Proteobacteria	Betaproteobacteria	Burkholderiales		0.961	0.905	0.932	0.007**
OTU128	Proteobacteria	Alphaproteobacteria	Rhizobiales		0.965	0.881	0.922	0.016*
OTU125	Proteobacteria	Alphaproteobacteria	Rhizobiales		0.983	0.762	0.865	0.024*
OTU117	Proteobacteria	Alphaproteobacteria	Rhizobiales		0.993	0.738	0.856	0.01**

Table 20. Continued.

0N-SG & 67N-SG & 0N-BB & 67N-BB & 202N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU32	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.979	0.976	0.978	0.002**
OTU36	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.991	0.952	0.971	0.002**
OTU101	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.985	0.952	0.968	0.002**
OTU48	Proteobacteria	Gammaproteobacteria	Pseudomonadales	0.978	0.952	0.965	0.006**
OTU109	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.959	0.857	0.907	0.044*
OTU148	Proteobacteria	Gammaproteobacteria	Pseudomonadales	0.984	0.833	0.906	0.003**
OTU140	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.956	0.810	0.88	0.028*
OTU114	Proteobacteria	Alphaproteobacteria	Rhodospirillales	0.993	0.762	0.87	0.017*
OTU168	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.963	0.762	0.857	0.043*
OTU146	Proteobacteria	Alphaproteobacteria	Rhodospirillales	0.985	0.714	0.839	0.025*
OTU86	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.997	0.691	0.83	0.019*
0N-SG & 202N-SG & 0N-BB & 67N-BB & 202N-BB							
OTU	*Phylum*	*Class*	*Order*	*A*	*B*	*stat*	*p. value*
OTU110	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.975	0.878	0.925	0.002**
OTU164	Proteobacteria	Alphaproteobacteria	Rhizobiales	0.979	0.805	0.888	0.02*

Significance level: * $0.01 < p\text{-value} \leq 0.05$; ** $0.001 < p\text{-value} \leq 0.01$; *** $p\text{-value} \leq 0.001$.

[†]0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

Table 21. Spearman correlation coefficients among abundances of *nifH* genes and transcripts, alpha-diversity metrics of *nifH* genes, and soil properties.

	pH	SWC[†]	NH_4^+-N	NO_3^--N	DOC	DON	TOC	TN	C: N ratio	*nifH* gene copies gdw^{-1}	*nifH* transcript copies gdw^{-1}
nifH gene copies gdw^{-1}	-0.043	-.781**	-.510**	-.387**	.728**	.510**	-0.127	0.039	-.347*	1	-.349*
nifH transcript copies gdw^{-1}	.423**	.590**	.320*	0.002	-.280*	-0.219	-0.092	-0.076	0.011	-.349*	1
Chao1 estimator	0.130	-.404**	-.339*	-.332*	.425**	0.187	-0.243	-0.158	-0.118	.690**	-0.041
Observed OTUs	0.144	-.324*	-0.265	-.436**	.403**	0.080	-0.180	-0.173	0.049	.614**	0.038
Phylogenetic diversity	0.198	-.355*	-0.246	-.414**	.377**	0.060	-0.201	-0.172	0.019	.600**	-0.005
Shannon index	0.046	0.041	0.196	-.359*	0.122	-0.166	-0.039	-0.168	.422**	-0.093	0.235
Pielou's evenness	0.014	0.221	.320*	-0.180	-0.119	-0.240	0.025	-0.125	.454**	-.381**	0.243

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

[†]SWC, soil water content; DOC, dissolved organic C; DON, dissolved organic N; TOC, total organic C; TN, total N.

Table 22. Specific standardized regression weight (path coefficient) for each pathway in the structural equation modeling for diazotrophs and soil properties.

From	To	Path coefficient	From	To	Path coefficient
Grass species	Observed OTUs	0.104	Diazotrophic community	TN	-0.130
Season	SWC	-0.600	SWC	DOC	-0.618
Observed OTUs	SWC	-0.368	SWC	*nifH* gene abundance	-0.334
N fertilization	Nitrate	0.689	Season	Ammonium	-0.325
SWC[†]	Nitrate	0.354	SWC	Ammonium	0.404
Grass species	Nitrate	-0.224	Season	DOC	-0.205
SWC	DON	-0.604	N fertilization	DOC	-0.297
Observed OTUs	Shannon	0.481	Diazotrophic community	Rhodocyclales	0.463
Nitrate	DON	0.375	SWC	*nifH* transcript abundance	0.602
SWC	Diazotrophic community	0.677	Observed OTUs	*nifH* gene abundance	0.427
Grass species	Diazotrophic community	0.233	TN	DOC	0.370
Grass species	pH	-0.443	Diazotrophic community	Rhizobiales	0.821
Observed OTUs	Chao1	1.044	Diazotrophic community	Sphingomonadales	-0.732
Nitrate	Chao1	0.109	Diazotrophic community	Rhodospirillales	-0.931
Season	Chao1	0.078	DON	*nifH* gene abundance	0.154
DON	pH	-0.303	Diazotrophic community	*nifH* gene abundance	-0.364
TOC	pH	-0.243	Evenness	*nifH* gene abundance	-0.177
Nitrate	TN	0.147	Chao1	DOC	0.301
TOC	Chao1	-0.081	Grass species	DOC	0.149
Shannon	Chao1	-0.190	Evenness	Rhodocyclales	0.321
Evenness	TN	-0.107	pH	Rhodocyclales	-0.248
Nitrate	pH	-0.369			

[†]SWC, soil water content; DOC, dissolved organic C; DON, dissolved organic N; TOC, total organic C; TN, total N.

Table 23. Standardized total effects of structural equation modeling for agricultural season, grass species, N fertilization rate, and soil physicochemical properties on diazotrophs abundance, activity, diversity, and major community composition.

Factors	Grass species	Nitrogen fertilization	Season	SWC†	nitrate	TOC	DON	pH
Observed OTUs	0.104	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Shannon index	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Chao1 index	0.074	0.075	0.055	0.039	0.109	-0.081	0.000	0.000
Diazotrophic community	0.207	0.000	-0.406	0.677	0.000	0.000	0.000	0.000
Rhodospirillales	-0.193	0.000	0.378	-0.630	0.000	0.000	0.000	0.000
Sphingomonadales	-0.151	0.000	0.297	-0.495	0.000	0.000	0.000	0.000
Rhizobiales	0.170	0.000	-0.333	0.555	0.000	0.000	0.000	0.000
Rhodocyclales	0.179	0.082	-0.186	0.310	0.120	0.060	0.075	-0.248
***nifH* transcript abundance**	-0.023	0.000	-0.361	0.602	0.000	0.000	0.000	0.000
***nifH* gene abundance**	-0.028	0.040	0.392	-0.654	0.058	0.000	0.154	0.000

†SWC, soil water content; TOC, total organic C; DON, dissolved organic N.

Table 24. Pearson correlation coefficients between alpha-diversity of diazotrophic microbial community and *nifH* gene abundance (copies gdw^{-1}) within different treatment combinations.

Treatment	Group	Chao1 estimator	Observed OTUs	Phylogenetic diversity	Shannon index
Overall		**0.675****	**0.596****	**0.595****	-0.055
Season	G†	**0.659****	**0.646****	**0.715****	**-0.516***
	H1	**0.580***	**0.579***	**0.660****	0.024
	H2	**0.544***	0.360	0.235	-0.072
Nitrogen	0N	**0.621****	**0.558***	**0.608****	-0.372
	67N	**0.731****	**0.689****	**0.756****	-0.146
	202N	**0.708****	**0.616****	**0.588***	0.062
Grass	SG	**0.798****	**0.741****	**0.669****	0.003
	BB	**0.506***	**0.462***	**0.519****	-0.060
Nitrogen × Grass	0N-SG	**0.803****	**0.800****	**0.806****	-0.447
	67N-SG	**0.943****	**0.948****	**0.903****	0.578
	202N-SG	**0.773***	**0.713***	0.643	0.203
	0N-BB	0.403	0.175	0.267	-0.320
	67N-BB	0.221	0.217	0.499	-0.562
	202N-BB	0.633	0.604	0.641	0.084
Season × Nitrogen	G-0N	0.632	0.630	0.696	-0.763
	G-67N	**0.968****	0.820	0.862	-0.588
	G-202N	0.779	0.734	0.737	-0.452
	H1-0N	0.811	0.798	**0.892***	-0.832
	H1-67N	-0.354	-0.195	0.004	-0.611
	H1-202N	0.271	0.271	0.420	0.107
	H2-0N	**0.852***	0.648	0.591	-0.708
	H2-67N	**0.855***	**0.938****	0.766	0.574
	H2-202N	0.002	-0.190	-0.292	-0.257
Season × Grass	G-SG	**0.798****	**0.858****	**0.805****	-0.489
	G-BB	0.076	0.153	0.370	-0.550
	H1-SG	0.528	0.508	0.527	-0.169
	H1-BB	0.744	**0.758***	**0.812***	0.257
	H2-SG	0.309	0.249	0.260	0.023
	H2-BB	0.599	0.438	0.250	-0.049

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

†G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

Table 25. Four abundant *nifH* OTUs that significantly correlated to *nifH* gene abundance. Overall and within subgroup's Pearson correlation coefficients between relative abundance of these four OTUs and *nifH* gene copy numbers were shown.

Treatment	Group	OTU1	OTU5	OTU6	OTU18
Overall		**0.647****	**0.679****	**0.748****	**0.460****
Season	G†	**0.655****	**0.741****	**0.614****	**0.740****
	H1	-0.10741	0.120642	0.320678	0.437788
	H2	0.186774	**0.500***	0.139235	**0.598****
Nitrogen	0N	**0.825****	**0.724****	**0.661****	**0.547***
	67N	**0.773****	**0.680****	**0.883****	0.363997
	202N	**0.698****	**0.686****	**0.768****	**0.521***
Grass	SG	**0.666****	**0.783****	**0.904****	**0.466***
	BB	**0.624****	**0.564****	**0.606****	**0.447***
Nitrogen × Grass	0N-SG	**0.864****	**0.836****	**0.902****	0.52957
	67N-SG	**0.755***	**0.740***	**0.884****	0.39659
	202N-SG	0.653957	**0.860****	**0.955****	0.592485
	0N-BB	**0.859****	**0.784***	0.406305	0.606361
	67N-BB	**0.862***	**0.885****	**0.907****	0.528859
	202N-BB	**0.722***	0.615928	0.64939	**0.798****
Season × Nitrogen	G-0N	**0.963****	**0.877***	0.460604	**0.823***
	G-67N	**0.986****	**0.964****	**0.972****	**0.972****
	G-202N	0.546559	0.628226	0.613152	0.504257
	H1-0N	0.249714	-0.04258	0.377701	0.720973
	H1-67N	0.072526	0.022422	0.663093	-0.21497
	H1-202N	0.099182	-0.68123	-0.005	0.735011
	H2-0N	0.338518	0.37421	-0.67162	0.572458
	H2-67N	**0.855***	0.670957	0.748818	0.605936
	H2-202N	0.095948	0.583233	0.594919	0.59337
Season × Grass	G-SG	**0.698***	**0.798***	**0.835****	**0.700***
	G-BB	**0.747***	0.666658	0.510379	0.675521
	H1-SG	-0.25415	-0.29598	0.06856	0.006104
	H1-BB	-0.05527	0.556075	0.500261	**0.862***
	H2-SG	-0.09718	0.510852	0.298216	0.536049
	H2-BB	0.331461	0.402836	0.061172	0.573548

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

†G, grass green up; H1, initial grass harvest; H2, second grass harvest; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; 202N, 202 kg N ha^{-1} fertilization; SG, switchgrass; BB, big bluestem.

Table 26. Results of mixed model ANOVA (based on GLIMMIX procedure in SAS) testing effects of agricultural season, nitrogen fertilization rate, and grass species on the alpha-diversity of *amoA* genes. F-values are reported.

Alpha diversity	Chao1 estimator	Observed OTUs	Shannon index	Pielou's evenness
Season	**5.21***	**4.29***	0.26	0.34
Nitrogen	1.98	**17.26*****	**56.65*****	**52.52*****
Grass	**9.31****	**16.87*****	2.29	0.11
Season×Nitrogen	0.97	0.39	0.75	0.82
Season×Grass	2.40	0.98	0.10	0.06
Nitrogen×Grass	1.00	1.77	1.97	2.59
Season×Nitrogen×Grass	0.32	0.71	0.57	0.53

Significance level: * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001.

Table 27. Pearson correlation coefficients among *amoA* genes and transcripts, alpha-diversity of ammonia-oxidizing bacterial community, and soil properties.

	pH	SWC†	NH_4^+-N	NO_3^--N	DOC	DON	TOC	TN	C: N ratio	N_2O-N	NP	*amoA* gene	*amoA* transcript
amoA gene	-0.303*	-0.792**	-0.471**	-0.066	0.477**	0.445**	0.173	0.273	-0.241	-0.322*	0.530**	1	-0.215
amoA transcript	0.067	0.561**	0.448**	0.339*	-0.640**	-0.110	0.042	0.059	-0.050	0.228	0.005	-0.215	1
Chao1 estimator	-0.234	-0.190	-0.114	0.079	0.056	0.176	0.068	0.018	0.129	-0.221	0.235	0.402**	0.070
Observed OTUs	-0.352*	-0.195	-0.085	0.359*	-0.080	0.356*	0.028	0.082	-0.124	-0.036	0.531**	0.552**	0.124
Shannon index	-0.569**	-0.014	0.161	0.624**	-0.264	0.114	0.269	0.290*	-0.065	0.280	0.514**	0.309*	0.207
Pielou's evenness	-0.585**	0.040	0.225	0.652**	-0.295*	0.046	0.316*	0.328*	-0.047	0.349*	0.471**	0.217	0.220

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

†SWC, soil water content; DOC, dissolved organic C; DON, dissolved organic N, TC, total organic C; TN, total N; NP, nitrification potential.

Table 28. Specific standardized regression weight (path coefficient) for each pathway in the structural equation modeling for ammonia-oxidizing bacteria and soil properties.

From	To	Path coefficient	From	To	Path coefficient
Season	SWC†	-0.590	N fertilization	*amoA* gene abundance	0.223
N fertilization	AOB community	-0.679	TN	DOC	0.315
AOB community	Observed OTUs	-0.615	DON	DOC	0.184
N fertilization	Nitrate	0.317	TOC	*amoA* gene abundance	0.186
Season	ammonium	-0.348	AOB community	*Nitrosospira* sp. 56-18	-0.886
SWC	ammonium	0.354	AOB community	*Nitrosospira* sp. APG3	-0.177
SWC	Nitrate	0.427	AOB community	*Nitrosospira multiformis*	-0.842
AOB community	Nitrate	-0.474	AOB community	*Nitrosospira* sp. Nsp14	0.882
Grass	Observed OTUs	-0.329	SWC	N_2O emission	0.723
Grass	Nitrate	-0.193	Grass	pH	-0.394
SWC	DON	-0.552	Observed OTUs	Chao1 index	0.978
Observed OTUs	Evenness	0.297	AOB community	N_2O emission	-0.294
Ammonium	Evenness	0.268	SWC	*amoA* transcript abundance	0.433
Nitrate	DON	0.366	DOC	*amoA* transcript abundance	-0.493
AOB community	Evenness	-0.690	AOB community	Nitrification potential	-0.386
AOB community	Shannon index	0.049	Season	Nitrification potential	0.599
Observed OTUs	Shannon index	0.263	N fertilization	Nitrification potential	0.204
Evenness	Shannon index	0.836	Grass	Nitrification potential	-0.267
Nitrate	TN	0.132	Ammonium	N_2O emission	0.167
DON	TN	0.115	*amoA* gene abundance	*amoA* transcript abundance	0.541
SWC	DOC	-0.776	Ammonium	*amoA* transcript abundance	0.331
SWC	*amoA* gene abundance	-0.711	Nitrate	Chao1 index	-0.366
Season	DOC	-0.339	Season	Chao1 index	-0.266
N fertilization	DOC	-0.416	DON	pH	-0.329
Observed OTUs	*amoA* gene abundance	0.604	TOC	pH	-0.239
Shannon index	*amoA* gene abundance	-0.394			

†SWC, soil water content; DOC, dissolved organic C; DON, dissolved organic N; TOC, total organic C; TN, total N; AOB, ammonia-oxidizing bacteria.

Table 29. Standardized total effects of structural equation modeling for agricultural season, grass species, N fertilization rate, and soil physicochemical properties on ammonia-oxidizing bacterial abundance, activity, diversity, and major community composition, as well as nitrification potential and N_2O emission.

Factors	Grass species	Nitrogen fertilization	Season	SWC†	Nitrate	Ammonium	DON	TOC	TN	DOC
Observed *amoA* OTUs	-0.329	0.417	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Evenness	-0.098	0.592	-0.149	0.095	0.000	0.268	0.000	0.000	0.000	0.000
Shannon index	-0.168	0.572	-0.125	0.079	0.000	0.224	0.000	0.000	0.000	0.000
Chao1 index	-0.251	0.174	-0.173	-0.157	-0.366	0.000	0.000	0.000	0.000	0.000
***amoA* gene abundance**	-0.132	0.250	0.469	-0.742	0.000	-0.088	0.000	0.186	0.000	0.000
***amoA* transcript abundance**	-0.060	0.302	-0.265	0.565	-0.060	0.283	-0.108	0.101	-0.155	-0.493
N_2O emission	0.000	0.199	-0.520	0.782	0.000	0.167	0.000	0.000	0.000	0.000
Nitrification potential	-0.267	0.466	0.599	0.000	0.000	0.000	0.000	0.000	0.000	0.000
AOB community	0.000	-0.679	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
***Nitrosospira* sp. Nsp14**	0.000	-0.598	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Nitrosospira multiformis	0.000	0.572	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
***Nitrosospira* sp. APG3**	0.000	0.120	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
***Nitrosospira* sp. 56-18**	0.000	0.601	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

†SWC, soil water content; DON, dissolved organic N; TOC, total organic C; TN, total N; DOC, dissolved organic C; AOB, ammonia-oxidizing bacteria.

CHAPTER III

EFFECTS OF WINTER COVER CROPS AND NITROGEN FERTILIZATION ON MICROBIAL NITROGEN CYCLING CAPACITY IN A NATIVE PASTURE IN MIDDLE TENNESSEE

Abstract

Soil microbial transformation of nitrogen (N) in low N required native C_4 pastures can be affected by pasture management practices. Here, we reported *in situ* seasonal dynamics of the population size (gene copy abundances) of five functional genes involved in soil N cycling (*nifH*, bacterial *amoA*, *nirK*, *nirS*, and *nosZ*) in a switchgrass (*Panicum virgatum*) pasture field experiment with three different winter cover crops [no cover, cereal rye, and multispecies cover crop blend of cereal rye/rape (*Brassica rapa*)/turnips (*B. napus*)/crimson clover (*Trifolium incarnatum*)] and two N fertilization rates (0 and 67 kg N ha^{-1}). Our results showed that the distribution of N-cycling microbial population changed significantly with sampling seasons but was not affected by N fertilization and winter cover crops. The population size of ammonia-oxidizing bacteria (AOB) and nitrous oxide-reducing bacteria were higher during the warm season than in cool season. N fertilization promoted the population size of AOB under all cover crop types. *nirS*-type denitrifiers were more responsive than *nirK*-type denitrifiers to sampling season and management practices. The population size of *nirS*-type denitrifiers were affected by the complex interaction of season, winter cover crops, and N fertilization. The abundances of soil total bacteria and *nirK*-type denitrifiers did not vary with season and pasture management practices. Compared to a hay harvest system in chapter II, the response of N-cycling microbial abundance to N fertilization was mitigated in this pasture, which may be due to the influence of cattle excreta from grazing management, but this needs further study.

Introduction

Grasslands account for approximately half of all agricultural lands in the United States. In recent years, perennial C_4 grasses, such as switchgrass (*Panicum virgatum*), have become the preferred grass species for regional bioenergy development and forage production due to their high adaptability, exceptional drought-tolerance, high productivity with low nitrogen (N) requirement (Fike et al., 2006; Parrish & Fike, 2005). Switchgrass may also improve soil stability, reduce greenhouse gas emissions, and mitigate water pollution due to their high volume of below-ground biomass (McLaughlin & Walsh, 1998).

The eco-friendly characteristics of switchgrass may be due to their interaction with the soil microbes responsible for N transformations. For example, root-associated diazotrophic bacteria have been demonstrated to provide a large amount of N to switchgrass. In turn, the high productivity of switchgrass can increase the belowground flow of C, which can support energy demands for associative N fixation by diazotrophs (Rodrigues et al., 2017). Nitrification and denitrification are two major processes regulating N_2O emissions and nitrate leaching in soils (Bárta et al., 2017; Khalil et al., 2004; Liu et al., 2016; White & Lehnert, 2016). Previous research have demonstrated that ammonia-oxidizing bacteria (AOB) rather than ammonia-oxidizing archaea were the major producer of N_2O emissions in fertilized switchgrass plots (Pannu et al., 2019). Moreover, soil denitrification was increased and nitrate pollution was reduced by the cultivation of switchgrass in over-fertilized soils (Giannoulis et al., 2011).

In recent years, winter annual cover crops have been applied in cropland to improve soil health and agricultural productivity by promoting soil nutrient transformations regulated by soil microbes, especially N-cycling microbes (Dabney et al., 2001; White et al., 2012). The interaction effect of winter cover crops and N fertilization regimes on N-cycling microbes has been investigated in a cotton field, which demonstrated that leguminous cover crops like hairy vetch can promote activity of N cycling populations through the growing season, equaling or exceeding the effects of inorganic N fertilizer (Hu et al., 2021; Mbuthia et al., 2015). Moreover, compared to hay production scenarios, livestock grazing may increase nutrient cycling through animal excreta and would help to reduce the fertilizer application. However, little work related to the effects of application of winter cover crops on N-cycling microbes has been conducted in native C_4 forage production systems under cool-season grazing, and less is known about the interaction effect of cover crops and N fertilization regimes on N-cycling microbes in native C_4 grass systems. Therefore, in this chapter, we investigated the effects of winter cover crops and N fertilization on microbial N-cycling capacity in native C_4 forage production systems under cool-season grazing in Middle Tennessee by quantifying soil physicochemical properties and functional marker genes of microbes involved in N fixation (*nifH*), nitrification (bacterial *amoA*) and denitrification (*nirK*, *nirS*, and *nosZ*) using quantitative polymerase

chain reaction (qPCR). We hypothesized that: 1) The microbial N-cycling capacity in warm season will be higher than in cool season; and 2) The application of multispecies winter cover crop blend will increase microbial N-cycling capacity and may have the same effect with N-fertilized no cover cropping systems.

Materials and Methods

Study site, experimental design, and sampling

This study was conducted at a native pasture with three successive-year cattle grazing treatments (2016-2018) at the University of Tennessee Middle Tennessee Research and Education Center (MTREC) located near Spring Hill, Tennessee (35.71° N, 86.95° W), which is situated on karst topography. Soils at this site are classified as Maury silt loam (fine, mixed, active, mesic Typic Paleudalfs). In order to mitigate the bias caused by the heterogeneous edaphic properties of the large area of this field, this study implemented an incomplete block design with a strip-plot treatment arrangement and four replications (Figure 32). Switchgrass [*Panicum virgatum*, SG] was used as the model forage due to past work indicating its superior productivity and economic returns (Brandes et al., 2018). There were 8 blocks in this field experiment with 1.2 ha per block established in 2008. The first factor of the strip-plot treatment was three levels of winter cover crops: 1) cereal rye (*Secale cereale*), 2) a multispecies cover crop blend of cereal rye/rape (*Brassica rapa*)/turnips (*B. napus*)/crimson clover (*Trifolium incarnatum*); and 3) an unseeded control. Each incomplete block included one of the two cover crops and an unseeded control. The second factor was urea fertilization with two levels (0 or 67 kg N ha^{-1}; 0N or 67N) and was aligned to include all possible combinations of planting and fertilization within each block. Cool-season grazing occurred from November to December, and from February to early April based on availability of forage produced by winter cover crops. Warm-season switchgrass was mowed for hay in August. Annual inter-seeding took place in October following dormancy of the native grasses. Weed control was conducted prior to the initiation of the experimental setting in spring 2016.

Soil samples were collected from each sub-plot (n = 32) in 2020: cool-season (early

May) and warm-season (mid-August). Six soil subsamples (2.5 × 10 cm cores) were collected from each experimental strip-plot at random locations but at least 4 m from the boundary of adjacent plots. Soil probes were sterilized with 70% ethanol after each strip-plot sampling. Soil sub-samples within each strip-plot were composited and stored in Ziplock® bags and transported to laboratory on ice immediately after sampling. Then, the composited sample from each sub-plot was sieved through 2-mm mesh. A subsample of 10 g sieved soil per strip-plot was stored in -80℃ freezer for DNA extraction, and the remainder of the soil sample was used for the analysis of soil physicochemical properties.

Soil physicochemical properties

Soil pH was measured in 1:2 soil:water suspension using pH electrode (Ultrabasic, Denver Instrument, Bohemia, NY, USA). Soil water content (SWC) was determined gravimetrically as, SWC (%) = [(fresh soil weight – dry soil weight$_{(105℃, 48h)}$)/dry soil weight] × 100%. Air-dried soil was pulverized and analyzed for total organic carbon (TC) and total nitrogen (TN) by dry combustion using an Elementar vario MAX cube (Elementar, Langenselbold, Germany). Dissolved organic C (DOC) and N (DON) were extracted in Milli-Q water and analyzed using an Elementar vario TOC cube in liquid mode. Soil NH_4^+-N and NO_3^--N were extracted in 0.5 M K_2SO_4 solution and analyzed by a Berthelot reaction-based spectrophotometric method (Doane & Horwáth, 2003; Rhine et al., 1998) using a microplate reader (Synergy HT, BioTek, Winooski, VT, USA).

Soil nitrification potential was measured using the chlorate block method (Belser & Mays, 1980). Nitrification potential was determined in a chlorate slurry followed by nitrite detection over six hours. The upper limit of nitrification was determined in an ammonium

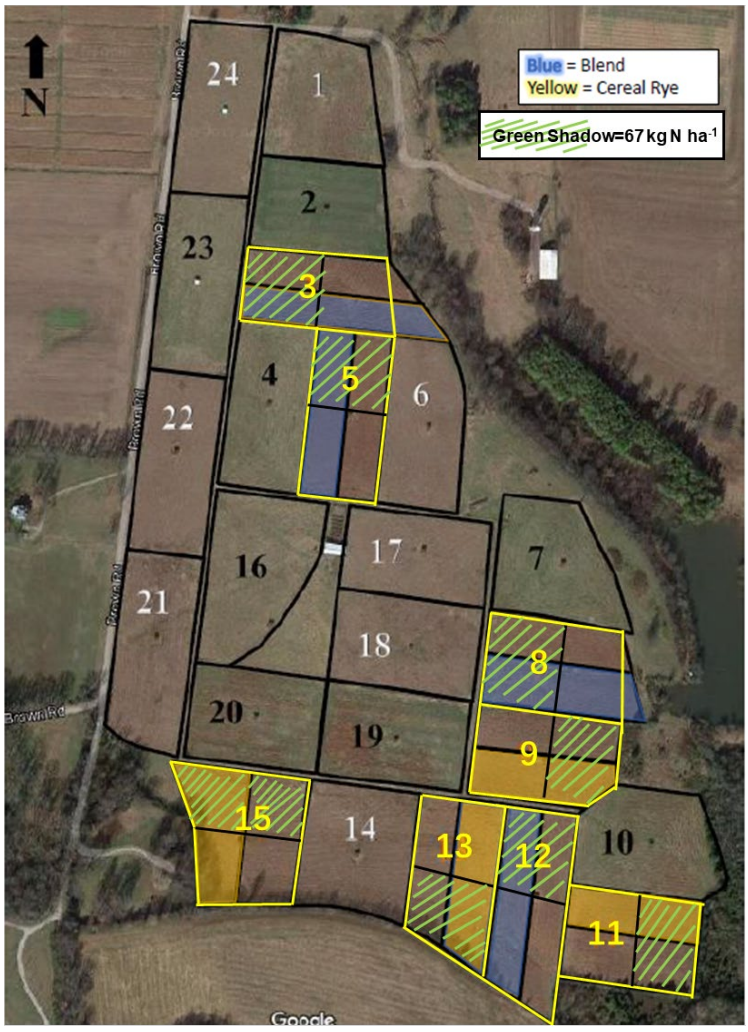

Figure 32. Field map of the experimental strip-plots located at the University of Tennessee Middle Tennessee Research and Education Center in Spring Hill, Tennessee, USA. The yellow boxes are the plots used in this study. The green line-shaded sub-plots are fertilized with 67 kg N ha^{-1}. The non-shaded sub-plots are not fertilized with nitrogen. The yellow shaded sub-plots planted cereal rye as winter cover crops. The blue shaded sub-plots planted a multispecies cover crop blend as winter cover crops.

and phosphate-rich medium in the presence of chlorate to prevent complete conversion of ammonium to nitrate. To do this, soil samples (2g) were mixed with 14 mL nitrification potential medium (4 mL 0.2M K_2HPO_4, 0.5 mL 0.2M KH_2PO_4, 2.5 mL 0.2M $(NH_4)_2SO_4$ and 10 mL 1M $NaClO_3$ in 983 mL deionized water) in 50 ml tubes and gently shaken for 4-6 hours (175 rpm, 25℃), with 1 mL slurry subsamples removed at 3 h intervals to measure nitrite accumulation rates. The 1 mL subsamples were transferred to 1.5 mL micro-centrifuge tubes and centrifuged before nitrite determination so that soil particles did not interfere with the colorimetric determination of NO_2^- and to stop the reaction (most of the nitrifiers will be associated with the soil particles). The NO_2^- concentrations in the filtrate were determined by adding 50 μL of NO_2^- color reagent (4 g of sulfanilamide and 0.1 g N-(1-naphthyl)-ethylenediamine hydrochloride and 17 mL 85% phosphoric acid in 87.6 mL deionized water) to 200 μL of centrifuged samples in a 96-well plate. After 15 min color reaction time, the absorbance is measured in a microplate reader spectrophotometrically at $\lambda = 543$ nm. Using the absorbance values for the nitrite standards compute the nitrite concentration for each sample, subtracting the non-soil controls. Subsamples of slurry were assayed at five minutes (T0), 3 hours (T1) and 6 hours (T2) for NO_2^-. At the end of the 6 h assay, the potential nitrification rates were calculated as μg NO_2^--N g^{-1} soil h^{-1}.

Soil DNA extraction and quantitative analysis of N-cycle genes

Soil genomic DNA was extracted from 0.25 g soil from each sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentrations were quantified using the NanoDrop One spectrophotometer (NanoDrop Technologies, Wilmington, DE). Extracted DNA samples were stored at -20℃ for further qPCR amplification and sequencing. The primer sets, reaction systems, and thermal cycle parameters of qPCR and qRT-PCR of *nifH*, *bacterial amoA*, *nirK*, *nirS*, and *nosZ*, as well as the quantification of 16S rRNA genes and 16S rRNA were described in detail in Materials and Methods in Chapter I (Hu et al., 2021).

Statistical analysis

A mixed model ANOVA within the GLIMMIX procedure in SAS 9.4 (SAS Inst., Cary, NC, USA) was performed to test the effects of grass growing season, cover crop, and N fertilization on population size of N-cycling microbes and total bacteria. The abundances of the N-cycle genes were log-transformed to achieve normal distributions. The fixed effects included season, cover crops, N fertilization, as well as their interactions. Block, interaction of block and cover crop, and interaction of block, cover crop, and N fertilization were included as random effects. A post-hoc least significant difference (LSD) method was used to compare the means of groups. Pearson correlation analysis was performed in IBM SPSS Statistics v26 to evaluate the correlation among the abundance of N-cycling microbes and total bacteria and soil physicochemical parameters. A heatmap based on Pearson correlations was used to visualize the significant positive and negative correlations among measured soil properties and N-cycle gene abundance and was performed by pheatmap package in R 3.6.1. Independent samples t-test was performed in IBM SPSS Statistics v26 to test whether there are any significant difference of soil physicochemical properties and abundances of N-cycle genes and 16S rRNA genes between the grazing native grass forage system at MTREC and hay production system at ETREC (data in Chapter II).

Non-metric Multi-Dimensional Scaling (NMDS) based on Bray-Curtis distances was performed in R 3.6.1 with packages vegan 2.5-5 and phyloseq to reveal the distribution patterns of N-cycling functional groups based on the relative abundance of five N-cycle functional genes (*nifH*, *amoA*, *nirK*, *nirS*, *nosZ*) among sampling seasons, cover crops, and N fertilization. Analysis of similarities (ANOSIM) was also performed by R with the function 'anosim' in vegan to compare groups and test the null hypothesis that the similarity between groups is greater than or equal to the similarity within the groups. In this study, statistically significant differences were accepted at p-value < 0.05 unless otherwise noted.

Results

Soil physicochemical properties

Dissolved organic C (DOC), total organic C (TC), total N (TN), and nitrification

potential (NP) showed significant differences in cool and warm season ($p < 0.01$ for both) (Table 30; Appendix Table 33). The DOC was higher (216.42 ± 6.60 μg g^{-1} dry weight soil [gdw^{-1}]) in cool season and lower (197.81 ± 4.69 μg gdw^{-1}) in warm season whereas both TC, TN, and NP were higher in warm-season (21.21 ± 0.55 mg gdw^{-1}, 2.15 ± 0.06 mg gdw^{-1}, and 0.20 ± 0.02 μg NO_2-N gdw^{-1} hr^{-1}, respectively) than in cool-season (19.15 ± 0.56 mg gdw^{-1}, 1.96 ± 0.05 mg gdw^{-1}, and 0.14 ± 0.01 μg NO_2-N gdw^{-1} hr^{-1}, respectively) (Figure 33; Appendix Table 33).

Winter cover crops had a significant effect on soil nitrate (NO_3^--N) ($p < 0.05$), which was highest under the cereal rye (3.92 ± 0.72 μg gdw^{-1}) and lowest under no cover crop (2.18 ± 0.31 μg gdw^{-1}). With N fertilization (67N), soil pH significantly decreased from 6.19 ± 0.04 to 6.03 ± 0.32 ($p < 0.01$), and ammonium (NH_4^+-N) decreased from 13.44 ± 0.96 to 11.69 ± 0.72 μg gdw^{-1} ($p < 0.05$) (Table 30 and Figure 33; Appendix Table 33).

The effect of cover crops on soil pH and NH_4^+-N depended on sampling season (Table 30; Appendix Table 33): in cool season (May), pH was highest under cereal rye (6.36 ± 0.06) and lowest under multispecies cover crop blend (B) (5.90 ± 0.17), whereas in warm season (August), pH remained stable (Figure 33; Appendix Table 33). NH_4^+-N was lower under no cover treatment (12.57 ± 0.83 μg gdw^{-1}) than under both cover crop treatments (16.02 ± 1.26 μg gdw^{-1} and 17.01 ± 2.07 μg gdw^{-1}, respectively) at the cool-season sampling date but remained unchanged under different cover crops in warm-season sampling date (Figure 33; Appendix Table 33).

Soil water content (SWC) was affected by the interaction of sampling seasons, winter cover crops, and N fertilization ($p < 0.01$) (Table 30). In general, SWC was higher in cool-season samples (27.6 ± 0.81%) than warm-season samples (20.59 ± 0.44%) for all the six treatment combinations of cover crops and N fertilization (Figure 33; Appendix Table 33). SWC remained unchanged under different treatments in warm-season samples but was higher under cereal rye with no N fertilization (0N) than other treatments in cool-season samples (Figure 33; Appendix Table 33).

N-cycle functional gene abundances

The total bacterial population size estimated by the abundance of 16S rRNA genes,

showed no significant difference between cool and warm seasons (Table 31 and Figure 34A; Appendix Table 34). The effects of sampling season, winter cover crops, and N fertilization on N-cycle genes were shown in Table 31. The absolute abundance (per gram dry weight soil) of N fixation gene *nifH* genes was not affected by sampling season and management practices.

The relative abundance of *nifH* (normalized to 16S rRNA genes) was affected by the interaction of season and cover crops, which was highest under cereal rye in cool season but lowest under cereal rye in warm season (Figure 34B; Appendix Table 34). The absolute and relative abundances of bacterial ammonia oxidation gene *amoA* and nitrous oxide reduction gene *nosZ* were both affected by agricultural season, which were higher in warm season than in cool season (Figure 34; Appendix Table 34). Compared to no N addition, N fertilization significantly increased absolute abundance of bacterial *amoA* (Figure 34A; Appendix Table 34). Both absolute and relative abundances of nitrite reduction gene *nirK* were not affected by sampling season and management practices. But the absolute abundance of nitrite reduction gene *nirS* was significantly affected by the interaction of season, cover crops, and N fertilization, which was highest under the blend with 67N in warm season and lowest under no cover with 67N in cool season. Moreover, within cool-season samples, the *nirS* absolute abundance was similarly high under cereal rye with 0N and blend with 67N than under other treatment combinations (Figure 34A; Appendix Table 34). Winter cover crops had no significant main effect on either absolute or relative abundances of N-cycle genes (Table 31).

Variability of N cycling microbial functional potential

Non-metric Multi-Dimensional Scaling (NMDS) showed the variability of the N-cycling microbial populations in terms of functional potential based on the five gene relative abundances (Figure 35). Analysis of similarities (ANOSIM) testing indicated that the population distribution by functional potential was only significantly affected by agricultural season ($R = 0.052$; $P = 0.034$), which was visualized by NMDS analysis (Table 32; Figure 35).

Table 30. Results of mixed model ANOVA (based on GLIMMIX procedure in SAS) testing effects of season, cover crop, and N fertilization on soil properties. F-values are reported.

Effect	pH	SWC†	NH_4^+-N	NO_3^--N	DOC	DON	TC	TN	C: N	NP
Season (S)	1.63	158.39***	27.05***	0.96	8.97**	1.99	9.60**	7.89**	2.43	10.51**
Cover (C)	0.23	2.67	2.18	4.00*	0.08	0.40	1.27	1.22	0.57	0.07
Nitrogen (N)	7.78**	4.14	4.31*	1.09	2.84	1.81	0.26	0.25	0.01	0.05
S×C	6.13**	3.78*	4.01*	1.67	0.14	0.03	0.45	0.53	0.05	0.02
S×N	1.21	11.61**	1.13	0.15	1.18	0.33	1.79	1.60	0.20	0.06
C×N	0.37	3.57*	0.07	2.01	0.20	1.51	1.01	0.77	0.73	1.42
S×C×N	0.32	5.84**	0.18	0.70	0.05	1.05	0.43	0.32	0.50	0.21

Significance level: * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001.

†SWC, soil water content; DOC, dissolved organic C; DON, dissolved organic N; TC, total organic C; TN, total N; NP, nitrification potential.

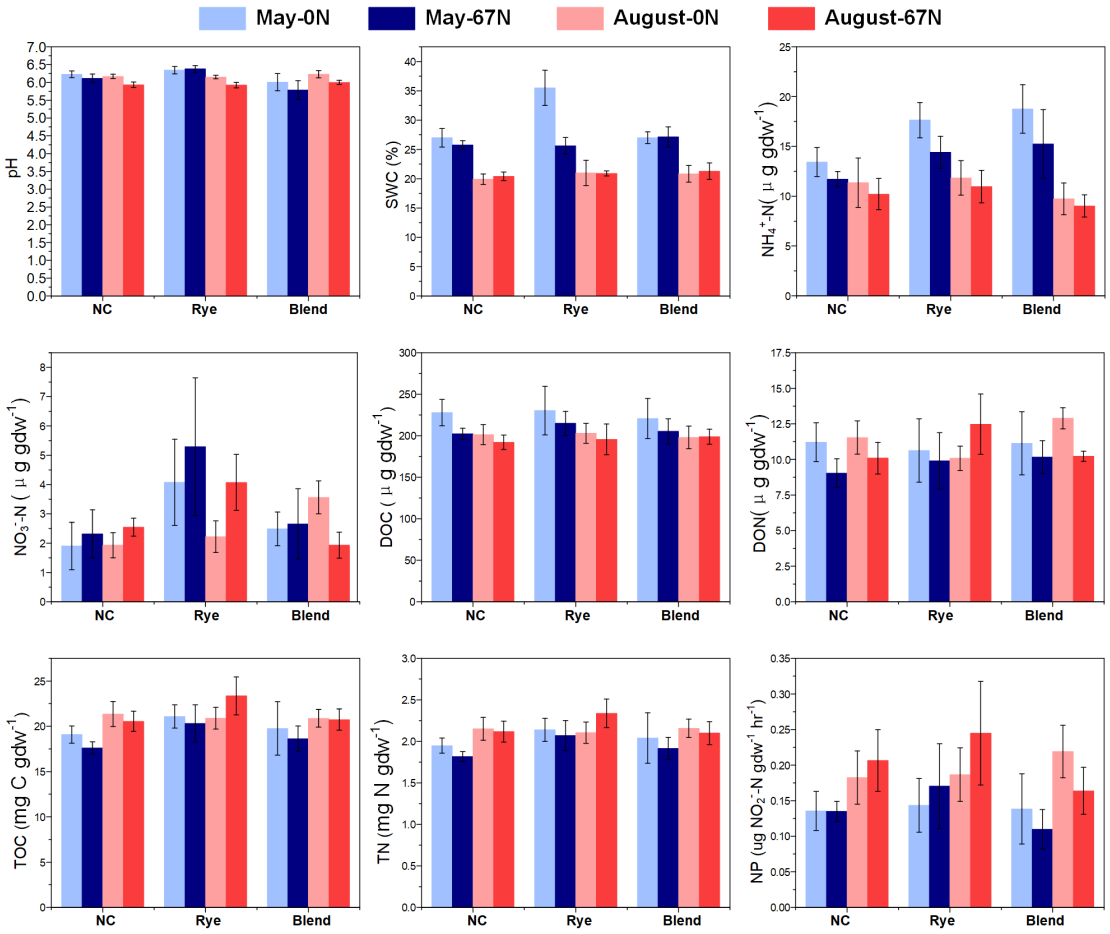

Figure 33. Soil physic-chemical properties in relation to agricultural season and N fertilization under different treatment of winter cover crops. NC, no cover crop; Rye, cereal rye; Blend, multispecies cover crop blend; SWC, soil water content; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; TOC, total organic carbon; TN, total nitrogen; NP, nitrification potential.

Table 31. Results of mixed model ANOVA (F values), based on GLIMMIX procedure in SAS, testing effects of season, cover crop, and N fertilization on the relative (R) and absolute (A) abundances of N-cycle genes. Significant values are indicated with bold fonts and asterisks.

Factor		*nifH*	AOB[a] *amoA*	*nirK*	*nirS*	*nosZ*	16S rRNA gene
Season	R	1.18	**5.65***	2.51	1.44	**6.94***	-
	A	0.35	**10.29****	0.08	**23.25*****	**30.91*****	3.44
Cover crop	R	0.19	3.28	0.13	0.21	0.97	-
	A	1.43	3.05	1.26	2.88	0.03	0.73
Nitrogen	R	2.47	3.85	0.43	1.31	0.00	-
	A	1.39	**4.93***	2.15	0.29	1.05	0.57
Season×Cover crop	R	**4.99***	0.64	1.83	3.30	2.91	-
	A	2.86	1.02	0.35	1.09	0.04	2.85
Season×Nitrogen	R	1.82	0.59	2.19	1.33	2.20	-
	A	0.06	1.59	0.09	0.01	0.33	1.27
Cover crop×Nitrogen	R	2.00	0.31	1.86	3.31	0.35	-
	A	1.33	0.58	1.38	**3.98***	0.33	0.78
Season×Cover crop×Nitrogen	R	3.37	0.86	0.53	2.62	0.88	-
	A	3.36	1.93	0.32	**6.28****	1.26	1.09

Significance level: * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001.

[a]AOB, Ammonia oxidizing bacteria.

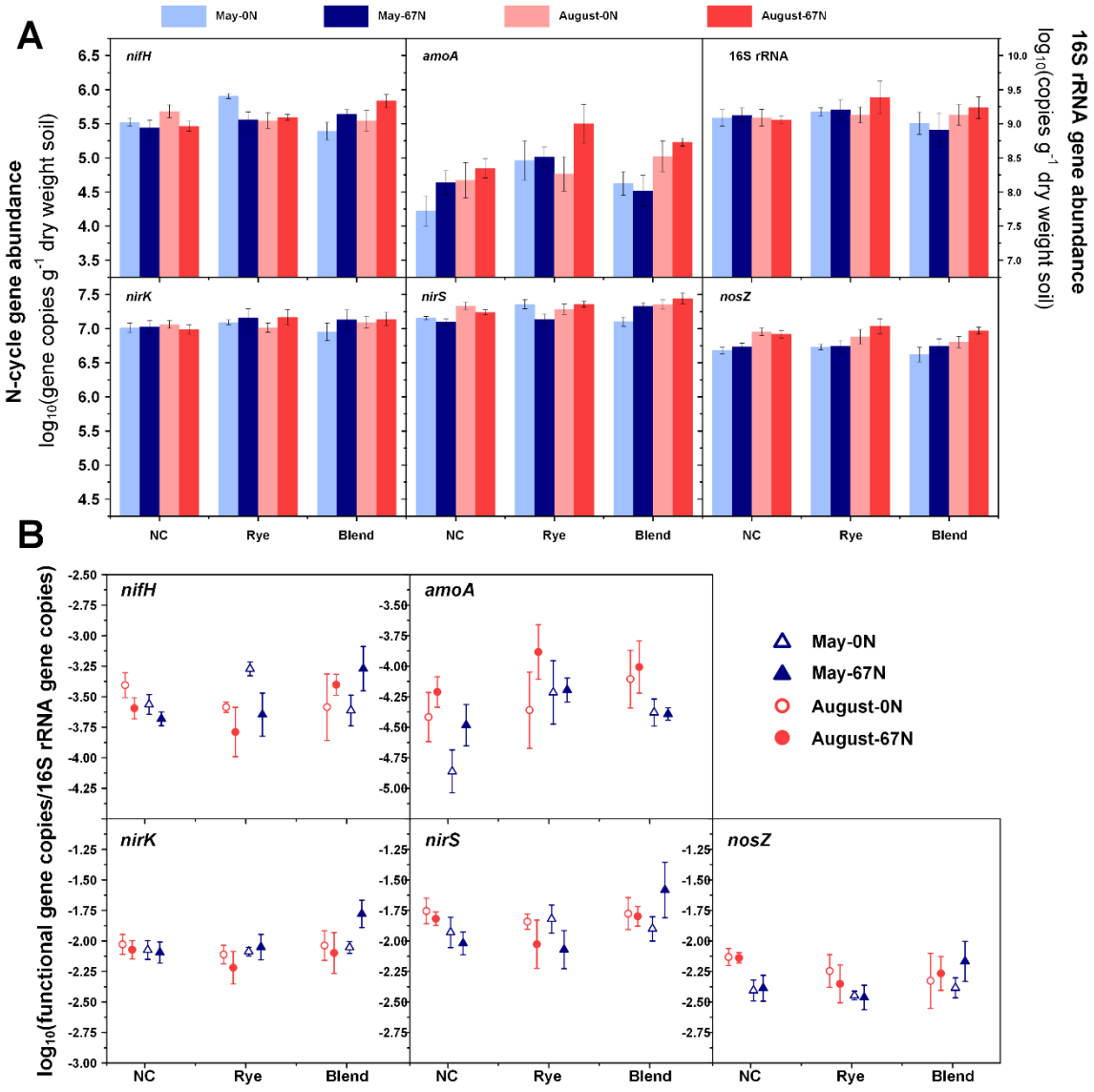

Figure 34. Absolute (A) and relative (B) abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes in relation to agricultural season and N fertilization under different cover crop treatments. Points represent the mean ± standard error. 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization; NC, no cover crop; Rye, Cereal rye; Blend, multispecies cover crop blend.

Relationship between soil properties and functional gene abundances

The correlation heatmap showed significant correlations among absolute and relative abundances of N-cycle genes and measured soil properties (Figure 36). In general, the absolute abundances of five N-cycle genes and 16S rRNA genes were significantly positively correlated to each other (Figure 36; Appendix Table 35). The relative abundances of N fixation gene (*nifH*) and denitrification genes (*nirK*, *nirS*, and *nosZ*) were also positively correlated to each other (Figure 36; Appendix Table 35). The absolute and relative abundances of N-cycle genes showed different patterns of correlations with soil properties (Figure 36). In general, the absolute abundances of N-cycle genes and 16S rRNA gene were mostly positively correlated to soil properties whereas the relative abundances of N-cycle genes were mainly negatively correlated to soil properties. However, both absolute and relative abundances of bacterial *amoA* had significant positive correlation with nitrification potential (NP) ($R = 0.516$, $p < 0.01$ and $R = 0.248$, $p < 0.05$, respectively) and nitrate (NO_3^--N) ($R = 0.653$, $p < 0.01$ and $R = 0.514$, $p < 0.01$, respectively). Moreover, absolute abundance rather than relative abundance of bacterial *amoA* was positively correlated to ammonium (NH_4^+-N) ($R = 0.260$, $p < 0.05$). In addition, the 16S rRNA gene abundance was significantly positively correlated to soil C and N availability (Figure 36; Appendix Table 35).

Grazed pasture versus hay production systems

The soil physicochemical properties and the abundances of N-cycle and 16S rRNA genes at different C_4 grass systems (grazing native grass forage system at Middle Tennessee Research and Education Center [MTREC] and hay production grass system at East Tennessee Research and Education Center [ETREC] mentioned in Chapter II) were compared in this study to investigate whether grazing can improve the sustainability of native grass forage system compared to hay production scenarios (Figure 37 and Figure 38). The samples collected from MTREC and ETREC under same C_4 grass species (switchgrass [*Panicum virgatum*, SG], management practices (0N and 67N under no winter cover crops), and sampling seasons (cool-season sampling in late April or early May; warm-season sampling in mid-August) were used for comparison.

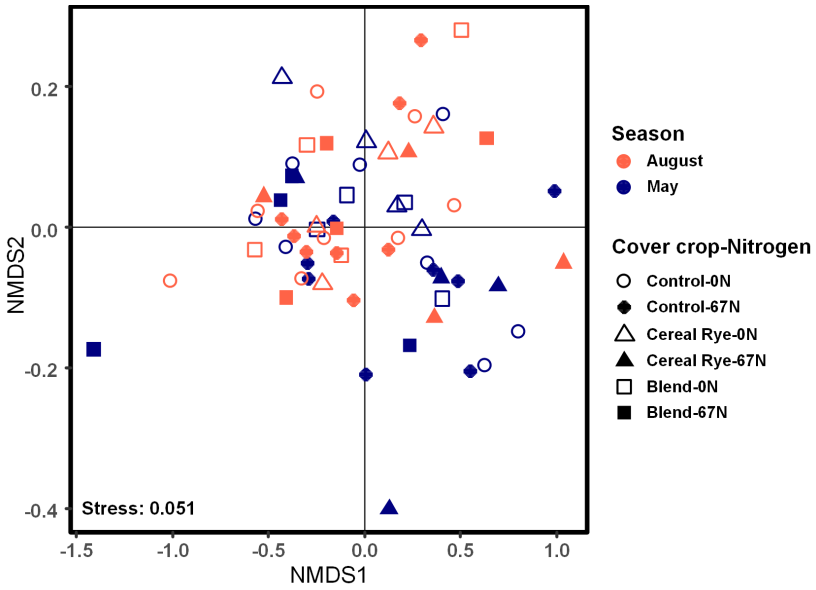

Figure 35. Non-metric Multi-Dimensional Scaling (NMDS) of Bray-Curtis distances between N-cycling communities based on relative abundances of five N-cycle genes. 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization.

Table 32. Results of ANOSIM examining the importance of the effects of season, cover crop, and N fertilization on N-cycling microbial communities.

Factor	*R*	*p*-value[†]
Season	**0.052**	**0.034**
Cover crop	-0.014	0.592
Nitrogen	-0.008	0.579
Season×Cover crop	0.011	0.332
Season×Nitrogen	0.018	0.186
Cover crop×Nitrogen	-0.014	0.620
Season×Cover crop×Nitrogen	-0.018	0.595

[†]Bold values indicate *p*-value ≤ 0.05.

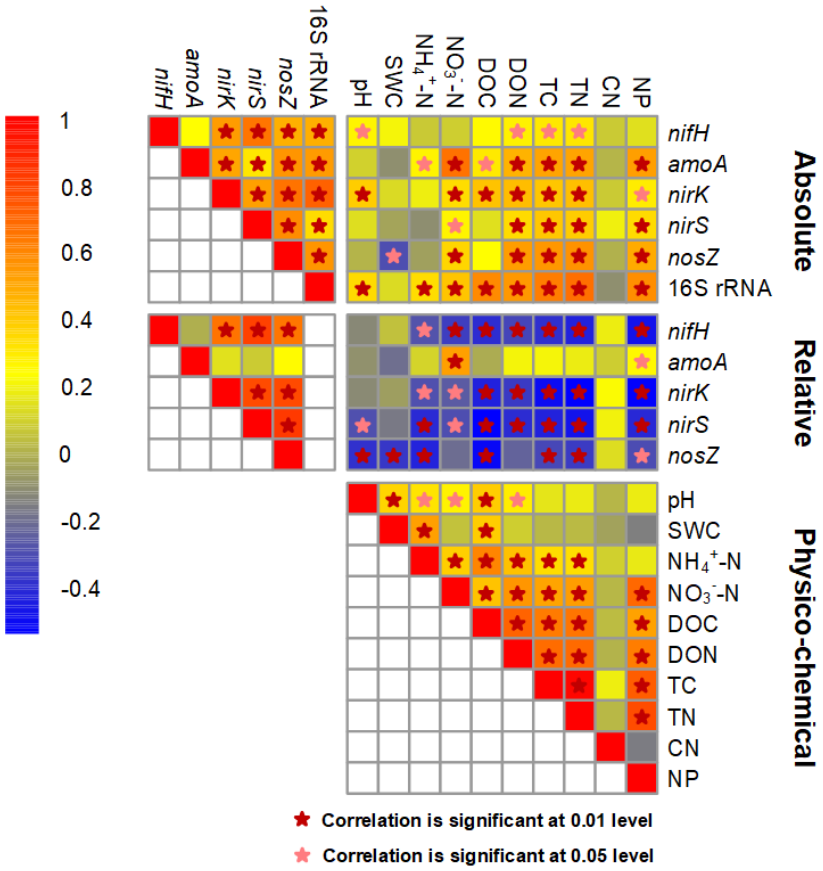

Figure 36. Heatmap showing correlation among absolute and relative abundance of marker genes and soil properties. SWC, soil water content; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; TC, total organic carbon; TN, total nitrogen; CN, C-to-N ratio; NP, nitrification potential.

For soil physicochemical properties, soil pH was lower at MTREC than at ETREC under 67N in warm season. SWC was lower at ETREC than MTREC in warm season. Nitrate was lower at ETREC than MTREC under no N fertilization treatment (0N) in warm season. DON and C:N ratio were generally higher at ETREC than MTREC. Nitrification potential was lower at ETREC than MTREC in cool season (Figure 37).

For the absolute abundance of N-cycle genes and 16S rRNA genes, *nifH*, bacterial *amoA*, *nosZ* and 16S rRNA genes were both more abundant at ETREC than MTREC regardless of season and N fertilization (Figure 38A). The nitrite reduction gene *nirK* was more abundant at ETREC than MTREC in warm season for both 0N and 67N, whereas *nirS* was more abundant at ETREC than MTREC under 67N in cool season and under both 0N and 67N in warm season (Figure 38A). The relative abundances of *nifH* and bacterial *amoA* were higher at ETREC than MTREC regardless of season and N fertilization (Figure 38B). Under 0N treatment, the relative abundances of *nirK* and *nirS* were lower at ETREC than MTREC in cool season and warm season, respectively (Figure 38B). The relative abundance of *nosZ* showed no significant difference between ETREC and MTREC (Figure 38B).

The change in soil physicochemical properties and N-cycle and 16S rRNA genes under 67N compared to 0N were compared between the grazing and hay production systems using Mann-Whitney U test (Figure 39 and Figure 40). N fertilization with 67 kg N ha^{-1} increased nitrate content, *nirS* and *nosZ* absolute abundances in the hay production system but had no effect in the grazing system in warm season (Figure 39, Figure 40A). But the influence of N fertilization on the relative abundances of N-cycle genes showed no significant difference between hay production system and grazing system (Figure 40B).

Discussion

The primary objective of this chapter was to investigate the impact of winter cover crops and N fertilization on the seasonal population size of soil microbial communities involved in N transformations by using *nifH*, bacterial *amoA*, *nirK*, *nirS*, and *nosZ* as marker genes.

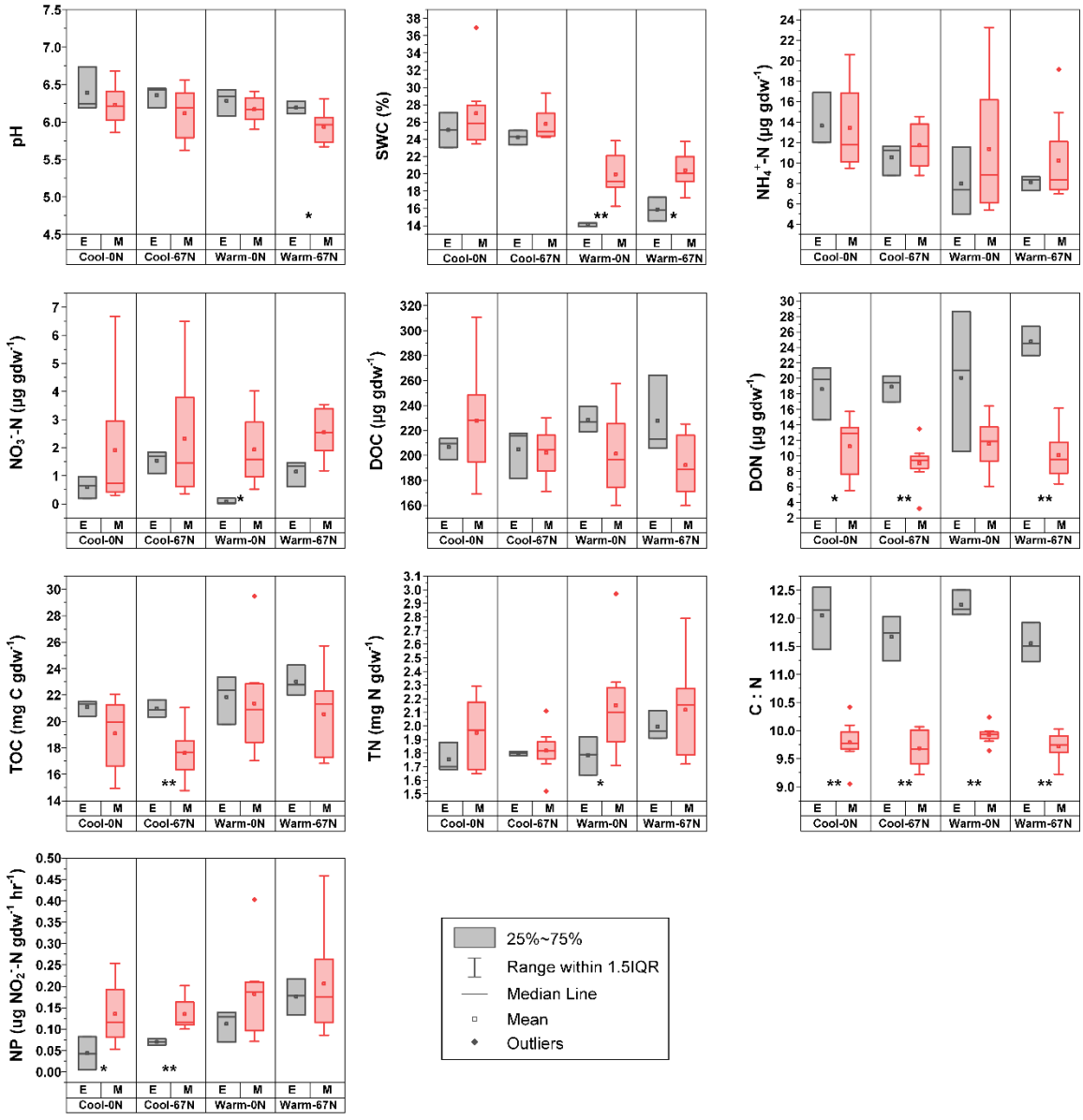

Figure 37. Comparison of soil physicochemical properties in relation to agricultural season and N fertilization between hay production system at ETREC (E) and grazing system at MTREC (M). 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization. SWC, soil water content; DOC, dissolved organic C; DON, dissolved organic N; TOC, total organic C; TN, total N; NP, nitrification potential. Significance level: * p-value ≤ 0.05; ** p-value ≤ 0.01.

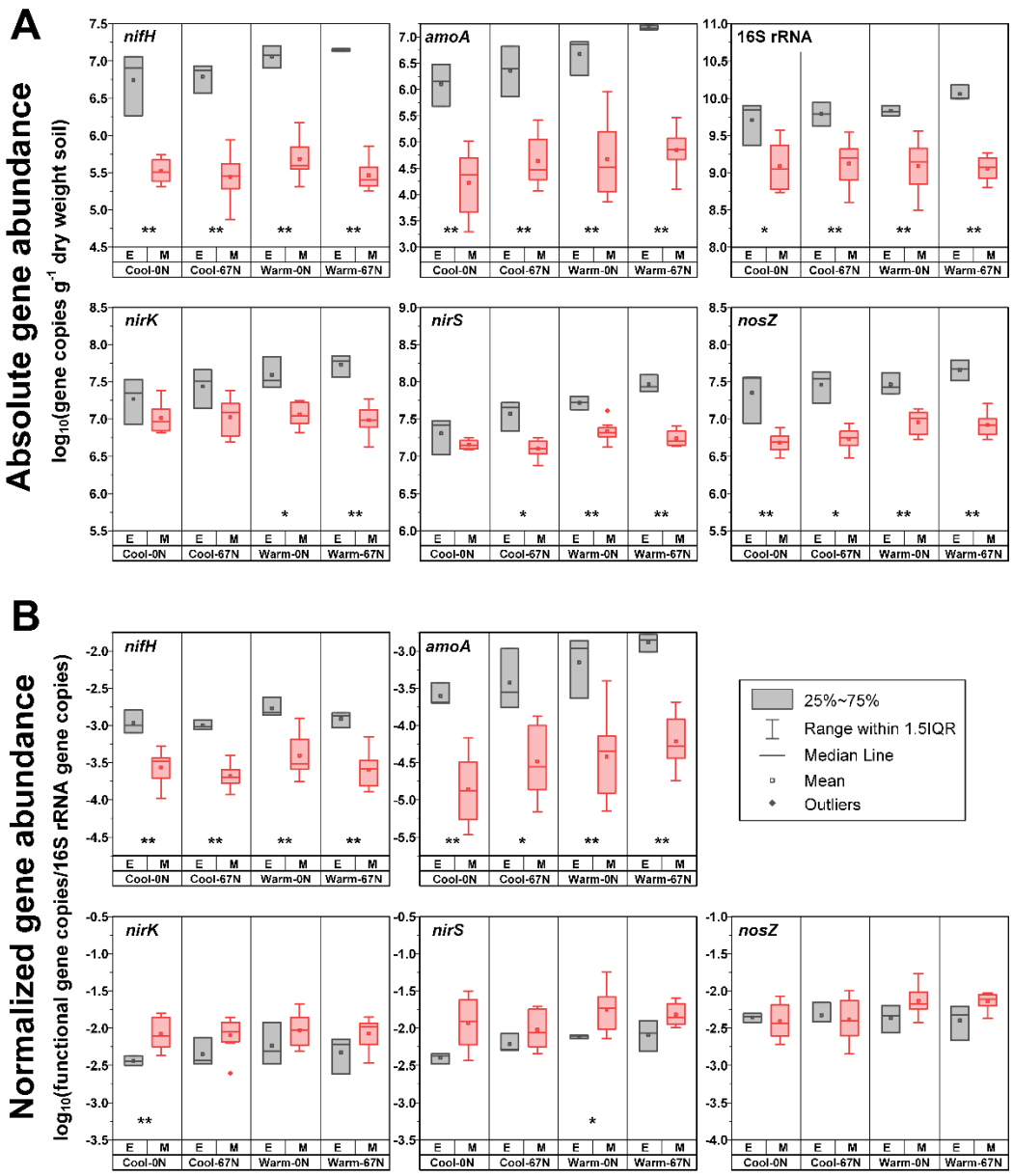

Figure 38. Comparison of absolute abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes (A) and 16S rRNA gene normalized *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* gene abundances (B) in relation to agricultural season and N fertilization between hay production system at ETREC (E) and grazing system at MTREC (M). 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization. Significance level: * p-value ≤ 0.05; ** p-value ≤ 0.01.

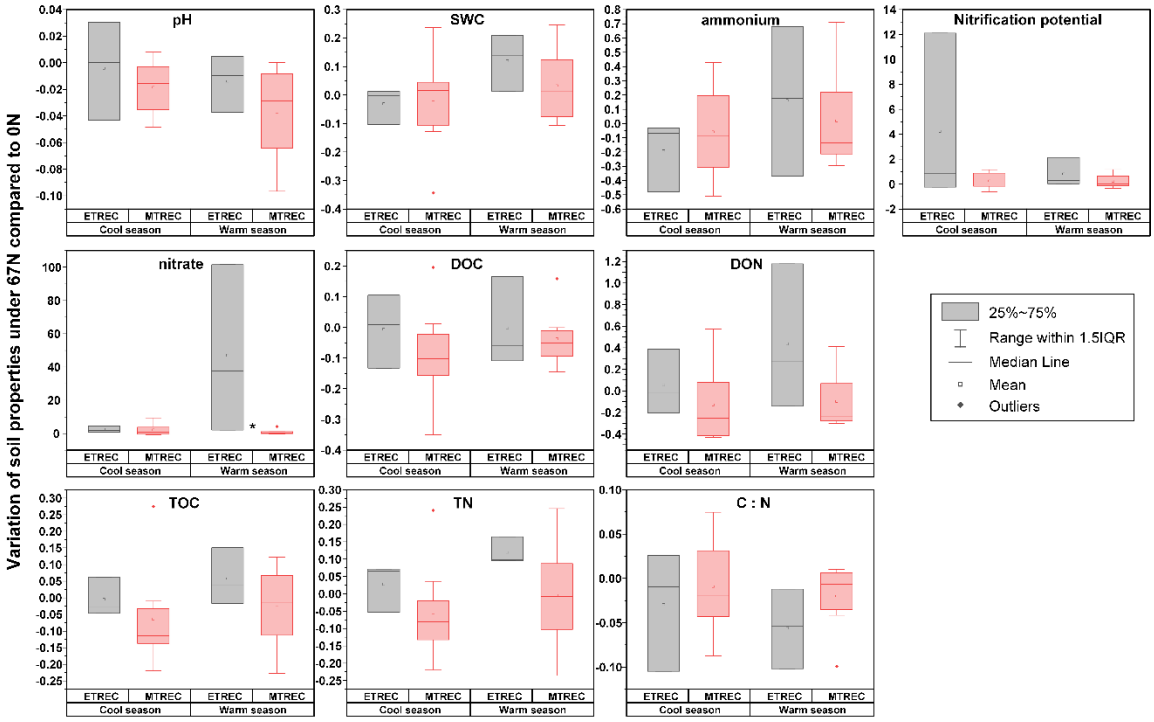

Figure 39. Comparison of the variation of soil properties under 67N compared to 0N in relation to agricultural season between hay production system in ETREC and grazing system in MTREC. SWC, soil water content; DOC, dissolved organic C; DON, dissolved organic N; TOC, total organic C; TN, total N; NP, nitrification potential. Significance level: * p-value ≤ 0.05; ** p-value ≤ 0.01.

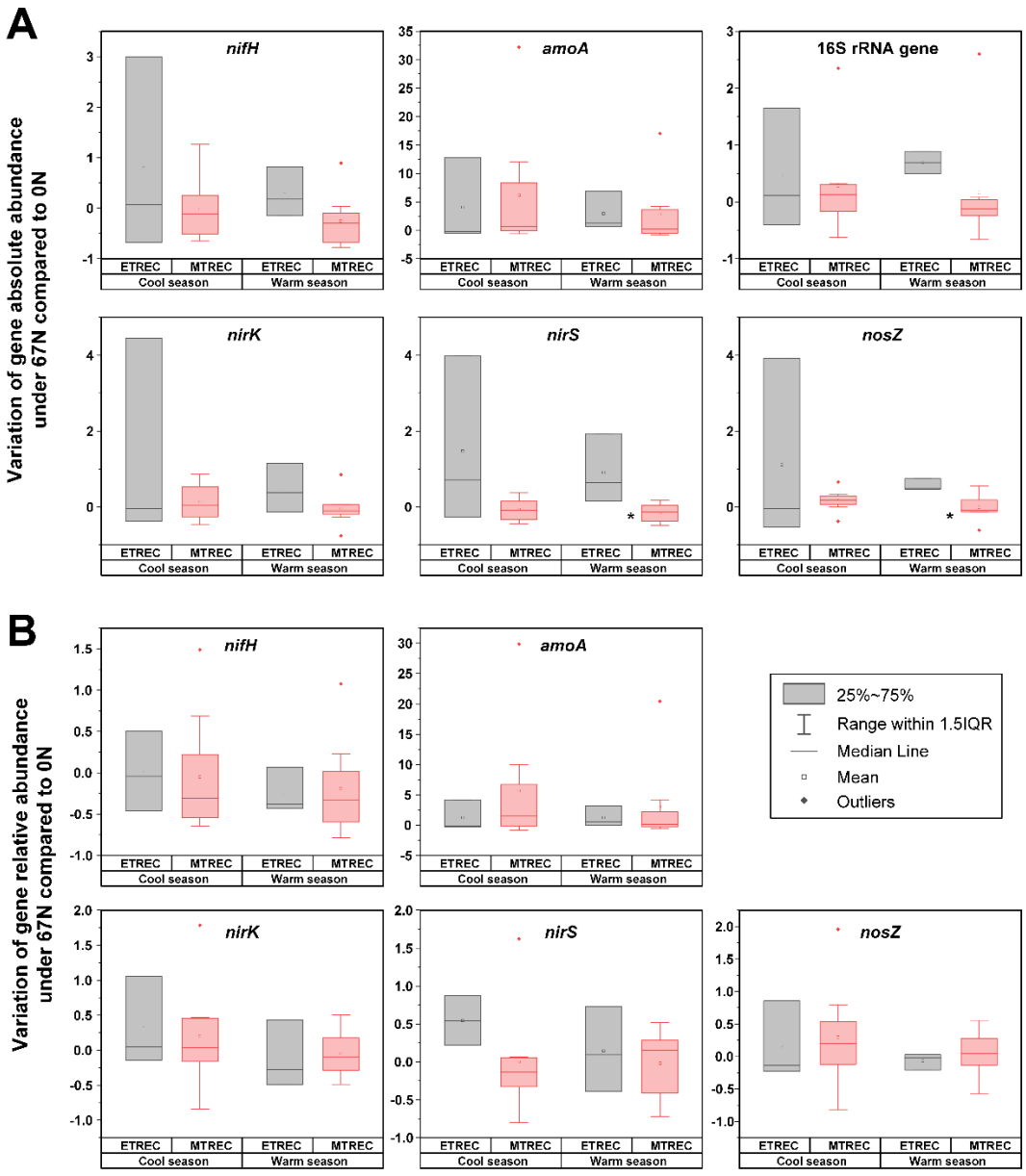

Figure 40. Comparison of the variation of absolute abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes (A) and 16S rRNA gene normalized *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* gene abundances (B) under 67N compared to 0N in relation to agricultural season between hay production system in ETREC and grazing system in MTREC. Significance level: * p-value ≤ 0.05; ** p-value ≤ 0.01.

We found that the distribution of N-cycling microbial populations showed seasonal changes regardless of winter cover crops and N fertilization (Figure 35), indicating that climatic factors and plant traits may play the key role in driving the variability of N-cycling microbial communities. This was consistent with a previous landscape-scale study which found that climatic factors and plant traits explained the major variation of soil microbial communities (de Vries et al., 2012). In addition, a previous continental-scale study reported that climatic factors indirectly affected soil microbial communities by impacting plant productivity, soil mineralogy, and soil pH whereas soil organic matter had a direct effect on microbial community composition (Waldrop et al., 2017).

In our study, the population size of total bacteria did not vary with sampling season and pasture management practices, but the population size of N-cycling bacteria, such as AOB, *nirS*-type nitrite-reducing bacteria, and nitrous oxide-reducing bacteria, were higher in warm season than in cool season, which may be due to the higher temperature and the growth of switchgrass in warm season. Soil denitrification was observed to increase in switchgrass plots in a previous study (Giannoulis et al., 2011), which was consistent with our study.

Compared to *nirS*-type nitrite-reducing bacteria, *nirK*-type nitrite-reducing bacteria did not vary between seasons and pasture management practices, suggesting that *nirS*-type denitrifiers were generally more responsive than *nirK*-type denitrifiers to soil management practices, which has been observed in other soil systems as well. For example, the research conducted in native C_4 grass systems at East Tennessee Research and Education Center (ETREC) (described in chapter II) also found that both population size and functional activity of *nirK* type denitrifiers were not affected by N fertilization rate whereas *nirS*-type denitrifiers were. Similarly, *nirS*-type denitrifiers were found to be more responsive than *nirK*-type denitrifiers to organic-chemical fertilization in a red paddy soil (Xiao et al., 2021) and to organic fertilizers in drip-irrigated calcareous soil (Tao et al., 2018). However, another study reported that the abundance of *nirK*-type rather than *nirS*-type denitrifers were more sensitive to inorganic and organic mixed fertilization in paddy soils (Yang et al., 2020). Therefore, the response of *nirK*-type or *nirS*-type denitrifers to agricultural management practices may have different patterns, which might depend on climatic

factors, soil properties, and plant species, and geographical factors at different terrestrial ecosystems.

In general, N fertilization can promote AOB abundance but not affect diazotroph abundance. The higher abundance of AOB under fertilized plots observed in this study was also detected in the study conducted in native C_4 grass systems at ETREC mentioned in chapter II and in other soil ecosystems, such as an alkaline sandy loam soil (Shen et al., 2008) and in unmanaged wildland soils by a meta-analysis (Carey et al., 2016). The significant positive correlation between AOB abundance and nitrification rate shown in this study was also observed in native C_4 grass systems at ETREC (Chapter II) and other soil systems (Carey et al., 2016; Shen et al., 2008). The no influence of N fertilization on the abundance of diazotrophs was also observed in ETREC (chapter II) and a meta-analysis by Ouyang et al. (Ouyang et al., 2018).

Contrary to our hypothesis, winter cover crops had no significant main effect on microbial N-cycling capacity reflected by the absolute abundance of five N-cycle marker genes. But we observed significant interaction effect of season, winter cover crops, and N fertilization on the population size of *nirS*-type denitrifiers. In winter cover crop growing season (cool season), the abundance of *nirS*-type denitrifiers was not affected by 67 kg N ha^{-1} fertilization in no cover systems but was decreased in cereal rye systems and increased in multispecies cover crop blended systems, suggesting the different denitrification potential in different cover cropping systems. In addition, the relative abundance of diazotrophs in cereal rye cover cropping systems was highest in cool season (May) but was lowest in warm season (August). One explanation is that cereal rye as a non-leguminous cover crop with high C:N residues, takes up additional N from soil for growth, which may decrease soil N availability and promote the expression of nitrogenase gene *nifH* for N fixation, therefore, potentially increase the competitiveness of diazotrophs in cereal rye cover cropping systems.

In this study, we assessed the relationship between population size of N-cycling microbes, total bacteria, and soil properties. In general, the absolute abundances of N-cycling microbes and total bacteria were both positively correlated to soil C and N availability. In contrast, the relative abundance of diazotrophs and denitrifiers were

negatively correlated to soil nutrient availability. One possible explanation is that diazotrophs and denitrifiers are mainly anaerobic and/or facultative anaerobic bacteria, which are less competitive than aerobic microbes for soil organic matter decomposition when nutrient availability becomes higher.

We compared the microbial N-cycling capacity at the Middle Tennessee Research and Education Center (MTREC, this chapter) with cool-season grazing at ETREC (chapter II) with mowing at harvest seasons. Generally, the hay production system in ETREC had higher N-cycling microbial population size than the cool-season grazing pastures in MTREC. However, this difference may be mainly explained by different climatic factors (temperature, precipitation, etc.), soil characteristics (soil physicochemical properties, soil texture, etc.), topography factors, and grass harvest method (grazing versus mowing). In addition, approximately 90% of the N in C_4 native grassland ecosystems stays in grass biomass rather than in soil. Therefore, the different plant biomass in ETREC and MTREC would be another important factor affecting N-cycling capacity. Notably, we observed that fertilization had different effects in the two systems; specifically, 67 N-rate promoted *nirS*-type nitrite-reducing bacteria, nitrous oxide-reducing bacteria, as well as soil nitrate in the ETREC hay production system but had less effect in the MTREC grazing system. Other than the difference of environmental factors, one possible explanation is that the lag effect of cattle excreta in grazing system might mitigated the impact of urea fertilization.

Conclusions

In summary, our results showed that the distribution of N-cycling microbial populations changed significantly between sampling seasons but was not affected by N fertilization and winter cover crops. N fertilization promoted the population size of AOB, and the population size of AOB and nitrous oxide-reducing bacteria were higher in warm season than in cool season. *nirS*-type denitrifiers were more responsive than *nirK*-type denitrifiers to season and management practices. Only the population size of *nirS*-type denitrifiers were affected by the interaction of season, winter cover crops, and N fertilization. The abundances of soil total bacteria and *nirK*-type denitrifiers did not vary with season and pasture management practices. Compared to hay harvest system, there was

less impact of N fertilization on N-cycling microbes in grazing system, which may potentially relate to the chromonic and lag effect of cattle excreta but need to be further studied. The less effect of pasture management practices on N-cycling microbial population size observed in this large-scale pasture study, suggesting complex influence of environmental factors, such as climatic factors, soil characteristics, and topography factors.

References

Bárta, J., Tahovská, K., Šantrůčková, H., & Oulehle, F. (2017). Microbial communities with distinct denitrification potential in spruce and beech soils differing in nitrate leaching. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-08554-1

Belser, L. W., & Mays, E. L. (1980). Specific inhibition of nitrite oxidation by chlorate and its use in assessing nitrification in soils and sediments. *Applied and environmental microbiology*, *39*(3), 505-510. https://doi.org/10.1128/AEM.39.3.505-510.1980

Brandes, E., Plastina, A., & Heaton, E. A. (2018). Where can switchgrass production be more profitable than corn and soybean? An integrated subfield assessment in Iowa, USA. *GCB Bioenergy*, *10*(7), 473-488. https://doi.org/10.1111/gcbb.12516

Carey, C. J., Dove, N. C., Beman, J. M., Hart, S. C., & Aronson, E. L. (2016). Meta-analysis reveals ammonia-oxidizing bacteria respond more strongly to nitrogen addition than ammonia-oxidizing archaea. *Soil Biology and Biochemistry*, *99*, 158-166. https://doi.org/10.1016/j.soilbio.2016.05.014

Dabney, S. M., Delgado, J. A., & Reeves, D. W. (2001). Using winter cover crops to improve soil and water quality. *Communications in Soil Science and Plant Analysis*, *32*(7-8), 1221-1250. https://doi.org/10.1081/CSS-100104110

de Vries, F. T., Manning, P., Tallowin, J. R. B., Mortimer, S. R., Pilgrim, E. S., Harrison, K. A., Hobbs, P. J., Quirk, H., Shipley, B., Cornelissen, J. H. C., Kattge, J., & Bardgett, R. D. (2012). Abiotic drivers and plant traits explain landscape-scale patterns in soil microbial communities [https://doi.org/10.1111/j.1461-0248.2012.01844.x]. *Ecology Letters*, *15*(11), 1230-1239. https://doi.org/https://doi.org/10.1111/j.1461-0248.2012.01844.x

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.

Giannoulis, K., Molla, A., Domirkou, A., & Danalatos, N. (2011). *Switchgrass as a promising biomass crop with soil improvement characteristics*.

Hu, J., Jin, V. L., Konkel, J. Y. M., Schaeffer, S. M., Schneider, L. G., & DeBruyn, J. M. (2021). Soil health management enhances microbial nitrogen cycling capacity and activity. *mSphere*, *6*(1), e01237-01220. https://doi.org/10.1128/mSphere.01237-20

Khalil, K., Mary, B., & Renault, P. (2004). Nitrous oxide production by nitrification and denitrification in soil aggregates as affected by O2 concentration. *Soil Biology and Biochemistry*, *36*(4), 687-699. https://doi.org/10.1016/j.soilbio.2004.01.004

Liu, R., Hu, H., Suter, H., Hayden, H. L., He, J., Mele, P., & Chen, D. (2016). Nitrification is a primary driver of nitrous oxide production in laboratory microcosms from different land-use soils. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01373

Mbuthia, L. W., Acosta-Martínez, V., DeBruyn, J., Schaeffer, S., Tyler, D., Odoi, E., Mpheshea, M., Walker, F., & Eash, N. (2015). Long term tillage, cover crop, and fertilization effects on microbial community structure, activity: implications for soil quality. *Soil Biology and Biochemistry*, *89*, 24-34.

https://doi.org/10.1016/j.soilbio.2015.06.016
McLaughlin, S. B., & Walsh, M. (1998). Evaluating environmental consequences of producing herbaceous crops for bioenergy. *Biomass and Bioenergy*, *14*(4), 317-324.
Pannu, M. W., Meinhardt, K. A., Bertagnolli, A., Fransen, S. C., Stahl, D. A., & Strand, S. E. (2019). Nitrous oxide emissions associated with ammonia-oxidizing bacteria abundance in fields of switchgrass with and without intercropped alfalfa. *Environmental Microbiology Reports*, *11*(5), 727-735. https://doi.org/10.1111/1758-2229.12790
Parrish, D. J., & Fike, J. H. (2005). The biology and agronomy of switchgrass for biofuels. *BPTS*, *24*(5-6), 423-459.
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. https://doi.org/10.1111/gcbb.12396
Shen, J. P., Zhang, L. M., Zhu, Y. G., Zhang, J. B., & He, J. Z. (2008). Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. *Environmental Microbiology*, *10*(6), 1601-1611. https://doi.org/10.1111/j.1462-2920.2008.01578.x
Smercina, D. N., Evans, S. E., Friesen, M. L., & Tiemann, L. K. (2020). Impacts of nitrogen addition on switchgrass root-associated diazotrophic community structure and function. *FEMS Microbiology Ecology*, *96*(12). https://doi.org/10.1093/femsec/fiaa208
Tao, R., Wakelin, S. A., Liang, Y., Hu, B., & Chu, G. (2018). Nitrous oxide emission and denitrifier communities in drip-irrigated calcareous soil as affected by chemical and organic fertilizers. *Science of the Total Environment*, *612*, 739-749. https://doi.org/10.1016/j.scitotenv.2017.08.258
Waldrop, M. P., Holloway, J. M., Smith, D. B., Goldhaber, M. B., Drenovsky, R. E., Scow, K. M., Dick, R., Howard, D., Wylie, B., & Grace, J. B. (2017). The interacting roles of climate, soils, and plant production on soil microbial communities at a continental scale [https://doi.org/10.1002/ecy.1883]. *Ecology*, *98*(7), 1957-1967. https://doi.org/https://doi.org/10.1002/ecy.1883
White, C. J., & Lehnert, N. (2016). Is there a pathway for N2O production from hydroxylamine oxidoreductase in ammonia-oxidizing bacteria? *Proceedings of the National Academy of Sciences*, *113*(51), 14474-14476. https://doi.org/10.1073/pnas.1617953114
White, P. J., Crawford, J. W., Díaz Álvarez, M. C., & García Moreno, R. (2012). Soil management for sustainable agriculture. *Applied and Environmental Soil Science*, *2012*, 1-3. https://doi.org/10.1155/2012/850739
Xiao, X., Xie, G., Yang, Z., He, N., Yang, D., & Liu, M. (2021). Variation in abundance, diversity, and composition of nirK and nirS containing denitrifying bacterial communities in a red paddy soil as affected by combined organic-chemical fertilization. *Applied Soil Ecology*, *166*, 104001. https://doi.org/https://doi.org/10.1016/j.apsoil.2021.104001

Yang, Y., Nie, J., Wang, S., Shi, L., Li, Z., Zeng, Z., & Zang, H. (2020). Differentiated responses of *nirS*- and *nirK*-type denitrifiers to 30 years of combined inorganic and organic fertilization in a paddy soil. *Archives of Agronomy and Soil Science*, *67*(1), 79-92. https://doi.org/10.1080/03650340.2020.1714032

Appendix

Table 33. Soil properties in relation to grass growing season, cover crop, N fertilization, and their combinations. Different lowercase letters indicate significant differences between treatment levels within groups. The letters were only shown in groups that have significant effects. Means were compared using Least Significant Difference (LSD) tests ($\alpha = 0.05$).

Factor[†]	pH	SWC[‡] (%)	NH_4^+-N	NO_3^--N	DOC	DON	TOC	TN	C: N ratio	Nitrification potential ($\mu g\ NO_2$-N $gdw^{-1}\ hr^{-1}$)
			($\mu g\ gdw^{-1}$)				($mg\ gdw^{-1}$)			
May	6.15±0.06	27.6±0.81^{a}	14.54±0.79^{a}	2.87±0.48	216.42±6.6^{a}	10.29±0.61	19.15±0.56^{b}	1.96±0.05^{b}	9.75±0.06	0.14±0.01^{b}
August	6.07±0.04	20.59±0.44^{b}	10.59±0.79^{b}	2.59±0.23	197.81±4.69^{b}	11.12±0.51	21.21±0.55^{a}	2.15±0.06^{a}	9.85±0.05	0.2±0.02^{a}
Control (NC)	6.11±0.05	23.27±0.75	11.68±0.83	2.18±0.31^{b}	205.88±5.9	10.47±0.59	19.65±0.57	2.01±0.06	9.78±0.05	0.16±0.02
Cereal rye (CR)	6.2±0.06	25.76±1.77	13.71±1.01	3.92±0.72^{a}	211.01±9.46	10.77±0.88	21.41±0.82	2.16±0.08	9.89±0.08	0.19±0.03
Blend (B)	6.01±0.09	24.07±1	13.19±1.47	2.66±0.37^{ab}	205.7±7.71	11.11±0.65	20±0.84	2.05±0.09	9.75±0.08	0.16±0.02
0N	6.19±0.04^{a}	24.77±1.1	13.44±0.96^{a}	2.5±0.32	213.8±7.01	11.28±0.58	20.44±0.58	2.08±0.06	9.82±0.04	0.17±0.02
67N	6.03±0.32^{b}	23.41±3.51	11.69±0.72^{b}	2.96±2.38	200.43±24.74	10.13±2.99	19.92±3.32	2.04±0.32	9.78±0.37	0.17±0.1
May-NC	6.17±0.08^{ab}	26.38±0.86^{b}	12.57±0.83^{b}	2.11±0.56	215.01±8.97	10.13±0.87	18.35±0.6	1.88±0.06	9.73±0.09	0.14±0.01
May-CR	6.36±0.06^{a}	30.57±2.42^{a}	16.02±1.26^{a}	4.69±1.3	222.65±15.43	10.26±1.39	20.7±1.13	2.11±0.11	9.83±0.12	0.16±0.03
May-B	5.9±0.17^{b}	27.06±0.92^{ab}	17.01±2.07^{a}	2.57±0.62	213.01±13.49	10.65±1.17	19.2±1.52	1.98±0.16	9.71±0.14	0.12±0.03
August-NC	6.05±0.06^{ab}	20.17±0.57^{c}	10.78±1.42^{b}	2.24±0.27	196.75±7.24	10.82±0.8	20.95±0.87	2.13±0.09	9.82±0.06	0.19±0.03
August-CR	6.04±0.06^{b}	20.96±1.02^{c}	11.4±1.12^{b}	3.15±0.62	199.36±10.31	11.28±1.15	22.12±1.21	2.22±0.11	9.95±0.12	0.22±0.04
August-B	6.12±0.07^{ab}	21.07±0.93^{c}	9.38±0.91^{b}	2.75±0.45	198.39±7.58	11.56±0.63	20.81±0.71	2.13±0.08	9.79±0.08	0.19±0.03
May-0N	6.2±0.08	29.12±1.41^{a}	15.82±1.16	2.59±0.57	226.68±11.45	11.05±0.97	19.75±0.88	2.02±0.09	9.78±0.07	0.14±0.02
May-67N	6.1±0.1	26.07±0.63^{b}	13.27±1.02	3.15±0.78	206.16±5.93	9.54±0.72	18.55±0.7	1.91±0.06	9.72±0.1	0.14±0.02
August-0N	6.18±0.04	20.43±0.73^{c}	11.07±1.32	2.41±0.32	200.92±7.07	11.52±0.67	21.12±0.76	2.14±0.08	9.87±0.04	0.19±0.02
August-67N	5.95±0.04	20.75±0.5^{c}	10.1±0.89	2.77±0.34	194.71±6.29	10.72±0.77	21.3±0.82	2.17±0.08	9.83±0.08	0.21±0.03
NC-0N	6.2±0.06	23.46±1.26^{b}	12.39±1.41	1.92±0.44	214.58±10.16	11.38±0.87	20.22±0.86	2.05±0.08	9.86±0.07	0.16±0.02
NC-67N	6.03±0.07	23.08±0.86^{b}	10.96±0.87	2.43±0.42	197.19±5.54	9.56±0.74	19.08±0.74	1.97±0.08	9.7±0.07	0.17±0.02
CR-0N	6.25±0.07	28.26±3.23^{a}	14.74±1.59	3.15±0.8	216.69±15.55	10.36±1.11	20.99±0.81	2.12±0.09	9.89±0.04	0.17±0.03
CR-67N	6.16±0.1	23.27±1.12^{b}	12.69±1.24	4.68±1.2	205.32±11.49	11.19±1.43	21.84±1.47	2.2±0.13	9.88±0.17	0.21±0.05
B-0N	6.12±0.13	23.92±1.41^{b}	14.25±2.17	3.02±0.42	209.36±13.5	12.02±1.13	20.32±1.45	2.1±0.15	9.69±0.04	0.18±0.03
B-67N	5.9±0.13	24.21±1.51^{b}	12.13±2.05	2.3±0.61	202.04±8.28	10.2±0.56	19.69±0.93	2.01±0.1	9.82±0.15	0.14±0.02

Table 33. Continued.

Factor†	pH	SWC‡ (%)	NH_4^+-N	NO_3^--N	DOC	DON	TOC	TN	C: N ratio	Nitrification potential ($\mu g\ NO_2$-N $gdw^{-1}\ hr^{-1}$)
			($\mu g\ gdw^{-1}$)				($mg\ gdw^{-1}$)			
May-NC-0N	6.23±0.1	26.99±1.57^{b}	13.43±1.46	1.9±0.81	227.82±15.8	11.22±1.37	19.09±0.95	1.95±0.09	9.79±0.14	0.14±0.03
May-NC-67N	6.12±0.12	25.76±0.76^{b}	11.71±0.78	2.32±0.82	202.21±6.93	9.04±1.01	17.61±0.68	1.82±0.06	9.68±0.12	0.14±0.01
May-CR-0N	6.35±0.11	35.51±3.01^{a}	17.64±1.78	4.08±1.47	230.38±29.31	10.63±2.23	21.08±1.28	2.14±0.14	9.86±0.05	0.14±0.04
May-CR-67N	6.38±0.09	25.63±1.39^{bc}	14.41±1.59	5.29±2.35	214.93±14.57	9.9±1.98	20.32±2.05	2.07±0.18	9.79±0.25	0.17±0.06
May-B-0N	6.01±0.24	27±1^{b}	18.77±2.43	2.49±0.57	220.73±24.05	11.13±2.22	19.76±2.94	2.04±0.3	9.69±0.05	0.14±0.05
May-B-67N	5.79±0.26	27.13±1.72^{b}	15.24±3.45	2.66±1.2	205.3±15.18	10.17±1.16	18.64±1.39	1.92±0.13	9.74±0.3	0.11±0.03
August-NC-0N	6.17±0.06	19.93±0.9^{d}	11.35±2.48	1.93±0.43	201.34±11.94	11.55±1.17	21.36±1.39	2.15±0.14	9.92±0.06	0.18±0.04
August-NC-67N	5.94±0.08	20.4±0.75^{d}	10.22±1.56	2.55±0.3	192.16±8.73	10.09±1.12	20.55±1.12	2.12±0.13	9.72±0.09	0.21±0.04
August-CR-0N	6.15±0.05	21±2.16^{d}	11.84±1.74	2.22±0.54	203.01±12.05	10.09±0.85	20.89±1.19	2.11±0.13	9.93±0.05	0.19±0.04
August-CR-67N	5.93±0.08	20.91±0.44^{d}	10.96±1.64	4.07±0.96	195.71±18.5	12.47±2.12	23.36±2.09	2.34±0.17	9.97±0.25	0.24±0.07
August-B-0N	6.23±0.1	20.84±1.41^{d}	9.74±1.59	3.56±0.56	197.99±13.61	12.9±0.75	20.88±0.97	2.16±0.11	9.68±0.06	0.22±0.04
August-B-67N	6±0.05	21.29±1.41^{cd}	9.02±1.12	1.93±0.44	198.79±9.08	10.23±0.35	20.74±1.17	2.1±0.14	9.89±0.13	0.16±0.03

† NC, no cover crop; CR, cereal rye; B, blend; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization.

‡SWC, soil water content; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; TOC, total organic carbon; TN, total nitrogen; NP, nitrification potential.

Table 34. Absolute abundances of *nifH*, *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes as well as relative abundances of *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* genes in relation to grass growing season, cover crop, N fertilization, and their combinations. Different lowercase letters indicate significant differences between treatment levels within groups. The letters were only shown in groups that have significant effects. Means were compared using Least Significant Difference (LSD) tests ($\alpha = 0.05$).

Factor†	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*	16S rRNA	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*
	Log(functional gene copies g^{-1} dry weight soil)						Log(functional gene copies/16S rRNA gene copies)				
May	5.55±0.05	4.61±0.1[b]	7.05±0.04	7.18±0.03[b]	6.71±0.03[b]	9.09±0.06	-3.54±0.05	-4.48±0.08[b]	-2.04±0.04	-1.91±0.06	-2.38±0.04[b]
August	5.6±0.04	4.94±0.1[a]	7.06±0.03	7.32±0.02[a]	6.93±0.03[a]	9.15±0.05	-3.55±0.05	-4.2±0.09[a]	-2.08±0.04	-1.82±0.04	-2.22±0.04[a]
Control (NC)	5.53±0.04	4.59±0.1	7.02±0.03	7.21±0.02	6.82±0.03	9.09±0.05	-3.56±0.04	-4.49±0.09	-2.07±0.04	-1.88±0.05	-2.27±0.04
Cereal rye (CR)	5.65±0.05	5.06±0.13	7.11±0.04	7.28±0.04	6.85±0.05	9.22±0.07	-3.57±0.08	-4.16±0.11	-2.12±0.05	-1.94±0.07	-2.38±0.06
Blend (B)	5.6±0.07	4.85±0.11	7.08±0.05	7.31±0.04	6.78±0.05	9.07±0.09	-3.47±0.09	-4.22±0.09	-1.99±0.06	-1.76±0.07	-2.29±0.07
0N	5.6±0.05	4.65±0.11[b]	7.04±0.03	7.26±0.03	6.79±0.03	9.1±0.05	-3.5±0.05	-4.45±0.09	-2.06±0.03	-1.84±0.05	-2.31±0.05
67N	5.55±0.04	4.9±0.09[a]	7.08±0.04	7.24±0.03	6.85±0.03	9.14±0.06	-3.58±0.05	-4.23±0.07	-2.06±0.04	-1.89±0.05	-2.29±0.05
May-NC	5.48±0.06	4.43±0.15	7.02±0.06	7.13±0.03	6.71±0.04	9.1±0.08	-3.62±0.05[ab]	-4.67±0.13	-2.08±0.06	-1.97±0.08	-2.4±0.07
May-CR	5.73±0.09	4.99±0.15	7.12±0.06	7.25±0.06	6.74±0.05	9.19±0.07	-3.46±0.11[a]	-4.21±0.13	-2.07±0.05	-1.95±0.1	-2.46±0.05
May-B	5.52±0.08	4.57±0.13	7.04±0.09	7.22±0.06	6.68±0.07	8.96±0.14	-3.44±0.12[ab]	-4.39±0.06	-1.92±0.08	-1.74±0.13	-2.28±0.09
August-NC	5.57±0.06	4.76±0.14	7.02±0.04	7.29±0.03	6.94±0.04	9.07±0.07	-3.5±0.07[ab]	-4.31±0.12	-2.05±0.05	-1.79±0.06	-2.14±0.04
August-CR	5.57±0.06	5.13±0.22	7.09±0.07	7.32±0.04	6.96±0.08	9.26±0.13	-3.69±0.1[b]	-4.12±0.2	-2.17±0.07	-1.93±0.1	-2.3±0.1
August-B	5.69±0.1	5.13±0.11	7.11±0.06	7.4±0.05	6.89±0.06	9.18±0.1	-3.49±0.14[ab]	-4.06±0.15	-2.07±0.1	-1.79±0.07	-2.3±0.12
May-0N	5.59±0.06	4.51±0.15	7.02±0.05	7.19±0.03	6.68±0.04	9.09±0.07	-3.5±0.06	-4.58±0.13	-2.07±0.04	-1.9±0.07	-2.41±0.05
May-67N	5.52±0.07	4.7±0.11	7.09±0.06	7.17±0.04	6.74±0.04	9.09±0.09	-3.57±0.08	-4.39±0.09	-2±0.06	-1.92±0.09	-2.35±0.07
August-0N	5.61±0.07	4.78±0.15	7.06±0.04	7.33±0.03	6.9±0.04	9.11±0.07	-3.5±0.08	-4.33±0.13	-2.05±0.05	-1.78±0.06	-2.21±0.07
August-67N	5.59±0.06	5.11±0.12	7.07±0.05	7.32±0.03	6.96±0.04	9.18±0.08	-3.6±0.07	-4.08±0.1	-2.12±0.06	-1.87±0.06	-2.22±0.06
NC-0N	5.6±0.06	4.45±0.17	7.04±0.04	7.24±0.03[ab]	6.82±0.05	9.09±0.08	-3.48±0.07	-4.64±0.14	-2.05±0.05	-1.84±0.08	-2.27±0.06
NC-67N	5.45±0.06	4.74±0.11	7.01±0.06	7.17±0.03[b]	6.83±0.04	9.09±0.06	-3.64±0.05	-4.35±0.11	-2.08±0.06	-1.92±0.06	-2.26±0.06
CR-0N	5.72±0.09	4.86±0.18	7.05±0.04	7.32±0.05[a]	6.81±0.06	9.15±0.06	-3.43±0.07	-4.29±0.19	-2.1±0.04	-1.83±0.06	-2.35±0.07
CR-67N	5.58±0.06	5.26±0.17	7.16±0.08	7.25±0.06[ab]	6.89±0.08	9.3±0.13	-3.72±0.13	-4.04±0.13	-2.13±0.08	-2.05±0.12	-2.41±0.09
B-0N	5.47±0.1	4.82±0.15	7.02±0.08	7.23±0.07[ab]	6.71±0.07	9.07±0.11	-3.6±0.14	-4.24±0.13	-2.04±0.06	-1.84±0.08	-2.36±0.11
B-67N	5.74±0.06	4.87±0.17	7.13±0.08	7.38±0.05[a]	6.86±0.07	9.07±0.15	-3.34±0.1	-4.2±0.13	-1.94±0.11	-1.69±0.12	-2.22±0.1

Table 34. Continued.

Factor†	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*	16S rRNA	*nifH*	*amoA*	*nirK*	*nirS*	*nosZ*
	Log(functional gene copies g^{-1} dry weight soil)						Log(functional gene copies/16S rRNA gene copies)				
May-NC-0N	5.52±0.06	4.22±0.22	7.01±0.07	7.16±0.02^{cd}	6.68±0.05	9.09±0.12	-3.56±0.08	-4.86±0.18	-2.07±0.08	-1.93±0.12	-2.41±0.09
May-NC-67N	5.44±0.11	4.64±0.17	7.03±0.09	7.1±0.04^{d}	6.73±0.05	9.12±0.11	-3.68±0.06	-4.48±0.17	-2.09±0.09	-2.02±0.09	-2.39±0.11
May-CR-0N	5.91±0.03	4.96±0.29	7.09±0.04	7.36±0.07^{ab}	6.73±0.04	9.18±0.06	-3.27±0.06	-4.22±0.26	-2.09±0.04	-1.82±0.12	-2.45±0.04
May-CR-67N	5.56±0.11	5.01±0.15	7.16±0.13	7.14±0.07^{cd}	6.74±0.09	9.21±0.14	-3.65±0.18	-4.2±0.1	-2.05±0.1	-2.07±0.16	-2.46±0.1
May-B-0N	5.39±0.13	4.63±0.17	6.95±0.13	7.11±0.07^{cd}	6.62±0.11	9.01±0.16	-3.61±0.13	-4.38±0.11	-2.05±0.05	-1.9±0.1	-2.38±0.08
May-B-67N	5.64±0.07	4.52±0.23	7.13±0.14	7.33±0.05^{ab}	6.74±0.11	8.91±0.25	-3.27±0.18	-4.39±0.05	-1.78±0.11	-1.58±0.23	-2.17±0.16
August-NC-0N	5.68±0.1	4.67±0.26	7.06±0.06	7.33±0.05^{ab}	6.96±0.06	9.09±0.12	-3.41±0.1	-4.42±0.2	-2.03±0.08	-1.75±0.1	-2.13±0.07
August-NC-67N	5.46±0.07	4.85±0.14	6.99±0.07	7.24±0.04^{bc}	6.92±0.06	9.06±0.06	-3.59±0.09	-4.21±0.12	-2.07±0.08	-1.82±0.05	-2.14±0.04
August-CR-0N	5.54±0.12	4.77±0.25	7.02±0.07	7.29±0.08^{abc}	6.88±0.11	9.13±0.12	-3.59±0.04	-4.36±0.31	-2.11±0.08	-1.84±0.06	-2.25±0.13
August-CR-67N	5.6±0.04	5.5±0.29	7.17±0.11	7.36±0.05^{ab}	7.03±0.11	9.38±0.24	-3.79±0.2	-3.88±0.22	-2.22±0.13	-2.03±0.2	-2.35±0.16
August-B-0N	5.54±0.15	5.02±0.22	7.09±0.09	7.35±0.07^{ab}	6.8±0.08	9.13±0.15	-3.59±0.27	-4.11±0.24	-2.04±0.12	-1.78±0.13	-2.33±0.22
August-B-67N	5.83±0.09	5.23±0.06	7.14±0.1	7.44±0.08^{a}	6.97±0.05	9.24±0.16	-3.4±0.09	-4.01±0.21	-2.1±0.17	-1.8±0.08	-2.27±0.14

† NC, no cover crop; CR, cereal rye; B, blend; 0N, no N fertilization; 67N, 67 kg N ha^{-1} fertilization.

Table 35. Pearson correlation coefficients between absolute and relative abundances of N-cycle genes and soil properties.

		nifH	*amoA*	*nirK*	*nirS*	*nosZ*	16S	pH	SWC†	NH_4^+-N	NO_3^--N	DOC	DON	TOC	TN	C:N	NP
Absolute abundance	*nifH*	1.000	0.230	0.526**	0.644**	0.463**	0.468**	0.259*	0.198	0.066	0.082	0.242	0.271*	0.296*	0.301*	0.068	0.136
	amoA	-	1.000	0.494**	0.301*	0.549**	0.530**	0.090	-0.110	0.260*	0.653**	0.295*	0.456**	0.501**	0.500**	0.007	0.516**
	nirK	-	-	1.000	0.514**	0.649**	0.704**	0.378**	0.118	0.176	0.328**	0.410**	0.427**	0.399**	0.422**	0.070	0.286*
	nirS	-	-	-	1.000	0.574**	0.332**	0.130	-0.044	-0.114	0.261*	0.136	0.335**	0.397**	0.380**	0.184	0.333**
	nosZ	-	-	-	-	1.000	0.547**	0.003	-0.306*	-0.058	0.351**	0.228	0.522**	0.542**	0.557**	-0.004	0.481**
	16S	-	-	-	-	-	1.000	0.333**	0.121	0.328**	0.402**	0.580**	0.537**	0.619**	0.661**	-0.110	0.569**
Relative abundance	*nifH*	1.000	-0.011	0.651**	0.800**	0.639**	-	-0.129	0.042	-0.289*	-0.353**	-0.403**	-0.334**	-0.398**	-0.438**	0.174	-0.483**
	amoA	-	1.000	0.139	0.072	0.240	-	-0.105	-0.207	0.099	0.514**	-0.021	0.197	0.198	0.170	0.078	0.248*
	nirK	-	-	1.000	0.771**	0.772**	-	-0.123	-0.063	-0.300*	-0.265*	-0.441**	-0.365**	-0.505**	-0.543**	0.218	-0.537**
	nirS	-	-	-	1.000	0.824**	-	-0.300*	-0.174	-0.413**	-0.304*	-0.544**	-0.406**	-0.436**	-0.485**	0.188	-0.418**
	nosZ	-	-	-	-	1.000	-	-0.393**	-0.376**	-0.433**	-0.211	-0.517**	-0.243	-0.325**	-0.364**	0.128	-0.312*
Physicochemical properties	pH	-	-	-	-	-	-	1.000	0.354**	0.306*	0.265*	0.378**	0.282*	0.152	0.167	0.010	0.172
	SWC	-	-	-	-	-	-	-	1.000	0.515**	0.043	0.379**	0.081	0.015	0.020	-0.052	-0.157
	NH_4^+-N	-	-	-	-	-	-	-	-	1.000	0.373**	0.595**	0.415**	0.330**	0.324**	0.089	0.161
	NO_3^--N	-	-	-	-	-	-	-	-	-	1.000	0.410**	0.546**	0.502**	0.523**	0.014	0.684**
	DOC	-	-	-	-	-	-	-	-	-	-	1.000	0.699**	0.626**	0.642**	0.025	0.507**
	DON	-	-	-	-	-	-	-	-	-	-	-	1.000	0.672**	0.678**	0.001	0.619**
	TC	-	-	-	-	-	-	-	-	-	-	-	-	1.000	0.979**	0.177	0.717**
	TN	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	0.016	0.767**
	C:N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	-0.166
	NP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000

Significance level: * p-value ≤ 0.05; ** p-value ≤ 0.01.

† SWC, soil water content; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; TOC, total organic carbon; TN, total nitrogen; NP, nitrification potential.

CONCLUSION

This study focused on investigating the effects of agricultural management practices on microbial assemblages involved in nitrogen (N) transformations in agroecosystems, including high-input cropland (cotton) and low-input native grassland in Tennessee.

For cropland, the focus was to compare the influence of conservation (no tillage, winter cover crops, no N fertilization) and conventional (tillage, no winter cover crops, inorganic N fertilization) agricultural management practices on N-cycling microbial population sizes and functional activity, to reveal the biotic mechanisms of N transformations under conservation management regimes. For low N-required native C_4 grass ecosystems, one major focus was on the effect of N fertilization rate on N-cycling microbes, especially diazotrophs which can potentially supplement N to C_4 grass systems by fixing N in atmosphere, as well as ammonia-oxidizing bacteria (AOB) which play important role in N losses through N_2O emission and nitrate leaching. Another focus in native grass ecosystems was the effects of winter annual cover crops on soil health from the perspective of soil N-cycling functional microbes.

The results of this study showed that for both cropland soils and grassland soils, the statistical dispersion, or variability, of the N-cycling microbial populations by activity (i.e., transcripts) was consistently and significantly higher than for functional potential (i.e., gene copies). Both the functional potential and activity of N-cycling microbial populations varied seasonally in all agroecosystems studied. This study revealed that both genetic and transcriptional assessments can provide important information about the microbial populations and functions under agricultural management practices. But the N-cycling microbial populations based on functional potential and activity under agricultural management practices were different between cropland soils and grassland soils: in the long-term cotton field, larger and more significant variation of N-cycling microbial activity than their population size was observed under different season and management practices. In contrast, in native C_4 grassland soils in East TN, more significant variation of N-cycling microbial population size rather than their activity under different season and management practices was observed.

N fertilization significantly affected N-cycling microbes for both cropland and native grasslands. Leguminous cover crops like hairy vetch can promote functional activity of N-

cycling populations through the growing season, equaling or exceeding the effects of inorganic N fertilizer in cotton land ecosystem, suggesting the potential of leguminous cover crops for replacing inorganic N fertilization in high-input cropland from the perspective of microbial ecology. High N fertilization rate inhibited both functional activity, diversity, and changed community composition of diazotrophs but promoted both abundance, activity, diversity, of ammonia-oxidizing bacteria as well as N_2O emission and nitrification potential in native C_4 grassland, indicating that excessive N fertilization had negative influence on soil functional microbial diversity, N fixation and reservation, and soil and downstream environmental health. Therefore, the significance of reduced N fertilization may be substantial economically and environmentally.

Compared to big bluestem, switchgrass systems had a less stable diazotrophic community structure and higher AOB population size and activity, nitrification potential and N_2O emission under N fertilization. This can be explained by the higher fertilizer N use efficiency and C:N ratio of big bluestem than switchgrass, which can potentially mitigate the N impact on diazotrophs for N fixation but also decrease the substrate (ammonium) for nitrification under big bluestem. This difference between big bluestem and switchgrass suggested a higher potential for N losses in switchgrass systems than in big bluestem systems.

In addition, although it would be difficult to compare grazing management and hay harvest management in native grasslands at different regions due to the complex effects of environmental factors, this study speculated that compared to hay harvesting, grazing may have the potential to mitigate the impact of N fertilization on soil nutrient content and N-cycling populations, which need to be further studied.

In summary, this study used traditional molecular techniques (qPCR and qRT-PCR) and high-throughput amplicon sequencing technology to investigate the biotic mechanisms of N-cycling under agricultural management practices in cropland and native grassland at both genetic and transcriptional level, which has higher resolution than only focused on one level in examining the effects of agricultural management on N-cycling microbial communities. From the perspective of soil N-cycling microbial ecology and taken both environmental health and economic costs into consideration, we suggested that leguminous

winter cover crops and reduced N fertilization rate can be considered as preferred agricultural management practices, and big bluestem system may have lower N loss potentials than switchgrass system.

The three major microbial N transformation processes in soil ecosystems, N fixation, nitrification, and denitrification, have been well studied, especially in agroecosystems. However, other important N-cycling processes in soils, such as N mineralization, dissimilative reduction of nitrate to ammonia (DRNA), and anaerobic ammonium oxidation (Anammox), also need to be studied due to that they are also significant processes of N transformations in terrestrial ecosystems and play important roles in the fate of N. In addition, it should be recognized that the major source of N in soils is crops or plants rather than fertilizers. Therefore, in future research of nitrogen cycles, both agricultural management and plant characteristics (such as yields and C:N ratio of tissues) need to be consideration to better assist in guiding the agricultural management regimes.

VITA

Jialin Hu was born and raised in an ordinary working-class family in Shandong, China. She got her Bachelor of Engineering degree in Environmental Engineering from China Jiliang University, China. After graduating, she decided to pursue advanced studies and got a Master of Science degree in Environmental Science (environmental microbiology focus) from Nanjing University, China. She then came to the University of Tennessee to pursue her Ph.D. in Plant, Soil, and Environmental Science in Department of Biosystems Engineering and Soil Science. Jialin plans to start her postdoctoral studies in North Carolina State University for about two years and then return to her country where she hopes to become an independent principal investigator in a university or research institute and dedicates herself to environmental health.