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A cross-scale nexus: Microbial functional genes in tandem with molecular-level nitrogen fluxes and implications for field-scale biogeochemical processes

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A cross-scale nexus: Microbial functional genes in tandem with molecular-level nitrogen fluxes and implications for field-scale biogeochemical processes

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Abstract

Nitrogen (N) biogeochemical cycling entails close networking between soil nutrients, plant species and resulting microbial communities, and abiotic factors such as temperature, moisture, and pH. To better understand transformations of N in soil microsites, which are known to be rich in N-cycling behavior, the microdialysis technique was employed to generate in situ data on temporal alterations of N during wetting-drying conditions in both the field and laboratory. The objectives were to 1) determine the lifespan of the microdialysis technique in field conditions, 2) evaluate the ability of the method to yield N fluxes that would be differential between the two plant communities, and 3) better understand N fluxes in soil associated with changing soil water content (wetting and drying). Microdialysis was deployed for approximately five months in the field and microdialysis sampling generated N fluxes that were distinct in soils of the two plant communities. While KCl extraction data resulted in greater inorganic N in orchardgrass soil in June, microdialysis, qPCR analysis of *amoA* gene abundances, and other physicochemical data such as pH supported the notion that native grass soil provided greater nitrification potential which could indicate the greater potential for N uptake in soil with native plants. Nitrogen fluxes were measured for five days in a lab study to provide greater detail in temporal data on the fluxes of nitrate-N, ammonium-N, and amino acid fluxes upon rewetting of field-moist and air-dried soil. There were also some significant parallels when comparing diffusive flux results of the field and laboratory studies. Frequent rainfall and drying events in the field resulted in increased nitrate-N fluxes which were analogous to the results of the lab study when using field-moist soil, signifying that the presence of water in the soil increased mineralization rates, which led to increased nitrification processes and a decrease of detectable ammonium-N and amino acids in soil solution. Nitrogen fluxes measured in the field during

drought where similar to the flux patterns observed in the air-dried soil in the lab study. The addition of water resulted in a flush of mineralization and increased ammonium-N and amino acid fluxes. In this way, expansion of the current knowledge base of N cycling by the use of a tandem approach utilizing microdialysis flux and microbial functional genes could provide more insights into microsite N processes which dictate larger biogeochemical processes. Furthering our understanding of the effects of wetting/drying cycling on N movement in the soil will only become more pertinent as more frequent and extreme climatic conditions persist.

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Preface

This thesis is a compilation of manuscripts in preparation for publication in peer-reviewed scientific journals. Individual chapters are formatted according to journal guidelines for publication.

CHAPTER 2. Maddala, S., Savin, M. C., & Philip, D. 2022. A cross-scale nexus: Use of microdialysis to monitor nitrogen fluxes in tandem with physicochemical and microbial analysis of grassland soil. (In preparation)

CHAPTER 3. Maddala, S., Savin, M. C., & Philip, D. 2022. Elucidating transient effects of wetting-drying cycles in soil by utilizing the in situ, nondestructive microdialysis technique. (In preparation)

CHAPTER 1: Literature Review

1. The Nitrogen Cycle and Microorganisms

Soil nitrogen (N) is among the substantial limiting nutrients for plant biomass production and species composition in terrestrial ecosystems due to its essential role in biochemical compounds and processes (Inselsbacher et al., 2011). Although the atmosphere, which is 78% gaseous nitrogen (N_2), is an abundant source of N, the very stable triple bond between the N atoms in N_2 makes it inaccessible to most organisms, and if energy and carbon needs are met, regulates primary productivity in many ecosystems (Bernhard, 2010; Pajares & Bohannan, 2016; Weil & Brady, 2016). Another level of complexity associated with nitrogen is that it exists in multiple oxidation states and chemical forms, undergoing transformations as microorganisms catalyze nitrogen for growth and energy (Bernhard, 2010; Pajares & Bohannan, 2016). Soil microorganisms play a pivotal role in processes involved in biogeochemical cycling, and microbial community dynamics can aid in understanding temporal and spatial variations in nitrogen cycling (Pajares & Bohannan, 2016). Microbial activity, enzymes, and the environmental conditions that regulate their activity are interconnected. Measuring soil enzyme activity directly or indirectly by analyzing key enzyme-encoding genes related to nitrogen cycling processes, and correlating them to environmental parameters such as pH, temperature, and moisture can lead to critical findings related to the global nitrogen cycle (Lammel et al., 2015). While assays to directly measure soil enzymatic activity exist (Kandeler et al., 2011), recent studies have shown the potential of using marker genes as indicators of associated biogeochemical N processes (Lammel et al., 2015; Levy-Booth et al., 2014; Rocca et al., 2015). The quanitification of genes using the qPCR technique enables approximate quantification of microorganisms that belong to taxonomic groups or functional communities such as those carrying out N processes (Lammel et al., 2015), making this analysis more powerful than

observing nutrient dynamics exclusively. Use of qPCR can provide insight into how management and conditions of the abiotic environment are connected to the functional community, thus affecting services provided within and from an ecosystem.

Nitrogen cycling involves microbiological processes: nitrogen fixation, immobilization, mineralization, nitrification, dissimilatory nitrate reduction to ammonia, anammox and denitrification, with fixation, nitrification, and denitrification being the most commonly studied processes in the soil (Hayatsu et al., 2008; Pajares & Bohannan, 2016). Several genes encoding for enzymes that play critical roles in each of these three N processes have been identified and used as markers to determine the abundance of microbial functional groups in various soils (Angel et al., 2018; Gaby & Buckley, 2012; Hayatsu et al., 2008; Lammel et al., 2015; Levy-Booth et al., 2014; Pajares & Bohannan, 2016).

1.1. Mineralization

In terrestrial ecosystems, most of the N input into the soil are plant and microbial residues in the form of polymers, and have to be degraded into smaller units by extracellular enzymes (Geisseler et al., 2010). Extracellular depolymerases such as proteases, chitinases, and peptidoglycan hydrolases are required to degrade complex organic polymers into oligmers followed by small, soluble organic molecules such as simple amino amino compounds or amine groups (Weil & Brady, 2016). These small organic molecules can either be taken up directly or be subjected to extracellular mineralization using enzymes such as urease, resulting in ammonium ions which are considered the preferred sources of N for bacteria and fungi (Geisseler et al., 2010).

1.2. Nitrogen Fixation

Gaseous nitrogen (N_2) in the atmosphere is extremely stable due to the presence of a triple bond, which requires a large amount of energy to break (8 electrons and at least 16 ATP molecules), and is the reason for nitrogen's decreased bioavailability to most organisms (Bernhard, 2010). However, nitrogen-fixing (diazotrophic) microorganisms have the ability to reduce N_2 to reactive and biologically available forms, and are significant in that they provide the natural biological sources of fixed nitrogen in the biosphere (Bernhard, 2010; Gaby & Buckley, 2012). Symbiotic and free-living diazotrophs accomplish nitrogen fixation using the nitrogenase enzyme, whose multiple subunits are encoded by the genes *nifH*, *nifD*, and *nifK*, of which the *nifH* gene (encoding the nitrogenase reductase subunit) is the most sequenced and has become the marker gene for studying the diversity and abundance of diazotrophs (Gaby & Buckley, 2012; Levy-Booth et al., 2014; Pajares & Bohannan, 2016).

Phylogenetic analyses of *nifH* gene sequences have revealed five primary clusters of genes homologous to *nifH*, with Cluster I consisting of aerobic N fixers, Cluster II as the alternative nitrogenase cluster, Cluster III consisting of anaerobic N fixers from the domains *Bacteria* and *Archaea*, and Clusters IV and V that are not involved in nitrogen fixation (Gaby & Buckley, 2012). Due to the presence of a wide variety of PCR primer sets from multiple N-fixers, there have been many studies with the aim of evaluting the quality and specificity of the available primer pairs (Gaby & Buckley, 2012). Gaby and Buckley (2012) reported that the combinations of F2-R6, IGK3-DVV, and Ueda19F-388R performed well with DNA from a diversity of phylogenetic groups, with the primer pair IGK3-DVV performing the best. However, Angel et al. (2018) reported that only the primer pairs of F2-R6, Ueda19F-R6, and IGK3-DVV produced PCR fragments of the correct size, and that primer pairs should be chosen depending

on the investigated environmental sample. This was due to the fact that F2-R6 had the propensity to discriminate against cyanobacteria, yet captured many sequences in subclusters from Cluster III, and resulted in an increased amplification of genes from root and rhizosphere samples in comparison to the other two primer pairs. The IGK3-DVV discriminated against Alphaproteobacteria but amplified sequences within the subculture IIIC, which is affiliated with Clostridia. Primer pair Ueda19F-R6 exhibited the least bias in the Angel et al. (2018) study, but discriminated against Firmicutes and the subcluster IIIC.

1.3. Nitrification

Nitrification processes include aerobic oxidation of ammonia (NH_3) to nitrite (NO_2^-) and oxidation of nitrite to nitrate (NO_3^-) and can be further divided into two pathways: autotrophic nitrification and heterotrophic nitrification based on the carbon source of the nitrifying organisms. Chemautotrophic ammonia-oxidizing bacteria (AOB), represented by *Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus* spp. (in the ß- and γ- subclasses of the *Proteobacteria* phylum), perform ammonia oxidation in which ammonia is oxidized to hydroxylamine by the ammonia monooxygenase (AMO) enzyme (Hayatsu et al., 2008; Pajares & Bohannan, 2016; Rotthauwe et al., 1997). The α-subunit of the AMO enzyme is encoded by the *amoA* gene, which has been designated as the marker gene for molecular studies of AOB communities (Rotthauwe et al., 1997) because of its highly conserved nucleotide sequence and its essential role in energy generating metabolism (Levy-Booth et al., 2014).

However, recent 16S rRNA surveys have found that though Archaea were previously thought to inhabit only extreme environments, Crenarchaeota, one of the four phyla of Archaea, have been found in various moderate environments such as soils (Hayatsu et al., 2008).

Metagenomic studies of the Sargasso Sea revealed that the AMO gene is also present on an archaeal-associated scaffold (Venter et al., 2004) and that archaea are capable of oxidizing ammonia, which have since been included into the first step of nitrification and are called ammonia-oxidizing archaea or AOA. Phylogenetic analysis showed low similarity between AOB and AOA *amoA* genes, allowing for the distinction between them using PCR techniques (Hayatsu et al., 2008). Leininger et. al (2006) investigated the abundance of the *amoA* gene in 12 pristine and agricultural soils of three climatic zones and found that archaeal *amoA* genes were up to 3000-fold more abundant than bacterial *amoA* genes. Conversely, other studies have reported that AOB contribution to nitrification activity in the soil is extremely large, leading to inconclusive findings on whether archaeal or bacterial nitrification are the major contributors to soil nitrification (Hayatsu et al., 2008), as it seems to rely heavily on the management of, nutrient availability in, and inorganic and organic inputs to the ecosystem. The second portion of ammonia oxidation is the conversion of the hydroxylamine, produced in the first segment, into nitrite using the hydroxylamine oxidoreductase (HAO) enzyme (Levy-Booth et al., 2014). Interestingly, Crenarchaeota lack HAO homologs, leading to the hypothesis that AOA use a different pathway of ammonia oxidation that is distinct from the AOB pathway (Levy-Booth et al., 2014). Differences between bacterial and archaeal ammonia oxidizers are exemplified by the fact that AOA populations appear to be insensitive to oxygen availability; they can tolerate extended periods of low oxygen and can also react faster than AOB under alternating between aerobic/anaerobic conditions (Levy-Booth et al., 2014; Pajares & Bohannan, 2016). In this way, nitrifying ammonia oxidation can still occur during anaerobic conditions.

The second nitrification process – oxidization of nitrite to nitrate –occurs using the nitrite oxidoreductase (NOX/NXR) enzyme, and the organisms that carry out this reaction are called

nitrite-oxidizing bacteria (NOB) (Levy-Booth et al., 2014). Based on 16S rRNA gene sequences, NOB have been classified into four genera: *Nitrobacter* (α-proteobacteria), *Nitrospina* (δ-proteobacteria), *Nitrococcus* (ß-proteobacteria), and *Nitrospira* (class Nitrospira, phylum Nitrospirae) (Hayatsu et al., 2008).

Heterotrophic nitrification is performed by a wide range of bacteria and fungi that can oxidize ammonia to hydroxylamine, nitrite, and nitrate. Bacteria such as *Paracoccus denitrficans*, *Alcaligenes faecalis*, and *Pseudomonas putida* and fugal species such as *Aspergillus wentii* and *Penicillium* spp. have all been shown to oxidize ammonia to nitrite or nitrite to nitrate (Hayatsu et al., 2008). Though the physiological role of heterotrophic nitrification is not yet clear, this process generally plays a predominant role in nitrification activity under extreme conditions such as strongly acidic soil due to the inhibition of autotrophic nitrifier activity (Hayatsu et al., 2008; Zhang et al., 2015).

1.4. Denitrification

Denitrification is the full or partial dissimilative process by which nitrate is sequentially reduced to dinitrogen gas (N_2) via the intermediates NO and N_2O (Levy-Booth et al., 2014; Pajares & Bohannan, 2016). The process begins with the activation of the nitrate reductase (NAR), which is encoded by the *nas*, *nar*, and *nap* operons, of which the *narG* and *napA* genes are the utilized the most in nitrate reduction studies (Levy-Booth et al., 2014). After nitrate has been converted to nitrite, nitrite is converted to NO by the nitrite reductase (NIR) enzyme. The Nir enzyme is the key enzyme of denitrification, since it catalyzes the rate limiting step of converting NO_2^- to gaseous products (Pajares & Bohannan, 2016). Two structurally different NIRs, a result of convergent evolution, are found among bacteria and evolution and generally do

not occur in the same organism: one contains copper (Cu-NIR) and is encoded by the *nirK* gene, and the other contains heme c and heme d1 (cd1-NIR) and is encoded by the *nirS* gene (Hayatsu et al., 2008; Levy-Booth et al., 2014). The NO produced is then converted to N_2O by the nitric oxide reductase (NOR) enzyme, which is encoded for by the *norB* gene. Finally, N_2O is reduced to N_2 by the cytochrom *bc* nitrous oxide reductase (NOS) enzyme, which is frequently studied using the *nosZ* gene (Levy-Booth et al., 2014).

Denitrifying organisms are prevalent in soil and distributed among taxonomically diverse groups of microorganisms such as bacteria, archaea, and fungi, which have acquired the ability to denitrify via different evolutionary mechanisms (Levy-Booth et al., 2014; Pajares & Bohannan, 2016). Fungi including Ascomycota (e.g. *Fusarium oxysporum*, *Fusarium solani*, *Cylindrocarpon tonkinense*, and *Gibberella fujiuroii*) and Basidomycota (e.g. *Trichosporon cutaneum*) contribute to the denitrification potential in soils and play a significant role in N_2O production (Hayatsu et al., 2008; Levy-Booth et al., 2014; Pajares & Bohannan, 2016). The fungal denitrification system is localized in the mitochondria and functions similarily to its bacterial counterpart. The properties of fungal NAR are thought to be similar to the dissimilatory NARs of *Escherichia coli* and other denitrifying bacteria; fungal NIR contains copper and is an ortholog of bacterial NIR-K (Hayatsu et al., 2008; Levy-Booth et al., 2014). However, fungal NOR is distinct from bacterial NOR; fungal NORs have been classified to be in the cytochrome P450 (P450) super-family, while bacterial NORs contain cytochromes *bc* (Hayatsu et al., 2008).

Fungi do not contain an enzyme orthologous to NOS, leading to the hypothesis that fungi are responsible for a large portion of N_2O emissions from soil (Levy-Booth et al., 2014). Gene analyses have revealed that P450-NOR is widely distributed among fungi, and that fungal denitrification has been found to be dominant in forest soils, grasslands, and semiarid regions,

making it a larger contributor to global denitrification rates than initially expected (Hayatsu et al., 2008). A study conducted by Laughlin and Stevens (2002) revealed that up to 89% N_2O and 23% N_2 in a grassland soil was produced by fungal denitrification. McLain and Martens (2006) found that fungal denitrification produced 63% of N_2O in semiarid soil and that streptomycin stimulated N_2O production.

The polyphyletic distribution of denitrifying genes can result in co-occurrence with N-fixation as well as ammonia-oxidation genes in many microbes (Hayatsu et al., 2008). Many diazotrophic bacteria such as some species of *Azospirillum* and *Bradyrhizobium* have been found to have the ability to denitrify (Hayatsu et al., 2008). The AOBs such as *Nitrosospira* and *Nitrosomonas* has also been shown to be capable of denitrification – the denitrification enzymes NIR and NOR were identified in the genome of *Nitrosomonas europaea* (Hayatsu et al., 2008; Pajares & Bohannan, 2016). In fact, it has been shown that AOB can contribute up to 80% of total soil N_2O emissions, depending on soil type, temperature, and water content (Shaw et al., 2006). Although the mechanism for N_2O production by nitrifiers is not complelely known, two different routes have been proposed: 1) hydroxylamine oxidoreductase (HAO in the second step of nitrification) can catalyze the oxidation of hydroxylamine to N_2O and 2) nitrite is reduced to NO and N_2O, which is also called "nitrifier denitrification" (Shaw et al., 2006).

The physiological role of nitrifier denitrification is not completely clear. Three hypotheses have been proposed: 1) it may be a strategy to reduce competition for oxygen from NOB by removing their substrate nitrite, 2) AOB use nitrite as an electron acceptor to obtain energy for growth in low-O_2 environments, and 3) nitrifier denitrification is used to protect AOB cells from toxic nitrite produced during ammonia oxidation (Hayatsu et al., 2008; Shaw et al., 2006). Upon studying eight different strains of AOBs, Shaw et al. (2006) reported that all eight

strains produced N_2O at detectable levels, and along with the previously known denitrifying activity of nitrosomonads, concluded that the ability to denitrify is a widespread, if not ubiquitous trait in ammonia oxidizers.

1.5. The Role of Soil Water

Microbial enzymes are regulated by environmental factors such as temperature, oxygen, and available water (Lammel et al., 2015). The presence or absence of oxygen in the soil pores is an especially important factor when studying N cycling processes due to the role of oxygen as an electron acceptor in metabolic reactions. Each of the three nitrogen processes discussed require different levels of oxygen – which is indirectly affected by soil moisture content. The inundation of soil pores with water regulates the diffusivity of gases, availability of dissolved substrates, and most importantly the availability of oxygen, which either stimulates or impedes microbial activity within nitrogen fixation, nitrification, and denitrification. The majority of the southeastern United States, including the state of Arkansas, is categorized as being a humid subtopical climate by the Köppen classification system and is subjected to episodic weather patterns such as extreme precipitation and droughts, which undoubtedly impacts N cycling (Chen & Chen, 2013). For this reason, the effects of soil water content must also be monitored in tandem with nutrient cycling and microbial functional groups.

The nitrogenase enzyme needed for nitrogen fixation is deactived in the presence of oxygen, which allows for increased biological N fixation in anaerobic conditions (Bernhard, 2010; Pajares & Bohannan, 2016). According to Pajares and Bohannan (2016), tropical ecosystems are characterized by N fixation hotspots that may reflect the small-scale spatial heterogeneity of abiotic factors – especially soil water content – that affect the diazotroph

community and its ability to fix N. Conversely, both steps of nitrification require oxygen simply due to its dependency on oxidation of ammonia and conversion into nitrate, the most oxidized form of nitrogen. Ammonium monooxygenase (AMO), the first enzyme of nitrification requires O_2 for activation. According to Linn and Doran (1984), soil moisture contents around 60% water filled pore space (WFPS) are optimal for nitrification because neither the diffusion of substrates nor diffusion of gases are restricted (Bollmann & Conrad, 1998).

Soil moisture contents above 60% favor anoxic processes such as denitrification since O_2 diffusion into the soil becomes restricted, and denitrifying enzyme activities are inhibited by O_2 and the expressions of the encoding genes are suppressed (Hayatsu et al., 2008). Similar to conventional denitrification, nitrifier denitrification is stimuled under anaerobic conditions, which could be explained by the reduced competition with nitrite from O_2 as a preferred electron acceptor. However, some species of bacteria (e.g. *Paracoccus denitrificans*, *Mesorhizobium* sp., and *Burkholderia cepacia*) reduce nitrite/nitrate to gaseous nitrogen compounds N_2O and N_2 in the presence of O_2 (Hayatsu et al., 2008). Bateman and Baggs (2005) conducted a study using different WFPS percentages and a combined stable isotope and acetylene inhibition approach to distinguish the contributions of nitrification and denitrification to N_2O production. The use of acetylene inhibits ammonia oxidation by autotrophic nitrifiers at concentrations lower than 10 Pa, but does not impact ammonia oxidation by heterotrophic nitrifiers. The acetylene method combined with the use of the ^{15}N isotope allows for accurate determination of the source of N_2O where several processes are contributing to emissions. The results from their study suggested that aerobic denitrification occurred in soil with a water content as dry as at 20% WFPS.

2. Further Implications to Land Use and Ecosystem Resiliency

Due to the intimate relationship between plant species and resulting rhizosphere interactions with microorganisms, plant species composition is an important factor in determining the distribution and abundance of soil N functional genes. Reverchon et al. (2015) conducted a study to determine how the establishment of mixed plantations would influence soil N pools and transformations, and their results show that soil properties and microbial functional gene abundances were both influenced by the composition of the species of plants used in the mixed plantation type. An example from their study is that interplanting teak trees with a local tree species (*Fluegga flexuosa* Muell. Arg.) decreased soil pH, which reduced soil bacterial activities and nutrient cycling rates, which in turn lessened N losses from the system. In this way, modifying the composition of plant species in location can have many unforeseen effects at the soil level. Bonsall et al. (2020) further exemplify the close relationship between plants, soil nutrients, and soil microorgansims – plants require soil nutrients for growth, plant-soil feedbacks affect nutrient cycling, and soil microorganisms such as ectomycorrhizal fungi (ECMs) influence plant access to soil resources and contribute to other aspects of nutrient cycling such as nitrification and denitrification. The authors hypothesize in their study that mycorrhizal associations modify interactions between plants and available nutrients, which contribute to promoting resilience of plant populations and ecosystem functioning. Moreover, various studies have suggested that deforestation of tropical forests for agriculture and agroforestry decreases microbial biomass and alters the soil microbial composition (Lammel et al., 2015; Pajares & Bohannan, 2016). This in turn changes soil nutrient cycling and affects both the health of the overall system as well as surrounding systems.

Understanding the link between biogeochemical N processes and microbial community dynamics can provide a more mechanistic understanding of the N cycle and of how the ecosystem responds to a changing environment (Singh et al., 2010). Quantification and characterization of microbial functional genes in the N-fixation, mineralization-immobilization, nitrification, and denitrification pathways can inform models of N cycling process rates, reactive N availability, and N_2O emissions, and provide scope for improved management techniques and mitigation strategies for leaching, fertilizer loss, and greenhouse gas emissions (Levy-Booth et al., 2014). According to Singh et al. (2010) and Richardson et al. (2009), in order to tackle the current problems of greenhouse gas emissions and climate change, there is a need to better understand terrestrial microbial feedback responses across ecosystems and along environmental gradients. With a more holistic view of the interplay between soil nutrients and enzyme activity, mitigating greenhouse gases such as N_2O emissions would be made possible, since this newly emerging knowledge of enzymology can be used to increase the reduction of N_2O to N_2, bringing the denitrification pathway to completion (Richardson et al., 2009).

Understanding the close interplay between microbial functional groups and nutrient dynamics associated with different processes of the N biogeochemical cycle will pave the way for further understanding of ecosystem resiliency along with mitigation efforts, both of which are needed to combat the effects of climate change.

3. Microdialysis as a Monitoring Device

Plant nutrient and water uptake – which is predominantly reliant on the creation of a concentration gradient along the soil-root surface interface – is limited by soil supply rates and not bulk soil N concentrations (Brackin et al., 2017; Inselsbacher & Näsholm, 2012b;

Inselsbacher et al., 2014; Oyewole et al., 2016). During N acquisition, the movement – or flux – of N from the surrounding soil to root surfaces is what determines plant growth and performance, and according to Inselsbacher and Näsholm (2012b) and Oyewole et al. (2014) should be the parameter used for plant N nutrition. However, the current methods for determining plant-available N are typically destructive and are expressed in terms of soil concentrations due to the inability of these methods to directly measure diffusion (Inselsbacher et al., 2014; Mulvaney, 1996; Oyewole et al., 2014).

Extraction of free and exchangeable forms of N using water and salts such as potassium chloride (KCl) and potassium sulfate (K_2SO_4) are the conventional methods of sampling nitrogenous ions such as nitrate-N and ammonium-N from the soil. Soil extractions require destruction of the soil structure and necessitate significant periods of time between sampling and analysis, allowing for error from transformation, contamination, or losses of N (Inselsbacher et al., 2014). Most importantly, analysis of soil extractions only provides bulk concentrations of N, which neglects the mechanics of the delicate interplay between root uptake and soil nutrient supplies.

Microdialysis is a diffusion-based sampling method that has been used extensively as research tool in biomedical and pharmacokinetic research (Duo et al., 2006; Kehr, 1993; Stenken, 2006). A perfusion fluid is passed through the device at μL/min flow rates, which induces the diffusion of analytes from the sample medium into the probe according to their concentration gradient. Unlike soil extractions, microdialysis relies on the same mode of action as plant roots during nutrient acquisition (diffusion), and according to Brackin et al. (2015) and Oyewole et al. (2016), soil N fluxes during plant uptake can be measured reliably by employing microdialysis in the rhizosphere. The small size of the microdialysis probes (0.5-mm diameter)

and their ability to function using simple diffusion makes it possible for minimal disturbance, making it suitable for sampling soil microsites and providing real-time, in situ data with greater temporal and spatial resolutions than salt-solution extraction methods. Continuous sampling of soil solution allows measurement of N flux, to provide insight into the mechanisms driving the availability of nutrients to plants, mobility, and turnover rates on site (Brackin et al., 2017).

Due to the highly dynamic turnover of N in soils and simultaneous uptake and immobilization of N by plants and soil microbes, N fluxes are epected to vary significantly both on a short-term and long-term basis (Inselsbacher et al., 2014). The ability of microdialysis to provide temporally and spatially resolved data on nutrient fluxes has resounding implications for microbial functional gene studies and for the soil-plant-microbial continuum as a whole. In order to examine the intimate relationship between nutrient availability and microbial activity, prior studies have relied on salt extraction of nitrate and ammonium ions (Bateman & Baggs, 2005; Bollmann & Conrad, 1998; Bremer et al., 2007; Lammel et al., 2015; Reverchon et al., 2015). These extractions provide a bulk concentration of nitrogen in the soil used for analysis and because of their destructive nature, do not distinguish between nutrients in soil solution – which is the fraction of nutrients that are readily available for uptake by plants and microbes – and those bound to soil exchange sites. Moreover, microdialysis N flux data are on the same scale as microbial activity – the microscale. Rocca et al. (2015) mention that spatial and temporal heterogeneity under environmental conditions such as anoxic/oxic and water and/or nutrient availability may not track the variability of corresponding gene abundance in space and time due to differences in residence times. Microdialysis has the ability to address the problem of monitoring nutrient availability and can provide insight into hotspots and areas of depletion, which are especially common in microsites of the soil such as the rhizosphere. This application

of diffusive collection within the pore network allows for nutrient fluxes to be correlated to microbial functional genes due to their presence on the same scale, which both have implications on larger scales such as the field-level and eventually on the ecosystem-level. Therefore, the use of microdialysis to study N fluxes with simultaneous qPCR analysis of the soil for microbial functional genes will provide a link between biogeochemical N processes and microbial community dynamics, which would provide a more mechanistic understanding of the N cycle than studying N fluxes or microbial activity and diversity individually (Pajares & Bohannan, 2016).

4. Research objectives, hypotheses, and justification

The goal of this thesis research was to relate microdialysis flux data with changes in soil moisture both in the field and laboratory setting. Supplementing the current knowledge base provided by soil-extraction data with changes in N movement and the contribution of bacteria and archaea to nitrogen fluxes at the scale of the soil pore network can improve understanding of N cycling with changes in soil water content.

Study 1 Objectives:

1. Determine the longevity of microdialysis as a continuous, in situ monitoring device.
2. Temporally resolve in situ N flux in soil growing differing plant species within a single growing season.
3. Supplement N flux data with the collection and analysis of soil samples for physicochemical and microbial functional community changes.

Study 2 Objectives:

1. Continuously sample N fluxes using microdialysis in field-moist and air-dried soils that were wet or rewet to differential water-filled pore space percentages.
2. Evaluate timing of and potential mechanisms involved in the short-term changes in N fluxes after wetting/rewetting.

Hypotheses:

1. Given the differing growing seasons for orchardgrass and native grass species, N fluxes and potential microbial activity are expected to be larger in soil growing during periods when respective plant biomass is increasing during the study period.
2. Abundances of microbial functional genes related to nitrogen cycling processes (i.e., bacterial *amoA* and archaeal *amoA*) will be affected by environmental variables such as precipitation and temperature.
    a. Genes associated with nitrification (bacterial *amoA* and archaeal *amoA*) are predicted to be more abundant under presumably aerobic conditions between precipitation events (e.g. periods following rewetting with greater soil water contents). However, archaeal *amoA* may also be prolific during periods of decreased water content due to archaea's ability to tolerate extreme conditions such as drought.
    b. Periods of high temperature and greater frequency wetting and drying cycles are predicted to result in greater abundances of the nitrogen cycling genes studied.

3. Nitrogen flux patterns are expected to differ between the field-moist and air-dried soils in the laboratory experiment due to differential levels of water stress present on the microorganisms which in turn effects the timing and types of N cycling processes occurring upon the addition of water.

Justification:

The use of microdialysis in the soil is an emerging technique. Because of the method's ability to directly access the soil pore network and provide information on movement of plant available nutrients, the technique has the potential for expanding current knowledge on the dynamics and processes taking place in soil microsites such as the rhizosphere. Nutrients in the soil directly influence the microbiota of the rhizosphere, which in turn influences plant growth. The soil microbiome substantially influences nutrient cycling in the soil, while plant physiology influences the composition and function of the microbiota in its root zone. Since these three factors – nutrient cycling, plant species and physiology, and rhizosphere community composition and function – are in unmistakable synergy with each other, pairing microdialysis sampling of N fluxes with temporally-resolved qPCR techniques to quantify abundances of N-cycling genes in locations with native vs. introduced plant species would provide valuable insight into the inner workings of this delicate but robust interplay. Improving understanding of the soil-microbiome-plant synergisms has the potential to provide a more holistic knowledge of ecosystem resiliency functions – which would greatly aid in combating and adapting to climate change – along with new management and mitigation strategies to alleviate problems such as fertilizer runoff and greenhouse gas emissions.

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CHAPTER 2:

A cross-scale nexus: Use of microdialysis to monitor nitrogen fluxes in tandem with physicochemical and microbial analysis of grassland soil[§]

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A cross-scale nexus: Use of microdialysis to monitor nitrogen fluxes in tandem with physicochemical and microbial analysis of grassland soil

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Abbreviations: N, nitrogen; qPCR, real-time quantitative polymerase chain reaction; KCl, potassium chloride; EC, electrical conductivity; SOM, soil organic matter; LOI, loss-on-ignition; AOB, ammonia-oxidizing bacteria; AOA, ammonia-oxidizing archaea.

Abstract

Microbial processes are the drivers of biogeochemical processes such as nitrogen (N) cycling. However, integrating microbial N dynamics with system-level processes proves to be difficult due to the difference in scales. How can molecular-level processes occurring in soil microsites be amalgamated with N-cycling on the field or even system scale? The tandem use of microdialysis, which can provide inorganic and organic N diffusive flux data, and molecular analyses of soils for N-cycling functional genes can bridge the current knowledge gap by providing a comprehensive understanding of the interplay between bioavailable soil nutrients and nitrogen cycling. The overall objectives of this study were to implant microdialysis probes in the field to 1) determine the longevity of the technique under field conditions, 2) continuously monitor and evaluate differences in organic and inorganic N diffusive fluxes with 3) simultaneous soil sampling for physicochemical and microbial analysis. Experimental plots were established in areas growing orchardgrass (introduced, cool season) and a mix of native grasses (warm season) in June – October 2021. Soil moisture, temperature, pH, EC, and KCl extract data were obtained during the study. Bacterial and archaeal *amoA* genes were quantified using qPCR to supplement microdialysis flux data. In this study, the microdialysis apparatus was sustained in the field for ~5 months with proper care and protection of equipment. Using microdialysis to sample also resulted in nitrate-N fluxes significantly differentiating the plant communities during June, and importantly, flux data were consistent with changes in *amoA* abundances – an agreement that was not observed when comparing KCl extract data with qPCR results. In conclusion, studies which employ simultaneous analysis of microdialysis N fluxes and microbial functional gene abundances may provide a more holistic understanding of the N cycle than sole

reliance on current, conventional soil extraction-based methods to measure soil available nutrients.

1. Introduction

The role of nitrogen (N) in the environment is a crucial one. Nitrogen is the backbone of many biochemical processes, and while it is considered a renewable resource due to its prevalence in the atmosphere as N_2 gas, N availability is limited in many ecosystems (Bernhard, 2010; Inselsbacher et al., 2011; Pajares & Bohannan, 2016). Because of its status as a limiting nutrient, monitoring of N is necessary for determining soil health and productivity, not just for crop production but also for determining ecosystem wellness and resiliency. However, tracking the movement of N through a system as diverse as the soil is difficult due to nitrogen's ability to exist in multiple chemical forms and oxidation states that depend on environmental factors such as pH, temperature, oxygen availability, and moisture levels, along with biological activity as microorganisms catalyze nitrogen for growth and energy (Bernhard, 2010; Pajares & Bohannan, 2016). For these reasons, recent studies have investigated the use of marker genes as indicators of biogeochemical N processes in place of attempting to directly measure N dynamics (Lammel et al., 2015; Levy-Booth et al., 2014; Rocca et al., 2015). The use of qPCR reactions enables quantification of specific targeted gene fragments, which can be used to approximate relative abundances of microorganisms involved in N cycling. Quantification of genes using the qPCR technique can provide insight into how management and abiotic conditions affect the functional community and ensuing services within and from an ecosystem.

While presence and relative quantities of microbial genetic potential dictate the level and occurrence of specific N-cycling processes, multiple researchers have reported the influence of

plant species composition on the distribution and abundance of microbial biomass and activity due to the intimate relationship between plant species and resulting rhizosphere interactions with microorganisms (Bonsall et al., 2020; Reverchon et al., 2015). Understanding the interplay between microbial community dynamics and biogeochemical cycling of N can provide a more holistic understanding of the N cycle and how the ecosystem responds to a changing environment – an essential area of focus, especially in this era of efforts to mitigate climate change and greenhouse gas emissions (Richardson et al., 2009; Singh et al., 2010).

When attempting to amalgamate microbial dynamics and system-level biogeochemical processes for a more comprehensive understanding of the N cycle, the issue of scale comes to the forefront. How can researchers fill the gap between processes occurring on the molecular level and others occuring on the field or even system scale? Microdialysis is a sampling technique with origins in biomedical research and has been used extensively in studying neurotransmitter levels and drug delivery in human and animal tissue (Duo et al., 2006; Stenken, 2006). Due to microdialysis' capabilities of providing continuous, in situ data, it is emerging as a method to sample nutrients in soil microsites, and has been the center of multiple research studies to test its efficacy in diverse soil environments while sampling for various forms of N (Brackin et al., 2015; Buckley et al., 2020; Buckley et al., 2017; Inselsbacher & Näsholm, 2012b; Inselsbacher et al., 2014; Inselsbacher et al., 2011; Maddala et al., 2020, 2021; Oyewole et al., 2014; Oyewole et al., 2017; Oyewole et al., 2016).

Although extraction-based soil sampling methods can be used for obtaining smaller-scale N measurements, the results do not provide information on the movement of N (Inselsbacher et al., 2014; Mulvaney, 1996; Oyewole et al., 2014). Nitrogen from extraction-based methods is reported in terms of bulk concentration, typically as mass N per mass of soil, which neglects the

delicate interplay between root uptake and soil nutrient supplies and also blurs the heterogeneity present in soil microsites. The use of microdialysis alleviates problems associated with point-in-time, extraction-based soil sampling. Because of the technique's dependence on simple diffusion, microdialysis provides data on the basis of flux, or movement of analyte across the surface area of the semipermeable membrane of the microdialysis probe.

Microdialysis samples soil solution, the pool of soil that contains readily available nutrients for use by plant roots and microbes (Brackin et al., 2017; Inselsbacher & Näsholm, 2012b; Oyewole et al., 2016). By excluding sampling of nutrients bound to soil particles, microdialysis provides reliable data on N turnover and mobility rates, while also deeming sample cleanup and particle removal from soil solution unnecessary (Oyewole et al., 2017). Moreover, microdialysis functions at the same scale as microbial processes – the molecular level – making it possible to integrate N flux data with microorganism dynamics.

Since microdialysis orginated for use in the sterile, laboratory setting, adapting the method to field applications presents challenges. A long-term temporal scale study is important to evaluate the effects of the range of soil temperature and water content fluctuations on the continued functionality of probes under field conditions. All previous published research has been conducted in the lab or in the field for a maximum of 25 days of continuous sampling located in a boreal forest setting (Inselsbacher et al., 2014). By incorporating soil microbial analysis with nutrient cycling and uptake in the rhizosphere, an integrative understanding of the dynamics between soil nutrients, plant roots, and the microbial community in the root zone will be possible. Therefore, the objectives of this study were to (1) implement microdialysis in the field and to determine its longevity as a continous monitoring device, (2) evalute N fluxes in sites of differing plant communities to observe if microdialysis is capable of capturing potential

differences, and (3) obtain data on microdialysis, soil properties, and microbial dynamics on a temporally-resolved scale.

2. Materials and methods

2.1. Site description

Field studies were located at the University of Arkansas Milo J. Shult Main Agricultural Research and Extension Center in Fayetteville, AR, USA (36°05'30.6" N 94°11'19.0" W). The two locations included grassland plots growing, respectively, a mix of native, perennial, warm season grasses (big bluestem [*Andropogon gerardii* Vitman], little bluestem [*Schizachyrium scoparium* (Michx.) Nash], and Indiangrass [*Sorghastrum nutans* (L.) Nash]) and an introduced, perennial, cool-season grass [orchardgrass (*Dactylis glomerata* L.)]. These grassland sites are located in a field designated for active, long-term research activities and is adjacent to an experimental agroforestry site (Figure 1) that was established in 1999 and has been thoroughly studied since establishment (Adhikari et al., 2018; Sauer et al., 2015). The soil from both the orchardgrass and native grass sites is classified as a Captina silt loam (fine-silty, siliceous, active, mesic Typic Fragiudults) (Sauer et al., 2015; *Web Soil Survey*). The grassland is not irrigated, and plant growth relies on natural precipitation events. Continuous precipitation and air temperature data were obtained from the USDA weather station located within the research site approximately 20 m from plots established for this study.

2.2. Microdialysis apparatus and field set-up

The microdialysis system was set-up according to Maddala et al. (2020, 2021) and adapted from Inselsbacher et al. (2011). For employment in the field, eight syringe pumps

(containing three syringes each, MD-1001, BASi, Lafayette, IN, USA) were equipped with a total of 24 gas-tight syringes (MDN-0250, 2.5 mL, BASi), which delivered MilliQ (MQ) water at a flow rate of 2.0 µL/min. Each syringe was connected to 2 m of extra tubing and a CMA 20 Elite probe (CMA8010436, membrane length of 10 mm, PAES membrane, and 20 kDa molecular weight cut-off, Harvard Apparatus, Holliston, MA, USA). An equilibration time of 15 minutes was used at the beginning of every sampling. All the probes were calibrated prior to field studies by placing into a solution of 10 µg/mL nitrate-N and 10 µg/mL ammonium-N with stirring (Maddala et al., 2020).

All plants in the experimental area were cut to a height of approximately 10-12 cm with a mower on May 27, 2021. Plots (n = 12) with dimensions of 1.25 m by 1.25 m, were established in each of the two communities of grasses. Alleyways 0.33-m wide were also established around each plot to act as a buffer zone and for walking. One microdialysis probe was implanted approximately in the center of each plot to a depth of 12 cm (distance of 1.58 m between probes; Figure 2). Two meters of extra tubing was connected to each probe and syringe. All microdialysis equipment (two controllers, 8 syringe pumps, 24 syringes, and tubing connecting syringes and probes) were weatherproofed and kept in the field for the duration of the field study. This was accomplished by placing the controllers, syringe pumps, and syringes in weighted insulated Styrofoam boxes. To protect the two meters of tubing from sun damage, insects, and small animals, tubing was placed in 1.27-cm CPVC piping leading from the implantation site to the syringe boxes. To protect the probe outlet and tubing connection from warping or kinking, a protective “bridge” using CPVC piping was constructed and attached to the CPVC pipes with the tubing (Figure 3). Metal mesh caging was placed around each

implantation site to prevent birds or small animals from pulling the microdialysis probes out of the soil.

2.3. Soil and data collection

Upon implantation, dialysates were collected on ice for 90 minutes at a flow rate of 2.0 µL/min and were stored at 4 °C until further analysis. Simultaneous to microdialysis collection, soil cores (diameter of 1.5 cm) were used to aseptically sample soil from each plot at a depth of 12 cm (n = 24) on selected sampling dates (Table 1). After homogenizing and mixing the collected core samples, approximately 2 g of each soil sample was aliquoted to be stored at -80 °C for DNA extraction and downstream molecular analysis. The remainder of all soil samples were utilized for soil analyses.

Additional soil cores from both the native grass and orchardgrass areas (n = 3) were used to sample soil for gravimetric determination of soil water content on each sampling day. Soil temperature (n = 3) at a depth of 12-cm was measured using a handheld thermometer in each of the grass areas outside of the established plots on each sampling day. Aboveground biomasses (n = 3) were collected monthly from outside of the established plots to monitor total biomass in the vicinity of the experimental area. Biomass samples were dried at 55 °C until no further weight loss was detected. Total biomass production was determined by loss-of-water upon drying.

Microdialysis sampling began on June 5, 2021 and continued for five consecutive days. After daily collection, samplings tapered off to alternating days for two weeks, and then to three times a week for the next six weeks. Continued samplings proceeded on a weekly basis for the next nine weeks, with the last collection day 2 weeks after the previous collection day. All dialysate samples were stored at 4 °C until analysis. Soil samples were not collected every day

that microdialysis samples were obtained because prolonged periods of little to no precipitation prevented soil collection. The sampling timeline for both microdialysis and soil collection is outlined in Table 1.

2.4. Soil analyses

Before the start of field experimentation, soil core samples (diameter = 5 cm; n = 3) were obtained from the upper 10 cm of the soil profile in each plant community, dried at 55 °C for five days, and used for determining the volumetric soil moisture and bulk density (Blake & Hartge, 1986). Soil collected (n = 12 from each plant community) on the first day of field experimentation – June 5, 2021 – was oven-dried at 105 °C overnight and analyzed for soil organic matter (SOM) percentage using the loss-on-ignition method at 450 °C (Nelson & Sommers, 1996) and particle size analysis by the micropipette method (Miller & Miller, 1987). Soil collected on June 8, 2021 was oven-dried at 105 °C, extracted using a 1:10 (w/v) soil-to-Mehlich-III solution ratio, and extracts were analyzed by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Zhang et al., 2014)

All soils that were collected on sampling days (Table 1; n = 12 from each plant community) were oven-dried at 105 °C overnight and analyzed for pH and electrical conductivity (EC) by the electrode method (1:2 (w/v) soil-to-water ratio) (Sikora & Kissel, 2014). Dry soil was extracted for inorganic nitrogen using a 1:10 (w/v) soil-to-2*M* potassium chloride (KCl) ratio. Soil results are reported on a per g dry soil basis (Mulvaney, 1996). All extraction samples were stored at 4 °C until analysis.

2.5. qPCR

Soils were analyzed for quantities of the bacterial *amoA* gene utilized by ammonia-oxidizing bacteria (AOB) and the archaeal *amoA* gene utilized by ammonia-oxidizing archaea (AOA) in the first step of the nitrification pathway. DNA was extracted from 0.25 g soil using the DNeasy® PowerSoil® Pro kit (Qiagen, Hilden, Germany) and the manufacturer's protocol was followed. Extracted DNA was quantified using UV-Vis spectroscopy with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to a concentration of 10 ng μL^{-1} from which further dilutions were made if required. Quantitative PCR was performed in a CFX Opus 96 device (Bio-Rad, Hercules, CA, USA) in 96-well plates. Each reaction was composed of 1 µL of extracted DNA (10 ng μL^{-1} for AOB and 1 ng μL^{-1} for AOA), 10 µL of 2X Forget-Me-Not™ EvaGreen® qPCR Master Mix (Biotium, Fremont, CA, USA), and molecular-grade water. 0.5 µM (AOB) or 0.4 µM (AOA) of the needed primer pairs was added to each reaction to a final volume of 20 µL (Lammel et al., 2015). Additionally, 3.75 mM of $MgCl_2$ was added to each 20 µL reaction for AOB quantification.

The primer pairs used for the *amoA* bacterial gene were AmoA1F/AmoA2R (5'-GGG GTT TCT ACT GGT GGT- 3') (3'-CCC CTC KGS AAA GCC TCC TCC- 5') and the pairs for the archaeal *amoA* gene were Arch-amoAF/Arch-amoAR (5'-STA ATG GTC TGG CTT AGA CG-3') (3'-TGT ATG TCT ACC TAC CGG CG- 5'). The thermal profiles for the studied genes are listed in Table 2. G-block standards (Integrated DNA Technologies, Coralville, IA, USA) were designed for the aforementioned primer pairs using the genomes of *Nitrosomonas europaea* and *Nitrosoarchaeum koreensis MY1* for the bacterial *amoA* and archaeal *amoA* genes (Masrahi et al., 2020; Prosser & Nicol, 2012). G-block standards, negative controls, and all samples were all microplated in duplicate. The qPCR runs maintained standard curves with correlation

coefficients ($r^2 \geq 0.99$) and amplification efficiency (> 90% or ≤ 110%), and samples were quality checked with a melt curve after each run. Gel electrophoresis using 1% agarose gels was used the ascertain specificity and size of PCR products using a T100 thermal cycler (Bio-Rad). Each PCR reaction (5 μL) was mixed with 1 μL loading dye and loaded into an agarose gel well. Gels were electrophoresed in 0.5X TBE buffer at 85 V for 45 min. To visualize DNA bands in gels and confirm PCR product, a UV trans-illuminator was used, and gels were digitally pictured. DNA size standards (VWR, Radnor, PA, USA) were used with each agarose gel to confirm distance of migration.

2.6. Chemical analyses

Nitrate-N in microdialysis samples and KCl extracts was analyzed by reduction using vanadium chloride (VCl_3), and the Griess reaction based on the technique described by Miranda et al. (2001). Ammonium-N in the dialysate samples and KCl extracts were analyzed using a microplate adaptation of the indophenol Berthelot reaction described by Baethgen and Alley (1989). Absorbance values were measured at 540 nm and 650 nm for the Griess and indophenol Berthelot reactions, respectively. Total amino acids (Leucine-equivalents) in the dialysate samples were analyzed using a microplate adaptation of the method described by Jones et al. (2002) (Darrouzet-Nardi et al., 2013). Fluorescence values were obtained using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The concentrations of nitrate-N, ammonium-N, and amino acids in the dialysate samples obtained from microplate analysis were used to calculate the diffusive flux (nmol N cm^{-2} h^{-1}) of inorganic and organic forms of N across the semipermeable membrane of the probe. All microplate analyses consisted of standards and blanks in triplicate. For KCl extracts, samples were also microplated in triplicate. Absorbance

and fluorescence values were measured using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, LLC, San Jose, CA, USA) and Tecan Infinite m200 Plate Reader (Tecan, Männedorf, Switzerland). Detection limits for nitrate-N, ammonium-N, and amino acid fluxes were 4.16, 2.79, and 1.64 nmol N cm^{-2} h^{-1} and detection limits for nitrate-N and ammonium-N concentrations were 0.08 and 0.05 μg g^{-1} soil. Values below detection limits were reported as ND on graphs and were put in as zeroes for statistical analyses.

2.7. Statistical analyses

Statistical analyses on microdialysis fluxes, KCl extracts, pH, EC, and qPCR results were performed by a two-way repeated measures ANOVA followed by the Holm-Sidak method as a post-hoc test. Mehlich-3, SOM, aboveground biomass, and bulk density data were analyzed using a Student's t-test. All statistical analyses were performed using a 95% confidence interval; differences were considered significant at $P \leq 0.05$. All reported values are means (± SE).

3. Results

3.1. Grassland site soil comparisons

This study was conducted in 24 field plots established in a grassland growing orchardgrass in one set of plots that were adjacent to a mix of native, big bluestem, little bluestem, and indiangrass in a second set of plots. The whole area is mapped as a Captina silt loam, and average sand/silt/clay% was 23.5/63.8/12.7 (0.7/0.8/0.6)% and 20.7/65.8/13.5 (0.5/0.3/0.4)% in orchardgrass and native grass soil, respectively. The overall average soil fractions of both soils were 20/65/15%, which experimentally confirmed the particle size silt loam. At the start of the experiment, bulk density of orchardgrass soil was determined to be 1.21

(0.09) g cm^{-1} with 3.42 (0.13)% soil organic matter. Bulk density of soil from the native grass plots was 1.28 (0.04) g cm^{-1} and organic matter content was 3.81 (0.20)%, with no significant differences present between the two soils ($P = 0.513$ and $P = 0.114$ when comparing bulk density and SOM, respectively). Background inorganic N content was 6.26 (0.16) µg ammonium-N g^{-1} soil and 0.94 (0.04) µg nitrate-N g^{-1} soil in orchardgrass soil and 5.89 (0.16) µg ammonium-N g^{-1} and 1.05 (0.07) µg nitrate-N g^{-1} soil in native grass soils, and neither form of N differed in the two soils ($P = 0.577$ and $P = 0.206$ for ammonium-N and nitrate-N comparisons, respectively). Soil pH was 5.29 (0.07) in the orchardgrass soil and 5.39 (0.11) in the native grass ($P = 0.317$). The EC was 250.89 (16.35) µhos cm^{-1} and 240.78 (7.08) µhos cm^{-1} in orchardgrass and native grasses, respectively, with a significant difference present between the two systems ($P = 0.043$).

Mehlich-3 phosphorus (P) concentrations of 5.67 (0.43) and 4.68 (0.36) µg g^{-1} soil in the orchardgrass and native grasses, respectively, did not differ significantly ($P = 0.095$) in June at the beginning of experiments. Calcium (Ca) of 86.89 (7.31) µg g^{-1} soil in orchardgrass compared to 88.72 (7.60) µg g^{-1} soil in native grasses ($P = 0.863$), and Mg concentrations of 4.90 (0.44) in orchardgrass compared to 5.65 (0.31) µg g^{-1} in the native grasses did not significantly differ ($P = 0.174$). Potassium (K) was 5.27 (0.31) and 10.40 (0.63) µg g^{-1} soil in the orchardgrass and native grass communities and was statistically different ($P < 0.001$). Sulfur (S) concentrations also significantly differed between the two plant communities, with 4.45 (0.24) µg g^{-1} in the orchardgrass soil and 3.88 (0.15) µg g^{-1} in the native grass soil ($P = 0.015$). Concentrations of sodium (Na) and iron (Fe) were also statistically different in the two soils: 0.80 (0.05) µg Na g^{-1} and 16.02 (2.22) µg Fe g^{-1} in the orchardgrass and 0.50 (0.02) µg Na g^{-1} and 10.71 (0.96) µg Fe g^{-1} in the native grass ($P < 0.001$ and $P = 0.033$ for Na and Fe, respectively) at the beginning of the study. The concentrations of manganese (Mn), zinc (Zn), copper (Cu), and boron (B) were

not significantly different between the two types of grasses ($P = 0.099$, $P = 0.558$, $P = 0.449$, and $P = 0.07$, respectively). Concentrations of Mn were 24.86 (2.24) and 20.44 (1.25) μg g^{-1} soil in the orchardgrass and native grass, and Zn concentrations were 0.61 (0.05) and 0.66 (0.06) μg g^{-1} soil. Copper concentration was 0.14 (0.01) and 0.15 (0.01) μg g^{-1}, and B values in the soil were 0.03 (0.01) and 0.02 (0.00) μg g^{-1} in the orchardgrass and native grass, respectively.

Aboveground orchardgrass and native biomass was 12627 (935) and 6120 (843) kg ha^{-1} in the month of June, which statistically differed ($P = 0.007$). Overall, at the beginning of experimentation, the soils from the two plant communities exhibited differences in electrical conductivity, K, S, Na, Fe concentrations, and aboveground biomass production.

Soil temperature measurements ranged from 26.7 to 16.6 °C (end of June – end of October; Figure 4a). Monthly rainfall values for the months of June, July, August, September, and October were 103-, 149-, 14-, 27-, and 99-mm. Average soil moisture (experimentally measured volumetric water content at a depth of 12 cm) was 0.324 and 0.313, 0.309 and 0.284, and 0.153 and 0.165 m^3 m^{-3} in the orchardgrass and native grass soils, respectively, for the months of June, July, and August (n = 14, 2, and 4, respectively). Only one set of soil samples were collected in October and soil moisture was 0.221 and 0.224 in orchardgrass and native grass soil.

Over the span of the next four months of sampling, pH values differed significantly on June 26 where the native grasses exhibited a pH of 5.06 and the soils in the orchardgrass were of pH 4.84 ($P < 0.05$; Figure 5a). Other than that time point, the pHs of the two types of grasses were closely paired ($P > 0.05$). Unlike pH, EC trends in the different plant communities were more dynamic. The EC values were significantly different between plant communities on June 5, 16, 24, 26, July 9, August 5, 11, 25, and October 20 ($P < 0.05$; Figure 5b).

Total orchardgrass aboveground biomass values were 9951 (2263), 6960 (600), 10333 (1948), and 3720 (212) kg ha^{-1} for the months of July, August, September, and October. Biomass production in the native grasses was 13063 (1245), 12040 (1323), 9867 (825), and 5987 (464) kg ha^{-1}. After differential growth in June, there were again statistical differences in aboveground biomass production between the two plant communities in the months of August and October ($P = 0.025$ and $P = 0.011$; Figure 6).

3.2. Microdialysis fluxes and KCl

When comparing nitrate-N flux in the orchardgrass and native grass plant communities, there were significant differences on most sampling days in June (6, 7, 9, 12, 14, 16, 18, 24, 26, and 30) during times of frequent rainfall ($P < 0.05$; Figures 4b and 4c). However, after June 30, any differences in nitrate flux between native grasses and orchardgrass were numerical and not significant. Within plant communities, nitrate-N fluxes in orchardgrass did not differ at any of the sampling dates ($P > 0.05$). Within the native grasses, most of the fluxes at the beginning of sampling, namely in the month of June, were significantly increased compared to the native fluxes in the remainder of sample collection ($P < 0.05$). There also were significant time by plant community interactions, meaning that the effect of the different plant communities depended on the sampling day, suggesting that factors present on individual days such as soil moisture, occurrence and magnitude of precipitation events, soil temperature, and other chemical, physical, and biological influences were affecting nitrate-N fluxes ($P = 0.001$).

Ammonium-N fluxes were not significantly different at any sampling time point in the months of June – October ($P > 0.05$; Figure 4d). Total amino acid (Leu-equivalent) fluxes were statistically different between the two plant communities on June 14 and 22 ($P < 0.001$ on both

days; Figure 4e). When comparing amino acid fluxes within the orchardgrass plots, the fluxes exhibited on June 14 and 22 were significantly larger than any of the fluxes on any other sampling day ($P < 0.05$). Flux in the native grasses on June 14 was also greater than the fluxes on other days ($P < 0.05$). One interesting observation was that both the ammonium-N and amino acid fluxes exhibited "hot moment" behavior – short periods of time with increased fluxes – at the beginning of experimentation during increased rainfall events. After approximately August 2, fluxes in both ammonium-N and total amino acids leveled out and did not display sudden spikes (Figure 4d and 4e).

Nitrate-N concentrations in KCl extracts were statistically different in the two plant communities on June 16, 18, and 22 ($P < 0.01$, $P = 0.034$, and $P = 0.002$, respectively; Figure 7a). Concentration was greater in the orchardgrass soil on June 16 and 18, while the soil in the native grass plots contained increased nitrate-N on June 22. Ammonium-N concentrations obtained from KCl extracts were significantly increased in the orchardgrass on June 6, 8, 12, 22, August 5, 18, and 25 ($P < 0.05$; Figure 7b). Both forms of inorganic N displayed increased concentrations at the beginning of the sampling period, which then decreased significantly in the months of August and October when comparing within each plant species community ($P < 0.05$).

3.3. Gene abundances over time

Soil samples from June 8, June 12, June 14, June 22, July 9, and August 25 were analyzed for quantities of the bacterial *amoA* and the archaeal *amoA* gene fragments, which provide an approximation for the relative abundances of bacterial and archaeal nitrifiers in the soils of the two plant communities. Across the six sampling dates, the native grass soil contained a significantly increased number of bacterial *amoA* copies until June 14 ($P < 0.05$; Figure 8a).

However, after June 14, there were not significant differences between the two soils on individual sampling dates ($P > 0.05$). On August 25, the soil from the orchardgrass and native grass sites contained virtually the same number of bacterial *amoA* copies. Within the orchardgrass soil, the number of copies of the bacterial *amoA* gene was significantly increased on June 22, July 9, and August 25 when compared to abundance of copies on June 8 and 12 ($P < 0.05$). The number of *amoA* genes on June 14 served as the intermediate between the two groups and did not statistically differ from the number of genes on any other sampling date ($P > 0.05$). The bacterial *amoA* counts did not differ at any time point in the native grass soil ($P > 0.05$).

Quantity of archaeal *amoA* differed between the orchardgrass and native grass plots on June 8, June 12, June 14, July 9, and August 25, with the native grass soil demonstrating a significant increase in gene copies on all of these days ($P < 0.05$; Figure 8b). On June 22 there was no statistical difference between the number of gene copies in the soils of the two plant communities ($P > 0.05$). Archaeal *amoA* counts in the orchardgrass remained statistically unchanged until August 25, where copy numbers increased significantly ($P < 0.05$). Quantities of archaeal *amoA* in the native grass remained unchanged until June 22 where total amounts of gene copies decreased significantly from the levels measured on the first 3 sampling days ($P = 0.003$, $P < 0.001$, and $P = 0.006$ when comparing June 22 average counts with counts on June 8, 12, and 14, respectively). After the all-time low of approximately $10^{8.66}$ copies g^{-1} observed on June 22, archaeal *amoA* gene copies increased numerically to $10^{8.78}$ copies g^{-1} on July 9 ($P = 0.200$), and then increased significantly on August 25 to $10^{9.18}$ copies g^{-1}. The value of copy numbers observed in the native grass plots on August 25 was statistically greater than the copy numbers in the prior sampling point – July 9 ($P < 0.001$) and was considerably greater than the copy numbers measured at any of the other time points ($P < 0.05$).

4. Discussion

4.1. Longevity of the microdialysis technique in field conditions

One of the objectives of this study was to determine the longevity of the microdialysis technique in field conditions. With proper care and protection of the microdialysis equipment – particularly the electrical equipment such as the syringe pumps and controllers – the technique was successfully sustained in the field for almost five months (June – October). However, there were some challenges that were presented as the microdialysis equipment was utilized in field conditions. While all microdialysis tubing was shielded from direct sunlight, most insects, and animals using CPVC piping, the tubing and tubing connectors were subject to – sometimes drastic – changes in temperature. At times, due to expansion and shrinking of the plastic, these temperature changes would cause clogs to form in the tubing or in the tubing connectors and would need to be closely monitored to alleviate clogging. It is also acknowledged that some microdialysis probes were likely at the end of their lifespan and would have been replaced if field studies had progressed. The continued use of microdialysis was discontinued due to the approach of freezing conditions (fall and approaching winter seasons). Equipment fortification had been designed for warm weather seasons and not cold weather seasons. Future studies should focus on the prolonged use of the technique to take advantage of its capabilities of providing minimally-destructive, continuous, in situ data.

4.2. Evaluating N fluxes in different plant communities and tandem biophysicochemical analyses

The utility of the microdialysis technique in differentiating bioavailable pools of N in differing plant communities was investigated as part of the second objective of this study. Just as microdialysis data are obtained in a temporally-resolved manner, other properties such as

precipitation (and resulting moisture), bulk soil nitrate-N and ammonium-N concentrations, pH, EC, and microbial nitrifier abundances were measured on a temporal basis to better explain the observed diffusive fluxes and further implications.

When comparing N fluxes with data from KCl extracts – the usual norm for monitoring inorganic N – there were some interesting observations that came to light. Of the sampling days in June, amid frequent wetting and drying changes in the soil, microdialysis data yielded significant increases in native grass soil. On the contrary, only three sampling days indicated significance in nitrate-N concentration, of which two presented greater concentrations in orchardgrass soil, which did not agree with microdialysis' data of significant increases of nitrate-N in native grass soil. However, after the end of June and a decrease in rainfall, KCl data showed similar patterns of nitrate-N in both soils, which coincided with there being no significant differences present between nitrate-N fluxes in the plant communities starting in July. By studying KCl extract data alone, nitrate availability in June would appear to either be greater in the presence of orchardgrass or similar in both soils; whereas in contrast, microdialysis data and qPCR analysis of bacterial and archaeal nitrifier abundances imply that greater nitrification and increased movement of nitrate-N was occurring in the soil growing native grasses. When considering the two types of ammonium-N data, KCl extraction data yielded significant increases of ammonium-N concentrations (substrate needed for nitrification) in the orchardgrass soil, signifying greater nitrification potential, though microdialysis did not show any differentiation in flux data.

The disparity in inorganic N concentrations yielded by KCl extractions and the data provided by microdialysis may be explained by the N pools that these methods are capable of measuring. Soil extractions depend on complete dispersal of soil colloids, releasing N present in

soil solution and bound to colloidal surfaces. Due to removal of both N fractions from soil, KCl extractions yield total inorganic N concentrations on a per gram soil basis – there is no further information on pockets of heterogeneity that would be expected soil microsites. In contrast, microdialysis sampling results in collection of compounds present only in their aqueous forms – contained in soil solution (Inselsbacher et al., 2014; Oyewole et al., 2014). Therefore, the method is only sampling the movement and forms of N in soil microsites that would be available for plant and microbe uptake. Instead of presenting the total amounts of N present in the entire gram of soil analyzed, microdialysis provides fluxes based on the net amounts of N present in soil solution during and after the occurrence of N cycling processes. Microorganisms such as bacterial and archaeal nitrifiers rely on N cycling that occurs on a micro-scale and reside in soil microsites that are sampled by microdialysis, resulting in the ability of qPCR and flux data to be amalgamated. In this way, tandem analysis of microdialysis flux and microbial functional genes may provide a novel, elucidative approach to understanding N cycling on a microscale and can be further used to augment the current KCl knowledge base.

Since microdialysis is a diffusion-based sampling technique, it was expected that soil moisture availability would have an effect on measured diffusive fluxes. Experimental data may have illustrated the dependence of the technique on moisture; frequent rain and drying events which kept the volumetric water content around 0.3-0.35 m^3 m^{-3} in the month of June yielded increased nitrate-N fluxes in both soils and “hot moment” behavior in ammonium-N and amino acid fluxes. After drying events and prolonged drought conditions starting in the beginning of August, nitrate-N fluxes decreased, and ammonium-N and amino acid fluxes showed a decrease in hot moment behavior, which could be attributed to decreases in soil moisture and less connectivity of soil pores, reducing the sphere of influence of the microdialysis technique.

However, significant differences in the magnitude of N fluxes – especially nitrate-N – in the month of June can also be attributed to differences in plant-induced growth responses.

The two plant communities involved– orchardgrass and mix of native grasses – exhibit different physiologies, metabolic pathways, growing seasons, and nitrogen needs (Brown, 1978; Singer & Moore, 2003; Wedin & Tilman, 1990). Orchardgrass is a cool season grass with a fall growth period and uses the C_3 pathway of photosynthesis. Orchardgrass has been used in prior studies for N removal, and according to Singer & Moore (2003), maximum N removal (from ammonium-nitrate fertilizer inputs) occurred approximately 40 days after harvest of orchardgrass. Since the orchardgrass plants were mowed on May 27, mimicking a harvesting event, nitrogen removal was expected to increase over the next month to reach maximum N uptake in the beginning of July. In contrast, the mix of native grasses in the experimental plots are warm season grasses that utilize the C_4 pathway. These grasses are known for their ability to thrive in low-N environments due to their efficiency in N use. According to Wedin & Tilman (1990), peak mineralization rates in big bluestem and little bluestem plants occur in early – mid June, with lower mineralization rates also occurring in mid-August. However, these peaks may have been slightly delayed in this study owing to the mowing of grasses at the end of May. Since the two plant communities were subject to the same rainfall and drying events, the differences in magnitude of N flux in June can be explained by rapid uptake by orchardgrass and increased mineralization rates in the native grasses. Ideal moisture conditions (periods of wetting and drying with fairly consistent moisture content) may have led to mineralization of soil organic matter and subsequent uptake by the orchardgrass plants, resulting in a net removal of mineralized N available for conversion to nitrate-N through nitrification. Increased mineralization rates in the soil growing native grasses, paired with ideal moisture contents ideal

for aerobic processes such as nitrification may have resulted in rapid conversion of the mineralized N to nitrate-N.

Since microdialysis functions on the principle of diffusion, there was some concern on whether or not the technique would be able to successfully measure ammonium-N and amino acids which are known for readily adsorbing to the soil solid phase (Cao et al., 2016). While these two forms of N did exhibit inconsistent, hot moment behavior in the initial two months of sampling, both ammonium-N and amino acid fluxes leveled out after approximately August 2, signifying that the responses of ammonium-N and amino acid were not solely dependent on the efficacy of the microdialysis technique. This can be further supported by the fact that while increasing soil temperature can lead to larger contributions of small compounds such as amino acids to diffusive fluxes, soil temperature decreased significantly ($P < 0.001$) from the end of June to the beginning August, which shows that diffusion rates were not being adversely affected by the typical binding behavior of amine compounds and that they were being influenced more by moisture availability and underlying biological processes such as rapid mineralization followed by nitrification or immobilization (Inselsbacher & Näsholm, 2012a).

An alteration of nitrifier abundances occurred on August 25, which was most likely due to sustained lower soil moisture contents during a period of prolonged drying starting in the beginning of August. Slower regeneration rate and mixotrophic growth of the AOA may have led to sustained and consistent nitrification rates, resulting in the level nitrate-N, ammonium-N, and amino acid diffusive fluxes (Figure 4) observed after approximately August 2 in both orchardgrass and native grass soil (Chen et al., 2017). However, while there was a significant increase in archaeal *amoA* gene copies in soils of both plant communities after drought conditions, the native grass soil revealed gene counts that were unprecedently high, in

comparison to past gene copy numbers from both native and orchardgrass soil (Figure 8). Greater abundance in the native grass after environmental stress can be explained by the ability of archaeal nitrifiers to adapt to extreme conditions such as prolonged drought, and these organisms, through symbiotic relationships, may confer native grasses such as big and little bluestem the ability to thrive in low-fertility and drought conditions (Chen et al., 2017; Hu et al., 2021; Levy-Booth et al., 2014; Revillini et al., 2019).

5. Conclusion

The overall objective of this study was to implant microdialysis probes in the field to monitor organic and inorganic N diffusive fluxes in situ with selected dates of simultaneous soil sampling for physical, chemical, and microbial analysis. The goal was to measure soil moisture, temperature, pH, EC, and the N-cycling functional gene *amoA*, on a temporal basis to supplement temporally-resolved microdialysis flux data. The microdialysis technique was successfully employed under field conditions for almost five months and the data exemplified the method's ability to differentiate fluxes in the orchardgrass and native grass communities. While time-wise KCl extract data on nitrate-N and ammonium-N diverged from the trends observed in fluxes, especially during frequent wetting and drying events in the month of June, molecular analysis of the soils to quantify *amoA* gene copies agreed with microdialysis flux data. In conclusion, tandem analysis incorporating both microdialysis flux and quantification of microbial functional genes provides a novel, elucidative approach to understanding N cycling on a microscale.

Tables and Figures

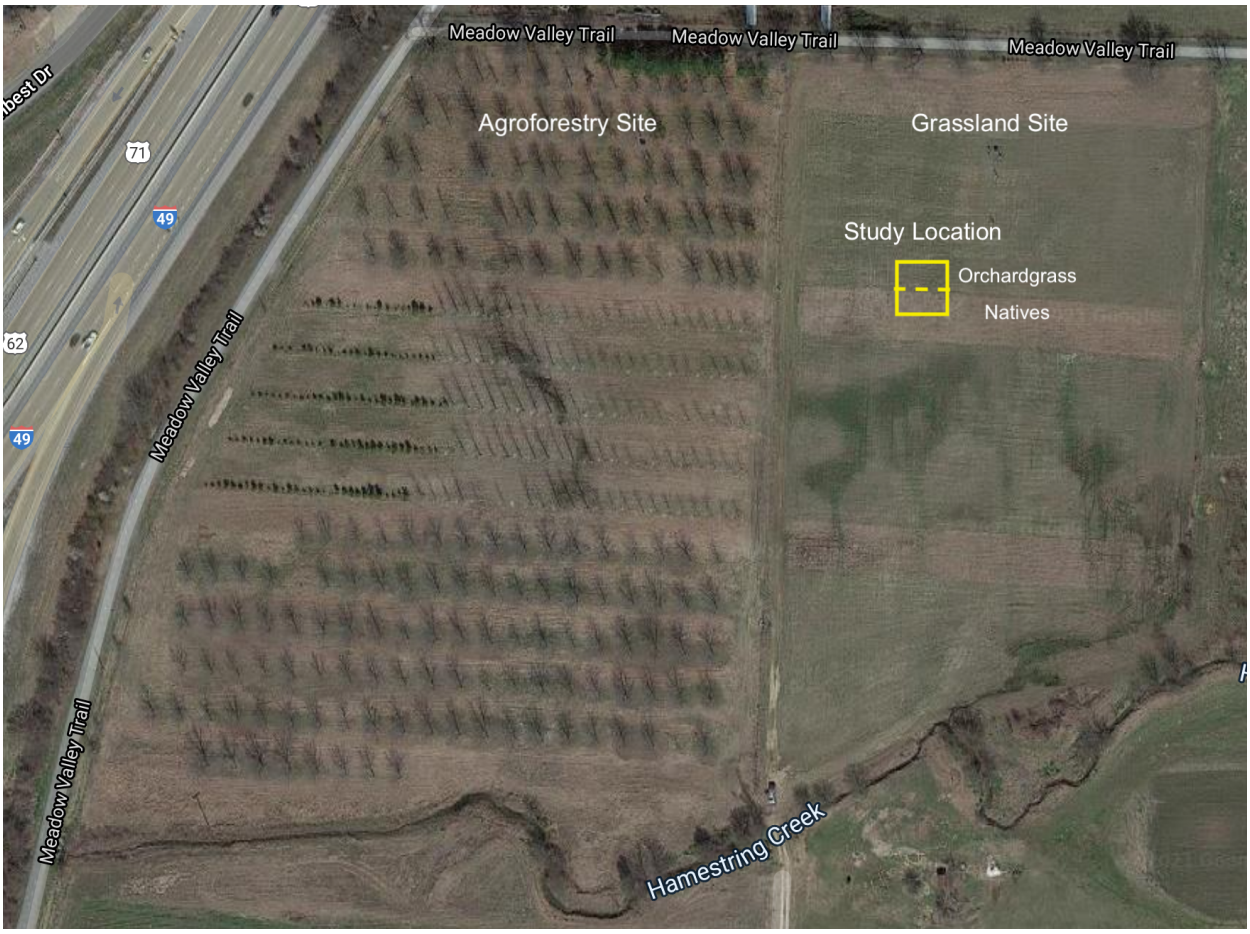

Figure 1. Aerial view of the grassland study site and adjacent agroforestry site. The boxed area represents the area where the orchardgrass and native grasses come together and where the experimental studies described in this chapter occurred.

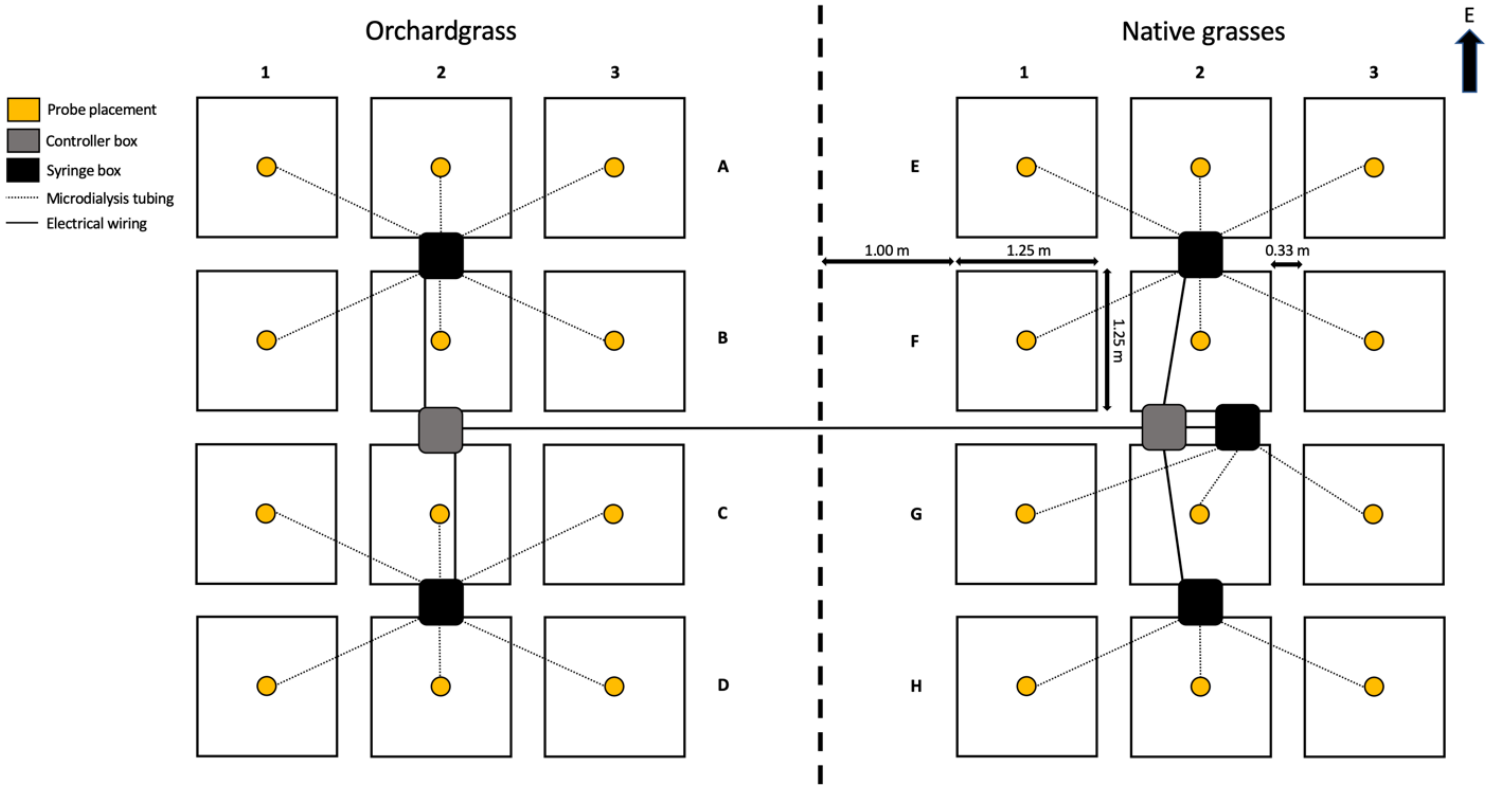

Figure 2. Experimental setup in the field. The approximate center of each plot (n = 12 in each grass type) was implanted with a microdialysis probe. The controllers and syringe pumps were housed in weighted Styrofoam boxes and all microdialysis tubing and electrical wiring was protected using CVPC pipes.

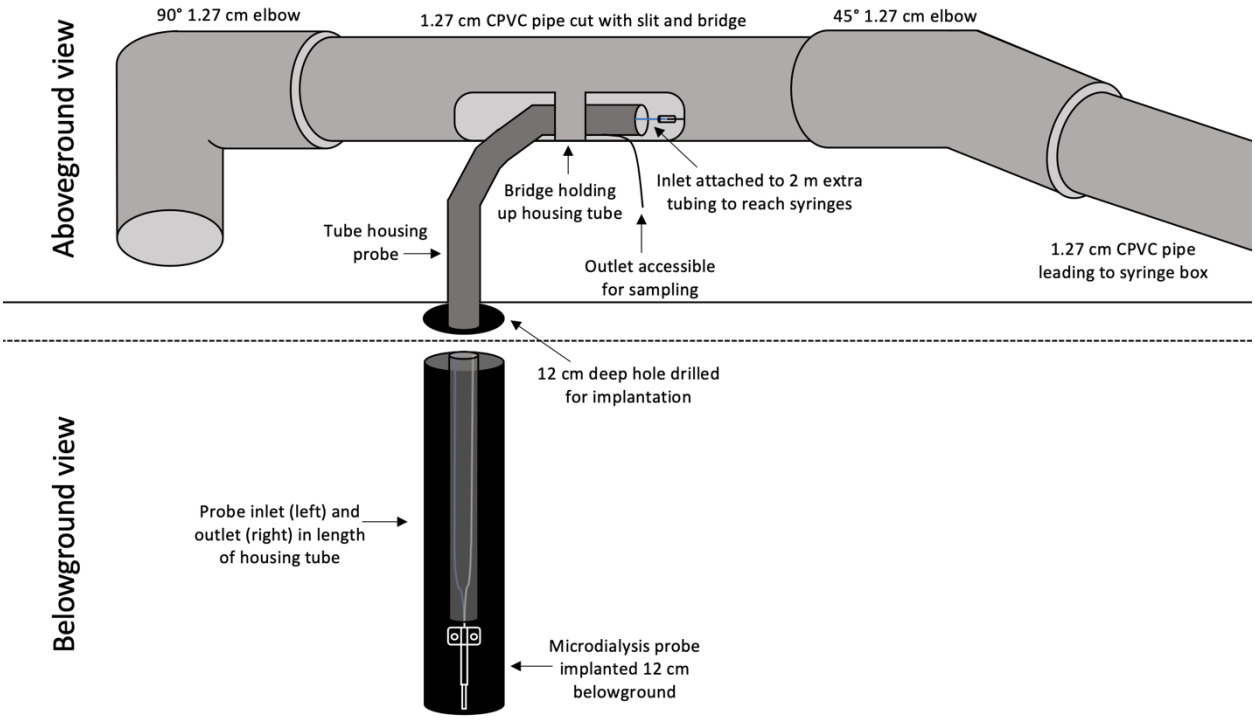

Figure 3. The protective bridge constructed to protect the probe outlet and tubing connection from warping or kinking.

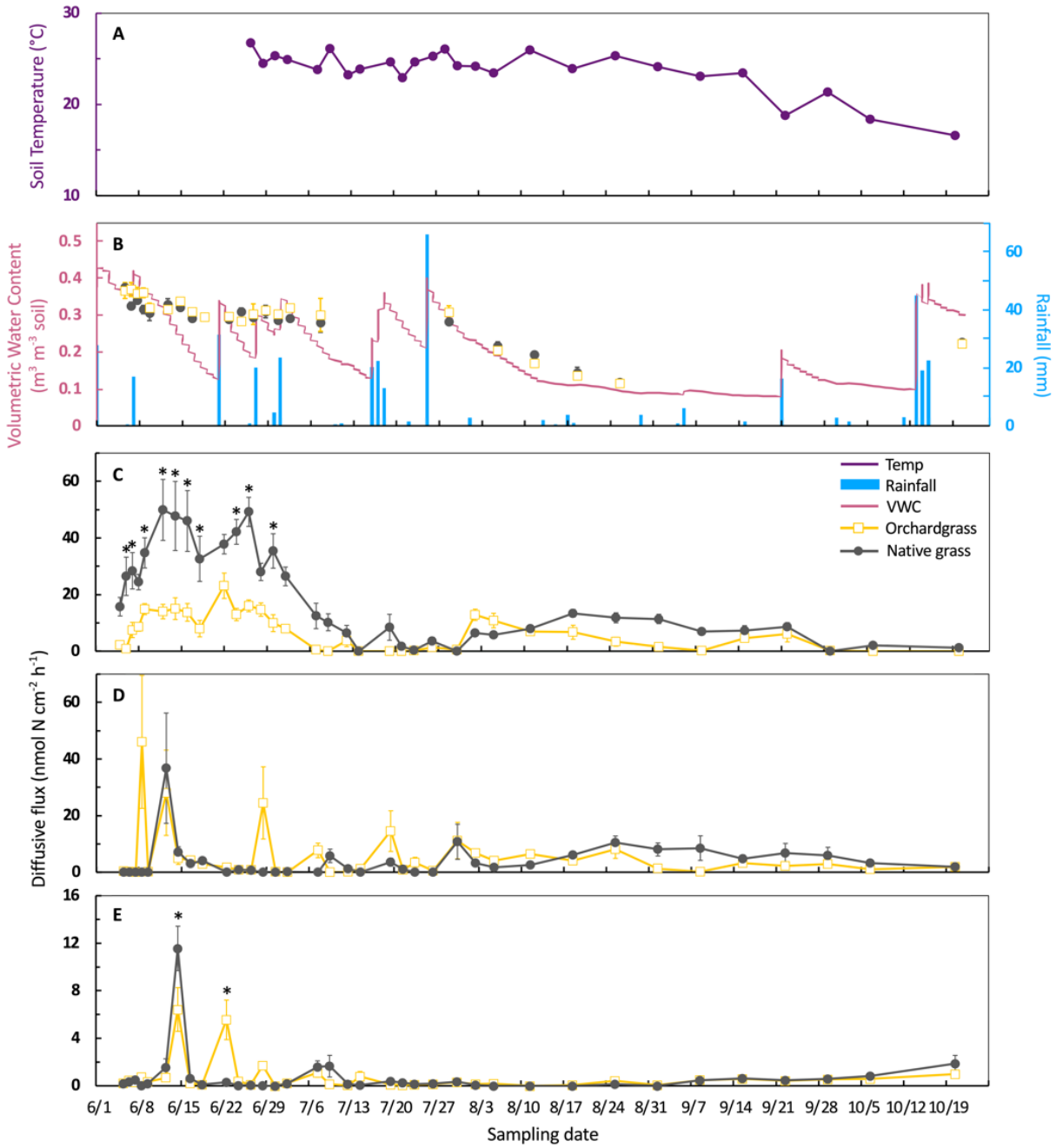

Figure 4. Overview of A) soil temperature (n = 3) at a 12 cm depth, B) hourly rainfall (mm) and volumetric water content (VWC; m^3 m^{-3} soil; 3 cm depth) data obtained from the USDA weather station with an overlay of VWC at 12 cm in plots growing orchardgrass (yellow squares) and a mixture of native grasses (gray filled circles), C) nitrate-N, D) ammonium-N, and E) total amino acid diffusive flux (nmol N cm^{-2} h^{-1}) data obtained from microdialysis probes implanted in plots of orchardgrass and native grasses in the months of June – October, 2021. Flux data points signify average diffusive fluxes and error bars represent SE (n = 12). Asterisks (*) denote sampling dates on which diffusive fluxes were significantly different between the two plant communities ($P < 0.05$).

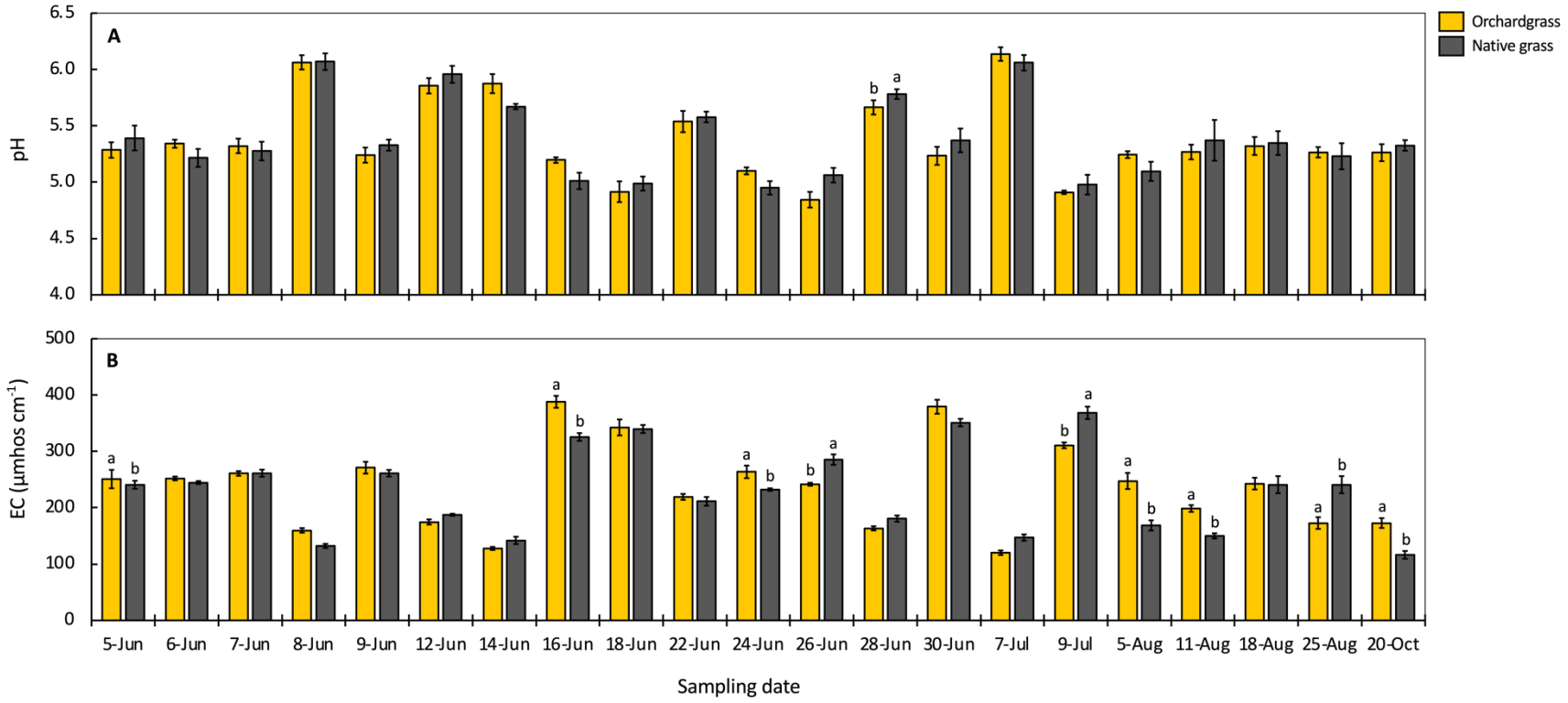

Figure 5. A) Soil pH and B) electrical conductivity (EC; µmhos cm^{-1}) of the orchardgrass (yellow) and native grass (gray) plant communities over time. Bars represent average pH and EC values ± SE with different lowercase letters showing days on which the pH and EC values are statistically different between the plant communities (n = 12; $P < 0.05$).

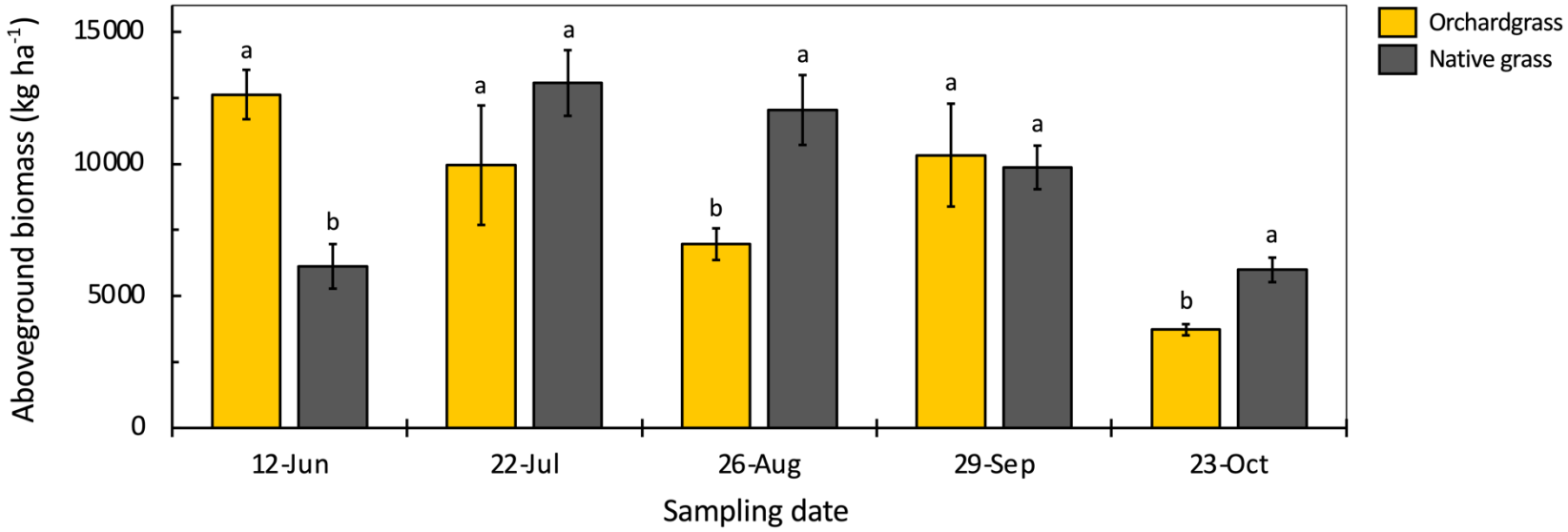

Figure 6. Monthly aboveground biomass production (kg ha^{-1}) in orchardgrass (yellow) and native grass (gray) plants during field experimentation in 2021. Bars denote average concentrations ± SE (n = 3), and different lowercase letters represent sampling dates on which biomass production were statistically different between the two plant communities ($P < 0.05$).

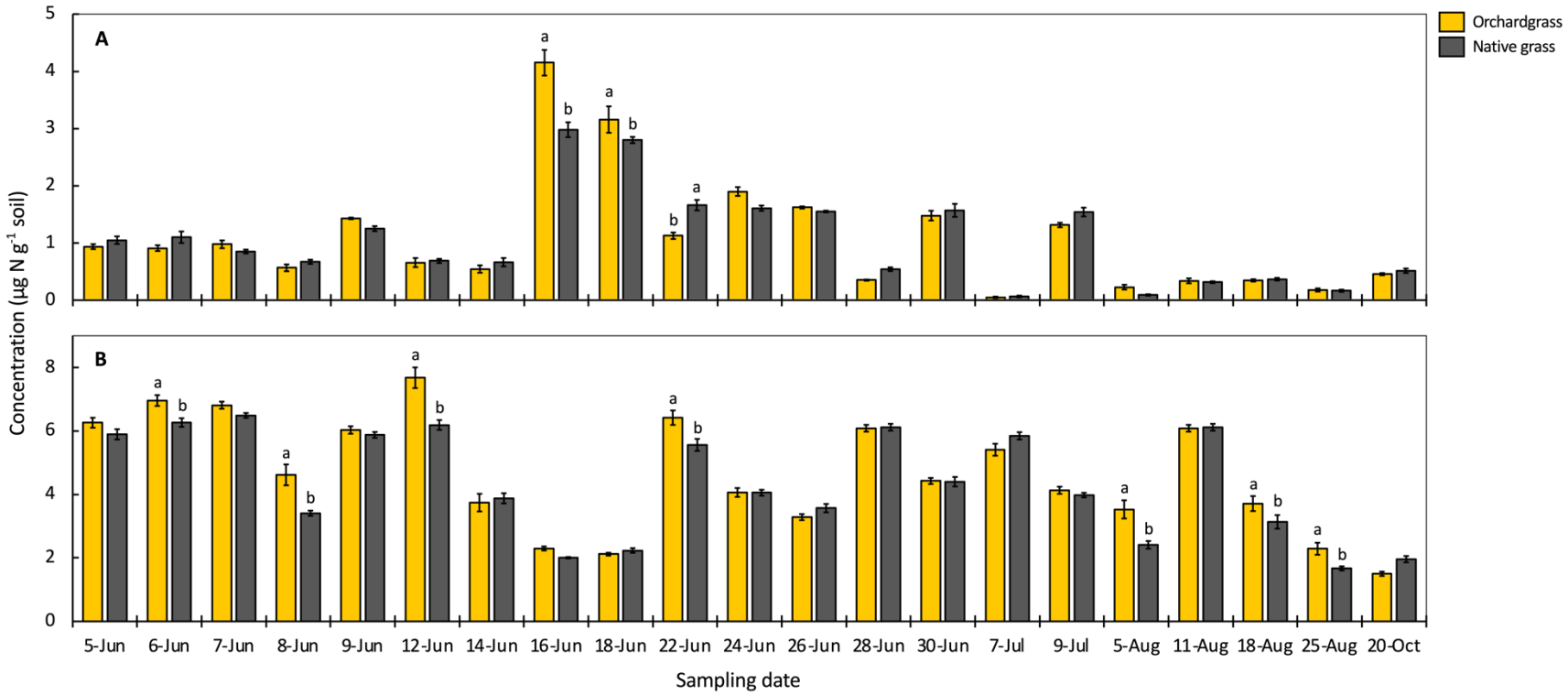

Figure 7. Concentrations of A) nitrate-N and B) ammonium-N (µg N g^{-1} soil) in orchardgrass (yellow) and native grass (gray) soil sampled in the months of June – October, 2021. Bars denote average concentrations ± SE (n = 12), and different lowercase letters represent time points on which concentrations are statistically different between the orchardgrass and native grass soils ($P < 0.05$).

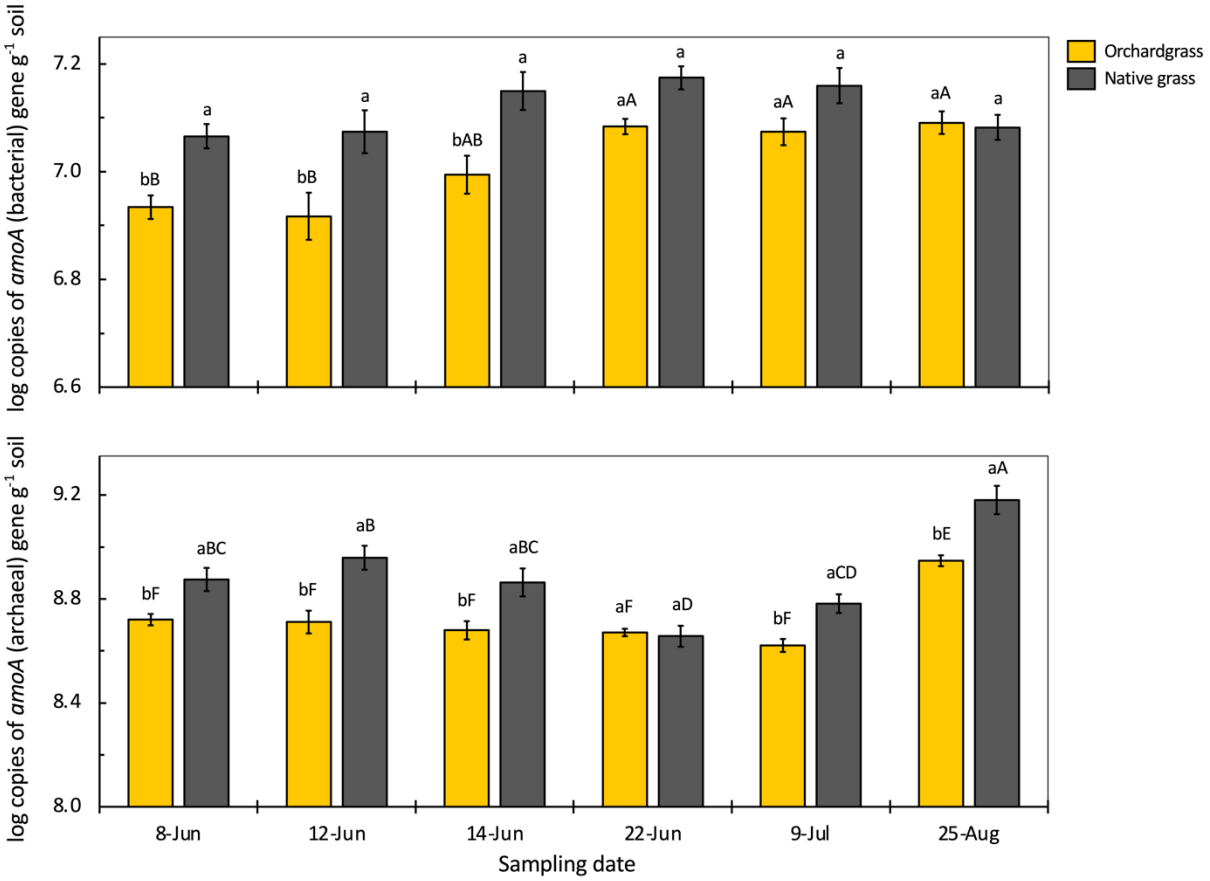

Figure 8. Copies of A) bacterial *amoA* gene g^{-1} soil and B) archaeal *amoA* gene g^{-1} soil on a log scale in plots growing orchardgrass (yellow) and native grasses (gray). Bars denote average log copy numbers ± SE (n = 12). Different lowercase letters within each graph indicate significant differences in number of gene copies between the two plant communities within the same sampling date. Different uppercase letters indicate differences in gene copies in the same plant community over time ($P < 0.05$).

Table 1. All microdialysis sampling days. The days indicated with asterisks (*) are days on which soil cores were also collected for soil analyses.

Month	Sampling date
June	5*, 6*, 7*, 8*, 9*, 12*, 14*, 16*, 18*, 22*, 24*, 26*, 28*, 30*
July	2, 7*, 9*, 12, 14, 19, 21, 23, 26, 30
August	2, 5*, 11*, 18*, 25*
September	1, 8, 15, 22, 29
October	6, 20*

Table 2. Overview of the target genes their respective thermal profiles.

Target gene	Primer	Product length (bp)	Reference	Initial denaturation	Number cycles	Denaturing	Annealing	Elongation	Final elongation
amoA (AOB)	AmoA1F/ AmoA2R	491	He et al., 2018	95 °C, 10 min	40	95 °C, 15s	60 °C, 120s	72 °C, 60s	
amoA (AOA)	Arch-amoAF/ Arch-amoAR	635	Szukics et al., 2012	95 °C, 5 min	45	95 °C, 45s	53 °C, 60s	72 °C, 60s 78 °C, 60s	72 °C, 10 min

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CHAPTER 3:
Elucidating transient effects of wetting-drying cycles in soil by utilizing the in situ, nondestructive microdialysis technique[§]

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Elucidating transient effects of wetting-drying cycles in soil by utilizing the in situ, nondestructive microdialysis technique

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Abbreviations: N, nitrogen; KCl, potassium chloride; WFPS, water-filled pore space; ND; not detected.

Abstract

Soils undergo rapid changes in biological, chemical, and physical properties as they endure wetting-drying cycles. As climate models project more frequent and extreme precipitation/drought events in coming years, it has become essential to better understand the effect of soil-water availability, as well as rapid changes in water content, on N cycling. Conventional methods of soil N sampling entails the use of extractions using salt or water, which are destructive in nature and result in total loss of soil structure. More importantly, extraction-based methods provide little information on the heterogeneity of soils, usually found in microsites. To circumvent the issues encompassing soil extractions, microdialysis was used to provide temporally-resolved data. In this study, soil cores repacked with field-moist or air-dried soils to a specified bulk density were implanted with microdialysis probes and either wet or rewet to 50, 60, 80, or 100% water-filled pore space (WFPS). The cores were incubated and microdialysis sampling was utilized to provide a temporal distribution of inorganic and organic forms of N for a total of 120 hours. The outcome of this study was that previously field-moist and air-dried soils exhibited distinct patterns of diffusive fluxes of nitrate-N, ammonium-N, and amino acids, demonstrating the importance of antecedent moisture and impact of drying on immediate N fluxes and immediate N availability in soil of humid regions after periods of drying. Gaining insight into the forms, transformations, and cycling of bioavailable N present in soil solution proves to be invaluable knowledge as this otherwise transient information that was unattainable in the past can bridge current knowledge gaps present in understanding soil wetting-drying cycles.

1. Introduction

Fluctuations in soil-water availability is typical as soil undergoes wetting-drying cycles throughout the year. Soils of Arkansas are subjected to episodic weather patterns such as extreme precipitation and droughts due to the state's Köppen classification as a humid subtropical climate (Chen & Chen, 2013). Analyses of climatic data project more frequent extreme weather events, making it paramount to understand the effects of wet-dry periods on shifts in underlying physical, chemical, and microbial processes (Leitner et al., 2017; Siebielec et al., 2020). Investigating the role of soil-water availability in nitrogen (N) cycling can lead to better comprehension of nitric oxide (NO) and nitrous oxide (N_2O) production – an air pollutant and a potent greenhouse gas, respectively – as well as fluxes of bioavailable nutrients in the form of organic nitrogen (amino acids) and inorganic nitrogen [ammonium-N (NH_4^+-N) and nitrate-N (NO_3^--N)] (Cui & Caldwell, 1997; Leitner et al., 2017). While inorganic N is generally more abundant in soil, amino acids are the second most abundant class of compounds exuded by plant roots after sugars, making them easily available for rhizospheric microbial uptake (Moe, 2013; Vargas et al., 2019). According to Hu et al. (2017), polymer-bound amino acids account for 30-60% of total soil nitrogen, however, free amino acids only contribute to less that 1% of the total pool but is highly dynamic with residence times ranging in minutes to hours. Since amino acids are involved in both plant root and microorganism activity – are also the chemical precursor for ammonium-N – understanding amino acid fluxes would also prove beneficial to elucidating changes in the rates and pathways of N cycling (Hu et al., 2017; Moe, 2013).

The inundation of soil pores with water regulates microsite connnectivity, diffusivity of gases, and most importantly, the availability of oxygen. The amount of oxygen present in soil pores – which is indirectly affected by soil moisture content – either stimulates or impedes

microbial activity within N processes such as nitrification and denitrification, which is related to the role of oxygen as an electon acceptor in metabolic reactions (Lammel et al., 2015). The entire process of nitrification requires the presence of oxygen on account of the oxidization of ammonia to nitrate, the most oxidized form of N (Hayatsu et al., 2008). Additionally, ammonium monooxygenase (AMO), involved in the first step of nitrification, requires oxygen for activation. Historically, soil moisture contents around 60% water filled pore space (WFPS) were considered optimal for nitrification because neither the diffusion of substrates nor diffusion of gases are restricted (Bollmann & Conrad, 1998; Linn & Doran, 1984). On the other hand, moisture contents above 60% were thought to favor anaerobic processes such as denitrification since denitrifying enzyme activities are deactivated or inhibited by O_2 and the expression of the encoding genes are suppressed (Bernhard, 2010; Hayatsu et al., 2008; Pajares & Bohannan, 2016). However, the discovery of archaeal nitrification among other novel N cycling processes such as archaeal denitrification, anammox, nitrifier denitrification, and aerobic bacterial denitrification, brought light to the oversights of prevailing theories on the interplay between physical soil characteristics and N dynamics (Hayatsu et al., 2008).

A multitude of studies, each with their own focus, have been conducted to further understand the intricacies of nitrogen cycling. Some emphasized the role of soil moisture on N_2O and NO gas emissions, some investigated the effects of differences in soil moisture on plant root nutrient availability and uptake, and some took a more molecular approach and studied gene copies and transcript abundances upon rewetting (Bateman & Baggs, 2005; Cui & Caldwell, 1997; Leitner et al., 2017; Placella & Firestone, 2013). Yet, to understand availability of small molecules of N that can be taken up by organisms in soil, conventional analysis of inorganic N levels rely on destructive sampling of soil using salt (KCl or K_2SO_4) extractions due to difficulty

in monitoring nitrogen in intact soil to determine rewetting dynamics (Bateman & Baggs, 2005; Cabrera, 1993; Cui & Caldwell, 1997; Fierer & Schimel, 2002; Placella & Firestone, 2013; Siebielec et al., 2020). Disturbing the soil during sampling for extractions can overestimate bioavailable N, resulting in altered N concentrations and inaccurate estimations of microbial processes and lead to additional convolutions of understanding and studying N dynamics (Leitner et al., 2017). The use of microdialysis, which has the ability to provide real-time data in a minimally invasive manner can alleivate the problems posed by extraction-based sampling methods (Inselsbacher et al., 2014; Inselsbacher et al., 2011; Leitner et al., 2017; Maddala et al., 2020, 2021). Continuous *in situ* sampling of nitrogen dynamics by utilizing the microdialysis technique can provide data about N in soil solution, bioavailable N, in a temporally resolved manner.

This study seeks to augment an experiment conducted by Maddala et al. (2020), which entailed the use of microdialysis to detect and measure fluxes of nitrate-N and ammonium-N in a soil upon rewetting to 50% and 100% saturation. Nitrate-N flux in the 50 and 100% saturated soils did not differ statistically in a previous experiment in which soils were equilibrated for 72 hours to allow the flush of microbial activity after re-wetting to subside (Maddala et al., 2020). In contrast, ammonium-N fluxes in soil at the two levels of saturation did differ significantly. Since other past studies also state that rewetting dynamics are concentrated in the first four days upon watering, the timeline of this current study was designed with the intention of capturing most of the ephemeral N dynamics, especially in the first few hours upon rewetting (Cabrera, 1993; Fierer & Schimel, 2002). Because microdialysis probes can be implanted and left in soil for the duration of experimentation, the technique offers the ability to sample N from the same

soil every couple of hours, providing more mechanistic insight into the "flush" of activity upon rewetting instead of utilizing soil extractions to measure inorganic N.

The objectives of this study were to 1) continuously monitor inorganic nitrogen (NH_4^+-N and NO_3^--N) and organic nitrogen (total amino acids) fluxes in soil cores that were constructed with soil that was kept field-moist or soil that was air-dried, and to 2) determine the differences in timing of and mechanisms of N flux after watering the field-miost and air-dried soil cores to bring the water-filled pore space (WFPS) to 50, 60, 80, and 100%. It was hypothesized that the previously field-moist and air-dried soils would exhibit different trends in N dynamics when comparing the same soil saturations. Air-dried soils would contain reduced diversity of soil microorganisms, surviving microbes would be more resistant to water stress, and rewetting is likely to display a larger flush of activity (greater availability of N) due to the rapid mineralization of necromass by the surviving community.

2. Materials and methods

2.1. Soil description

Soil was obtained from a location growing plots of orchardgrass (*Dactylis glomerata* L.) adjacent to mixed native grasses (big bluestem [*Andropogon gerardii* Vitman], little bluestem [*Schizachyrium scoparium* (Michx.) Nash], and Indiangrass [*Sorghastrum nutans* (L.) Nash]) at the Milo J. Shult Main Agricultural Research and Extension Center in Fayetteville, AR, USA (36°05'30.6" N 94°11'19.0" W). The soil is classified as a Captina silt loam (fine-silty, siliceous, active, mesic Typic Fragiudults). Volumetric soil moisture and bulk density were determined by collecting soil core samples (diameter = 5 cm; n = 3) from the upper 10 cm of the soil profile in each plant community and dried at 55 °C for five days (Blake & Hartge, 1986). Soil was

analyzed for pH and electrical conductivity (EC) by the electrode method (1:2 (w/v) soil-to-water ratio) (Sikora & Kissel, 2014), soil organic matter (SOM) percentage using the loss-on-ignition method at 450 °C (Nelson & Sommers, 1996), particle size analysis by the micropipette method (Miller & Miller, 1987), and total soil N and C by combustion analysis (Provin, 2014). Soil was extracted using a 1:10 (w/v) soil-to-Mehlich-III solution ratio and extracts were analyzed by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Zhang et al., 2014). Inorganic nitrogen was extracted using a 1:10 (w/v) soil-to-2*M* potassium chloride (KCl) ratio and all results were reported on a per g dry soil basis (Mulvaney, 1996). All extraction samples were stored at 4 °C until analysis.

2.2. Core construction and microdialysis probe implantation

Soil from the orchardgrass, native grasses, and transition area between the two grass communities was collected in equal portions (approximately 5 kg from three sections to yield 15 kg in total), sieved using a 4.75-mm sieve, and homogenized. Approximately half of the homogenized soil was set aside to air dry at room temperature. The remaining soil was kept field moist and repacked into soil cores (diameter of 5-cm and 10-cm height) with a bulk density of 1.2 g cm^{-3}. Since the diameter of the soil cores would not allow for simultaneous undisturbed microdialysis sampling and destructive sampling for KCl extraction, a total of 12 soil cores were prepared for microdialysis sampling while another set of 12 were prepared exclusively for soil sampling (Figure 1).

The microdialysis apparatus was set-up similar to Maddala et al. (2020, 2021), which was adapted from Inselsbacher et al. (2011). One microdialysis probe was implanted into each of the 12 designated soil cores to a depth of 5 cm. MilliQ (MQ) water was perfused at a flow rate of 2

$\mu L\ min^{-1}$ for the duration of all experimentation in the field-moist and air-dried soils. An equilibration time of 15 minutes was used at the beginning of every sampling, and all probes were calibrated by placing into a solution of 10 µg/mL nitrate-N and 10 µg/mL ammonium-N with stirring prior to experimentation (Maddala et al., 2020).

2.3. Water addition and data collection

The volume of MQ water needed to saturate the soil to 50%, 60%, 80%, or 100% WFPS was calculated using a series of equations based on bulk density, total pore space, volumetric water content, and gravimetric water content (Weil & Brady, 2016) and added to each designated core for the specified WFPS (Table 1). Microdialysis samples were collected every 3 hours for 12 hours, every 6 hours for the next 12-hour period, every 12 hours for the next 48 hours, and then every 24 hours for the next 48 hours. At each time point, approximately 1 g of soil was collected from the set of extraction cores, at approximately the same depth as probe implantation, and stored for further analysis at -80 °C. Of the 1 g collected, 0.5 g was used for a scaled-down version of KCl extractions (Mulvaney, 1996). The same procedure was repeated with the air-dried soil, with the volume of MQ water required for re-saturation being added adjusted for air-dry conditions (Table 1).

2.5. Sample chemical analysis

All microdialysis samples were analyzed by vanadium chloride (VCl_3) reduction and the Griess reaction based on the technique described by Miranda et al. (2001) for nitrate-N and the microplate adaptation of the indophenol Berthelot reaction described by Baethgen and Alley (1989) for ammonium-N. Absorbance values were measured at 540 nm and 650 nm for the

Griess and indophenol Berthelot reactions, respectively. Additionally, the dialysate samples were analyzed for total amino acids (Leucine-equivalents) (Darrouzet-Nardi et al., 2013; Jones et al., 2002). Fluorescence values were obtained using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Standards and blanks were microplated in triplicates. Absorbance and fluorescence values were all measured using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, LLC, San Jose, CA, USA) and Tecan Infinite m200 Plate Reader (Tecan, Männedorf, Switzerland). The concentrations of nitrate-N, ammonium-N, and amino acids obtained from the dialysate samples were used to calculate the diffusive flux of the analytes across the semipermeable membrane of the probe (surface area 0.159 cm^2) and were reported in units of nmol N cm^{-2} h^{-1}. Detection limits for nitrate-N, ammonium-N, and amino acid fluxes were 4.16, 2.79, and 1.64 nmol N cm^{-2} h^{-1}. Values below detection limits were reported as ND on graphs and were put in as zeroes for statistical analyses.

2.6. Statistical analysis

Statistical analyses on microdialysis fluxes were performed by a two-way repeated measures ANOVA followed by appropriate mean separation for pairwise comparisons using SigmaPlot 14.5 (Systat Software, Inc.). Differences were considered significant at $P \leq 0.05$. All reported values are means ± SE.

3. Results

3.1. Soil characteristics

The bulk density of the homogenized soil was measured to be 1.23 ± 0.03 g cm^{-3}. The pH and EC of the soil was 5.26 ± 0.17 and 241.7 ± 7.6 μhos cm^{-1}, respectively. Inorganic N content

in the soil was 5.33 ± 0.10 μg NO_3^--N g^{-1} soil and 3.05 ± 0.07 μg NH_4^+-N g^{-1} soil. The texture of the soil was experimentally determined to be a silt loam with 14% clay, 64% silt, and 22% sand. Soil organic matter content was measured to be 2.53 ± 0.06% using the loss-on-ignition method.

3.2. Field-moist soil

Under field-moist conditions, nitrate-N diffusive fluxes were above detection limits and were measured consistently throughout the 120-hour experiment in all four water contents (Figure 2). During the duration of the experiment, the soils held at 50 and 60% WFPS did not differ statistically in nitrate-N flux ($P > 0.05$). Nitrate-N flux measured in the soils brought to 80 and 100% WFPS also did not differ between each other at any time point. The 80 and 100% WFPS soils displayed increased diffusive fluxes of nitrate-N in comparison to the 50 and 60% saturated soils in the first 12 hours after wetting ($P < 0.05$). At hour 18, the 100% WFPS soil exhibited greater nitrate-N flux than the soil at 50 and 60% WFPS, and the nitrate-N flux in the 80% WFPS soil was not significantly different than in soil at 60% WFPS ($P > 0.05$). At hour 24, nitrate-N flux significantly differed in soil at 50% and 100%, but not in soil at 50, 60, and 80% or in soil at 60, 80, and 100% WFPS. When comparing among the different WFPS percentages after 24 hours, nitrate-N fluxes in the soils did not differ.

When comparing nitrate-N fluxes over time within the same water content, there was no significant effect of water addition in the soil at 50% and 60% WFPS over the 120-hour period ($P = 1.00$). In other words, the diffusive fluxes within these treatments stayed consistent with values ranging from 0.36 ± 0.21 to 30.82 ± 9.60 nmol N cm^{-2} h^{-1} and 12.65 ± 0.66 to 44.97 ± 22.89 nmol N cm^{-2} h^{-1} in the 50% WFPS soil and the 60% WFPS soil, respectively. In the 80 and 100% WFPS soil, fluxes were significantly greater over time points 0-24 than they were over

hours 36-120. Within the 120-hour timeframe, a decrease in nitrate-N flux was observed during the first 24 hours such that flux was statistically slower after 36 hours compared to earlier time points during the first 24 hours after rewetting field-moist soil. Flux values in field-moist soil wet to 80% WFPS did not differ between hours 36 to 120. Nitrate-N flux in soil at 80% WFPS ranged from 74.31 ± 33.38 to 384.37 ± 38.95 nmol N cm^{-2} h^{-1}. In the soil that was watered to 100% WFPS, there were no significant differences in the first 24 hours of the experiment, or between hours 36 to 120. Mean nitrate-N fluxes in the 100% soil ranged from 21.89 ± 1.11 to 309.10 ± 129.75 nmol N cm^{-2} h^{-1}.

Ammonium-N fluxes were below detection limits until hour 36 and there were no significant differences between soil saturation treatments or within treatments over time (Figure 3; $P = 0.473$ for WFPS and $P = 0.388$ for time effects). Amino acid fluxes were also small throughout most of the experiment and were not significant in either treatment or time (Figure 4; $P = 0.078$ for treatment effects and $P = 0.250$ for time).

3.3. Air-dried soil

The diffusive fluxes of inorganic and organic N differed in the air-dried soil upon rewetting compared to the field-moist soil after re-wetting. Nitrate-N diffusive flux, and at most of sampling times during the incubation, the fluxes in ammonium-N and total amino acids – in particular in the soil rewet to 80 and 100% WFPS – had diffusive flux measurable above detection limits (Figures 5, 6, and 7). Furthermore, significant differences were measured between sampling times and water saturations for nitrate-N, ammonium-N, and total amino acid diffusive fluxes.

When measuring nitrate-N fluxes, the 80 and 100% saturated soils were significantly increased when compared to the 50 and 60% saturated soils at time 0 (Figure 5). After 3 and 6 hours of incubation, nitrate-N flux at 50% WFPS was less than 80 and 100%, while flux at 60% WFPS did not significantly differ from 50 or 100% WFPS. From hour 9 through the remainder of the 120-hour incubation, soil at all four water saturations did not differ in nitrate-N diffusive fluxes at each sampling time except at hour 48, where there was a significant spike in flux in the 60% WFPS soil, resulting in a difference from the other three soil saturations ($P < 0.001$).

Within treatments, the 50% saturated soil did not significantly change throughout the experiment, with mean diffusive flux values ranging from 6.98 ± 0.41 to 53.73 ± 8.86 nmol N cm^{-2} h^{-1} during the 120-h period of experimentation ($P = 1.00$). The 60% soil also exhibited fluxes that did not change significantly throughout the experiment, with the only exception resulting from the sudden uptick in nitrate-N flux at time point 48. With the increase in nitrate-N flux at 48 hours, subsequent fluxes at hours 72, 96, and 120 were significantly reduced compared to the increased flux at time point 48 ($P = 0.026$, $P < 0.001$, and $P < 0.001$, respectively). However, when compared to the diffusive fluxes in the first 36 hours, the flux at hour 48 was not statistically different from any of them (hours 0-36), and the fluxes measured at 72, 96, and 120 hours were not statistically different from any sampling time other than hour 48. In the 60% WFPS, soil diffusive fluxes ranged from 15.15 ± 3.70 to 144.24 ± 66.93 nmol N cm^{-2} h^{-1}.

Within the 80% saturated soil, fluxes ranged from 14.66 ± 0.58 to 190.68 ± 44.56 nmol N cm^{-2} h^{-1}. Nitrate-N flux at time point 0 was significantly increased in comparison to hour 9 and all following time points ($P = 0.026$; $P < 0.05$). While diffusive fluxes at time points 0, 3, and 6 were not different statistically, there was a numerical decrease every 3 hours, which resulted in a significantly decreased flux at hour 12 when compared to the initial sampling times ($P < 0.05$).

After hour 12, fluxes in the 80% treatment did not differ statistically for the remainder of the sampling regime. The 100% saturated soil exhibited a similar pattern of significance as the 80% WFPS soil in that time point 0 also demonstrated increased flux when compared to hour 9 flux and all following time points thereafter ($P = 0.003$; $P < 0.05$). Time points 0, 3, and 6 were also not different. The range of flux values in the 100% saturated soil was 10.38 ± 0.52 to 149.91 ± 34.42 (nmol N cm^{-2} h^{-1}).

The trends observed in the ammonium-N fluxes showed an inverse pattern to those of nitrate-N results in the wetter soils (Figure 6). There were no measurable treatment effects in the first 24 hours of sampling ($P > 0.05$). Upon 36 hours of rewetting, both the 80 and 100% saturated soils exhibited greater fluxes than the 50 and 60% soils ($P < 0.05$). Fluxes in the 50 and 60% WFPS soils did not differ, nor did fluxes in the 80 and 100% soil ($P > 0.05$). These differences continued until hour 96 and 120, where ammonium-N flux became greater in the 100% saturated soil ($P = 0.016$ at hour 96 and $P < 0.001$ at hour 120), but flux in soil at 60 and 80% WFPS did not differ ($P > 0.05$) and flux at 50% WFPS was below detection. By the end of the experiment, at hour 120, the 100% saturated soil exhibited ammonium-N fluxes that were approximately 10 times greater than the 80% soil.

When comparing differences within each treatment, neither the 50% nor the 60% soils exhibited differential ammonium-N fluxes throughout the experiment ($P > 0.05$). Although not significantly different over time, ammonium-N fluxes in the 50% and 60% soils started at very low or below detection levels, steadily increased numerically until hour 18, and then gradually decreased to below detection levels at time point 120. Diffusive fluxes ranged from below detection limits to 16.12 ± 9.24 nmol N cm^{-2} h^{-1} and below detection limits to 18.74 ± 2.78 nmol N cm^{-2} h^{-1} in the 50% WFPS soil and 60% WFPS soil, respectively. In the 80% treatment,

ammonium-N flux was increased significantly at hour 36 when compared to hours 0, 3, 6, 9, and 12 ($P < 0.05$). Fluxes at time points 18 and 24 were intermediate between the two groups of significance in that they were not different to hours 0, 3, 6, 9, and 12 as well as hour 36. After 36 hours, ammonium-N fluxes plateaued and then decreased between hour 72 and 120, such that flux at 120 hours did not differ from those fluxes measured during the initial 24 hours.

Within the 100% saturated soil, ammonium-N fluxes did not increase within the first 24 hours and then increased slowly throughout the remainder of the incubation through 120 hours. Time point 36 demonstrated elevated ammonium-N flux when compared to hours 0, 6, 9, and 12 ($P < 0.05$). Time points 3, 18, and 24 were not different to hour 36 in flux, and flux at hour 36 was not different than fluxes at hours 48, 60, 72, and 96 ($P > 0.05$). At and after 60 hours, ammonium-N flux increased to an amount greater than any fluxes before time point 18 (range of $7.65 \pm 4.03 – 25.87 \pm 7.33$ nmol N cm^{-2} h^{-1} in the first 18 hours vs. 53.84 ± 8.04 at 60 hours). Following the pattern of steady increase, hour 96 exhibited larger flux values than what was measured at or before hour 24 ($P < 0.001$). Finally, after 120 hours of the rewetting event, ammonium-N fluxes were significantly greater in comparison to fluxes at or prior to 60 hours ($P = 0.031$).

Total amino acid (Leu-equivalents) fluxes were small, often below detection in soil at 50 and 60% WFPS especially after 36 hours of incubation, but were different across WFPS at 3, 18, 24, 36, 60, 96, and 120 hours (Figure 7). Fluxes at 80% WFPS were greater than amino acid fluxes at 50% at 3 hours ($P = 0.036$). At hours 18, 24, and 36, the 80 and 100% saturated soils displayed elevated amino acid fluxes in comparison to the 60% treatment ($P < 0.05$) and the 50% soil at hours 36 and 96. Fluxes in soil at 50% WFPS were below detection limits at hours 18, 24, 48, 60, 72, and 120. At hours 48, 72, and 96, diffusive fluxes in the 80% saturated soils

were not significantly different when compared to 100% saturation. In contrast, 80% WFPS displayed smaller fluxes than 100% WFPS at hours 60 and 120 ($P = 0.007$ and $P = 0.002$).

There were no significant differences in the fluxes of the 50%, 60%, and 80% saturated soils over time ($P > 0.05$). Within the 100% WFPS soil, flux values decreased to below detection limits at hour 3, and then increased at hour 6. However, the first significant difference was detected at hour 9, which exhibited an average flux of 0.34 ± 0.17 nmol N cm^{-2} h^{-1}, a decrease from the 0.72 ± 0.08 nmol N cm^{-2} h^{-1} measured at hour 0 ($P = 0.001$). Fluxes increased between hours 12 to 48, with a significant increase occurring between the fluxes below detection limits at hour 12 and 0.63 ± 0.10 nmol N cm^{-2} h^{-1} at hour 48 ($P < 0.001$). Amino acid diffusive fluxes in soil at 100% WFPS did not increase significantly again until hour 120 ($P < 0.001$).

4. Discussion

The objectives of this study were to 1) continuously monitor N dynamics resulting from the addition of water on a 5-day scale, and to use the flux data to 2) delineate the differences in N fluxes between soils rewet from either field-moist or air-dried conditions. These two objectives were fulfilled in that microdialysis yielded data that corroborated with previous wetting-rewetting studies and as hypothesized, there were distinct differences observed in nitrate-N, ammonium-N, and amino acid flux changes between the field-moist and air-dried soils.

Based on gravimetric water content data obtained at the time of field soil sampling and the total porosity of the soil (determined by the bulk density), the field-moist soil had a WFPS of 46.5% at the time of collection. Thus, a 3.5% increase in saturation resulted in 50% WFPS, and a 13.5% increase was needed for 60% saturation, resulting in little change in nitrate-N flux during the experiment at these two saturation levels from preexisting soil-water-microbial equilibrium.

There would be little expected water-stress present at the existing water contents, large pools of necromass would not exist and therefore, rapid mineralization would not occur after the addition of water, explaining the absence of ammonium-N fluxes with additional wetting of the field-moist soils (Siebielec et al., 2020). The lack of significant ammonium-N fluxes in field-moist cores throughout the duration of the 120-hour sampling period suggests that immobilization of ammonium-N by active soil microbes had already occurred prior to the experiment and that there was not a drastic change in ecological interactions occurring in the water-filled pore spaces (Cui & Caldwell, 1997).

The increased nitrate-N fluxes in 80% and 100% soil saturation at the beginning of the experiment could have resulted from improved connectivity of pores – thus diffusion – making a larger pool of nitrate-N available for sampling by the microdialysis probe. Prior to wetting of the field-moist soil – which was already at 46.5% WFPS – the presence of water, oxygen, and immobilized ammonium-N made conditions optimal for nitrification to occur, resulting in increased nitrate-N in soil solution that was easily accessible to microdialysis sampling upon wetting. As time elapsed, after approximately 36 and 48 hours in the 80 and 100% saturated soils respectively, nitrate-N fluxes significantly declined from flux levels observed in the first 24 hours, signifying that immobilization and subsequent denitrification to NO, N_2O, and N_2 gas occurred due to limited diffusion of oxygen (Scholes et al., 1997).

In the air-dried soil, diffusive ammonium-N flux at 80 and 100% saturation increased approximately 24 hours after rewetting, indicating a flush of mineralization that is typical of wetting dry soils (Cabrera, 1993; Leitner et al., 2017; Placella & Firestone, 2013). Rapid decomposition and mineralization following rewetting of a dry soil occurs due to the accumulation of labile organic matter from a myriad of sources: hydrologically disconnected

microsites can lead to separation between pores of mineralization from pores of immobilization, a sudden change in soil water potential may cause microbes to undergo osmotic shock and cell lysis, and necromass from organisms that could not survive drying conditions (Fierer & Schimel, 2002; Groffman & Tiedje, 1988; Leitner et al., 2017). Continued increase of ammonium-N, along with amino acids in the 80 and 100% saturated soils suggests prolonged mineralization, possibly due to microbes recovering from drought-induced stress, increased ecological interactions from connections between previously disconnected pores and aggregates facilitating mineralization of N, or soil N pool replenishment from recalcitrant/physically protected organic matter from cycling between air-drying and rewetting (Cabrera, 1993; Leitner et al., 2017; Savin et al., 2001; Wang et al., 2009). This relatively slower mineralization could help explain increasing ammonium-N and amino acid-N fluxes even at the end of the 120-hour incubation period in the soil rewet to 100% WFPS. Another explanation for prolonged mineralization in the 80 and 100% saturated soils is the decomposition-facilitation paradox described in Wang et al. (2009), which addresses the observation that the rate or extent of organic matter decomposition increases in the presence of bacterivores (grazers) that reduces bacterial abundances. Grazers can facilitate decomposition by releasing nutrients back into the soil environment, leading to increases in mineralization, thus ammonium-N and amino acid fluxes (Savin et al., 2001; Wang et al., 2009). Water content and the connectivity of aggregates, water films, and water-filled pores also influences the extent of mineralization and immobilization that occurs and how much N is available for measurement in the presence of ecological interactions (Savin et al., 2001).

Nitrate-N exhibited greatly increased fluxes immediately upon rewetting, especially at 80 and 100% soil saturation (Figure 4). A rapid decrease in diffusive flux may be attributed to immobilization and/or denitrification due to anaerobic conditions in the first 9 hours as typically

described (accounting for difference in soil type and variability in underlying microbial community adaptations) (Groffman & Tiedje, 1988; Scholes et al., 1997).

Conclusion

Shifting climatic conditions to more episodic patterns necessitates better understanding of microbial processes driving and resulting from wetting/drying cycles. The ability to nondestructively sample organic and inorganic forms of N during wetting-drying cycles and to differentiate timing of transient wet/dry responses would be very advantageous to elucidating N dynamics in soils. However, current methods of soil extractions rely on complete destruction of soil structure, making in situ measurement impossible. The use of microdialysis alleviates this problem and also makes continuous sampling attainable, providing temporally-resolved data on the predominant forms of bioavailable N upon wetting or rewetting to differential soil saturations. The use of microdialysis flux data and the further addition of microbial functional genes would offer insight into the mechanisms involved in soil N transformations.

Tables and Figures

Table 1. Volumes of MilliQ water (mL) required for specified water-filled pore spaces in the field-moist and air-dried soils.

	50%	60%	80%	100%
Field-moist	6.0	19.0	44.9	70.9
Air-dry	43.0	51.6	68.8	86.0

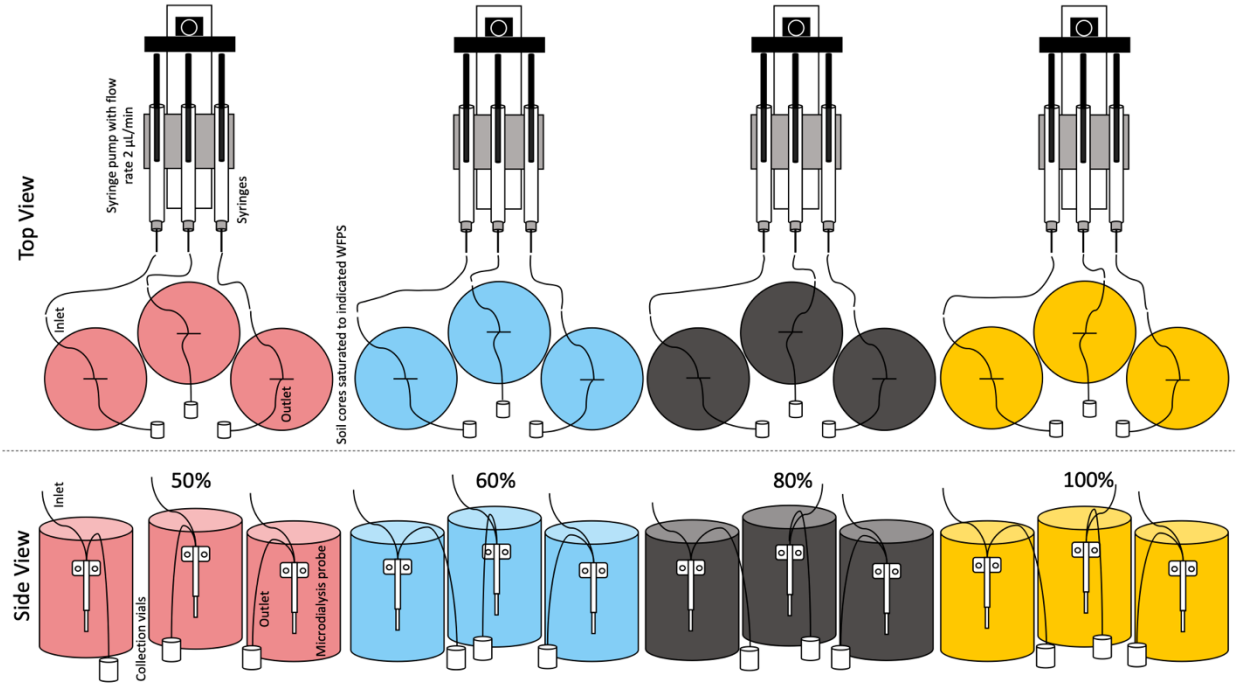

Figure 1. Diagram of laboratory experiment evaluating N fluxes and microbial communities for 5 days after rewetting field-moist and air-dried soil to 50, 60, 80 and 100% WFPS(water-filled pore space).

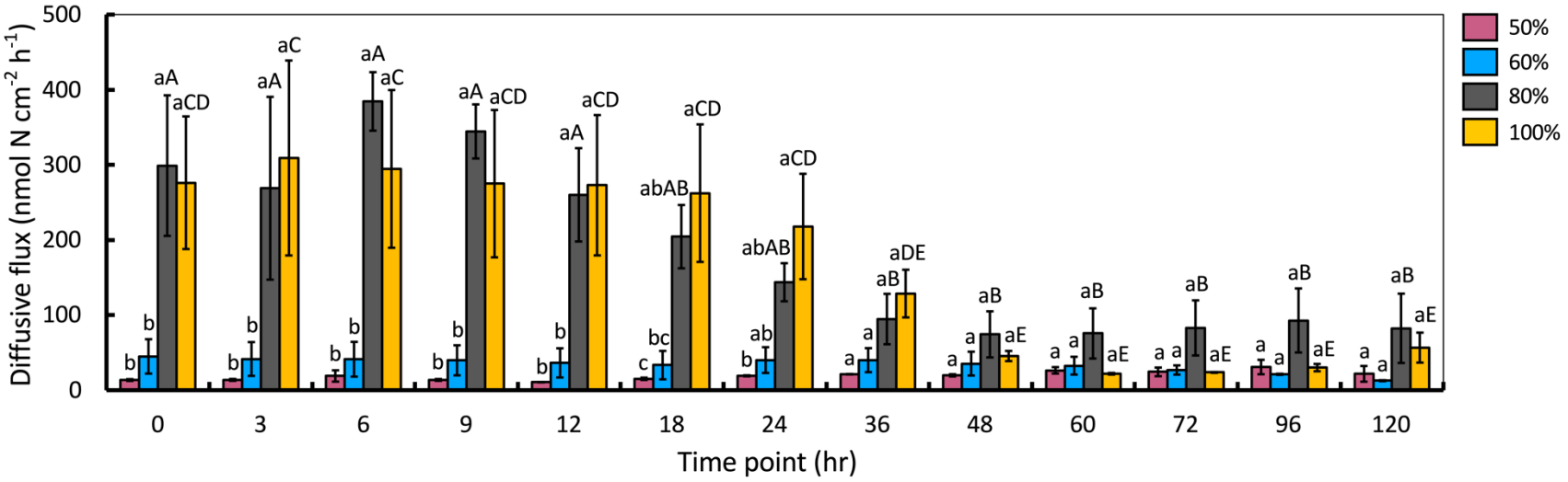

Figure 2. Mean (± SE) nitrate-N diffusive flux (nmol N cm^{-2} h^{-1}) collected using microdialysis (n = 3) in cores of field-moist silt loam soil wet to 50, 60, 80, and 100% water-filled pore space (WFPS) and incubated for 120 hours at room temperature. Different lowercase letters indicate statistical differences in flux between the soil saturation percentages within the same time point. Different uppercase letters indicate statistically different fluxes within the same soil saturation at different time points ($P < 0.05$).

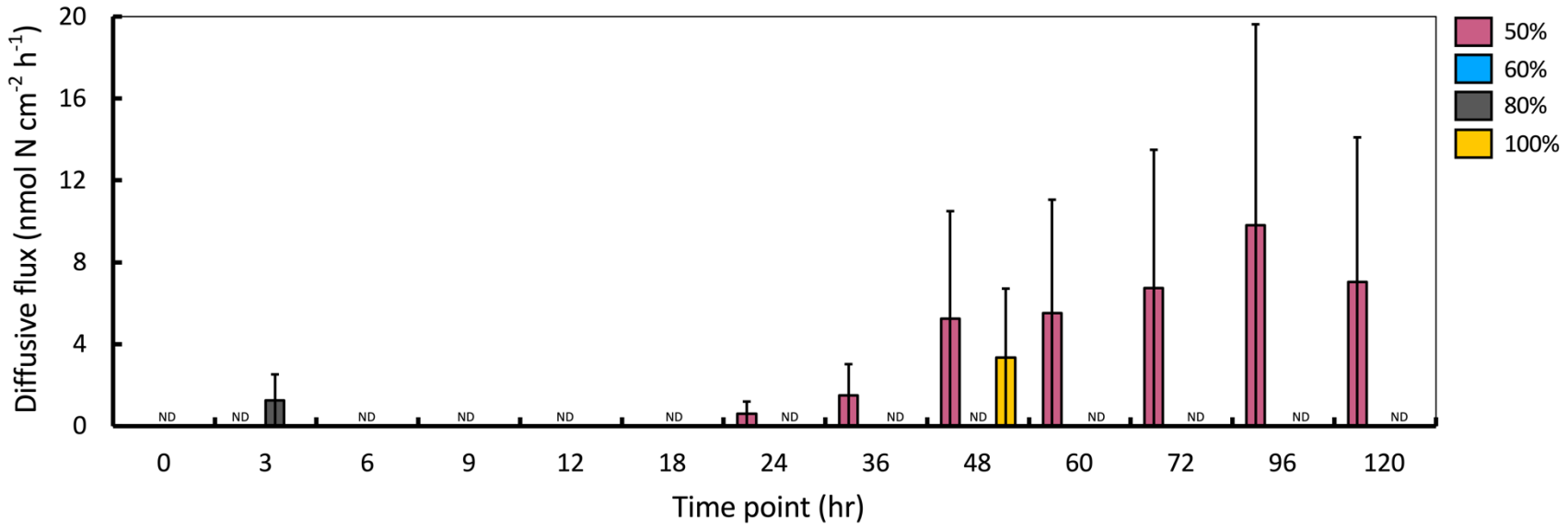

Figure 3. Changes in ammonium-N fluxes (nmol N cm^{-2} h^{-1}) in field-moist soil wet to 50, 60, 80, and 100% water-filled pore space (WFPS) and incubated for 120 hours at room temperature. Bars depict means ± SE (n = 3). ND indicates values below detection levels. Different lowercase letters indicate significant differences in flux between WFPS percentages within the same sampling time point. Different uppercase letters indicate differences in fluxes within the same soil saturation at different time points ($P < 0.05$).

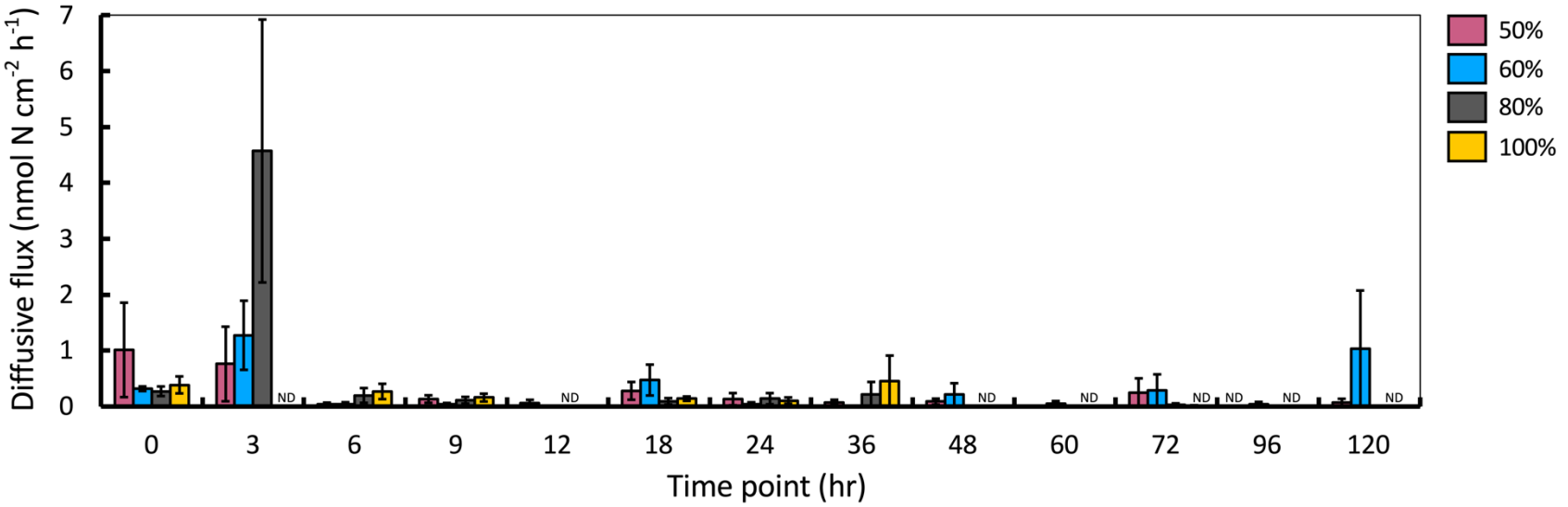

Figure 4. Total amino acid (Leucine-equivalents) fluxes (nmol N cm^{-2} h^{-1}) obtained from microdialysis sampling (n = 3) in field-moist soil wet to 50, 60, 80, and 100% saturation. Bars depict means ± SE. ND represents flux values below detection levels. Different lowercase letters indicate differences in flux between soil saturation percentages in the same sampling time point. Different uppercase letters signify statistically different fluxes within the same soil saturation at different time points ($P < 0.05$).

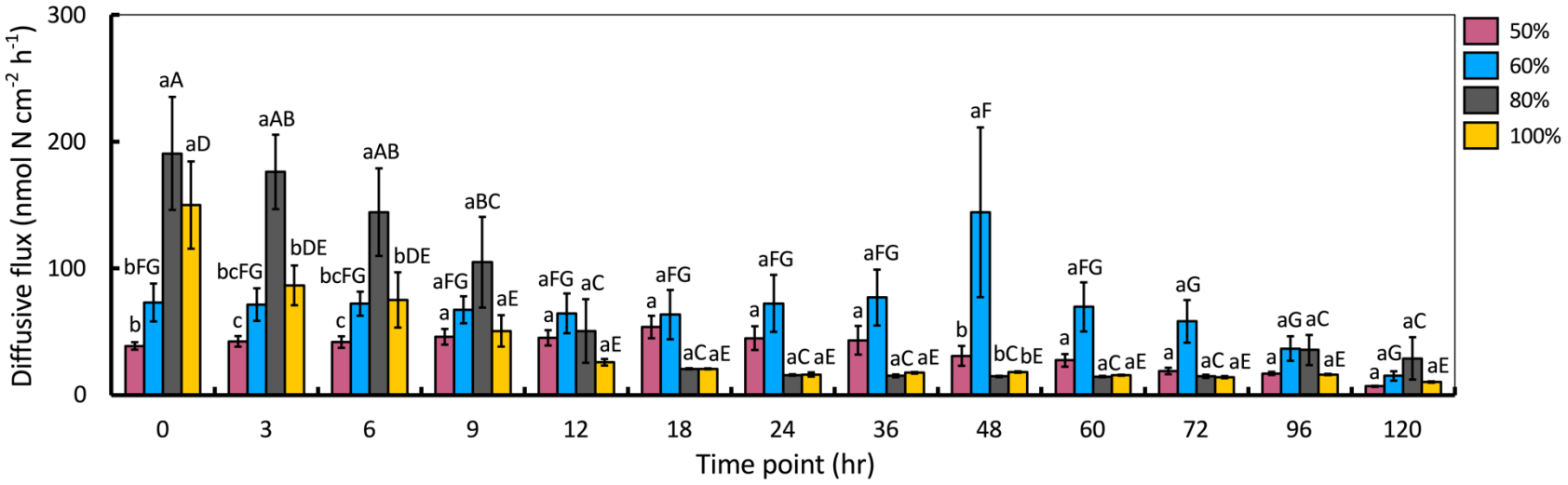

Figure 5. Nitrate-N fluxes (nmol N cm^{-2} h^{-1}) in air-dried silt loam soil rewet to 50, 60, 80, and 100% water-filled pore space (WFPS). Bars depict means ± SE (n = 3). Different lowercase letters indicate statistical differences in flux between saturation levels at the same sampling time. Different uppercase letters indicate significantly different fluxes within the same soil saturation at different time points (*P < 0.05*).

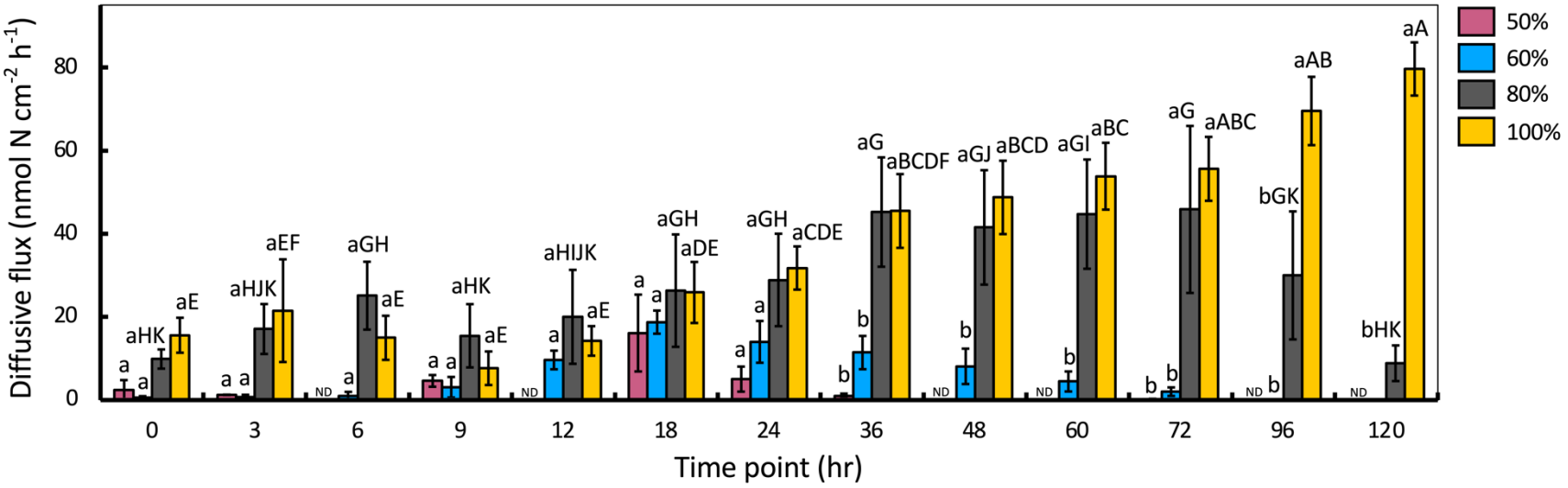

Figure 6. Mean (± SE) ammonium-N diffusive fluxes (nmol N cm^{-2} h^{-1}; n = 3) in air-dried soil rewet to 50, 60, 80, and 100% water-filled pore space (WFPS). ND indicates values below detection levels. Different lowercase letters indicate significant differences in flux between WFPS percentages within the same sampling time point. Different uppercase letters indicate differences in fluxes within the same soil saturation at different time points ($P < 0.05$).

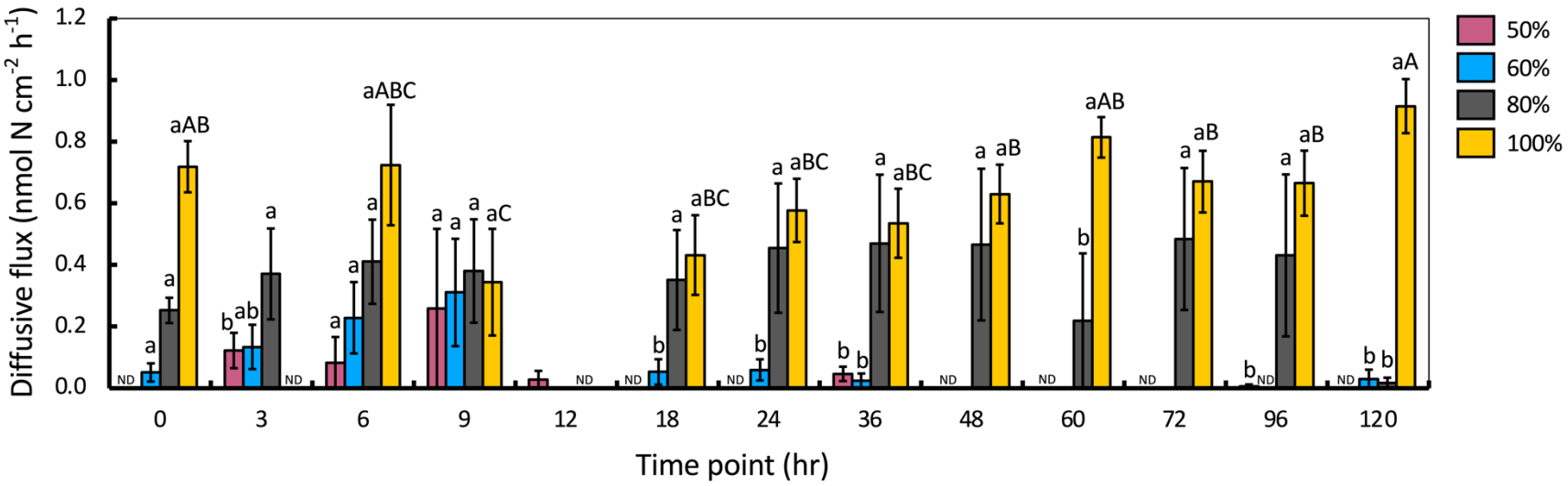

Figure 7. Total amino acid (Leucine-equivalents) fluxes (nmol N cm^{-2} h^{-1}) from microdialysis sampling (n = 3) in air-dried soil rewet to 50, 60, 80, and 100% saturation. Bars depict means ± SE. ND represents flux values below detection levels. Different lowercase letters indicate differences in flux between soil saturation percentages in the same sampling time point. Different uppercase letters signify statistically different fluxes within the same soil saturation at different time points ($P < 0.05$).

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CHAPTER 4: Discussion and Conclusions

1. Discussion

The delicate interplay between physical changes in soil conditions, biological and microbial communities and activities, and chemical shifts in the forms and quantities nitrogen (N) results in dynamic processes of N biogeochemical cycling. In lieu of utilizing the conventional methods of soil extractions to obtain chemical measurements of inorganic and organic N concentrations, the microdialysis technique was employed to generate data on temporal transformations of N. In both studies enclosed in this thesis, the effects of wetting-drying cycles on forms and recovery of bioavailable N were considered.

The first study entailed the prolonged use of the microdialysis method under field conditions and continuous monitoring of diffusive fluxes of inorganic and organic N in plots established in two differing plant communities – orchardgrass (introduced, cool season) and a mixture of native, warm season grasses (big bluestem, little bluestem, and indiangrass). The results of this study indicate that microdialysis could be employed in the field for approximately five months with the proper design to protect all equipment. Statistically differential nitrate-N fluxes were observed in native grass soil when compared to orchardgrass soil. Measurement of nitrate-N and ammonium-N concentrations using KCl extractions did not corroborate with microdialysis flux data. In fact, if only changes in inorganic N concentration were considered, extraction data suggested that orchardgrass soil displayed increased N dynamics. However, tandem molecular analysis of the soils using qPCR showed reconciliation between gene abundances and microdialysis fluxes. Bacterial and archaeal *amoA* gene copies in the native grass soil were consistently significantly elevated in comparison to copy numbers in orchardgrass soil, providing a microbial basis for the increased nitrate-N fluxes observed in the native grass soils in the month of June when soil was soil was consistently wet. The ability of

microdialysis to provide data on changes in soil microsites allows it to capture the heterogeneity of N processes that are innately present in soils, and to function at the same scale as microorganisms that are residing in and initiating these sites of heterogeneity. Native grass soil also exhibited increased archaeal *amoA* gene abundances, especially in times of prolonged drought. The ability of archaea to function and thrive in more extreme environments may have resulted in adaptations by the native grasses to encourage archaeal nitrifiers in their microbiome, leading to the competence of native grasses to grow in low N and drought conditions.

In the second study, microdialysis was employed in field-moist and air-dried soils that were wet or rewet to 50, 60, 80, and 100% water-filled pore space. The fluxes of organic and inorganic N were monitored over a period of 120 hours of incubation. The objective of the study was to take advantage of microdialysis' capability of providing continuous flux data to gain insight into the transient transformations of N in soils with differing initial starting saturations and which were treated with different amounts of water. The patterns of nitrate-N, ammonium-N, and amino acid fluxes were distinct between the field-moist and air-dried soil, and small-scale changes were successfully measured using microdialysis.

There were also some significant parallels when comparing diffusive flux results of the field and laboratory studies. In the field study, in the month of June, there were frequent rainfall and drying events which resulted in a consistent moisture content and increased nitrate-N fluxes over this wet period. This was analogous to the results of the lab study when using field-moist soil. The presence of water in the soil prior to the start of the study potentially resulted in mineralization, and with the addition of more water, immobilization and nitrification occurred, resulting in increased nitrate-N fluxes, along with ammonium-N and amino acid fluxes that

either were below detection limits or displayed sporadic peaks in movement at certain time points, much akin to the hot moment behavior observed in the field results.

Nitrogen fluxes measured during the drought period beginning in August in the field where similar to the flux patterns observed in the air-dried soil in the lab study. As drying conditions persisted and necromass accumulated, the addition of water resulted in a flush of mineralization which may have contributed to increased nitrification initially. However, as time elapsed, continued mineralization, possibly due to increased ecological interactions, resulted in increased ammonium-N and amino acid fluxes.

While the field and laboratory studies were vastly different in experimental set-up, they both displayed similar flux patterns which were dependent on precedent moisture conditions, providing a basis for further investigation of wetting/drying cycling and its impacts on the movement and fate of different forms of N.

2. Conclusion

The microdialysis technique has displayed the elevated capability to provide continuous, temporal data in soil under both field and laboratory conditions. In the field study, the sampling technique was successful in differentiating fluxes in areas of distinct plant communities with contrasting N needs, metabolisms, and growing seasons. Analysis of diffusive fluxes in tandem with microbial analyses also provided a more holistic view than exclusive use of soil extraction data. More insight into biogeochemical cycling of N and the types of organisms involved became possible with the tandem use of microdialysis and qPCR techniques, and these tandem studies have the potential to augment the current knowledge base relying on traditional soil sampling practices such as soil extractions. The lab study component also exemplified the ability of

microdialysis to capture transient processes resulting from wetting a dried soil, knowledge essential in today's climate with frequent extreme drought and precipitation events. Apparent overlap between the diffusive flux results of the field and lab studies also provided evidence for the hypothesis that antecedent water availability dictates N availability in solution and subsequent cycling through biogeochemical processes. In conclusion, the use of microdialysis provided novel information on available N forms in soil solutions and should be incorporated in more studies to understand underlying microscale N dynamics.