ABSTRACT

Title of thesis: THE DISTRIBUTION AND FUNCTION OF DENITRIFICATION GENES: EXPLORING AGRICULTURAL MANAGEMENT AND SOIL CHEMICAL IMPLICATIONS

Holly Bowen, Masters of Science, 2016

Thesis directed by: Professor Stephanie Yarwood
Department of Environmental Science and Technology

Denitrification is a microbially-mediated process that converts nitrate (NO_3^-) to dinitrogen (N_2) gas and has implications for soil fertility, climate change, and water quality. Using PCR, qPCR, and T-RFLP, the effects of environmental drivers and land management on the abundance and composition of functional genes were investigated. Environmental variables affecting gene abundance were soil type, soil depth, nitrogen concentrations, soil moisture, and pH, although each gene was unique in its spatial distribution and controlling factors. The inclusion of microbial variables, specifically genotype and gene abundance, improved denitrification models and highlights the benefit of including microbial data in modeling denitrification. Along with some evidence of niche selection, I show that *nirS* is a good predictor of denitrification enzyme activity (DEA) and N_2O:N_2 ratio, especially in alkaline and wetland soils. *nirK* was correlated to N_2O production and became a stronger predictor of DEA in acidic soils, indicating that *nirK* and *nirS* are not ecologically redundant.

THE DISTRIBUTION AND FUNCTION OF DENITRIFICATION GENES: EXPLORING AGRICULTURAL MANAGEMENT AND SOIL CHEMICAL IMPLICATIONS

By

Holly Bowen

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Advisory Committee:

Professor Stephanie Yarwood, Chair
Professor Andrew Baldwin,
Dr. Michel Cavigelli
Dr. Jude Maul

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Chapter 1: Introduction

Nitrogen (N) is essential for all living organisms and its quantity and form mediate many important ecosystem processes. Although N in its various forms is ubiquitous and critical in healthy plant production, overuse of N-containing fertilizer has led to serious issues for human and ecosystem health, climate change, and water quality. Denitrification leads to the loss of bioavailable N from the soil and can reduce soil fertility (Velthof *et al.*, 2009). Furthermore, incomplete denitrification in agricultural soils is the leading source of anthropogenic nitrous oxide (N_2O) emissions (EPA, 2013). Nitrous oxide is a potent greenhouse gas, 296 times more potent than carbon dioxide (CO_2), and is a leading source of stratospheric ozone depletion (Snyder *et al.*, 2009 and Ravishankara *et al.*, 2009). As a result, denitrification is often unwanted in agricultural settings. Plants are limited in their ability to take up N in agricultural soil due to differences in timing between fertilization and plant growth, and therefore inevitably N exits the system. If it is not denitrified, N leaves via overland runoff, soil erosion, and leaching. Excess N then enters nearby waterways, possibly causing eutrophication (Mosier *et al.*, 2004) or enters groundwater where it can negatively impact human health when consumed, especially in infants and young children by restricting O_2 transport in the body (Mosier *et al.*, 2004).

Although denitrification has potentially negative implication in agricultural uplands, it is valued in wetlands. Complete denitrification occurs in anaerobic wetland soil (Reddy *et al.*, 1989; Gillam *et al.*, 2008) meaning that these areas serve as buffers, removing excess N before it enters waterways. Modeling denitrification rates and predicting how land use will alter those rates is equally important for agricultural managers and for wetland scientists, but such models are complicated by spatial and temporal heterogeneity. Furthermore, measuring complete

denitrification is difficult, because of the high abundance of N_2 in the atmosphere. Denitrification relies on the actions of the microbial community, but attempts to correlate rates to biological parameters have met with mixed results (Boyer *et al.*, 2006; Powell *et al.*, 2015; Morales *et al.*, 2010; Cavigelli and Robertson, 2000; Attard *et al.*, 2011). Although the microbial communities that carry out denitrification differ in composition between agricultural soils and wetlands, the enzymes they use and the conditions that promote their activity are similar. Studies like those reported here, seek to further elucidate soil conditions that encourage or discourage denitrification and N_2O emissions and link the microbial communities in the soil to denitrification rates.

The first study explores the environmental and management factors driving the spatial distribution of N cycling genes in the soil profile. I modeled the spatial distribution of bacteria, fungi, and the denitrifier community within the soil profile as impacted by poultry litter placement (broadcast vs. subsurface-banded) and hairy vetch vs. cereal rye cover cropping during the corn growing season. I assessed gene quantity across the corn row and with soil depth in relation to other environmental variables to predict microbial distribution and to identify potential denitrification hotspots. I hypothesized that: 1) subsurface-banded poultry litter would create a hotspot of microbial activity, measured as an increase in N functional gene abundance immediately surrounding the band; 2) soil depth would be a strong regulator of bacterial and fungal genes, with decreased gene copies as depth increased due to decreasing soil resources; 3) soils planted with hairy vetch would have a higher abundance of denitrification genes compared to those planted with cereal rye due to higher N inputs from hairy vetch.

The second study looks closer at the effect of pH and soil moisture on denitrifying bacteria. Soils from an agricultural field and a freshwater wetland were manipulated to observe

the effect of pH and moisture on gene abundance, community compositions, and activity over time. In particular, this study examines possible niche selection and the ecological significance of the two nitrite reducing genes, *nirK* and *nirS*, and also determines the impact of abiotic and biotic variables on regression models predicting rates of potential denitrification, N_2O production, and the ratio of N_2O to N_2. We had three main hypotheses: 1) Manipulating pH and moisture content would result in changes in the denitrifying community and lead to differences in denitrification activity; 2) Including denitrifier community composition and gene copy would improve models predicting denitrification enzyme activity (DEA) and N_2O production; 3) *nirK* and *nirS* are not ecologically redundant, and *nirS* quantity to correlate more to rates of denitrification than *nirK*.

The remainder of this chapter is a brief review of the literature concerning N cycling. The review includes detailed descriptions of nitrification and denitrification, the microbes that mediate these processes, and the most current knowledge concerning their distribution. Chapter 2 describes the study that examined spatial scales. Supplemental information from this chapter is included in Appendix 1. Chapter 3 describe the mesocosms study that evaluated the importance of biotic parameters in modeling denitrificaiton and Appendix 2 includes supplements to that study. Appendix 3 includes data that will be written up at a later date describing the gene distribution of ammonia-oxidizing bacteria and archaea from both experiments.

Literature Review

The Nitrogen Cycle

Nitrogen is found in the soil in a number of different forms, both organic and inorganic, as well as soluble and gaseous forms. These different forms of N have substantially different properties and bioavailability. Transformations of N in the environment are refered to as the N cycle. While some N cylcing process are influenced by physical and chemical properties, much of this cycle is microbial-mediated through metabolic and enzymatic processes. Two of these processes, nitrification and denitrification, were the focus of this research.

Nitrification:

Nitrification is the general term for the transformation of reduced forms of N to nitrate (NO_3^-), an oxidized form. The most common process is the oxidation of ammonia (NH_3) to $NO3^-$. Like many other processes in N cycling, nitrification is a multi-step redox process. The first step is NH_3 oxidation, the conversion of NH_3 to nitrite (NO_2^-) (equation 1). Ammonia oxidization is further broken down into two steps: the conversion of ammonia to hydroxylamine (NH_2OH) (equation 1.1), and the conversion of NH_2OH to NO_2^- (equation 1.2). The second major step of nitrification is NO_2^- oxidation (equation 2).

(1) $NH_3 + 1.5O_2 \rightarrow NO_2^- + H^+ + H_2O$

(1.1) $NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$

(1.2) $NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$

(2) $NO_2^- + H_2O \rightarrow NO_3^- + 2e^- + 2H^+$

Nitrification can play an important role in soils through the transformation of a less mobile, cationic form of N (NH_4^+) to an extremely mobile anionic form of N (NO_3^-), which is

more easily lost through leaching. In addition, NO_3^- is the input for denitrification, which can lead to additional losses of N from the soil. Ammonia oxidation may also be important in soils because of the ability of N_2O to be produced. The relative contribution of nitrification to N_2O emissions is dependent on oxygen availability, soil moisture, and the abundance of NH_3 (Skiba and Smith, 2000). This process has been found to significantly contribute to N_2O emissions under aerobic conditions (Venterea, 2007), predominating from 35-60% water filled pore space (WFPS) (Bateman and Baggs, 2005). However, it contributes very little to N_2O emissions under wet conditions, > 70% WFPS, where denitrification dominates (Bateman and Baggs, 2005). Nitrification is generally thought to be less important than denitrification in terms of total N_2O produced (Vilain *et al.*, 2014).

The first step of ammonia oxidation (equation 1.1), is carried out by the ammonia monooxygenase enzyme. This enzyme is encoded by the genes *amoA*, *amoB*, and *amoC*. The ability to carry out nitrification occurs in a relatively small number of phyla, although the ability to carry out ammonia oxidation occurs in both bacteria (AOB) and archaea (AOA) (Venter *et al.*, 2004; Treusch *et al.*, 2005; Schleper *et al.*, 2005). In bacteria, most ammonia oxidizers are found in the β-Proteobacteria group, with a much smaller number of species found in γ-Proteobacteria class (*Nitrosococcus*) (Phillips *et al.*, 2000). These γ-Proteobacteria species have only been found in marine and brackish waters, therefore, they should not be a significant group in the scope of this research. Within β-Proteobacteria, common ammonia oxidizers include *Nitrosomonas, Nitrosolobus, Nitrosovibro,* and *Nitrosospira* (Koops and Pommerening-Röser, 2001). The majority of soil bacterial ammonia oxidizers belong to Nitrosospira clusters 2, 3, and 4 (Prosser and Nicol, 2008), with Nitrosopria cluster 3 and Nitrosomonas cluster 7 being the most common in tilled and fertilized agricultural soils (Phillips *et al.*, 2000).

Archaea are also significant contributors to the ammonia oxidizing community and have been isolated from a number of different ecosystems including marine, soils, hot springs, and bioreactors (Wuchter *et al.*, 2006; Leininger *et al.*, 2006; Weidler *et al.*, 2007; Park *et al.*, 2006). Ammonia oxidizing archaea are all found in the *Crenarchaeota*, clades 1.1a, 1.1b, 1.1c, and pSL12 (Prosser and Nicol, 2008; Francis *et al.*, 2007; Mincer *et al.*, 2007), although there has recently been a push to incorporate this group into a new archaeal phylum, the *Thaumarchaeota* (Spang *et al.*, 2010).

The relative contribution of bacteria and archaea to ammonia oxidation in terrestrial soil systems is still relatively unclear and much of the literature has contrasting results. For example, research completed by Leininger et al. (2006) and Gubry-Rangin et al. (2010) found that AOA predominate in terrestrial soil and agricultural environments. In contrast, other studies have found that AOB predominate (Jia and Conrad, 2009; Di, *et al.*, 2010a). As a result, it is still unclear in what ecosystems AOA or AOB are the most abundant and the most active.

Although it is still unclear, a couple of common themes have emerged in the literature that may help to explain the relative abundance of these two groups. The first is that AOA and AOB seem to occupy different niches in the soil. Nitrogen concentrations and availability play a large role in the niche partitioning between AOA and AOB, with AOA decreasing and AOB increasing as N levels increase (Di *et al.*, 2010b; Verhamme *et al.*, 2011; Sims *et al.*, 2012). This is most likely due to the fact that AOA have a higher affinity for NH_3 (Martens-Habbena *et al.*, 2009), and thus AOA are able to outcompete AOB in low N environments. Soil pH also plays a large role in the niche partitioning between AOA and AOB, with abundances of AOA increasing and AOB decreasing as soil acidity increases (Prosser and Nicol, 2008; Zhang *et al.*, 2012; Baolan *et al.*, 2014). One of the reasons this may occur is the increased conversion of NH_3 to

ammonium (NH_4^+) at a lower pH, which lowers the amount of NH_3, creating a low substrate environment for bacteria, again allowing for AOA to outcompete the usually more active AOB organisms (De Boer and Kowalchuk, 2001). Not only does pH seem to change the AOA:AOB ratio, but it also leads to different archaeal community structures as assessed by sequence data (Gubry-Rangin *et al.*, 2011). Additional factors that may contribute to niche partitioning between AOA and AOB include dissolved oxygen levels and salinity (Santoro *et al.*, 2008), temperature (Sims *et al.*, 2012), and soil type (Morimoto *et al.*, 2011).

Nitrite oxidization (equation 2), is the second step of nitrification. There are four distinct phylogenetic groups of bacterial nitrite oxidizers. The major group found in soils belong to the genus *Nitrobacter*, which belong to the α-Proteobacteria group. Other nitrite oxidizers, which belong to γ- and δ- Proteobacteria are found in marine environments (Koops and Pommerening-Röser, 2001). However, nitrite oxidation will not be specifically targeted by this research, since nitrite is often found in much smaller quantities in the soil, and the process of nitrite oxidation occurs relatively rapidly in the soil. Ammonia oxidation is usually the rate limiting step in nitrification. In addition, the primers for bacterial and archaeal ammonia oxidizers are more reliable than those for nitrite oxidizers.

Denitrification

Denitrification is a microbially mediated, multi-step reduction process that converts NO_3^- to N_2 gas. The process of denitrification is important in soils as it is a key pathway for the loss of NO_3^-. While this can be beneficial in some cases, leading to less N in nearby waterways, especially in oversaturated systems, this process can also be economically and environmentally damaging. For example, the conversion of NO_3^-, a plant available form of N, to a gaseous form which is emitted into the atmosphere leads to a reduction in the N pool in these systems, and can

reduce soil fertility. Furthermore, the denitrification process can lead to N_2O production and emission into the atmosphere.

Nitrous oxide is a greenhouse gas 296 times more powerful than CO_2 (IPCC, 2001). It is also currently the most dominant ozone depleting substance (Ravishankara *et al.*, 2009). N_2O has been increasing at a mean rate of 0.7 ppb yr^{-1} for the past 30 years to its current concentration in the atmosphere of 322 ppb (Montzka *et al.*, 2011). Globally, 40% of N_2O emissions are from anthropogenic activities, and 69% of the anthropogenic emissions are due to agricultural soil management (EPA, 2013). Agricultural soils are estimated to emit between 4.3 and 5.8 Tg N_2O-N yr^{-1} (Butterbach-Bahl *et al.*, 2013). Natural emissions of N_2O mainly occur in oceans and soils. The estimated annual emissions from natural soils is 6-7 Tg N_2O-N yr^{-1} (Butterbach-Bahl *et al.*, 2013).

There are four steps involved in the denitrification process, each involving a reduction of N, as each intermediate is used as a terminal electron acceptor in microbial metabolism (**Figure 1.1**). The first step is the reduction of NO_3^- to NO_2^-, and is carried out by the nitrate reductase enzyme. This enzyme is encoded by the genes *narG* or *napA*. The second step is the reduction of NO_2^- to nitric oxide (NO), and is carried out by nitrite reductase. There are also two, non-homologous forms of this enzyme (Jones *et al.*, 2008). One is encoded for by the *nirS* gene, and the other by the *nirK* gene. The third step is the reduction of NO to N_2O, and is carried out by nitric oxide reductase, encoded for by the *norB* gene. The last step is the reduction of N_2O to N_2, and is carried out by nitrous oxide reductase. This enzyme is encoded by the *nosZ* gene.

Denitrification is a community process. The diversity of organisms that are able to perform steps of denitrification is very high, representing over 60 genera, which are widespread across many taxonomic groups (Philippot *et al.*, 2007). These include members of the phyla

Aquificae, Deinococcus-Thermus, Firmicutes, Actinobacteria, Bacteroides, and Proteobacteria (Wallenstein *et al.*, 2006). The proportion of denitrifiers usually makes up ~5% of the total microbial communities in soil (Philippot *et al.*, 2007). Due to this diversity, it is much more effective to study functional genes, representing functional groups of organisms, rather than taxonomy. Techniques such as PCR, quantitative-PCR (qPCR), and terminal restriction fragment length polymorphism (TRFLP), using primers specifically targeted for genes in the denitrification pathway have been the most common way to study denitrifier ecology. The *nirK*, *nirS*, and *nosZ* genes have been most commonly targeted in denitrification studies.

Nitrite reductase genes are the key genes distinguishing true denitrifying microorganisms, since nitrate reduction can exist decoupled from the denitrification pathway (Zumft, 1997). Although the two nitrite reductase enzymes perform the same function, they are evolutionarily unrelated and structurally different. *nirS* encodes for a cytochrome cd_1 nitrite reductase. This enzyme has two identical subunits, each containing one heme *c* and one heme d_1 (Zumft, 1997). The *nirS* gene alone does not code for a functional enzyme, and a number of accessory proteins are needed. It is generally found as part of a gene cluster that contains *nir*MCFSTB (Philippot, 2002). The other enzyme is a Cu-containing nitrite reductase, encoded for by the *nirK* gene. Unlike *nirS, nirK* alone can encode for a functional protein (Philippot, 2002). Transcription for both genes is controlled by levels of oxygen and NO in the cell, with active transcription occurring in low O_2 and NO conditions (Philippot, 2002).

Both *nirK* and *nirS* are widely distributed across bacterial groups. They have been found in numerous strains within α-, β-, and γ- *Proteobacteria* (Heylen *et al.*, 2006), in addition to other phyla such as *Aquificae, Bacteriodetes, Firmicutes* (Philippot *et al.*, 2007; Graf *et al.*, 2014). It was eariler believed that *nirK* and *nirS* were mutually exclusive in a bacterial strain

(Zumft, 1997), however, a few strains of bacteria recently isolated from a wastewater treatment system had both genes present in their genomes (Graf *et al.*, 2014). In general, taxonomy is not a good indicator of *nirK* or *nirS,* as phylogenies for these genes are incongruent with that of 16S RNA (Jones *et al.*, 2008). It has been shown that closely related species and even similar bacterial strains can harbor different nitrite reductase genes (Heylen *et al.*, 2006). Habitat type seems to play a larger role than taxonomy, as *nirK* and *nirS* communities are generally phylogenetically clustered by habitat type (Jones *et al.*, 2010). This may be due to niche-based selection processes such as environment or habitat filtering (Jones *et al.*, 2010), indicating that closely related taxa share traits important for their persistence in a particular environment (Webb *et al.*, 2002. This in conjunction with horizontal gene transfer could lead to the phylogenetic clustering by habitat type (Jones *et al.*, 2008).

The *nosZ* gene, encoding for nitrous oxide reductase, is important as it regulates the consumption of N_2O. The reduction of N_2O to N_2 by this enzyme is the only known biological sink of N_2O, and the only mechanism to remove N_2O from the atmosphere, other than photolysis and oxidative reactions in the stratosphere (Montzka *et al.*, 2011). Recently it has been discovered that there is a second clade of this gene: *nosZ*-II, which is found in similar abundances to *nosZ*-I (Jones *et al.*, 2013). This second clade was found in a wide range of environments including arable soil, alpine soil, rice paddies, wetlands, lakes, and wastewater. These two clades have signal peptides coding for different protein secretion pathways. *nosZ*-I codes for the twin arginine translocation (tat) pathway (Jones *et al.*, 2013; Graf, 2015). This is the more energy intensive pathway in which proteins are transported across the membrane already folded. *nosZ*-II codes for the secretory (sec) pathway, where proteins are transported across the membrane unfolded (Jones *et al.*, 2013; Graf, 2015). This pathway uses much less

energy than the tat pathway, and the reason for the continued use of a costly process is still unknown. However, this may suggest an advantage of one pathway over the other in certain organisms or environments (Jones *et al.*, 2013). There is some evidence for this, as *nosZ*-I and II have ties to taxonomic affiliation. *nosZ*-I is found widespread across α-, β-, and γ-*Proteobacteria*. *nosZ*-II is also found within these organisms, but is also found throughout δ-, and ε- *Proteobacteria*. The nosZ-II clade also has strong affiliations with *Bacteriodetes, Firmicutes, Verrucommicrobia, Aquificae, Chloorflexi,* and others (Jones *et al.*, 2013). The two *nosZ* clades have also been found to be sensitive to different edaphic factors such as soil texture and pH (Jones *et al.*, 2014). The *nosZ*-I was also found in higher abundance in the rhizosphere, and governed by plant effects, while *nosZ*-II was higher in bulk soil (Graf, 2015).

For many years it was thought that only bacteria were able to carry out the process of denitrification. However, in 1991, *Fusarium oxysporum* and *Fusarium solani,* were shown to have the ability to denitrify using the p450nor gene (Shoun and Tanimoto, 1991). Since then, more fungal species have been found to have this capability and more knowledge about this process has been uncovered. There is now evidence that in some environments, especially in more aerobic conditions, that fungi can contribute to a large portion of the N_2O emission (Zhou *et al.*, 2001; Chen *et al.*, 2014). Additionally, some archaea have been shown to have the ability to denitrify (Zumft, 1997). However, fungi and archaeal denitrifiers are not a focus of this research.

Factors controlling denitrification

Overall, there are a number of factors controlling denitrification in soils. One of the most important is oxygen availability. Denitrification in bacteria is an anaerobic process, which takes place only when oxygen concentration is about one-tenth of atmospheric concentration. Oxygen

inhibits enzyme synthesis and activity of *nirK*, *nirS*, and *nosZ* (Sylvia *et al.*, 2005). Nitrous oxide accumulates when conditions allow for the first three steps of denitrification but inhibit the last step, the conversion of N_2O to N_2 (Sylvia *et al.*, 2005). This conceptualization of N_2O accumulation is generally termed the hole-in-the-pipe model (Firestone and Davidson, 1989). Therefore, oxygen concentration not only controls the overall process of denitrification, but also the ratio of gases that are produced. Oxygen concentration is influenced by soil texture and by soil moisture content, with O_2 concentrations decreasing as soil moisture increases due to substainailly lower diffusion rates of O_2 in water compared to air. Denitrification generally occurs when water filled pore space (WFPS) is greater than 70%, with N_2O production greatest between 70-80% WFPS (Butterbach-Bahl *et al.*, 2013). Like with many other enzymatic reactions, the rate of denitrification increases with increasing temperature. Denitrification rate is very sensitive to temperature, even more so then carbon (C) respiration rate (Butterbach-Bahl *et al.*, 2013). From 15 ºC to 35ºC denitrification rate increases, with a Q_{10} coefficient around 2. Below 10ºC denitrification rate declines rapidly (Stanford *et al.*, 1975).

Nitrate and C availability also control denitrification rate. Nitrate and subsequent N-oxides are needed as the terminal electron acceptors for the reaction. Organic C is often the reductant, donating electrons for the reaction. Numerous studies have found that increasing the amount of NO_3^- and C in the soil will increase denitrification rate (Weier *et al.*, 1992; Gillam *et al.*, 2008). Specific to work in this thesis, studies have found that increased fertilizer rates (Grant *et al.*, 2006; Bouwman *et al.*, 2002; McSwiney and Robertson, 2005), changes in fertilizer type (Pelster *et al.*, 2012; Lesschen *et al.*, 2011), and no-till agriculture (Rochette *et al.*, 2008) can increase denitrification rate and N_2O emissions. In addition, increased C and N loading to wetlands can increase N_2O production (Sirivedhin and Gray, 2006).

Often called a master variable, pH is another factor that controls many reactions, both chemical and biological. pH has a strong influence on denitrification rate, with optimal pH for denitrification around neutral (Bakken *et al.*, 2012). pH also effects denitrification end products, with the ratio of N_2O:N_2 increasing with decreasing pH (Šimek and Cooper, 2002). Mechanisms proposed for this effect include interference at low pH with the assembly of nitrous oxide reductase (Bakken *et al.*, 2012), and interference with translation or function of the enzyme (Liu *et al.*, 2010).

There is also large spatial and temporal variability associated with denitrification. Often a disproportionate amount of denitrification occurs in small patches, or a disproportionate amount occurs over a short period of time. These phenomena are often termed "hotspots" and "hot moments", respectively (Groffman *et al.*, 2009). The hotspot concept originated from Parkin (1987), who found that over 80% of denitrification occurred in only 1% of the soil core volume around a decaying plant leaf. Hotspots can also occur in the anaerobic centers of aggregates, along roots, and at oxic/anoxic interfaces (Groffman *et al.*, 2009). More recently, hotspots have also been considered at the field and landscape scales, for example riparian zones (Groffman *et al.*, 2009). Factors controlling denitrification hotspots likely differ depending on scale (van den Heuvel *et al.*, 2009; McClain *et al.*, 2003). Hot moments occur after events such as drying-rewetting, freezing-thawing, or major disturbances (Groffman *et al.*, 2009; McClain *et al.*, 2003). They often occur in anthropogenic systems. For example, large rates of fertilizer followed by a rainfall or irrigation event are known to result in a hot moment of denitrification and a pulse of N_2O from an agricultural field (Dobbie *et al.*, 1999; Markfoged *et al.*, 2011). A better understanding of hotspots and hot moments in denitrification have been identified as critical areas of research to be able to improve denitrification modelling (Groffman *et al.*, 2009).

The microorganisms carrying denitrification genes have been shown in a number of studies to be important in denitrification and N_2O emissions. However, it is still not clear how different environmental and soil conditions select for a particular denitrifying community in soil, and in turn, how community composition leads to ecosystem function seen in the field. A number of studies have found that community composition (Cavigelli and Robertson, 2000; Rich *et al.*, 2003) and diversity (Philippot *et al.*, 2013) are important indicators of ecosystem functioning, especially in regards to nitrogen cycling. Other studies have found that community composition is not always related to ecosystem functioning (Hallin *et al.*, 2009; Enwall *et al.*, 2005; Attard *et al.*, 2011; Dandie *et al.*, 2008). Hallin et al. (2009) found that the size of the microbial community and the abundance of denitrification genes may be more important to ecosystem function and nitrogen use efficiency then community composition. This is supported in the literature, as the ratio of genes, especially *nirK* and/or *nirS* to *nosZ* have been positively correlated to N_2O emissions (Morales *et al.*, 2010; Philippot *et al.*, 2011; Xue *et al.*, 2013). Still, questions remain as to whether changes in the composition of nitrogen cycling communities, physiological changes, or gene expression of the existing community are most important to ecosystem function (Morales *et al.*, 2010).

Factors controlling nitrite reducing genes

The two nitrite reductase genes are found across diverse taxonomic groups and were previously thought to be mutually exclusive in a given bacterial strain. We know now that there are a few organisms that carry both *nirK* and *nirS* (Graf *et al.*, 2014). It has been speculated that carrying both types of *nir* genes is advantageous if the genes are functional under different conditions (Graf *et al.*, 2014). This finding, along with evidence from numerous studies showing that the ratio of *nirK*/*nirS* changes under differing environmental conditions, that there is

dominance of one nitrite reductase over the other in certain environments, or the differing response of *nirK* and *nirS* to environmental gradients suggests the possibility of niche selection between the *nirK* and *nirS* communities.

Many studies have found differences in denitrification gene abundance and composition in different agricultural systems. *nirK* gene copies were shown to be less abundant and spatially more evenly dispersed in an organically managed agricultural system than in a conventional system. In constrast, *nirS* gene copies were higher in the organic system, but also exhibited more spatial variability than *nirK* (Enwall *et al.*, 2010). Differences have also been found between annual, and perennial agricultural systems, and agricultural successional sites (Morales *et al.*, 2010). Perennial sites and the early native successional site had higher levels of *nosZ,* and *nirS* was much higher in the annual and perennial agricultural systems then in the succession sites. Although not related to agriculture, an interesting study was done during primary succession of previously glaciated land (Kandeler *et al.*, 2006). The relative abundance of *nirS* (*nirS*/16S) decreasing with soil development, and total abudnaces of *nirK* and *nosZ* increasing with soil development. The changes in denitrification gene abundances in these different systems show that edaphic factors play a role in determining the composition and structure of the denitrifer community and that different members of this community may occupy the different niches created by these factors. There have been some studies that begin to dig deeper to determine how individual factors contribute to this niche selection and lead to the community of denitrifers in the soil.

pH

pH is one factor known to be a driving factor for microbial community diversity and populations, and may also play a role in the niche differentiation between *nirK* and *nirS*. Bárta et

al. (2010) found that *nirK* was found in higher abundance in the alkaline than acidic soils. In contrast, *nirS* had the opposite trend, being found in higher abundance in the acidic compared to the alkaline soils. This study also found evidence of a pH threshold level. Under a pH of 5, *nirK* numbers decreased rapidly. *nirK* also seemed to be much more sensitive to changes in pH than *nirS*. Results from other studies have found contrasting results. Chuel *et al*, (2010) found that both *nirK* and *nirS* gene copy numbers were highest in a neutral soil, however, the pH range in this study was not large (5.52-7.67), and did not include a soil with a pH under the proposed threshold of 5. Another study, which have a large pH range (4.0-8.0) have found that *nirK*, *nirS*, and *nosZ* all increased with increasing pH (Liu *et al.*, 2010). Enwall et al. (2010), while also having a narrow pH range (5.7-7.0) showed positive correlations of both *nirK* and *nirS* to pH, but *nirK* was more strongly correlated, indicating that it may be more sensitive to pH than *nirS*. Additional studies have shown that the *nirK/nirS* ratio was negatively correlated to pH (Philippot *et al.*, 2009). Although many of these studies have contrasting results, they do provide evidence that pH has an impact on nitrite reducering genes.

These contrasting studies highlight the need for the impact of pH on denitrification gene abundance to be further studied in order to clear up these inconsistencies. In addition, studies noted above were not controlled laboratory studies. As a result, there were a number of soil factors in addition to pH that changed between the sites including total carbon (TC), dissolved organic carbon (DOC), total nitrogen (TN), and phosphorus (P), in addition to unmeasured variables that could also influence gene abundance. This is the case for most studies discussed in this literature review, and highlights the need for controlled laboratory studies to assess the impact of pH on denitrifier abundance.

Soil Moisture

Soil moisture appears to play a large role in determining denitrifer abundance, yet again the literature provides contrasting results. Evidence of this is shown in a study of forest soils, in which *nirK* and *nirS* were both found in wet soils, whereas only *nirS* was found in dry soils (Katsuyama *et al.*, 2008). This would suggest that *nirK* organisms are not abundant in drier soil conditions and increasing soil moisture would increase *nirK* abundance. However, in a study of a rice paddy, Yoshida *et al*. (2009) found that *nirK* abundance significantly decreased after waterlogging while *nirS* gene abundance was not changed after waterlogging, and Phillippot *et al*. (2009) found the *nirS/nirK* ratio increased with increasing soil moisture. This suggests the opposite phenomenon. Along with gene abundance, soil moisture has also been shown to change the composition and diversity of the *nirK* communities (Smith and Ogram, 2008; Szukics *et al.*, 2010; Yoshida *et al.*, 2010).

Carbon

Carbon is an important factor controlling denitrification, as an available carbon source is needed for each step of the process. Bárta *et al.* (2010) found that *nirK* was positively correlated with DOC and *nirS* was positively correlated with TC. The *nirK/nirS* ratio was also positively correlated to DOC possibly suggesting that nitrite reducers respond differently to the amount of carbon in the soil. Again like pH, Bárta *et al*. (2010) found that there may be a C threshold for *nirK*: below 4.8 mol/kg DOC, *nirK* numbers decreased rapidly. Other studies have also found a positive correlation between these nitrite reducing genes and either organic matter content of the soil, the C content, or both (Chen *et al.*, 2010 and Kandeler *et al.*, 2006). Again, the literature provides inconsistent results, as other studies did not find a correlation between *nirK* or *nirS* and DOC (Enwall *et al.*, 2010; Philippot *et al.*, 2009).

Additional studies have eluded to C as a controlling factor of denitrifiers. For example, *nirK/nirS* ratio was much lower in an unfertilized bare soil compared to soils with crops, indicating the selection for Nir type by the habitat created by the presence or absence of plants and presumably C. In addition, the fallow plots also separated out from the vegetated plots in terms of *nosZ* community structure (Hallin *et al.*, 2009). *nirK* was found to be present in higher abundances in the rhizosphere soil while *nirS* was highest in the bulk soil, which could also possible be a function of C concentration and availability (Towe *et al.*, 2010). However, in an experiment looking at the effects of artificial root exudates on denitrifer abundance and structure, no significant results were found, although activities were stimulated (Henry *et al.*, 2008).

Soil Nutrients

nirS gene abundances have been positively correlated to NO_3^- concentrations (Philippot *et al.*, 2009; Chen *et al.*, 2010; Morales *et al.*, 2010), NH_4^+ concentrations (Philippot *et al.*, 2009), rates of mineral fertilizer (Chen *et al.*, 2010). However, other studies have found no correlation (Bárta *et al.*, 2010), or a negative correlation between *nir*S gene abundances and NH_3 (Chen *et al.*, 2010). *nirK* was found to be postiviely correlated with TN and P, while *nirS* was not (Bárta *et al.*, 2010). In a study that investigated the effect of fertilizer regime on denitrifer abundance and composition, it was shown that increasing the fertilizer application rate and especially of mineral fertilizer, increased *nirK* abundance, and altered the *nirK* community composition, suggesting that the *nirK* community may be very sensitive to changes in N concentrations and availability. It is less clear if *nirS* communities are as impacted by N, but evidence shows it may play a role in determining *nirS* abundance (Philippot *et al.*, 2009; Chen *et al.*, 2010; Morales *et al.*, 2010).

Other nutrients that may play a role in the abundance of denitrifers are P, potassium (K), calcium (Ca), and copper (Cu) (Enwall *et al.*, 2010). Copper has long been suggested to contribute to differences in *nirK* and *nirS* abundances in the soil since the NirK enzyme has a Cu cofactor, while NirS does not have a Cu requirement. The *nirK/nirS* ratio has been found to be positively correlated Cu (Enwall *et al.*, 2010). However, it seems that in the field differences in Cu concentrations in the soil are superceded by other differences such as soil moisture, pH, C, or N, and is either not measured (Philippot *et al*., 2009; Bárta *et al.*, 2010) or not correlated to gene abundances in most studies.

Microorganisms in denitrification models

Due to the complicated nature of measuring denitrifier communities and linking community structure to ecosystem function, microbial communities are often left out of denitrification models. Furthermore, microbial communities are often assumed to be functionally redundant (Wertz *et al.*, 2007) and thus are not an important component of models. When microorganisms are included in models it is often based on relatively old data on enzyme and growth kinetics (Hartel and Alexander, 1987 and Bakken *et al.*, 2012). However, not only has the functional capacity of denitrifier communities been shown to be variable, as cited above, but the inclusion of biotic parameters has improved model fit for denitrification and N_2O models in a number of cases (Reed *et al.*, 2014; Allison *et al.*, 2012; Kaiser *et al.*, 2014; Wieder *et al.*, 2013; Powell *et al.*, 2015). Powell *et al*., (2015), found that biotic factors such as the relative abundance of specific genotypes, *nirK* gene copy number, and *nirS* evenness were all significant variables for a model predicting potential denitrification. The inclusion of biotic variables in the model increased the R^2 from 0.50 to 0.76. Other microbially mediated processes have benefited

from the inclusion of biotic variables, such as nitrogen cycling in the Arabian Sea (Reed *et al.*, 2014), litter decomposition (Allison, 2012; Kaiser *et al.*, 2014), and soil carbon dynamics (Wieder *et al.*, 2013). More insight linking microbial communities to ecosystem function may lead to better predictive models for denitrification and N_2O emissions.

Tables and Figures

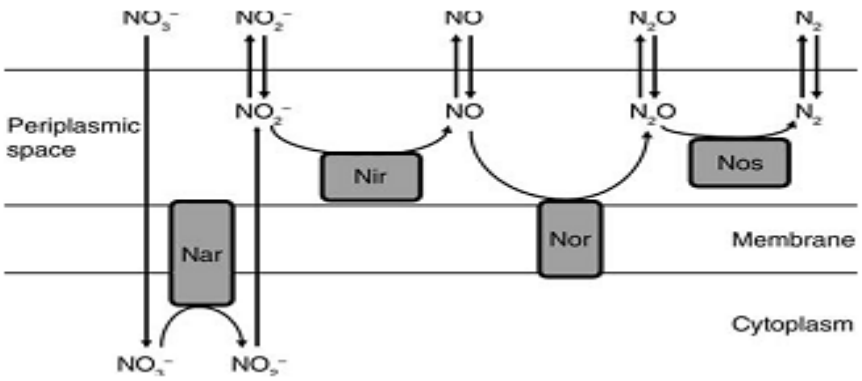

Figure 1.1. The four steps of the denitrification process and associated enzymes, showing the location of enzymes in the cell membrane. Source: (Wallenstein *et al.*, 2006).

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Chapter 2: Spatial patterns of microbial denitrification genes change in response to fertilizer placement and cover crop species in an agricultural soil

Abstract

Subsurface-banding manure and winter cover cropping are farming techniques designed to increase nitrogen use efficiency. Little is known, however, about the effects of these management tools on denitrifying microbial communities and the greenhouse gases they produce. Abundances of bacterial (16S), fungal (ITS), and denitrification genes (*nirK*, *nirS*, and *nosZ*) were measured in soil samples collected from a field experiment testing the combination of cereal rye and hairy vetch cover cropping with either surface broadcasted or subsurface-banded poultry litter. The spatial distribution of genes was mapped to identify potential denitrifier hotspots. Spatial distribution maps showed increased 16S rRNA genes around the manure band, but no denitrifier hotspots were observed. Bacteria carrying *nirK* versus *nirS* genes were found to be sensitive to different soil characteristics and management methods. Gene copies of *nirK* were higher under cereal rye than hairy vetch, while *nirS* gene copy number did not differ between cover crop species. The *nirS* gene copies increased when manure was surface broadcasted compared to subsurface-banded. Soil depth and nitrate concentration were the strongest drivers of gene abundance. Agricultural management differentially affects spatial distributions of genes coding for denitrification enzymes, leading to changes in the structure of the denitrifying community.

Introduction

Denitrification is a microbially-mediated, multi-step reduction process that converts nitrate (NO_3^-) to dinitrogen (N_2) gas, a key pathway for the loss of bioavailable nitrogen (N) from soil. In agricultural soils, up to 56% of fertilizer nitrogen (N) can be lost through denitrification (Velthof *et al.*, 2009). Globally, N losses from agricultural soils represent 22 to 87 Tg N yr^{-1} (Hofstra and Bouwman, 2005). Incomplete denitrification produces nitrous oxide (N_2O), a greenhouse gas that is 296 times more potent than carbon dioxide (CO_2) and contributes to stratospheric ozone depletion (Ravishankara *et al.*, 2009; Snyder *et al.*, 2009). Of the 40% of global N_2O emissions that are anthropogenic, 69% is released from agricultural soils (EPA, 2012). This portion is estimated to be 4.3 to 5.8 Tg of N_2O-N yr^{-1} (Butterbach-Bahl *et al.*, 2013), and has caused atmospheric concentrations of N_2O to increase over the last 30 years (Montzka *et al.*, 2011). Nitrite reductase (Nir) is the key enzyme that distinguishes denitrifiers and has two known forms: the Cu-containing enzyme encoded by the *nirK* gene and the cytochrome cd1 enzyme encoded by the *nirS* gene. The last step of denitrification, the reduction of N_2O to N_2, is catalyzed by nitrous oxide reductase, encoded by *nosZ*, which has recently been determined to have at least two distinct isoforms, Clade I and Clade II (Jones *et al.*, 2013).

The spatial distribution of C, N, and denitrifying microorganisms within the soil profile can substantially impact denitrification rate, but few studies have examined these factors spatially at the soil profile scale to understand these effects. Parkin (1987) found that 80% of denitrification occurred in only 1% of the volume in a soil core, concentrated around a decaying leaf. More recently, studies have reported changes in microbial community composition (Izquierdo and Nüsslein, 2006) and N_2O production among soil aggregate sizes (Sey *et al.*, 2008; Miller *et al.*, 2009), confirming microscale variations in microbial community structure and

function. These studies are difficult to scale up to the field level, however, where agricultural management might be an important driver of microbial communities and activity. At the soil profile or field scale it is unclear if spatial heterogeneity in denitrification rate is the result of changes in the size or composition of the denitrifying bacterial community. At the landscape scale, several studies have reported changes in *nirK*, *nirS*, and *nosZ* gene abundance due to land management, land use, and soil physio-chemical properties (Enwall *et al.*, 2010; Bru *et al.*, 2011), but these studies do not specifically address anthropogenic impact on denitrification gene abundance. Studies intermediate to the aggregate and landscape scales will improve our understanding of how anthropogenic disturbance and management, including agricultural practices, impact microbial community composition and function (Groffman *et al.*, 2009).

When animal manure is used in no-till agricultural systems it is typically applied by surface broadcasting, but this type of manure application may lead to relatively high N and P losses (Philippot *et al.*, 2007). For example when surface applied poultry litter was not incorporated within 48 hours, 66% of the N was lost via ammonia (NH_3) volatilization (Pote and Meisinger, 2014). Although subsurface-banding may decrease NH_3 volatilization, it may also increase N_2O production due to the formation of a denitrification hotspot due to high concentrations of C, N, and moisture in the band. Results concerning the impact of manure placement on N_2O emissions, however have been mixed (Smith *et al.*, 2012; Halvorson and Del Grosso, 2013; Nash *et al.*, 2012). Some studies have observed a decrease in N_2O emissions with banding (Smith *et al.*, 2012; Nash *et al*. 2012), but Halvorson and Del Grosso (2013) reported a ~50% increase in N_2O emissions from subsurface-banding compared to broadcasting manure. To our knowledge, no studies have evaluated the impact of manure placement on the soil microbial or denitrifier communities, even though the phylogenetic structure and total biomass of these

communities have been shown to affect denitrification rates and the amount of N_2O produced (Cavigelli and Robertson, 2000; Morales *et al.*, 2010; Philippot *et al.*, 2011).

Animal manure nutrient-use-efficiency may increase by using manure in combination with cover crops (Poffenbarger *et al.*, 2015a; Spargo *et al.*, 2016). Farmers commonly use non-leguminous crops such as cereal rye (*Secale cereal* L.) as cover crops, but the high C:N ratio of cereal rye biomass can lead to reduction of plant available N during decomposition due to microbial immobilization (Snyder and Meisinger, 2012). Nonetheless, cereal rye has been shown to increase microbial biomass following termination of the crop in early spring (Bossio *et al.*, 1998; Buyer *et al.*, 2010). Steenwerth and Belina (2008) also showed that rye cover cropping increased average daily N_2O emissions. Leguminous cover crops such as hairy vetch (*Vicia villosa* Roth) have been used to increase plant available soil N, but have also been shown to result in increased denitrification (Rosecrance *et al.*, 2000) and N_2O emissions (Kallenbach *et al.*, 2010 and Davis *et al*, in preparation). Studies exploring the linkages between cover cropping and N_2O emissions have focused on rates but have not evaluated shifts in microbial communities.

Here we modeled the spatial distribution of bacteria, fungi, and the denitrifier community within the soil profile as impacted by poultry litter placement (broadcast vs. subsurface-banded) and hairy vetch vs. cereal rye cover cropping during the corn growing season. We assessed gene quantity across the inter-row between corn rows and with soil depth in relation to other environmental variables to predict microbial distribution and to identify potential denitrification hotspots. We hypothesized that: 1) subsurface-banded poultry litter would create a hotspot of microbial activity, measured as an increase in denitrifier functional gene abundance immediately surrounding the band; 2) soil depth would be a strong regulator of bacterial and fungal genes,

with decreased gene copies with depth due to decreasing soil resources; and 3) soils planted with hairy vetch would have a higher abundance of denitrification genes compared to those planted with cereal rye due to higher N inputs from hairy vetch.

Materials and Methods

Site Description and Sampling

The experiment was conducted at the Beltsville Agricultural Research Center (BARC), Beltsville, MD, USA (39.02 N, 76.94 W) to investigate the effects of cover crops and poultry litter management on no-till (NT) corn (*Zea mays* L.*)* production, N use efficiency and soil N_2O emissions. The soils are fine-loamy, mixed, active, mesic Fluvaquentic Dystrudepts and fine-loamy, mixed, active, nonacid, mesic, Fluvaquentic Endoaquepts with a silt loam texture and a 0-2% slope (Web Soil Survey, 2015). Samples for this study were taken during the 2013 growing season.

Treatments sampled for this study were a factorial combination of two poultry litter placement methods, no-till surface broadcasting and no-till subsurface-banding, and two cover crop species, hairy vetch and cereal rye, in a randomized, blocked, strip-plot design with three replicates. The winter cover crop were planted in late fall, roll-killed in late spring, and left on the soil surface. Each plot was then planted with corn and received ~10 kg PAN ha^{-1} as starter poultry litter just below the soil surface. The poultry litter in surface broadcasted treatments was applied at corn planting 22 May, 2013. The poultry litter for the subsurface-banded treatments was applied on 25 June, 2013 when corn was at the fifth-leaf growth stage. The poultry litter bands were placed 10 cm below the soil surface 38 cm from and parallel to corn rows, which were planted with 76 cm spacing (**Figure 2.1B**). Both treatments received 3.4 Mg poultry litter

ha^{-1}, estimated to provide 67 kg plant available N ha^{-1}. Additional details pertaining to field management can be found in Poffenbarger et al. (2015b).

Soils were collected during the fifth-leaf growth stage of corn, on 26 and 27 June, 2013. This was the first sampling time point after the establishment of both treatments, and was chosen to coincide with anticipated peak N_2O emissions from the subsurface band, which have been shown to occur soon after manure application (Markfoged *et al.*, 2011). In the surface broadcasted plots, soil cores were taken halfway between the corn rows (i.e. inter-row center) and at a distance of 20 and 38 cm on both sides of the inter-row center (**Figure 2.1A**) using a 1.9 cm diameter push probe. The samples taken at 38 cm from the inter-row center were taken in the corn row. At each location in the broadcasted treatment plots, soil cores were taken to a depth of 30 cm and cut into four depth increments: 0-5, 5-10, 10-20, and 20-30 cm. The subsurface-banded plots were sampled at five distances from the inter-row center (0, 5, 10, 25, and 38 cm) and separated into five depth increments: 0-5, 5-10, 10-15, 15-20, and 20-30 cm. We collected more samples in the subsurface-banded plots in order to achieve better spatial resolution around the poultry litter subsurface-band. Sampling along transects was repeated in four randomly chosen locations within each plot and all cores within the plot were composited by distance from inter-row center and soil depth.

Soil Analyses

Soil samples were sieved fresh (64 mm), 10 g was subsampled for gravimetric moisture content determination, and 5 g was subsampled and immediately frozen at -80^{o}C for molecular work. The remaining soil was air-dried and passed through a 2 mm sieve. Two grams of air-dried soil from each sample were extracted using 20 mL of 1 M KCl by shaking for one hour on a

platform shaker. Filtered extracts were analyzed colorimetrically for ammonium (NH_4^+-N) and nitrate (NO_3^-- N) using an auto analyzer (Seal AQ2 Automated Discrete Analyzer, Mequon, WI).

DNA Extractions

DNA was extracted from 2g of each field moist soil sample using the MoBio PowerSoil DNA Extraction Kit, according to the manufacturer's protocol (MoBio Laboratory, Carlsbad, CA). DNA was quantified using a Qubit 2.0 Fluorometer (Invitrogen, Waltham, MA) and diluted to a final concentration of 2.5 ng μl^{-1} with autoclaved water. Extracts were stored at -80ºC until further analysis.

Real time PCR

Gene abundances were determined using real-time PCR (qPCR). Specifically, the denitrification genes *nirK*, *nirS* and *nosZ-I* were quantified, along with *16S* rRNA genes (to quantify total bacterial abundance) and ITS (to quantify fungal abundance) (**Table I.1**). Recently it has been shown that there is a second bacterial clade, *nosZ-II* (Jones *et al.*, 2013). We screened our samples using the Jones et al. (2013) primer set and conditions and found no evidence of *nosZ-II*. Each qPCR plate included a set of soil plasmid standards and a set of no template controls made with sterilized water. The primers, qPCR conditions, and the range of efficiencies for each gene are found in **Table I.1**. Gene copy numbers were corrected to account for PCR inhibition (Hargreaves *et al.*, 2013). Briefly, 36 soil DNA extracts were randomly selected and pooled. The pooled samples were serially diluted and run at the standard conditions for each gene (**Table I.1**). The efficiency and intercept of the pooled samples were calculated and used to correct for relative copy number differences between the plasmid standard curve and the pooled soil sample standard curve.

Statistical Analyses

The Geostatistical Analyst extension of ArcMap 10.0 was used to interpolate and visualize microbial gene spatial distributions (ESRI, Redlands, CA). Due to the limited number of data points in this study kriging could not be used. Non-normally distributed data were first log-transformed to meet the normality assumption (16S, *nirK*, *nirS*, *nosZ*, and ITS gene copy numbers, NH_4^+-N and NO_3^- -N concentrations); arithmetic means and SE of untransformed data were used for presentation. A first order global polynomial was modeled for each variable to map the linear depth trend. This was performed separately for each plot. The residuals of this model were found by computing the difference between observed and predicted values. The residuals were then analyzed with inverse distance weighted interpolation using the formula:

$$Z_j = \frac{\sum_i \frac{Z_i}{d_{ij}^n}}{\sum_i \frac{1}{d_{ij}^n}}$$

where Z_j is the predicted value at an unknown location, d is the distance from a known point (Z_i), and n is an exponent for weighting. These predicted values were added to the global model. Replicate plots were then averaged and back transformed when necessary to create the final spatial maps. The presence of spatial hotspots was tested with the Getis-Ord-Gi* statistic in Geostatistical Analyst extension of ArcMap 10.0, a statistic that identifies points with values higher in magnitude than expected by chance (ESRI, Redlands, CA).

ANOVA was used to determine the significance of treatment effects using the nlme package in R (R Development Core Team, 2015). ANOVA was run as a linear mixed model separately for each depth, cover crop and poultry litter placement as fixed effects, and block as a random effect. The predicted raster cell values from the model were averaged at depth increments (0-5, 5-10, 10-20, and 20-30 cm) for each plot when testing for the main effects. An

ANOVA was also run with pixels averaged across all depth. Principal components analysis (PCA) and graphing were completed in PC-ORD (MjM Software, Gleneden Beach, OR).

Results

Gene copy numbers

16S rRNA gene copy number averaged over all samples was 4.6 x 10^9 gene copies g^{-1} soil (**Figure 2.2**), and did not differ between surface broadcast and subsurface-banded manure treatments or between vetch and rye cover crops (**Table 2.1**) when analyzed with means of 16S across all depths. The spatial distribution of 16S rRNA genes did vary within the soil profile among the treatments (**Figure 2.3**). There was a significant increase in 16S bacterial gene copies around the subsurface band (Getis-Ord-Gi*, $p<0.002$) in both cereal rye and hairy vetch plots (**Figure 2.3B and 2.3D**). In addition, 16S gene abundance increased at the corn row in the cereal rye treatments under both poultry litter application regimes (**Figure 2.3A and 2.3B**). The abundance of 16S genes was positively correlated with NH_4^+-N, NO_3^- -N, and soil water content, and negatively correlated with soil depth (**Table 2.2**).

Gene copy numbers of the fungal ITS averaged over all samples was 3.48 x 10^7 gene copies g^{-1} soil (**Figure 2.2**), approximately 2 orders of magnitude lower than for bacteria. Depth was the most important driver for ITS gene distribution (**Table 2.2**, **Figure 2.3E-H**), with gene copy numbers decreasing with soil depth in each treatment. Gene copy numbers of fungal ITS were positively correlated with NH_4^+-N, NO_3^--N, soil moisture, and soil pH (**Table 2.2**), but did not vary due to cover crop or poultry litter placement (**Table 2.1**).

Total *nirK* gene copy number g^{-1} of soil was significantly altered by cover crop species ($P=0.01$) with *nirK* gene numbers higher in the cereal rye compared to the hairy vetch plots

(**Table 2.1**). Poultry litter placement did not affect overall *nirK* gene abundance (Getis-Ord-Gi*, p=0.96), however, *nirK* gene copy number g^{-1} of soil seemed to decrease slightly at the band, **Figure 2.4A-D**). Interestingly, *nirK* but not *nirS* gene copy number was positively correlated to soil moisture (**Table 2.2**). In contrast, *nirS* gene copy number was mainly influenced by poultry litter placement, with the highest abundance in the broadcast manure treatments (**Table 2.1**, **Figure 2.4E-H**). Like *nirK*, *nirS* gene copy numbers also tended to decrease near the subsurface band (**Figure 2.4F and 2.4H**). Both *nirK* and *nirS* were strongly negatively correlated with depth and positively correlated with NO_3^--N concentration (**Table 2.2**). The *nirS* abundance was also positively correlated to pH, but this was not true for *nirK* gene abundance.

The *nosZ* gene copy numbers were highest in the plots with hairy vetch and broadcast manure, with gene copies averaging 8.4×10^7 g^{-1} of soil (**Figure 2.2**, **Figure 2.4I-L**). Due to evidence of a potential interaction effect between cover crop and poultry litter placement on *nosZ* gene copy number (p=0.09), data were analyzed separately for each factor. However, in both analyses the *nosZ* gene copy numbers were not affected by cover crop treatment or poultry litter placement at any depth (**Table 2.1**, **Table I.2**). Poultry litter placement did not alter the distribution of *nosZ* (p>0.05), but *nosZ* abundance was strongly negatively correlated with depth and positively correlated with NO_3^- -N concentration (**Figure 2.4I-L**, **Table 2.2**). The average copy numbers of denitrification genes *nirK*, *nirS*, and *nosZ* were similar, 3.0×10^7, 1.8×10^7, and 2.2×10^7 gene copy number g^{-1} soil, respectively (**Figure 2.2**).

Edaphic Factors

Inorganic N concentrations were highest near the subsurface band of poultry litter (**Figure I.1**), driven primarily by high levels of NH_4^+-N rather than NO_3^--N. Manure placement had a significant effect on NH_4^+-N concentration, with higher NH_4^+-N concentration in the

subsurface-banded plots compared to the broadcasted plot at 5-10 cm, the depth at which the subsurface band was located ($p=0.01$). However, at all other depths poultry litter placement did not have an effect on NH_4^+-N concentration. Ammonium-N concentration was not significantly affected by cover crop at any depth, but cover crop had a significant impact on the NO_3^--N level in the soil at 20-30 cm depth (**Table 2.1**), with NO_3^- -N concentration 1.8 times higher in the hairy vetch than the cereal rye plots (**Figure I.1**). Additionally, cover crop type affected soil moisture (**Table I.1**), with gravimetric water content 1.2 times higher in the rye than the vetch plots (**Figure I.2**). Poultry litter placement did not affect the soil moisture content. Soil pH also did not differ between poultry litter placement or cover crop (**Table 2.1**). However, there is evidence for a localized increase in pH around the subsurface band of poultry litter (**Figure I.3**). There were also pH differences among experimental blocks due to a pH gradient across the field (data not shown).

A PCA ordination of all gene copy numbers g^{-1} soil explained a total of 63.5% of the variation across three axes (**Figure 2.5**). Axis 1 explained 32.3% of the variability with soil depth the most strongly correlated edaphic factor ($r=0.86$, $p<0.001$). All other measured edaphic features were correlated with depth (**Figure I.4**). Both the 16S rRNA and ITS gene copy numbers g^{-1} soil were significantly correlated to axis 1 ($r=0.87$ and 0.91, respectively). Therefore, depth and associated edaphic factors appear to influence the gene abundance of 16S and ITS within the soil profile. The gene abundances of *nirK*, *nirS*, and *nosZ* were also correlated to depth, but when axes 2 and 3 were plotted (excluding the strong depth effect) *nirS* positively correlated to pH along axis 2. In contrast, *nirK* was positively correlated to moisture content along axis 3 (**Figure 2.5**).

Discussion

Our study was designed to test for the creation of potential denitrification hotspots surrounding subsurface-banded poultry litter as determined by increased abundances of denitrifying genes. Spatial patterns of the bacterial 16S rRNA showed an increase in gene abundance both at the location of the poultry litter band and at the corn row (**Figure 2.3B and 2.3D**). This could be due to the proliferation of the native bacterial soil population in response to the increased NH_4^+-N (**Figure I.1**), or the input of bacteria directly from the poultry litter to the soil. Counter to our first hypothesis, however, the banding did not result in increased denitrification gene abundances at the site of poultry litter application (**Figure 2.4**). In fact poultry litter banding under the cereal rye cover crop appeared to decrease *nirK* and *nirS* at the location of the band (**Figure 2.4B and 2.4F**). Our results would suggest that poultry litter addition by subsurface-banding did not increase denitrifier populations to a greater extent than surface broadcasting within the 48 hr time-frame between poultry litter banding and soil sampling. We compared our gene copy numbers to a data sub-set of N_2O flux values from these experiments but found no relationship (data not shown). There are several possible explanations for why we failed to see a correlation. We sampled the poultry litter band two days after its placement, assuming that the local bacteria would respond quickly to this new input. A different timeframe may have yielded different results. Because we targeted soil DNA, our results do not exclude the possibility of increased denitrification activity by the resident community, nor does the quantification of genes account for potential variations in denitrification rate amoung different bacterial species. Other studies have also failed to document correlations between DNA gene copy numbers and N_2O flux (Dandie *et al.*, 2011, 2008). Future work targeting mRNA may shed light on the immediate response of the community to manure addition.

In support of our second hypothesis, microbial gene abundance significantly decreased with depth (**Figure 2.5**, **Table 2.2**). This decrease in gene abundance has been documented in a number of studies (Barrett *et al.*, 2016; Fierer *et al.*, 2003). The environmental mechanism(s) that lead to changes in microbial gene abundance with depth are not clear, however, because depth was correlated with all of the soil variables measured. Therefore, it is likely that the "depth effect" is a combination of differences in NO_3^--N and NH_4^+-N concentrations, pH, and soil moisture (**Figure 2.5**), and likely other factors such as C availability and O_2 levels also play a role, but those were not measured in this study.

Although all gene abundances decreased with soil depth, the abundance of each group of microbial genes had unique correlations to the measured environmental variables. In spite of a narrow pH range measured in our study (5-7), we observed positive correlations between pH and ITS and *nirS* gene copy numbers (**Figure 2.5**). Previously, pH has been noted as an important factor affecting microbial communities (Fierer and Jackson, 2006), but typically ITS abundance has been documented as negatively correlated to pH (Rousk *et al.*, 2010). There are likely different mechanisms at play over the large pH range (4.0-8.3) seen in the study by Rousk *et al*., (2010) and the narrow range seen in our study, possibly accounting for the conflicting results. Furthermore, the variability in pH in our study was associated with a gradient across the agricultural field. Other unmeasured variables may have also been associated with this gradient, such as soil texture, which has also been shown to have an influence on fungal abundance (Lauber *et al*., 2008). The relationship between *nirS* and pH is interesting because we did not see the same correlation between *nirK* and pH, which has been reported in several studies (Bárta *et al.*, 2010; Enwall *et al.*, 2010; Liu *et al.*, 2010). Several studies have suggested that organisms carrying *nirS* fill different niches compared to those harboring *nirK* (Bárta *et al.*, 2010; Cuhel *et*

al., 2010; Graf *et al.*, 2014; Enwall *et al.*, 2010). For example, some studies have speculated that organisms carrying *nirS* are better adapted to anoxic soil conditions and are more likely to also contain the genes for complete denitrification (Graf et al. 2015). Other studies have observed a positive correlation between *nirS* and pH, as in our study (Liu *et al.*, 2010). Enwall et al. (2010) reported that *nirK* was more sensitive to changes in pH compared to *nirS*, however, highlighting the need for additional research to better understand what controls the distribution and expression of these genes. The same study also reported that *nirS*/*nirK* ratio reflected differences in community functioning, suggesting that the two *nir* genes may not be ecologically redundant. This has been confirmed in recent meta-analysis performed by Graf *et al*. (2014).

When gene copy numbers from all soil samples were averaged, we observed almost equal amounts of *nirK*, *nirS*, and *nosZ* with all three gene copy numbers averaging in the 10^7 g^{-1} soil. This is atypical, as *nir* genes often exceed *nos* genes by an order of magnitude (Jones *et al.*, 2013; Hallin *et al.*, 2009). Assuming 1-15 copies of the 16S rRNA gene (Klappenbach *et al*., 2001) and one copy of each of the denitrification genes per bacterium (Kandeler *et al*., 2006), approximately 0.5-7.2% of the bacterial community carried denitrification genes. Although most bacteria appear to only have a copy of either *nirK* or *nirS*, some genomes have been shown to contain both genes (Graf *et al.*, 2014). Some researchers have observed the dominance of *nirS* over *nirK* in agricultural soils (Hallin *et al.*, 2009; Enwall *et al.*, 2010), but others report *nirK* to be more abundant than *nirS* (Clark *et al.*, 2012). Our observation thar *nirK* and *nirS* were present in almost equal quantities, suggests that soil properties, not just changes due to management, may determine the presence of *nirS* and *nirK*. Given the wide variation in the relative abundances of these two genes reported in the literature, it is important that researchers include both genes in any future surveys of soil denitrifier communities.

The gene copy numbers of *nosZ-I* were also similar to values reported in the literature (Henry *et al.*, 2006). Recently a second clade, *nosZ-II*, of *nosZ* has been reported (Jones *et al.*, 2013). According to that paper, *nosZ-II* appeared to be ubiquitous in soils, but variable across ecosystem and habitat types including agricultural soils. Our soils were screened for *nosZ-II*, but no evidence of its presence was found with the primer sets and conditions used in this study. Recent research on differences between *nosZ-I* and *nosZ-II* indicate that *nosZ-I* is favored in the plant rhizosphere, and the distribution of *nosZ-II* is dependent on edaphic features such as bulk density and pH (Graf, 2015). Further research is needed to understand why we were unable to detect *nosZ-II*, although it is possible that cover cropping may favor *nosZ-I* by increasing the amount of rhizosphere soil. It is also possible that the primer and PCR conditions used were not suitable. Further research will work on re-analyzing samples for *nosZ-II*.

We hypothesized that the increase in N inputs due to hairy vetch would lead to higher gene copy numbers of denitrification genes. Mineral N was greater in plots planted with hairy vetch immediately after cover crop termination (Poffenbarger *et al*, 2015a). However, we observed limited differences in the amount of mineral N between the cover crop species at the time of sampling, with higher concentration of soil NO_3^--N in the hairy vetch only in the 20-30 cm soil samples (**Table I.2**). Wet conditions may have caused leaching of NO_3^--N prior to our sampling. However, cover cropping with cereal rye versus hairy vetch did lead to distinct changes in soil properties and microbial communities, partially supporting our third hypothesis. Across all depths, soil moisture was higher under the cereal rye, compared to hairy vetch (**Table 2.1**, **Figure I.3**). This is likely due to a dense cover crop mulch layer formed at the soil surface after roller crimping, which was especially noticeable in the rye plots. *nirK* gene copy numbers, which were positively correlated with soil moisture, were higher under cereal rye than vetch (**Figure**

2.4, **Figure 2.5**). The abundance of *nirK* has been positively linked to soil moisture in forest soils (Katsuyama *et al.*, 2008; Szukics *et al.*, 2010). In contrast, *nirS* and *nosZ-I* did not differ between the two cover crop species. This again suggests that *nirK* and *nirS* gene abundance are controlled by different factors. Our data also indicates that *nosZ-I* and *nirS* were more strongly correlated than *nosZ-I* and *nirK* (**Figure 2.5**). This finding supports recent work examining dentirifying genomes that reported more co-occurrence of *nirS* and *nosZ* than *nirK* and *nosZ* (Graf *et al.* 2014).

Conclusion

Each gene analyzed in this study had a unique spatial distribution in the soil profile and was differently affected by agricultural management factors. Although bacterial 16S rRNA genes were abundant around the subsurface-banded poultry litter, there was no observed increase in the denitrification genes (**Figure 2.3 and 2.4**). Either the microbes containing the denitrifying genes did not have sufficient time to respond to this input before sampling or the conditions were not suitable for these organisms. In fact a decrease in *nirK, nirS,* and *nosZ* gene abundances was observed at the manure band, suggesting that poultry litter is not a source of large numbers of denitrifying organisms, and/or the poultry litter negatively affected the existing microbial community. Poultry litter placement did significantly affect *nirS* gene abundance and pH, however. In contrast, cover crop type had a larger effect than poultry litter placement on the overall community and on *nirK* gene abundance in particular, possibly due to higher soil moisture in cereal rye compared to hairy vetch plots. Depth was the most significant factor leading to decreased microbial abundances with increasing depth (**Figure 2.5**). These results show that agricultural management, as defined by choice of cover crop and manure application method, affects both gene abundances and patterns in the soil and can alter the community

composition and structure, possibly leading to changes in the functionality of the N-cycling microbial communities.

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Table 2.1. P-values for ANOVA used to test the effects of cover crop and poultry litter placement on genes, ammonium-N, nitrate-N, soil moisture, and pH at each soil depth. Bold numbers indicate p-values <0.05.

	Cover Crop					Poulty Litter Placement				
	All depths	0-5cm	5-10 cm	10-20 cm	20-30 cm	All depths	0-5cm	5-10 cm	10-20 cm	20-30 cm
16S	0.32	0.17	0.24	0.34	0.35	0.98	0.91	0.9	0.99	0.89
nirK	**0.01**	**0.00**	**0.01**	**0.02**	**0.03**	0.97	0.56	0.92	0.84	0.84
nirS	0.37	0.1	0.12	0.14	0.17	0.08	**0.05**	0.08	0.14	**0.04**
nosZ	0.14	0.19	0.14	0.16	0.14	0.14	0.11	0.13	0.27	0.11
ITS	0.52	0.14	0.11	0.86	0.58	0.91	0.3	0.21	0.67	0.1
Ammonium-N	0.95	0.07	0.21	0.29	0.28	0.36	0.31	**0.01**	0.85	0.59
Nitrate-N	0.24	0.66	0.66	0.17	**0.02**	0.46	0.33	0.37	0.62	0.54
Soil Moisture	**0.03**	**0.02**	**0.02**	**0.05**	0.09	0.94	0.88	0.74	0.9	0.99
pH	0.72	0.26	0.42	0.54	0.32	0.52	0.26	0.47	0.38	0.78

Table 2.2. Correlation coefficients (r) for 16S, ITS, and nitrogen cycling genes with soil covariates. An asterisk indicates a p-value < 0.05. Gene copy numbers, ammonium-N concentrations, and nitrate-N concentrations were log transformed.

Gene	NH_4^+-N	NO_3^--N	Soil Moisture	pH	Distance from Inter-row Center	Depth
16S	0.45*	0.30*	0.38*	0.13	-0.08	-0.73*
nirK	0.05	0.23*	0.52*	0.01	0.05	-0.54*
nirS	0.02	0.42*	-0.04	0.46*	0.06	-0.68*
nosZ	0.07	0.42*	0.10	0.13	0.03	-0.62*
ITS	0.30*	0.44*	0.28*	0.20*	-0.02	-0.82*

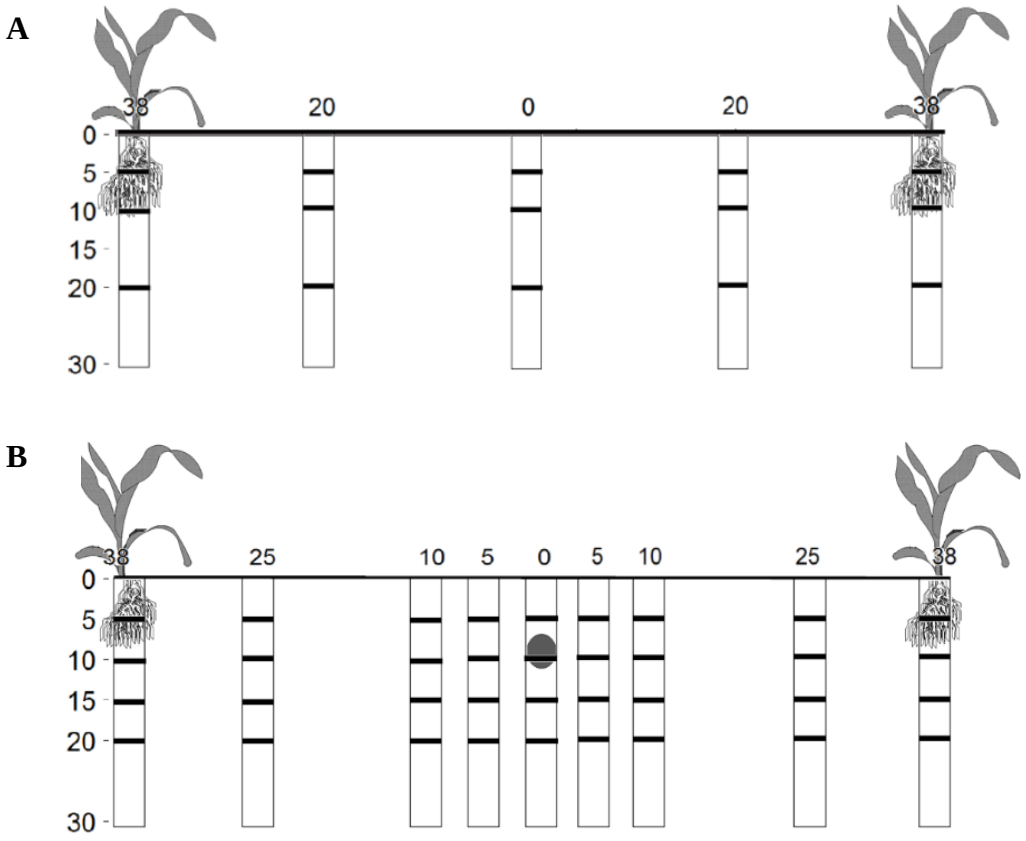

Figure 2.1. Diagram of soil sampling scheme for A) the broadcast poultry litter treatment (NTB) and B) the subsurface banded poultry litter treatment (SSB). Soil samples in the NTB plots were taken at 0, 20, and 38 cm from the inter-row center. The 38 cm distance is the corn row. Samples in these plots were separated at 0-5, 5-10, 10-20, and 20-30 cm depths, for a total of 12 samples per plot. The SSB plots were sampled at 0, 5, 10, 25, and 38 cm from the inter-row center and separated at 0-5, 5-10, 10-15, 5-20, and 20-30 cm depths, for a total of 25 samples per plot. The subsurface banded poultry litter, which is illustrated with a gray oval, was placed 10 cm below the soil surface.

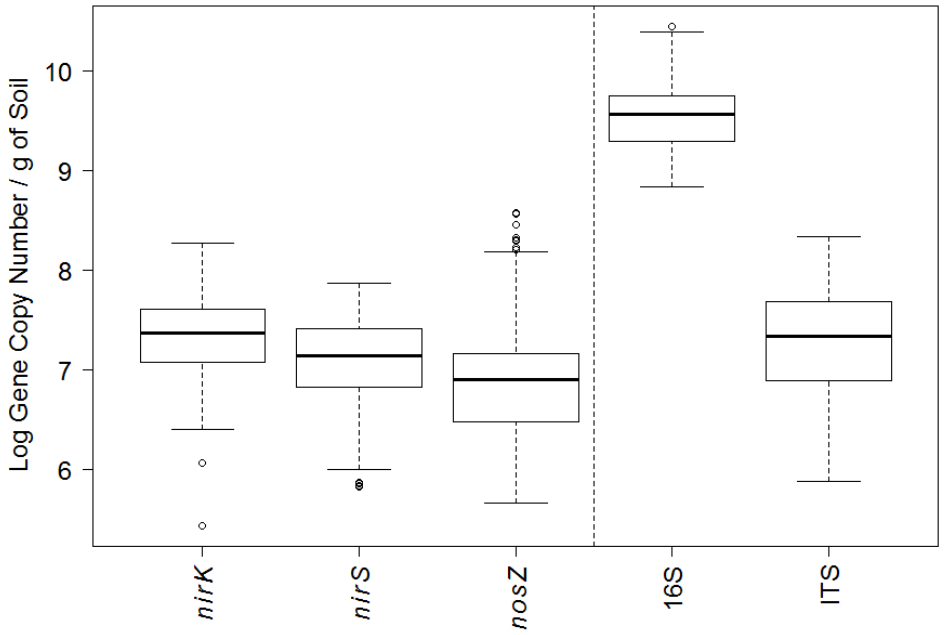

Figure 2.2. Box plots showing abundance of measured microbial genes from all treatments. The thick line indicates the median value, the upper and lower boundaries of each box indicate the 75th and 25th percentiles, respectively. Outliers are shown by open circles. The mean of non-transformed gene abundance is given for each gene. All values are in gene copy number g^{-1} fresh soil.

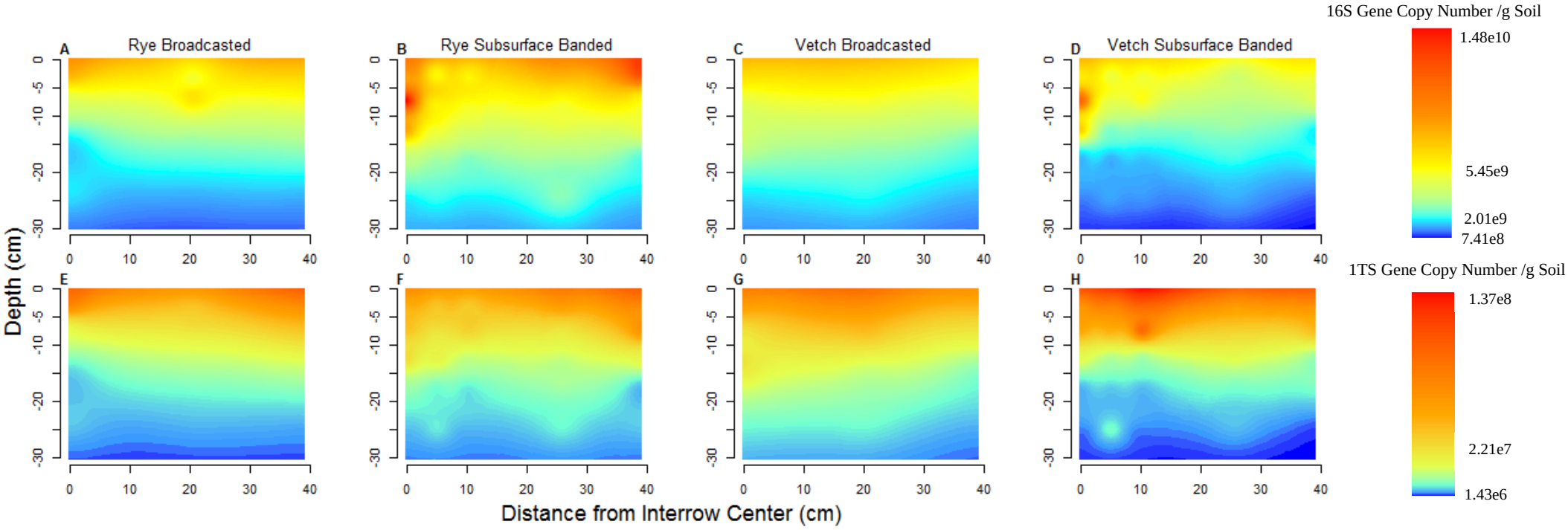

Figure 2.3. Spatial distribution of bacterial 16S rRNA genes (A-D) and fungal ITS genes (E-H) at the corn V5 growth stage: A) 16S cereal rye with broadcast poultry litter, B) 16S cereal rye with subsurface banded poultry litter, C) 16S hairy vetch with broadcast poultry litter, D) 16S hairy vetch and subsurface band poultry litter, E) ITS cereal rye with broadcast manure, F) ITS cereal rye with subsurface band, G) ITS hairy vetch with broadcast manure, and H) ITS hairy vetch and subsurface band. For each panel, the inter-row center is on the left and the corn row is on the right. Figures are based on inverse distance weighted interpolation.

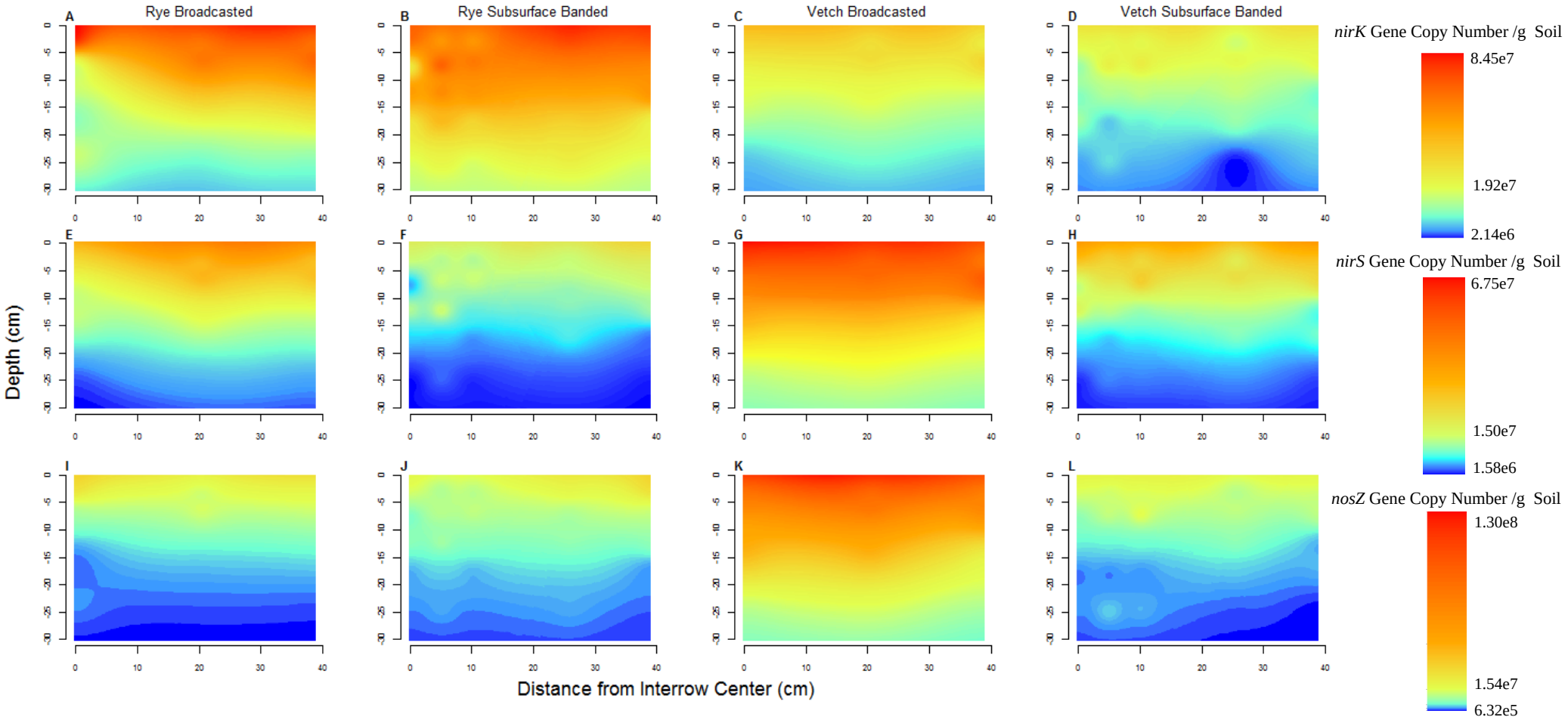

Figure 2.4. Spatial distribution of *nirK* (A-D), *nirS* (E-H), and *nosZ* (I-L). genes at the corn V5 growth stage: A) *nirK* cereal rye with broadcast poultry litter, B) *nirK* cereal rye with subsurface banded poultry litter, C) *nirK* hairy vetch with broadcast poultry litter, D) *nirK* hairy vetch and subsurface banded poultry litter, E) *nirS* cereal rye with broadcast manure, F) *nirS* cereal rye with subsurface band, G) *nirS* hairy vetch with broadcast manure, H) *nirS* hairy vetch and subsurface band, I) *nosZ* cereal rye with broadcast manure, J) *nosZ* cereal rye with subsurface band, K) *nosZ* hairy vetch with broadcast manure, and L) *nosZ* hairy vetch and subsurface band. For each panel, the inter-row center is on the left and the corn row is on the right. Figures are based on inverse distance weighted interpolation.

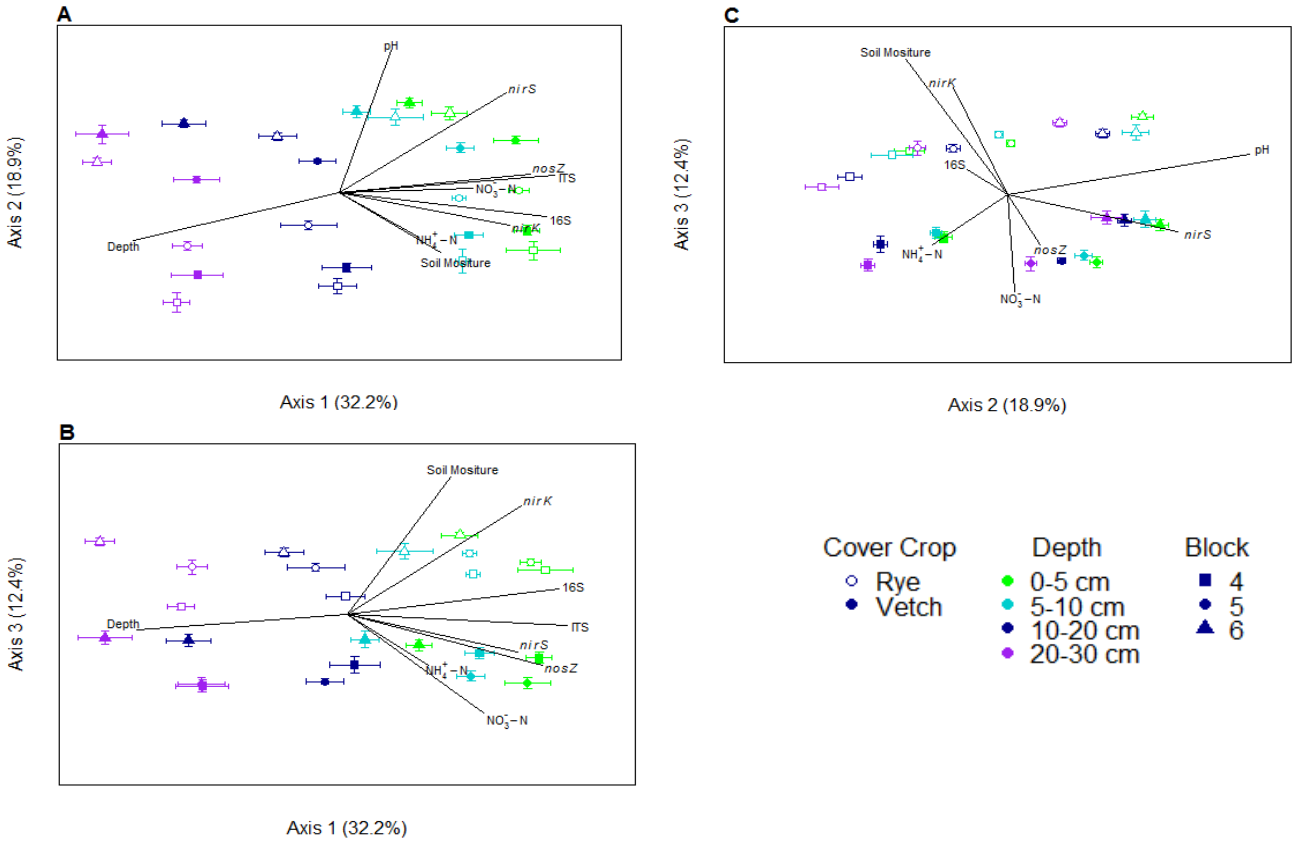

Figure 2.5. Principal component analysis (PCA) of gene copy numbers for 16S, *nirK*, *nirS*, *nosZ*, and ITS. Gene copy numbers were log transformed. Cover crop is represented by opened (rye) or closed (vetch) symbols. Depth is represented by the color of the symbol. Field replication is represented by the symbol shape. A) PCA axis 1 vs axis 2 in which observations group by depth along the first axis and by replicate along the second axis. B) PCA axis 1 vs axis 3 in which observations group by depth along the first axis and by cover crop along the third axis. C) PCA axis 2 vs axis 3 in which observations group by replicate along the second axis and by cover crop along the third axis. The total variability explained by the first 3 axes is 63.5%. Significant vectors (α=0.05) are plotted as biplots.

Chapter 3. Denitrifier abundance and community composition improves denitrification models

Abstract

The lack of data linking denitrifying microbial communities to nitrous oxide (N_2O) and dinitrogen (N_2) production has resulted in the exclusion of biotic variables from most denitrification models. This study examined denitrifier community abundance and composition in an agricultural and a wetland soil subjected to manipulations of pH and moisture content. Microbial community data were linked to potential N_2O and N_2 production, and their importance to denitrification models were tested. The *nirK* and *nirS* genes, which code for nitrite reductase, differed in composition between the wetland and agricultural field soils. Manipulations of soil pH resulted in significant changes in the denitrifier community; when soil pH was lowered to 3, denitrification gene abundance decreased and the composition of *nirK* and *nirS* changed. Regression models were created to predict denitrification rates. The inclusion of specific denitrifier genotypes and denitrifier gene abundance led to a better model than abiotic variables alone. The composition and abundance of *nirS* helped to predict denitrification enzyme activity (DEA) and N_2O:N_2 ratio, and was a better predictor of DEA under alkaline conditions and in wetland soils. The composition and abundance of *nirK* was correlated to potential N_2O production and became a realtively stronger predictor of DEA in acidic soils. These findings highlight the benefit of including microbial data in models predicting denitrification activity, but also show that *nirK* and *nirS* are not ecologically redundant.

Introduction

The relative importance of soil conditions and the denitrifying microbial community on denitrification rates is still contested in the literature (Attard *et al.*, 2011; Enwall *et al.*, 2005; Dandie *et al.*, 2011; Morales *et al.*, 2010; Hallin *et al.*, 2009; Philippot *et al.*, 2011). Some studies have found that abiotic variables are more important than biotic variables as predictors of denitrification (Attard *et al.*, 2011). The majority of denitrification models, especially at the landscape scale, do not include microbial variables (Boyer *et al.*, 2006; Groffman *et al.*, 2009). Examples include the ANIM, NTRM, and CENTURY models (Heinen, 2006). When microorganisms are included in models it is often based on relatively old data on enzyme and growth kinetics (Bakken *et al.*, 2012). However, biotic variables such as denitrifier gene abundance (Morales *et al.*, 2010; Hallin *et al.*, 2009; Philippot *et al.*, 2011), community composition (Cavigelli and Robertson, 2000; Rich *et al.*, 2003), and diversity (Philippot *et al.*, 2013) have been linked to rates of denitrification and nitrous oxide (N_2O) production. In fact, Powell et al. (2015), found that biotic factors, including the relative abundance of specific genotypes of *nir* genes (those that code for nitrite reductase), were significant variables in their denitrification model, and lead to a better model, in terms of model fit, than abiotic factors alone. These findings demonstrate that knowledge of the size and composition of the denitrifier community can be important for predicting function.

Two edaphic factors commonly used in denitrification models are pH and soil moisture (Wallenstein *et al.*, 2006). pH, often called a master variable, controls many reactions, both chemical and biological, and impacts both the rates of denitrification, with optimal pH for denitrification around neutral, and the end-products of denitrification, with the ratio of N_2O:N_2 increasing with decreaseing pH (Šimek and Cooper, 2002; Bakken *et al.*, 2012). Changes in soil

pH occur often in anthropogenic systems, such as agricultural fields. The addition of ammonium-based mineral fertilizer can lower the soil pH over time (Pierre *et al.*, 1971), whereas practices such as liming and the addition of organic manures can raise the pH (Whalen *et al.*, 2002). Soil moisture also has an impact on denitrification in bacteria since this is an anaerobic process, generally occurring when the water filled pore space (WFPS) is greater than 70% (Butterbach-Bahl *et al.*, 2013). In upland soils, such as agricultural fields, moisture can change rapidly due to irrigation events, plant uptake, rainfall, and drought. In wetland soils, moisture may be more constant in the short term, but could become drier seasonally. Climate change is likely to result in large-scale changes in precipitation patterns, which could increase rainfall and/or drought intensity, or the timing of precipitation events (IPCC, 2007; Wentz *et al.*, 2007; Min *et al*., 2011), resulting in higher variability in soil moisture for both uplands and wetlands. Due to the importance of denitrification to soil fertility, water quality, and climate change, it is important to have accurate models predicting these processes in soils of many types. Furthermore, the addition of microbial data to these models may be a way to further improve model accuracy. Experimental manipulations of pH and moisture will lead to a greater understanding of the role of these two factors on denitrification and N_2O production rates, and on the denitrifying community in the soil.

If it is important to include biotic variables in denitrification models, we are still faced with: What microbial measures are important? Microbial denitrification studies have largely focused on three genes. The gene *nosZ* encodes for nitrous oxide reductase, the last step in the pathway, and has been characterized in numerous studies (Zumft, 1997; Philippot, 2002; Henry *et al.*, 2006; Jones *et al.*, 2013). The genes *nirK* and *nirS* both encode for nitrite reductase. They are found across diverse taxonomic groups, and until recently were thought to be mutually

exclusive in a given strain (Graf *et al.*, 2014). It has been speculated, however, that it may be advantageous for a single bacterium to carry both *nir* genes if the genes are functionally divergent or active under differing conditions (Graf *et al.*, 2014). Numerous studies have reported that the ratio of *nirK*/*nirS* gene copy numbers changes under differing environmental conditions (Enwall *et al.*, 2010; Philippot *et al.*, 2009; Hallin *et al.*, 2009; Bárta *et al.*, 2010). Differences in these ratios suggest the possibility of niche selection between the *nirK* and *nirS* communities, although the environmental variables contributing to niche selection need to be further elucidated. Some studies have reported that decreased pH leads to a higher *nirK/nirS* gene abundance ratio (Philippot *et al.*, 2009), but others report an opposite effect (Bárta *et al.*, 2010). Because differences in pH can be linked to a variety of other soil characteristics such as metal availability and soil organic matter content, contrasting results in the literature are likely due to confounding factors across many different soil types. Similarly, the effect of soil moisture on the abundance of *nirK/nirS* varies across studies. One study reported that organisms carrying *nirK* are more important in wet or saturated conditions (Katsuyama *et al.*, 2008), but other studies have suggested a decrease in the *nirK/nirS* ratio under saturated conditions (Yoshida *et al.*, 2009, 2010; Philippot *et al.*, 2009).

There have been few controlled laboratory studies investigating the individual impacts of abiotic factors, such as pH or soil moisture on denitrification genes (Liu *et al.*, 2010) and to our knowledge no study has simultaneously examined denitrification community shifts and potential denitrification rates. Here we present a study evaluating the importance of abiotic vs. biotic variables in modeling denitrification activity. Soils from an agricultural field and a freshwater wetland were manipulated to observe the effect of pH and moisture on gene abundance, community composition, and activity over time. We had three main hypotheses: 1) Manipulating

pH and moisture content would result in changes in the denitrifying community and lead to differences in denitrification activity; 2) Inclusion of denitrifier community composition and gene copy numbers would improve models predicting denitrification enzyme activity (DEA) and N_2O production; 3) *nirK* and *nirS* are not ecologically redundant, and *nirS* abudance is more strongly correlatated than *nirK* abudance to rates of denitrification.

Materials and Methods

Soil Sampling and Site Description

Soils were collected from an agricultural and a wetland site located at Beltsville Agricultural Research Center (BARC) in Beltsville, MD. The wetland is a non-tidal freshwater wetland adjacent to Beaverdam Creek. Three replicate plots were established in the forested wetland approximately 100 m from the creek. Plots were chosen to occur under similar vegetation and along a similar elevation, and were speperated by at least 50 m. Soils were collected from each plot using a bucket augar from 0-10 cm after removing the Oi horizon. The agricultural soil was collected from the Farming Systems Project (FSP), a long-term agro-ecological research system established in 1996. Additional details pertaining to field management can be found in Cavigelli et al., (2008). Soils were collected from three replicate conventionally-managed tilled plots in the wheat/soybean rotation phase of a corn-soybean-wheat/soybean rotation. Soils were collected with hand held soil corers (1.9 cm diameter) from 0-10 cm depth. Sampling at both sites took place in April 2015. Details on soil physical and chemical properties from the two sites can be found in **Table 3.1**.

Experimental set-up

Soils were taken to the lab and were homogenized for each plot within 24 hours of collection. The agricultural soils were homogenized by sieving through a 4.75 mm sieve. The

wetland soils were too saturated to sieve and were homogenized in plastic bags by hand. Subsamples were collected for pH (≥ 10 g), gravimetric moisture content (≥ 10 g), inorganic and total nitrogen (TN) and dissolved organic carbon (DOC) (≥ 15 g), denitrification enzyme activity (DEA) assays (≥ 20 g), and DNA extraction (≥ 10 g). The remaining soil was allowed to air dry for two days, and then sieved through a 4.75 mm sieve, placed in sealed bags, and stored at 4°C for one week. After storage, additional soil was removed for soil moisture and pH measurements.

Both the pH manipulation and moisture manipulation experiments were performed in 236 ml jars. The jars remained capped during the experiment, but had a 1.7 cm diameter hole drilled in the cap. This hole was covered with 0.45 Whatman filter paper to allow the exchange of air while reducing water loss from the jars. The jars were weighed at the start of the experiment and re-weighed twice a week throughout the experiment to determine water loss via evaporation. Water was added to keep jars at the desired water content. All jars were kept in the dark and at 25°C for the duration of the experiment. Three jars per treatment were destructively sampled at 0, 7, 21, and 35 days (one jar per field replicate). The day 0 sampling occurred three hours after re-wetting and treatment addition.

Soil Moisture Experiment

Gravimetric soil moisture was determined after drying samples at 105°C for 24 h, and was calculated as:

$$\theta_g = \frac{(Wet\ soil - Oven\ dried\ soil)}{Oven\ dried\ soil}$$

Water-filled pore space (WFPS) was calculated due to the large difference in bulk density and water holding capacity between the agricultural and wetland soil:

$$\%WFPS = \frac{(\theta g * \rho_b)}{(1 - \frac{\rho_b}{\rho_d})} * 100$$

where:

θg = the gravimetric water content (g water g^{-1} soil)

ρ_b = bulk density (g cm^{-3})

ρ_d = particle density (g cm^{-3}), assumed to be 2.65 g cm^{-3}

Matric potentials were determined from soil water retention curves that were created for each plot using a WP4 Dewpoint Potentiometer (Decagon Devices Inc, Pullman, WA).

Each microcosm contained 100 g dry weight equivalent soil. Soils were incubated at 20%, 50%, and 90% WFPS. To determine the amount of water needed to bring soils up to the desired WFPS, the actual and desired gravimetric water content was determined for each soil. Grams of water was then converted to volume of water assuming a bulk density of 1g cm^{-3}.

$$\text{Water added to each jar (g)} = (\theta_g \text{desired} - \theta_g \text{actual}) * 100$$

pH Manipulation Experiment

Soils in each microcosm were maintained at 90% WFPS for the duration of the pH experiment once they were adjusted to pH 3, pH 7, or pH 9. The amount of water needed to bring the soils to 90% WFPS was calculated using the same method as described for the soil moisture experiment. Once the volume of water was determined, the moles of H^+ or OH^- ions needed to achieve the desired pH was determined from titration curves made prior to the experiment. An acid solution, using HCl or an alkaline solution, using NaOH, was then added to each soil to bring it up to 90% WFPS and the desired pH. The same samples that served as the soil moisture 90% WFPS treatments served for the pH experiment as control treatments. Twice a week over the course of the experiment, the pH 3 and pH 9 jars received HCl or NaOH solutions, respectively, diluted to maintain desired soil pH values. The control and pH 7 jars received

distilled water. These solutions was used unless the pH shifted. In that case a new dilution was made and added to the jar to return the soil to the desired pH.

Carbon and Nitrogen extraction

Soils were extracted with 2M KCl (5:1 v/w) for 1 h on a reciprocating shaker. The soil slurries were centrifuged at 6000 rpm for 10 min and filtered through Whatman 42 filter paper. The extractions were frozen at -20°C until analysis. Ammonium-N (NH_4^+-N) and nitrate-N (NO_3^--N) were analyzed on a Lachat (Lachat Instruments, Loveland, CO), using the salicylate (QuickChem Method 12-107-06-2-A) and cadmium reduction and reaction with sulfanilamide and N-1- napthylethylenediamine dihydrochloride (QuickChem Method 12-107-04-1-B), respectively. Total nitrogen and DOC were analyzed on a Shimadzu TOC-L (Shimadzu Cooperation).

Denitrification Enzyme Activity (DEA) and Potential N_2O Production

DEA assays were carried out based on Groffman et al. (1999), to determine potential N_2 and N_2O production. Ten grams (dry-weight) of soil were weighed into 60 ml serum bottles and 10 ml of DEA medium (1mM KNO_3, 1mM dextrose, and 0.10mg kg^{-1} chloramphenol) were added to each bottle. Bottles were sealed with rubber stoppers and flushed with N_2 gas for 4 mins to remove oxygen; then they were brought to atmospheric pressure with a syringe to relase excess gas. Bottles received either 6ml of acetylene (C_2H_2) for N_2 assays, bringing the concentration of C_2H_2 to 10% of the volume of the headspace, or 6 ml of N_2 gas for N_2O assays. Bottles were shaken on a reciprocating shaker. Three milliliter gas samples were taken at 45 min and 105 min and stored in 12 ml Extainers that had been flushed with N_2 gas (Labco, UK). N_2O

concentration was analyzed on a HP 6890 ECD gas chromatograph with an oven temperature of 50ºC and a flow rate of 50.8 ml/min.

Denitrification rates were calculated as follows:

$$DR = \frac{[(C_{105} * H) - (C_{45} * H)]}{(D * T)}$$

where:

DR= denitrification rate (µg N kg soil^{-1} h^{-1})

C_{105}= N_2O concentration at 105 mins (µg N_2O-N L^{-1} headspace)

C_{45}= N_2O concentration at 45 mins (µg N_2O- N L^{-1} headspace)

H= headspace volume (L)

D= soil dry weight (g)

T= time between sampling times (h)

DNA extraction, qPCR, and TRFLP

DNA was extracted from 0.25g of fresh soil using the MoBio PowerSoil DNA Extraction Kit, according to the manufacture protocol (MoBio Laboratory, Carlsbad, CA). DNA was quantified using a Qubit 2.0 Fluorometer (Invitrogen, Waltham, MA) and diluted to a final concentration of 2.5 ng µl^{-1} with autoclaved water. Extractions and dilutions were stored at -80ºC until further analysis.

Gene abundances for the 16S (bacteria), *nirK* and *nirS* (nitrite reduction), and *nosZ* (nitrous oxide reduction) genes were determined using real-time PCR (qPCR). Samples were also analyzed for the p450nor gene (fungal denitrification). We saw the presence of p450nor gene in almost all of the samples, but the quantities were below detection limit of our qPCR assay and are therefore not included in results. The qPCR conditions, efficiencies, and primers are found in **Table S1**. Each sample was run in triplicate. Each plate was run with a set of soil

plasmid standards in triplicate with gene copy numbers ranging from $1.1e^4$-1.1 e^8 for 16S, $1.1e^2$-$1.1e^7$ for *nirK,* $1.05e^3$-$1.05e^5$ for *nirS,* and $1.08e^2$-$1.08e^6$ for *nosZ* . Each plate was also included a set of no template controls made with sterilized water.

Gene copy numbers were corrected to account for PCR inhibition (Hargreaves *et al.*, 2013). Briefly, samples were randomly selected and pooled. The pooled samples were serially diluted and run at the standard conditions for each gene. The efficiency and intercept of the pooled samples were calculated and used to correct for relative copy number differences between the plasmid standard curve and the pooled sample standard curve. pH and soil type were tested separately to determine if conditions inhibited PCR, but no inhibition was observed.

The composition of genes *nirK*, *nirS*, and *nosZ* was determined using terminal restriction fragment length polymorphism (TRFLP) analysis. *nirK* was amplified with the primer set *nirK*1F (5'-6FAM GGMATGGTKCCSTGGCA) and *nirK*5R (5'-GCCTCGATCARTTRTGG). *nirS* was amplified with the primer set *nirS*1F(5'-6FAM CCTAYTGGCCGCCRCAR) and *nirS*6R (5'-CGTTGAACTTRCCGGT) (Braker *et al.*, 1998). *nosZ* was amplified with the primer set *nosZ*-F-1181 (5'-6FAM CGCTGTTCITCGACAGY) and *nosZ*-F-1180 (5'-ATGTGCAKIGCRTGGCAGAAC) (Rich *et al.*, 2003). Each 50µl PCR reaction consisted of 18.3µl H_2O, 10µl Promega PCR buffer, 3.5µl 25 mM $MgCl_2$, 1.0µl 10mM dNTPs, 2.5µl 10mM forward primer, 2.5µl 10mM reverse primer, 8µl 0.4% BSA, 0.25µl TAQ, and 4µl sample DNA. *nirK* and *nirS* PCR conditions were as follows: 95°C for 5 min, followed by 95°C for 30 s, 56°C for 40 s, and 72°C for 40 s. This was repeated for 10 cycles with a 0.5°C drop in the annealing temperature until 51°C, and then followed by 20 cycles with an annealing temperature of 54°C. The reactions were then held at 72°C for 7 min (Braker *et al.*, 2000). For *nosZ* the reactions were run at 94°C for 3 min, followed by 25 cycles of 94°C for 45 s, 56°C for 60 s, and 72°C for 2 min.

The reactions were then held at 72°C for 7 min (Rich *et al.*, 2003). For each gene 3-50µl reactions were run per sample and pooled for PCR clean-up. The PCR reactions were cleaned using the MO BIO UltraClean® PCR Clean-Up Kit, according to manufacturer protocol (Carlsbad, CA). A portion of the samples was checked for amplification and primer removal by running 10µl of cleaned PCR product on 1.1% agrose gel.

All products were digested with *MspI* FastDigest Enzyme (Thermo Scientifc, Waltham, MA). In addition *nosZ* products were also digested with *RsaI* FastDigest Enzyme (Thermo Scientifc, Waltham, MA). *MspI* digestions consisted of 8µl PCR product, 10µl water, 1.33µl 10x FastDigest buffer, and 0.66µl enzyme. *RsaI* digestions consisted of 8µl of PCR product, 9.5µl water, 1.5µl 10x FastDigest buffer, and 1µl enzyme. Reactions were incubated at 37°C for 10 min. Restrictions were cleaned using DTR Ultra 96-well plate kits according to manufacturer protocol. (EdgeBio, Gaithersburg, MD), and denatured at 95°C for 5 min. Restriction products were run on a 3730xl DNA Analyzer using the Liz-600 size standard (Applied Biosystems, Waltham, MA).

TRFLP profiles were analyzed with PeakScanner Software v 1.0 (Applied Biosystems, Waltham, MA). Only peaks greater than the primer length were included in the analysis, >18 bp for *nirK* and *nirS* and >20 bp for *nosZ*. Peak areas were normalized by dividing the area for each peak by the summed area for each sample. Peaks consisting of <1% of the total area (*nirK* and *nirS)* and <1.5% (*nosZ*) were removed. If peak sizes were >1 bp apart they were determined to be separated T-RFs. If more than three samples had the same double peak within a T-RF and there was a clear break, the peaks were separated and a new T-RF was created. Otherwise, double peaks were summed.

Statistical Analyses

To determine if treatments had an effect on gene abundance and denitrification measures, an ANOVA was run with treatment and time as fixed factors. Due to significant interactions between treatment and soil type, the agricultural and wetland soils were analyzed separately. Significant differences between treatments were determined with the Tukey honestly significant difference (HSD) post hoc test. All tests were performed in R (R Development Team, 2015).

Community composition data were analyzed with the vegan package in R (R Development Team, 2015). Non-metric multidimensional scaling (NMDS) ordinations of TRFLP profiles were created using Bray-Curtis similarity. All ordinations were constrained to two axes. Significance of treatment and group differences was found using multi-response permutation procedure (MRPP) with 999 permutations. Shannon's diversity, Pielou evenness, and richness based on the relative peak areas and numbers of T-RFs were used to assess denitrifier diversity.

Multiple regression was performed based on the methods used in Diaz *et al*. (2007) and Powell *et al.* (2015). Predictor variables were divided into four categories: 1) abiotic variables 2) microbial functional variables (qPCR data), 3) microbial diversity measures, and 4) genotypes. For the first three categories, all relevant predictor variables were included. Diversity measures used included Shannon's diversity, evenness, and species richness. For the fourth category, we conducted pairwise correlations between the genotype (relativized peak areas) with Bonferroni corrections and chose only significant correlations to add to the model. All possible combinations of predictors were tested and the importance of each predictor was found by summing the Akaike weights of all models including the predictor variable. Predictors with an important value greater than 0.5 were included in future models. Regression analysis was

performed with the MuMIn package in R (R Development Team, 2015). The importance of each category was tested by comparing the Akaike information criterion (AIC) corrected for sample size (AICc) and the corrected R^2 of models after the removal of variables from each category.

To determine the relative importance of nitrite reducers to denitrication, regression models using *nirK* and *nirS* gene copy numbers as predictor variables. Variable importance was defined as the percentage of the model explained by each variable. Realtive important was calculated as R^2 contribution averaged over orderings among regressors (lmg value) using the relaimpo package in R (R Development Team, 2015).

Results

DEA in freshly collected wetland soil (3.26 µgN kg soil $^{-1}$ h $^{-1}$) was three times higher than in the agricultural soils (0.96 µg N kg soil $^{-1}$ h $^{-1}$) (**Table 3.1**, p=0.05). The drying, rewetting, and application of treatments decreased DEA in the wetland soils, but tended to increase DEA in the agricultural soils, with the exception of the pH 3 treatments (**Figure 3.1**). The manipulation of agricultural soils to pH 3 significantly decreased DEA (p=0.001). The pH manipulations had no effect on DEA in wetland soils, however. Denitrification enzyme activity increased in the 90% WFPS treatment for the agricultural soil, but DEA in wetland soils were the same across moisture regimes.

Similar to DEA, N_2O production was highest in the soils taken directly from the field and decreased after drying, rewetting, and treatment application (**Table 3.1**, **Figure 3.1 and 3.2**). The pH manipulations had no effect on N_2O production in the agricultural soil. In wetland soils, pH 9 treated soil had the lowest rate of N_2O production (**Figure 3.1**). Moisture regime had no consistent effect on potential N_2O production in either soil type (**Figure 3.2**). The agricultural

soil had a consistently lower $N_2O:N_2$ ratio compared to wetland soils across all treatments, except in the pH 3 treatment and 50% WFPS on day 35 (**Figure 3.1**). In both the agricultural and wetland soils, pH 3 treatments had the highest $N_2O:N_2$ ratios compared to other manipulations. The driest agricultural soil had higher $N_2O:N_2$ than the wettest soils (**Figure 3.2**), but moisture did not affect $N_2O:N_2$ ratio in the wetland soil.

pH had a significant impact on gene copy numbers both in the wetland and agricultural soils for all the genes measured ($p<0.001$). In all cases manipulating soils to pH 3 lead to decreased copy numbers (**Table 3.2**). In agricultural soils pH 3 was the only treatment that impacted copy numbers of 16S, *nirK*, *nirS*, and *nosZ* genes (**Table 3.2**). In the wetland soil manipulated to pH3 all gene copy numbers were also reduced. In addition, pH 9 soils also tended to have lower gene copies then the control treatment, and this was a significant effect in the abundance of *nirK*.

The %WFPS did not affect gene copy numbers in the agricultural soils, except for *nosZ* (**Table 3.3**). In contrast, the gene copy numbers did differ between moisture treatments in the wetland soil. 16S gene copies were significantly lower in the 90% WFPS treatment compared to the pre and 50% WFPS treatments ($p<0.001$). The 50% WFPS treatment had higher levels of *nirK* and *nirS* gene copy numbers than the 20% and 90% WFPS treatments, and higher levels of *nosZ* than they 90% WFPS treatment (**Table 3.3**).

Overall the abundances of 16S, *nirK*, *nirS*, and *nosZ* were higher in the freshly sampled wetland soils compared to the agricultural soil (**Table 3.2 and 3.3** $p=0.002$, <0.001, 0.03, and 0.005, respectively). The composition of organisms containing *nirK*, *nirS*, and *nosZ* also varied most between soil types (**Table 3.4**), with distinct communities in the agricultural and wetland soils (**Figure 3.3**). Due to large differences in composition between the soils, agricultural and

wetland soils were analyzed separately to observe treatment effects on community composition. Manipulating the pH changed the composition of *nirK* in both agricultural and wetland soils (**Table 3.4**, **Figure 3.4A and B**). pH manipulation in the agricultural soil also resulted in compositional changes in *nirS* (**Table 3.4**, **Figure 3.4C**). This was not the case for *nirS* in the wetland soil, which did not vary among any of the pH treatments (**Table 3.4**, **Figure 3.4D**). The composition of *nosZ* did not vary between pH treatments in either soil (**Table 3.4**, **Figure 3.4E and F**).

Soil samples were taken at 7, 21, and 35 days after establishment to assess if composition shifted with incubation time. The composition of *nirK* changed with time during the pH experiments, but did not change during the moisture manipulations. The composition of *nirS* and *nosZ* both changed temporally in the pH and moisture experiments, however. These changes in composition were associated with increases in TN, NO_3^--N, and NH_4^+-N over time (data not shown).

Pairwise correlations were used to investigate the associations between gene abundance, chemical factors, and denitrification measures. The two strongest predictors of DEA were the log *nirS*/16S gene copy number g^{-1} dry soil (r^2= 0.334, p<0.0001), and log *nirS* gene copy number g^{-1} dry soil (r^2= 0.328, p<0.0001), which were both positively correlated to DEA, although there were a number of variables that showed significant correlations. The strongest predictor of N_2O production was the log *nirK* gene copy number g^{-1} dry soil, which was positively correlated to N_2O production (r^2= 0.136, p=0.001). The abundance of *nirS* genes did not significantly correlate to N_2O produced. The strongest predictor for N_2O:N_2 was pH, with the ratio of N_2O:N_2 increasing with decreasing pH (r^2= 0.198, p<0.0001), followed by log *nirS*/16S gene copy

number g^{-1} dry soil (r^2= 0.171, p<0.0001), and log *nirS* gene copy number g^{-1} dry soil (r^2= 0.168, p<0.0001) (**Table 3.5**).

To determine if community composition had an effect on denitrification, NMDS ordinations were constructed from TRFLP data representing the community, and soil variables and denitrification measurements were plotted as biplots (data not shown). The composition of *nosZ* was significantly correlated with DEA production (r^2=0.13, p=0.001), but was not correlated to N_2O production or N_2O:N_2. The compositon of *nirK* was significantly correlated to all three activity metrics (DEA r^2=0.12, p=0.001; N_2O r^2=0.12, p=0.001; N_2O:N_2 r^2=0.09, p=0.008). The *nirS* composition was also significantly correlated to all three denitrification measures (DEA r^2=0.30, p=0.001; N_2O r^2=0.10, p=0.002; N_2O:N_2 r^2=0.11, p=0.003). Denitrifier composition follows a similar pattern to the qPCR data with the *nirS* community explaining the most variability for DEA and N_2O:N_2, while the *nirK* community composition explained the most variability in N_2O production.

All predictors, including abiotic soil variables, gene abundances, community composition measures, and specific genotypes were included in a multiple regression analysis. All four classes of predictor variables were important predictors of DEA, N_2O production, and N_2O:N_2. For DEA the most important variables in the regression were %WFPS, DOC, *nirS* gene copy numbers, and three specific genotypes: *nosZ* rsaI 26, *nosZ* rsaI 26, and *nirS* 136 (**Figure 3.5**). Interestingly, DOC was negatively correlated to DEA. However, this is likely an indirect pH effect, as carbon concentrations were significantly altered by pH treatment (p<0.001). The adjusted AIC (AICc) and adjusted R^2 were used to evaluate the importance of each group of variables when dropped from the model. The most important group was genotype. Removing genotype from the regression reduced the adjusted R^2 from 0.71 to 0.49, and increased the AICc

from 193.0 to 240.9 (**Table 3.6)**. The second most important group was the abiotic soil variables, followed by the abundance of functional genes, and then diversity indices. In fact, removing diversity measures from the regression resulted in a better model.

The most important variables for predicting potential N_2O production were gravimetric soil moisture, DOC, *nirK* gene copy numbers, genotypes *nosZ* rsaI 655 and *nirS* 39, and *nirK* diversity (**Figure 3.5**). Again, genotype seems to play an important role in the regression model. Although abiotic variables alone led to a better model then genotype variables alone (AICc 274.9 and 278.0, respectively, **Table 3.6**), the exclusion of genotype from the full model led to a larger increase in AICc and decrease in R^2 than did the removal of abiotic variables (**Table 3.7**). Nonetheless, the importance of variable groups in predicting potential N_2O production followed a similar trend as for DEA, with genotype and abiotic variables being most important, followed by abundance of functional genes, and lastly diversity.

The regression for N_2O:N_2 only had six predictor variables included in the final model. Here the most important variables were pH, *nirS* gene copy number, and *nirS* genotype 101 (**Figure 3.6**). The abiotic variables were the most important group while diversity was least important (**Table 3.6**). Removing functional genes and genotype from the model resulted in a slight decreased predictive capacity of the model. Overall, diversity seems to be the least important in terms of modeling or predicting denitrification rate. Rather, the relative abundance of specific genotypes were often the best indicators of denitrification (**Table 3.6**). Regardless, the addition of biotic variables to the model increased the ability of the model to explain variation in multiple measures of denitrification.

The *nirK* and *nirS* gene copy number data were further analyzed to investigate possible niche differences based on pH and soil type. When soils were separated into acidic (pH<7) and

alkaline (pH>7), the alkaline soil regression model explained 51% of the variability in DEA with *nirS* being responsible for the majority of the total variability (44%). The abundance of *nirK* explained only (7%). In the acidic soils, the abundance of *nirK* and *nirS* only explained 27% of the variability in DEA (**Table 3.7**, $p<0.0001$). Although *nirS* was the better predictor in both cases, the relative importance of *nirK* and *nirS* to regression models predicting DEA had opposite trends: *nirK* was more predictive in acidic soils and *nirS* was more predictive in alkaline soils. The regression models predicting soil moisture were not significant.

A similar trend was found for regression models predicting the ratio of N_2O:N_2 (**Table 3.7**). Although both nir genes were better predictors in alkaline soils then acidic soils (R^2 = 33.4%, p=0.002), *nirS* was the most important predictor, explaining 27% and *nirK* explained 7%. In acidic soils, the total predictive power of nir genes decreased to 12% (p=0.012). In this case *nirS* predicted 6% and *nirK* predicted 6% of the variability in N_2O:N_2 produced. Due to the low levels of N_2O production in acidic conditions, the trend could not be tested on potential N_2O, as the regression model was not significant.

When the samples were divided into wetland and agricultural soil, regression analysis and variance decomposition also suggested possible differences between *nirK* and *nirS*. In general, nir gene abundance explained more variability in wetland soil DEA compared to agricultural soil DEA, but the relative importance of *nirK* and *nirS* to the model for DEA did not change. In the model for N_2O the relative importance of *nirK* was larger in the agricultural soil and lower in the wetland soil (**Table 3.9**). In contrast, the relative importance of *nirS* was higher in the wetland soil and lower in the agricultural soil.

Discussion:

Effects of pH and Moisture Regime

In partial support of our first hypothesis, manipulating pH significantly changed denitrification rates and microbial communities. The most consistent effect was observed in pH 3 treatments resulted in lower denitrification rates, but increased the ratio of N_2O:N_2 in both wetland and agricultural soils (**Figure 3.1**). This is consistent with other findings that show lower total gas emission in acidic soils, and an increasing ratio of N_2O:N_2 with decreasing pH (Šimek and Cooper, 2002). Unlike the pH 3 treatments that caused similar changes in wetland and agricultural soils, the pH 9 treatments only lowered denitrification rates in the wetland soils. It has been shown that long term acidic conditions can select for acid tolerant denitrifying bacteria, and these soils have a lower optimal pH for denitrification compared to soils that naturally have a higher pH (Parkin *et al.*, 1985). This suggests that ecosystem function in wetlands may be more sensitive than agricultural soils to increases in pH.

With few exceptions, the abundances of 16S, *nirK*, *nirS*, and *nosZ* were similar between wetland and agricultural soils when pH was manipulated (**Table 3.2**). Overall, pH 3 led to lower gene copy numbers for all denitrification genes, regardless of soil type (**Table 3.2**). In the wetland system, the pH 9 soils also had lower gene abundances for *nirK* than the control soils, and lower abundances for *nirS* than pH 7 soils (**Table 3.2**), but *nosZ* did not change. Similar results have been found in another study, in which *nosZ* abundance changed 20-fold with pH, while *nirK* and *nirS* changed 110 and 170-fold, respectively (Liu *et al.*, 2010), indicating that *nirK* and *nirS* are more sentive to changes in pH than *nosZ*. A previous study found that *nirK*, but not *nirS*, gene copy numbers decreased rapidly below a pH of 5, suggesting that *nirS* may have a lower pH threshold than *nirK* (Bárta *et al.*, 2010). If there were a difference in pH tolerance

between pH 3 and 5, our experiment would not have detected this difference, however. Our results were most similar to the results found in Chuel et al. (2010), which found the highest gene copy numbers in neutral conditions.

Although we hypothesized that shifts in water regimes would significantly alter denitrification rates and the denitrifying community, results were nuanced. Denitrification activity and N_2O production did not change in wetland soils under three substantially different moisture treatments. In the case of agricultural soils, there was also no effect of %WFPS on the amount of N_2O produced, but different moisture treatments led to differences in denitrification activity and the ratio of N_2O:N_2 produced (**Figure 3.2**). The agricultural soil incubated at 90% WFPS tended to have higher denitrification activity than at lower water contents, while agricultural soil at 20% WFPS had a higher N_2O:N_2 ratio than at higher water potentials.

Microbial communities in the agricultural and wetland soil were also influenced by soil moisture content, although only slightly in agricultural soil. In the agricultural soil, the only significant change in gene copy number was a higher abundance of *nosZ* genes under 90% WFPS than under drier conditions (**Table 3.3***)*. In contrast, gene copy numbers of 16S, *nirK*, *nirS*, and *nosZ* all differed in the wetland soil under different moisture treatments. The drier treatments had higher genes abundances, while the 90% WFPS treatment had lower copy numbers for all four measured genes (**Table 3.3**). This was counterintuitive, as we expected the wetland community to be better adapted to thrive in wet conditions. However, the osmotic stress of drying and rewetting during the pretreatment, which was most extreme for the wetland soils, may have resulted in cell death (Halverson *et al.*, 2000; Fierer *et al.*, 2003).

The most substaintial impacts of soil moisture were not during the treatment incubations, but during the first drying and rewetting event in preparation for the experiment. We observed a

marked reduction in N_2O produced in both the agricultural and wetland soils. This is somewhat surprising since N_2O flux generally increases after a wet-up event (Steenwerth *et al.*, 2005; Kim *et al.*, 2012), though decreases have also been reported (Song *et al.*, 2010). The decrease in rates also corresponded with decreased gene copy numbers (**Table 3.2 and 3.3**). Similar decreases in gene abundance have been previously reported in response to soil drying and rewetting (Gordon *et al.*, 2008) and are likely due to extreme osmotic stress on cells (Halverson *et al.*, 2000; Fierer *et al.*, 2003). We did not anticipate that our pretreatment would lead to these differences and suggest that future research consider such effects when examining denitrification in microcosm experiments.

All three denitrification genes showed distinct communities between agricultural and wetland soils. The composition of *nirK* and *nirS* were particularly distinct with several T-RFs unique to either wetland or agricultural soils (**Figure 3.3**). This suggests that *nirK* and *nirS* communities may be under greater selective pressure from the environment than *nosZ* communities (Dörsch *et al.*, 2012; Braker *et al.*, 2012). Both the *nirK* and *nirS* composition shifted with pH over time. The pH 3 treatment had the most distinct community composition in both agricultural and wetland soils (**Figure 3.4**). When comparing the MRPP values it appears that the composition of *nirK* changed the most and *nosZ* changed the least (**Table 3.4**), indicating that sensitivity to pH is in the order *nirK* > *nirS* > *nosZ* communities.

Models for Denitrification

Microbial measures, such as denitrifier gene abundance (Hallin *et al.*, 2009; Morales *et al.*, 2010), and denitrifier community composition (Cavigelli and Robertson, 2000; Cuhel *et al.*, 2010) have been linked to denitrification in several studies. Our study supports these findings, as we found that the combination of abiotic variables, gene abundance, diversity measures, and

specific denitrifier genotypes produced a significantly better predictive model than any of these factors alone. Although the specific variables and their importance were dependent on the measurement (i.e. denitrification activity versus N_2O production), microbial diversity was consistently the least important of the four groups. This is somewhat inconsistent with the results of Powell *et al.*, (2015), who found that *nirS* evenness was an important variable in predicting DEA. It is possible that variable importance is not consistent among ecosystem types or soil conditions. In addition, the study by Powell (2015) was a field study that looked only at agricultural soil which had narrower pH range (5.7-7.0) than that used in our study. In contrast, our study included lab manipulations, two very different soil types and a wide range of pH conditions, which caused a shift in the denitrifying communities. This shift resulted in the increase or decrease of particular genotypes able to survive in these environments. Rather than evenness, the dominance of particular genotypes in these conditions were a better measure of potential denitfication rate in this study.

Most modeling efforts have linked compositional changes or diversity to activity, but few studies have investigated specific OTUs or T-RFs to predict denitrification activity (Powell et al. 2015). We observed that specific genotypes had a significant role in models of denitrification activity.

Both gene abundances and community composition are important predictors of denitrification (**Table 3.5**). Interestingly, when we compared the relative importance of each denitrification gene, *nirS* tended to be a better and more important predictor of potential denitrification and of the end product ratio of denitrification. In contrast *nirK* was a better predictor of potential N_2O production in both soil types. Similar findings have been reported in a number of studies (Philippot *et al.*, 2009; Szukics *et al.*, 2010a; Cuhel *et al.*, 2010). One likely

explanation is that 70% of *nirK* carrying organisms lack the *nosZ* gene, while only 20% of *nirS* carrying organisms lacked the *nosZ* gene (Graf *et al.*, 2014). This suggests that *nirK*-carrying organism are less likely to carry out complete denitrification and contribute more to N_2O emissions than *nirS*-carrying organisms (Graf *et al.*, 2014). The abundance and composition of *nosZ* was not as strongly linked to denitrification as *nirK* and *nirS*. Previous experiments have shown pH to have the strongest effect on nitrous oxide reductase post-transcriptionally, thus the quantity of the *nosZ* genes may not be the best way to link the denitrifier community to function (Liu *et al.*, 2010; Bergaust *et al.*, 2010). Instead, presence of nitrous oxide reductase enzyme may be a better indication of function, however, pH may also alter the activity of enzyme (Liu *et al.*, 2010).

Niche Differences Between nirK and nirS

Due to evidence that there is niche selection between *nirK* and *nirS* (Graf *et al.*, 2014; Graf, 2015; Philippot *et al.*, 2009; Enwall *et al.*, 2010), we hypothesized that *nirK* and *nirS* gene abundance and composition would respond differently to changes in pH and soil moisture. The *nirK* and *nirS* gene copy numbers per gram of dried soil, however, responded similarly to pH and moisture manipulations (**Table 3.2**, **Table 3.3**, **Figure 3.4**). Additionally, the community composition for both genes were similarly altered by pH. This would suggest that there was not niche selection in our experiment. However, there is evidence for niche differences based on the relative contributions of *nirK* and *nirS* to the regression models for denitrification. When predicting denitrification activity, *nirS* contributed more to variance explained by the model, regardless of soil type. The *nirS* composition also became relatively more important in alkaline soils compared to acidic soils. The relative contribution of *nirK* also appears to be related to pH, but with higher relative importance of composition in acidic environments. Although the

nirK/*nirS* gene copy numbers did not change with pH, it is possible that the *nirS* community is less active in acidic soils, and that in these environments *nirK* is a more active and important driver of denitrification.

One of the challenges to incorporating microbial community data into denitrification models is that microbial communities can be functionally redundant (Wertz *et al.*, 2007). This is especially true when the function is widely distributed across taxa, as is the case with denitrification (Philippot *et al.*, 2007). However, in addition to showing that groups of denitrifying organisms are not ecologically redundant, we also show that particular T-RFs are more strongly correlated with potential denitrification activity and N_2O activity. This suggests that bacteria carrying denitrification genes do not always behave similarly, and do not equally contribute to the denitrification process.

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Table 3.1. Edaphic properties for the agricultural and wetland sampling sites, located at Beltsville Agricultural Research Center, MD. (n=3, mean ± SE). *only one sample due to loss of data

		$g\ cm^{-3}$	$g\ g^{-1}$	$mg\ L^{-1}$				$\mu g\ N\ g^{-1}$ dry soil h^{-1}		
Soil Type	pH	Bulk density	Moisture Content	NH_3-N	NO_3-N	Total N	Dissolved Organic Carbon	DEA	N_2O	N_2O:N_2
Agricultural	6.8 ± 0.3	1.4	0.26 ± 0.00	1.0 ± 0.2	0.3 ± 0.1	2.4 ± 0.5	9.0 ± 2.7	1.0 ± 0.3	0.5 ± 0.1	0.7 ± 0.3
Wetland	5.1 ± 0.1	0.7 ± 0.1	0.96 ± 0.12	2.1 ± 0.4	0.3 ± 0.1	3.7*	15.3*	3.3 ± 0.8	1.4 ± 0.1	0.5 ± 0.2

Table 3.2. Means (± standard errors) of log transformed gene copy numbers per gram of dry soil for the pH manipulation experiment.

	16S		*nirK*		*nirS*		*nosZ*	
	Agricultral	Wetland	Agricultral	Wetland	Agricultral	Wetland	Agricultral	Wetland
Pre	10.2 ± 0.0^{a}	10.7 ± 0.1^{a}	6.9 ± 0.0^{a}	7.6 ± 0.06^{a}	7.3 ± 0.0^{a}	7.5 ± 0.1^{a}	8.0 ± 0.1^{a}	8.4 ± 0.1^{a}
Control	10.0 ± 0.0^{b}	9.9 ± 0.1^{bc}	6.8 ± 0.1^{a}	6.6 ± 0.06^{b}	7.2 ± 0.0^{a}	6.4 ± 0.1^{bc}	7.8 ± 0.0^{a}	7.6 ± 0.1^{b}
pH 3	9.6 ± 0.1^{c}	9.7 ± 0.1^{c}	5.5 ± 0.2^{b}	5.7 ± 0.10^{d}	5.9 ± 0.2^{b}	5.6 ± 0.1^{d}	6.9 ± 0.2^{b}	6.9 ± 0.1^{c}
pH 7	10.0 ± 0.0^{b}	10.1 ± 0.1^{b}	6.7 ± 0.1^{a}	6.6 ± 0.12^{bc}	7.2 ± 0.1^{a}	6.6 ± 0.2^{b}	7.8 ± 0.0^{a}	7.7 ± 0.1^{b}
pH 9	9.9 ± 0.0^{b}	10.1 ± 0.1^{b}	6.5 ± 0.1^{a}	6.2 ± 0.50^{c}	7.1 ± 0.1^{a}	6.1 ± 0.1^{cd}	8.0 ± 0.0^{a}	7.6 ± 0.1^{b}

Letters denote statistical differences among treatments for a given soil type based on Tukey post-hoc analysis for each gene. Means and standard errors were calculated from pre (undisturbed soil samples taken directly from the field), Day 7, Day 21, and Day 35 samples. Wetland and agricultural soils were statistically analyzed separately.

Table 3.3. Means (± standard errors) of log transformed gene copy numbers per gram of dry soil for the soil moisture manipulation experiment.

	16S		*nirK*		*nirS*		*nosZ*	
	Agricultral	Wetland	Agricultral	Wetland	Agricultral	Wetland	Agricultral	Wetland
Pre	10.2 ± 0.0^{a}	10.7 ± 0.1^{a}	6.9 ± 0.0^{a}	7.6 ± 0.1^{a}	7.3 ± 0.0^{a}	7.5 ± 0.1^{a}	8.0 ± 0.1^{a}	8.4 ± 0.1^{a}
20% WFPS	10.0 ± 0.0^{b}	10.0 ± 0.0^{bc}	6.7 ± 0.1^{a}	6.7 ± 0.0^{c}	7.1 ± 0.0^{a}	6.5 ± 0.1^{c}	7.7 ± 0.0^{c}	7.7 ± 0.0^{bc}
50% WFPS	10.0 ± 0.0^{b}	10.1 ± 0.0^{b}	6.8 ± 0.1^{a}	6.9 ± 0.0^{b}	7.1 ± 0.1^{a}	6.9 ± 0.1^{b}	7.7 ± 0.0^{bc}	8.0 ± 0.0^{b}
90% WFPS	10.0 ± 0.0^{b}	9.9 ± 0.1^{c}	6.8 ± 0.1^{a}	6.6 ± 0.1^{c}	7.2 ± 0.0^{a}	6.4 ± 0.1^{c}	7.8 ± 0.0^{b}	7.6 ± 0.1^{c}

Letters denote statistical differences among treatments for a given soil type based on Tukey post-hoc analysis for each gene. Means and standard errors were calculated from pre (undisturbed soil samples taken directly from the field), Day 7, Day 21, and Day 35 samples. Wetland and agricultural soils were statistically analyzed separately.

Table 3.4. MRPP results for denitrification gene community composition. Results based on 999 permutations

			pH experiment		Moisture Experiment	
			pH	Time	Moisture	Time
nirK	Agricultral	p	0.001	0.002	0.466	0.001
		A	0.147	0.044	-0.001	0.083
	Wetland	p	0.001	0.001	0.034	0.026
		A	0.071	0.051	0.002	0.058
nirS	Agricultral	p	0.001	0.001	0.024	0.001
		A	0.991	0.140	0.045	0.202
	Wetland	p	0.007	0.001	0.468	0.001
		A	0.032	0.091	-0.001	0.095
nosZ	Agricultral	p	0.030	0.001	0.198	0.032
		A	0.029	0.144	0.017	0.049
	Wetland	p	0.683	0.001	0.681	0.002
		A	-0.009	0.133	-0.011	0.097

p is a the p-value based on the MRPP analysis. A is the size effect value, which is a chance-corrected estimate of the proportion of the distances explained by group identity; a value analogous to a coefficient of determination in a linear model (vegan package, R Development Team, 2015).

Table 3.5. Pairwise correlation coefficients (r) between soil parameters and gene abundances and denitrification gas fluxes. All data for genes are log transformed.

	DEA	N2O	N2O:N2
Soil	-0.32[c]	0.26	0.25
pH	0.29	-0.09	**-0.45[a]**
Grav. Soil Moisture	-0.16	0.23	0.14
% WFPS	0.21	-0.06	-0.11
Matric Potential	-0.15	-0.05	0.10
Ammonium-N	-0.36[b]	0.09	0.19
Nitrate-N	-0.13	-0.15	-0.07
Inorganic-N	-0.43[a]	0.07	0.21
TN	-0.37[b]	-0.02	0.15
DOC	-0.24	-0.06	0.06
16S gene copy number	0.25	0.36[b]	-0.20
nirK gene copy number	0.36[b]	**0.37[b]**	-0.34[b]
nirS gene copy number	0.57[a]	0.30[c]	-0.41[a]
nosZ gene copy number	0.43[a]	0.32[c]	-0.35[b]
nirK/nirS	-0.41[a]	0.04	0.17
nirK/nosZ	0.13	0.28	-0.22
nirS/nosZ	0.52[a]	0.18	-0.34[b]
(nirK+nirS)/nosZ	0.42[a]	0.27	-0.34[b]
nirK/16S	0.36[b]	0.33[c]	-0.36[b]
nirS/16S	**0.58[a]**	0.24	-0.41[a]
nosZ/16S	0.45[a]	0.25	-0.37[b]

*Soil type was coded as a dummy variable with the agricultural soil defined as 0 and the wetland soil defined as 1. Bonferroni corrections were used to adjust p-values for multiple comparisons. [a] $p<0.001$, [b] $P<0.01$, [c] $P<0.05$. The best predictor for each category is in bold.

Table 3.6. Summary of **DEA, N_2O,** and **N_2O:N_2** model fits, including the full model, and models in which groups of variables were dropped. Data are sorted by AICc.

DEA

Abiotic	Function	Diversity	Genotype	R2	Adj R2	AIC	AICc
x	x		x	0.7362	0.701	182.04	186.81
x	x	x	x	0.7563	0.7076	183.87	193.03
x		x	x	0.7288	0.6821	190.89	198.09
	x	x	x	0.6963	0.6519	198.56	204.08
			x	0.5965	0.5713	213.82	215.35
x	x	x		0.5422	0.4869	236.81	240.91
	x			0.353	0.3401	254.45	254.86
x				0.3743	0.3486	255.01	255.88
		x		0.2556	0.2172	274.9	276.08

N_2O

Abiotic	Function	Diversity	Genotype	R2	Adj R2	AIC	AICc
x	x		x	0.4803	0.442	241.89	243.82
x	x	x	x	0.5263	0.469	240.34	244.43
x		x	x	0.4888	0.4333	246.18	249.65
	x	x	x	0.4332	0.3914	250.83	252.76
x	x	x		0.4006	0.3426	260.58	263.48
x				0.2473	0.2166	274.04	274.91
			x	0.19	0.1738	277.59	278
	x			0.1342	0.1256	282.46	282.7
		x		0.1466	0.1118	286.96	287.84

N_2O:N_2

Abiotic	Function	Diversity	Genotype	R2	Adj R2	AIC	AICc
x	x		x	0.3286	0.3012	262.26	263.13
x	x	x	x	0.3504	0.3098	262.86	264.39
x		x	x	0.3365	0.2982	263.65	264.82
x				0.2736	0.2591	266.37	266.78
x	x	x		0.3098	0.2742	267.11	268.29
	x	x	x	0.2641	0.2341	271.71	272.59
	x			0.164	0.1557	278.85	279.09
			x	0.1375	0.129	282.06	282.3
		x		0.0451	0.026	294.5	294.95

Table 3.7. Summary of regression models and variance partitioning of the nitrite reductase genes *nirK* and *nirS*. Variable importance is the % explained by each variable (sum=R^2). Relative importance is the relative contribution of each variable to the model (sum=100%).

Denitrification Measurement	Soil condition	R^2	Nitrite reductase gene	Variable Importance (%)	Relative Importance (%)
DEA	Acidic Soil	27.4%	*nirK*	10.0	36.5
			nirS	17.4	63.5
	Alkaline Soil	51.2%	*nirK*	7.1	14.0
			nirS	44.0	86.0
Potential N_2O	Acidic Soil	19.7%	*nirK*	13.6	69.1
			nirS	6.1	30.9
	Alkaline Soil	N.S.	*nirK*	N.S.	N.S.
			nirS	N.S.	N.S.
$N_2O:N_2$	Acidic Soil	12.3%	*nirK*	6.7	54.0
			nirS	5.7	46.0
	Alkaline Soil	33.4%	*nirK*	6.9	20.7
			nirS	26.5	79.3
DEA	Agricultural Soil	22.5%	*nirK*	6.4	28.4
			nirS	16.1	71.2
	Wetland Soil	45.1%	*nirK*	13.0	28.8
			nirS	32.2	71.2
Potential N_2O	Agricultural Soil	15.0%	*nirK*	11.1	73.1
			nirS	4.1	26.9
	Wetland Soil	47.5%	*nirK*	12.6	26.5
			nirS	34.9	73.5
$N_2O:N_2$	Agricultural Soil	12.0%	*nirK*	6.7	55.5
			nirS	5.4	44.5
	Wetland Soil	14.2%	*nirK*	6.3	44.1
			nirS	8.0	55.9

N.S. (not significant)

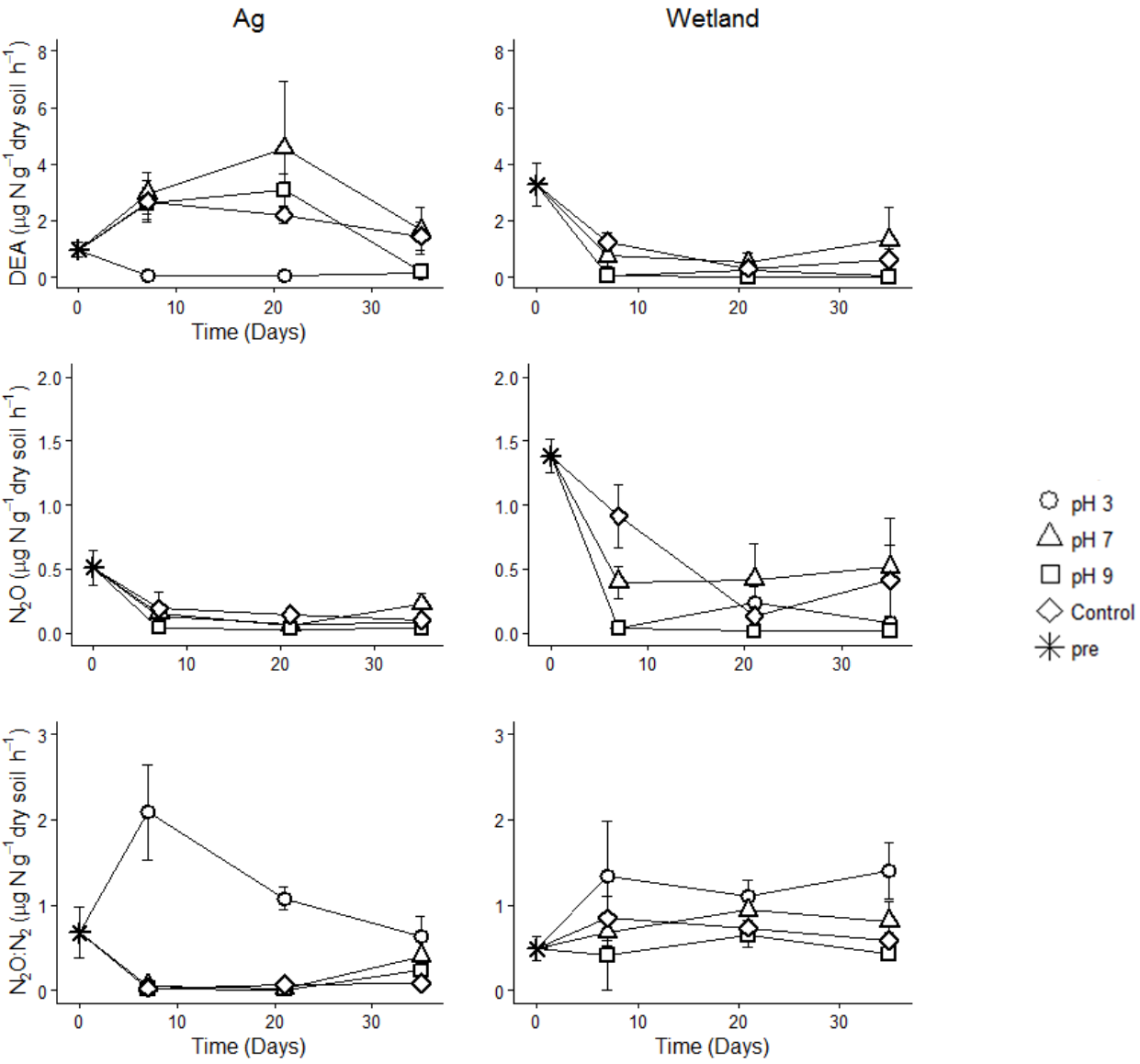

Figure 3.1. Line graph showing the effect of pH on DEA (A), N_2O (B), and N_2O:N_2 (C) over time. Points represent the mean of three replicated treatment jars with SE bars.

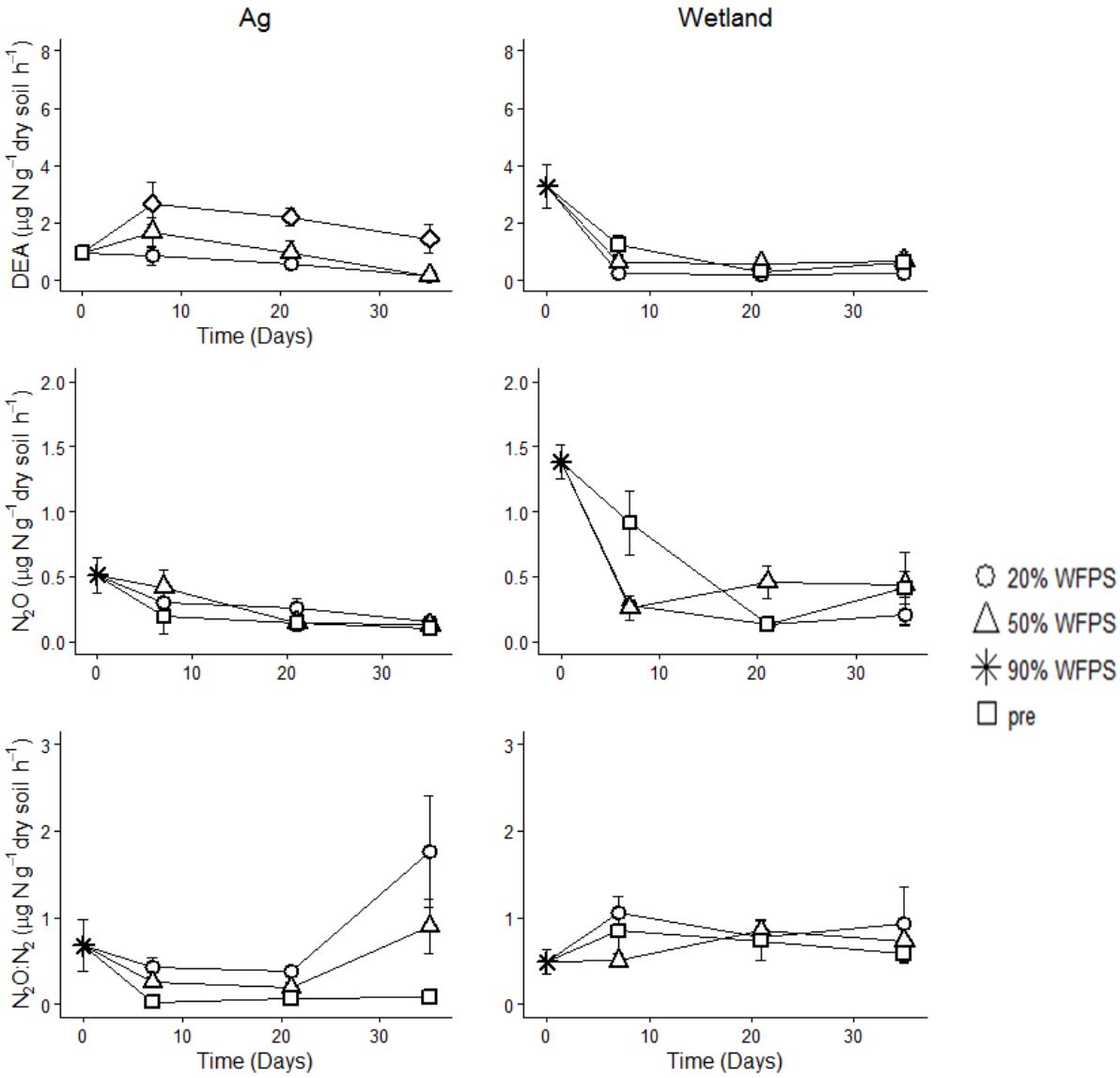

Figure 3.2. Line graph showing the effect of soil moisture on DEA (A), N_2O (B), and N_2O:N_2 (C) over time. Points represent the mean of three replicated treatment jars with SE bars.

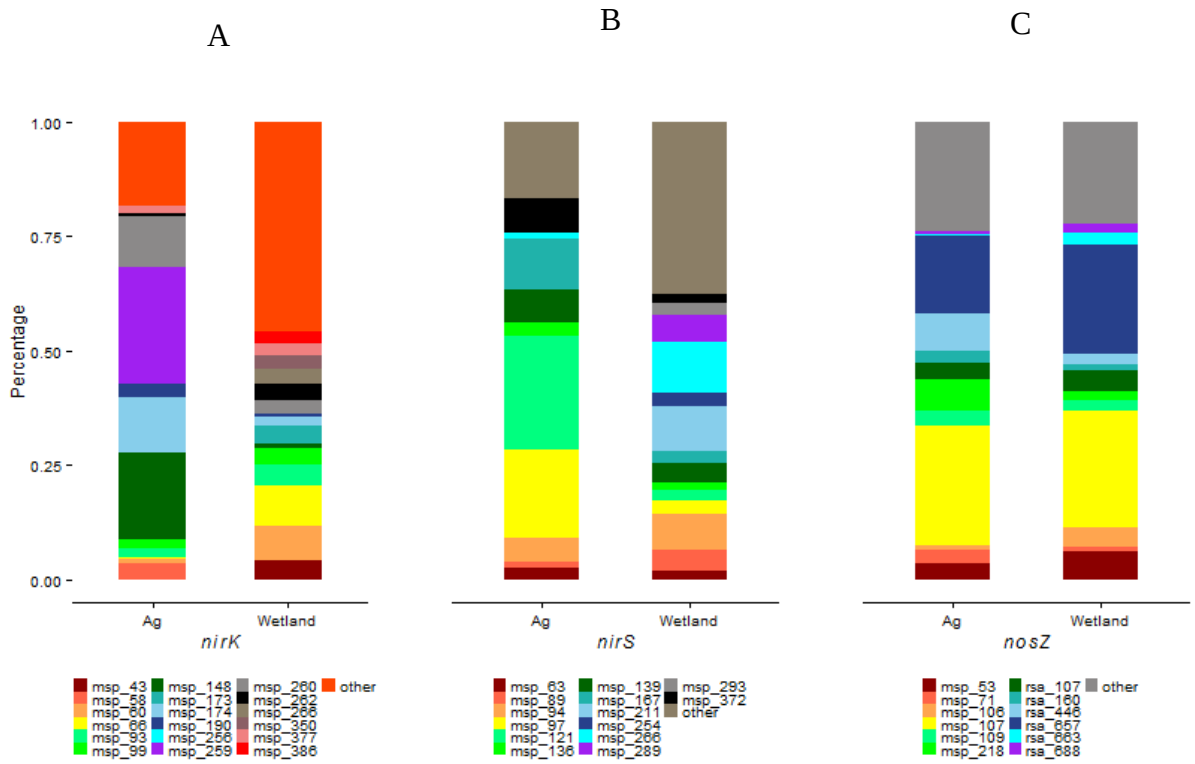

Figure 3.3 Most common TRFLP peaks for *nirK* (A) *nirS* (B) and *nosZ* (C), representing community composition for the agricultural and wetland soils.

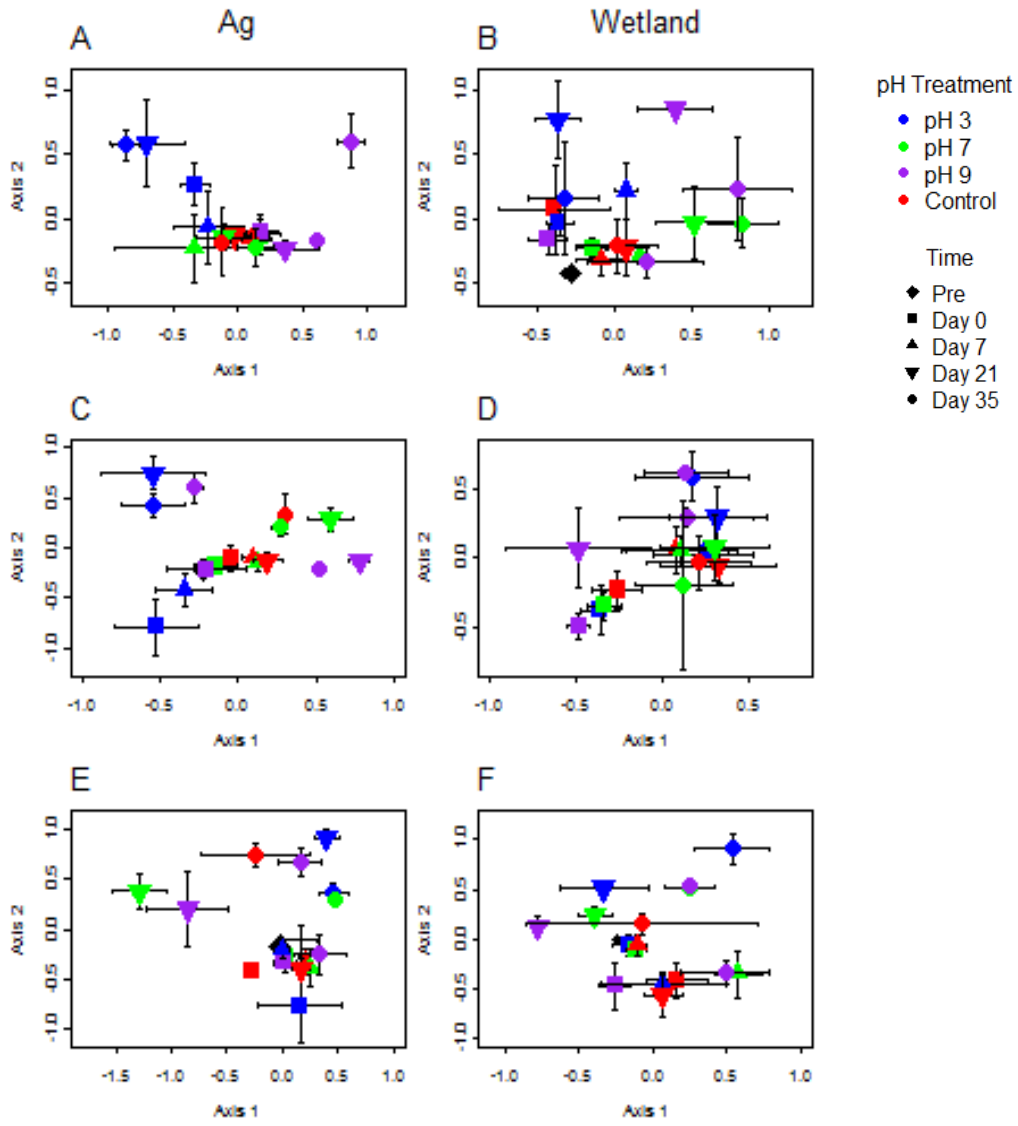

Figure 3.4. NMS ordination of *nirK* (A-B), *nirS* (C-D), and *nosZ* (E-F) community composition based on TRFLP profiles. Points represent the means of replicates with standard error bars.

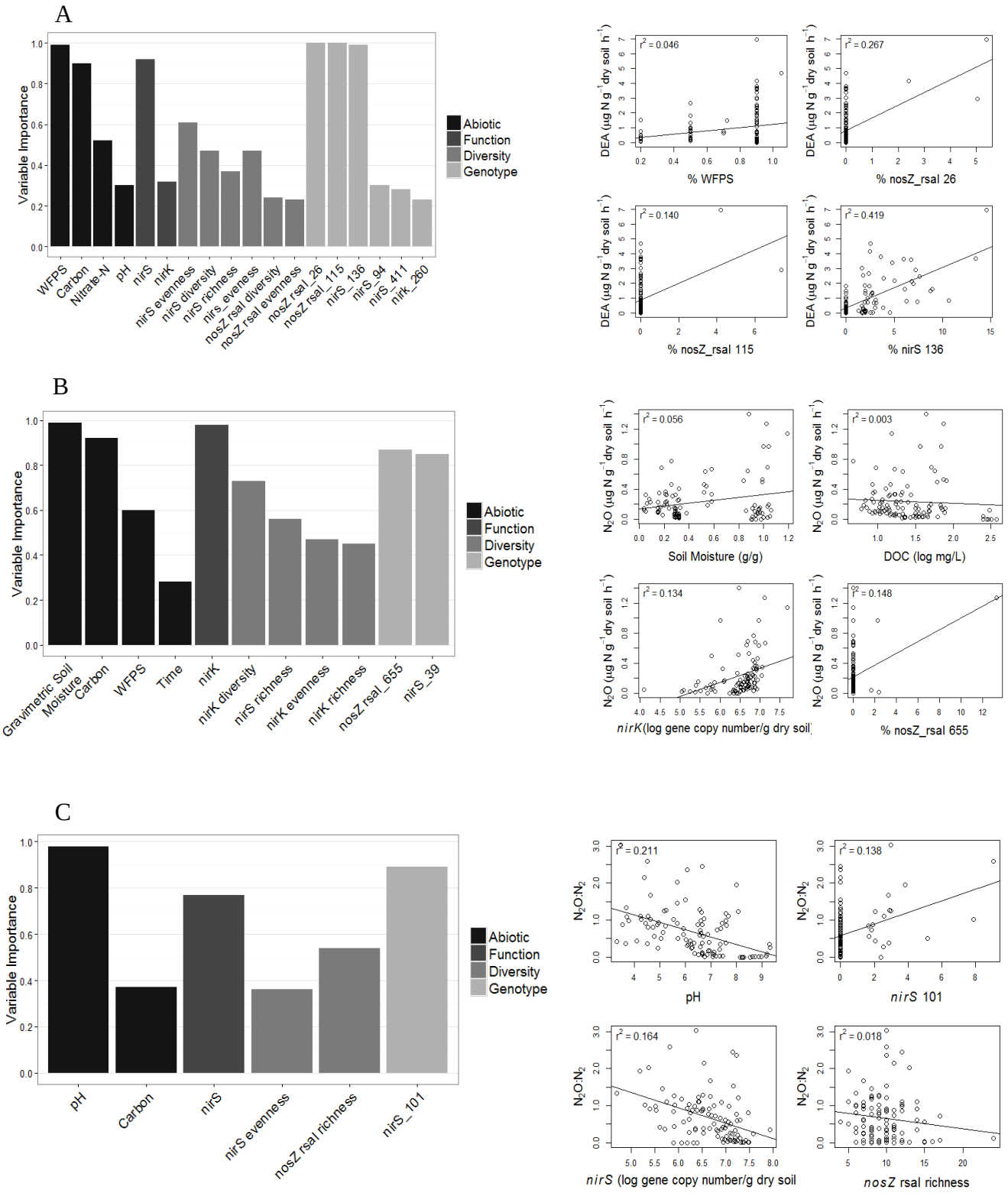

Figure 3.5. Estimates of the importance of variables to multiple linear regression models predicting DEA (A), potential N_2O production (B), and N_2O:N_2 (C). Variable importance was estimated from the sum of Akaike weights over all models containing the explanatory variable. Variables with a weight over 0.5 in each category (abiotic, functiona, diversity, and genotype) were tested for variable importance in the final models. Scatterplots of the top variables from each model are plotted with r^2 values.

Chapter 4. Conclusions

The overall goal of this research was to investigate the link between the denitrifying soil microbial community to ecosystem function. The first study evaluated the impact of edaphic factors such as NH_4^+ and NO_3^- concentrations, pH, and soil moisture; spatial factors, represented by depth in the soil and distance from corn rows; and agricultural management in the form of manure placement and cover crop as drivers of nitrogen cycling microbial communities. The second study took an in depth experimental approach to study the impacts of pH and soil moisture on denitrification genes. In addition, it linked the microbial communities in the soil to potential denitrification rates, N_2O production, and the ratio of N_2O:N_2 produced during denitrification, and evaluated the importance of microbial community data to regression models predicting these functions.

Environmental Drivers of Nitrogen Cycling Microorganism

- Soil type- Soil type was shown to play a large role in denitrifier abundance with higher abundances of denitrification genes (*nirK, nirS,* and *nosZ*) and bacterial genes (16S) in a wetland soil than in an agricultural soil. In addition, the agricultural and wetland soils showed distinct communities. The composition of *nirK* and *nirS* communities were particularly distinct with several T-RFs unique to either wetlands or agricultural soils. The composition of *nosZ* communitities also differed between the two soil types, but there was much more overlap in T-RFs. This suggests that *nirK* and *nirS* communities may be under greater selective pressure from the environment than *nosZ* communities.
- Nitrogen concentrations- Nitrate concentration was positively correlated to denitrification gene abundance, bacterial gene abundance, and fungal gene abundance. Ammonia concentration was linked to bacterial and fungal gene abundances. These results indicate that

an increase in NO_3^- concentration in the soil may increase the size of the denitrifier community, as well as cause a shift in denitrifier community composition.

- pH- The first study found that *nirS* was positively correlated to pH, but no correlations were observed with other genes. In contrast, all denitrification genes were linked to pH in the second study, with *nirK, nirS,* and *nosZ* gene abundances significantly lower in the pH 3 than other pH treatments. In addition, community compositions of *nirK* and *nirS* were significantly altered by pH 3 treatments. This sensitivity to pH was determined to be in the order: *nirK* > *nirS* > *nosZ*. The discrepancy between the two studies may be a result of the ranges of pH. The first study looked at a pH range of 5-7 while the second study looked at a range of 3-9. In addition, the second study was highly controlled, with pH level being the only experimental factor. In the first study, pH was correlated with other edaphic factors and causality cannot be confirmed. While pH was shown to alter denitrifier abundance and community composition in the second study, it is still not clear how pH impacts microbial community function.
- Soil moisture- In the first study, gravimetric soil moisture was positively correlated to *nirK* and 16S gene copy numbers only. In the second study, changes in the %WFPS did not affect gene copy numbers in the agricultural soils, but it did in wetland soils, with 16S gene copies lower in the 90% WFPS treatment, and highest levels of denitrifier gene copy numbers in the 50% WFPS treatment. Soil moisture did not alter the community composition of denitrification genes. Again these two studies deal with different ranges of soil moisture and different measurements of soil moisture. In addition, soil moisture in the first study covaried with other factors, such as cover crop and depth, possibly leading to the observed discrepancies. Soil moisture alters microbial abundances, but there is not a clear pattern

across different systems and genes. The wetting and drying of soil had a large unexpected impact and lead to the decrease of gene copy numbers. This effect should be taken into account in future studies.

- Spatial distribution- Depth impacted gene abundance in the soil with the highest abundance of microbial genes at the surface and decreasing gene abundance with depth. This was consistent for all genes measured including 16S, ITS, *ureC*¸ AOA, AOB, *nirK*, *nirS*, and *nosZ*. Horizontal distance was not significant for any genes.
- Agricultural management- The timing and placement of poultry litter fertilizer (broadcasted vs. subsurface banded) and winter cover crop type (hairy vetch vs. cereal rye) impacted each gene differently. Importantly, *nirK* and *nirS* responded differently, with *nirK* influenced strongly by soil moisture and cover crop, and *nirS* gene copies influenced by fertilizer timing and placement and pH. These results provide evidence of niche selection between the two groups of organisms. An additional implication of this study was the lack of denitrification genes around the subsurface band of poultry litter.

Future Research

Although community data derived from DNA was associated with function, the use of RNA, which captures the active community, may provide further insights to the environmental controls of microorganisms and may be more beneficial for use in N cycling models. Additionally, the sequencing of functional genes may be able to provide additional information on these communities.

Microbial samples from Chapter 2 and Chapter 3 will be further analyzed for the gene encoding the second bacterial clade of *nosZ*, *nosZ*-II. This gene has been found in numerous

environments, and its presence in the agricultural and wetland soils used here is likey. However, since it was recently discovered, there is still much to learn about its ecology. By re-analyzing samples for this gene, we will be able to determine if there are differeces in the spatial distubution of this gene in the soil profile, if there is evidence of niche selection, and possible differences in activity in different soil conditions.

Here, we provided evidence to support findings reported in the literature that *nirK* and *nirS* are not ecologically redundant and undergo niche selection. Most of the evidence in the literature comes from field studies and there is a need for more controlled laboratory studies to elucidate the effects, other than soil moisture and pH, on the niche selection of *nirK* and *nirS*, such as carbon concentrations, nitrate concentration, or temperature. These two studies also show that a number of environmental drivers impact nitrogen cycling genes. The most consistent patterns found were that gene abundance decreased with depth in the soil, and is strongly linked with nitrogen concentrations. pH and soil moisture were shown to affect denitrification genes, however the results were not consistent across studies. More research is needed to discern the effect of moisture and pH on N cycling communities, both experimentally and in the field, and using a range of soil types and ecosystems. Finally, our finding that microbial data can be used to improve models should be tested with currently available denitrification models to determine if, and to what extent, models can be enhanced. Including gene abundances, diversity, or genotype measures to an existing model may lead to better understanding and prediction of denitrification and N_2O emissions.

Appendices

Appendix I: Chapter 2 Supplemental Information

Gene	Function	Pure Culture	Primers	Thermocycler Conditions	Number of Cycles	Plasmid Standard and Soil Correction Efficiencies (%) (R^2 values)	Reference
16S	Bacterial biomass	*Escherichia coli*	Eub 338F/ Eub518R	95°C for 5 min 95ºC for 5 s / 55ºC for 15 s / **72 ºC for10 s**	1 40	97-105% soil = 102% All $r^2 > 0.99$%	Fierer *et al.*, 2005
ITS	Fungal biomass	*Haematonectria haematococca*	ITS1F/5.8S	95°C for 5 min 95ºC for 5 s / 55ºC for 15 s / **72 ºC for10 s**	1 40	98-101% soil = 102% All $r^2 > 0.99$%	Fierer *et al.*, 2005
*nir*K	Nitrite reduction	*Sinorhizobium meliloti*	nirK876/ nirK1040	95ºC for 5 min 95ºC for 15 s / 63-58ºC for 60 s 95ºC for 15 s / **58ºC for 60 s**	1 5 (TD) 40	98-102% soil = 94% All $r^2 > 0.99$%	Henry *et al*., 2004
*nir*S	Nitrite reduction	*Pseudomonas stutzeri*	nirSCD3aF/ nirSR3cd	95ºC for 5 min 95ºC for 15 s / 63-58ºC for 60 s 95ºC for 15 s / **58ºC for 60 s**	1 5 (TD) 40	95-101% soil = 96% All $r^2 > 0.99$%	Hristova and Six., 2006
*nos*Z2	Nitrous oxide reduction	*Pseudomonas stutzeri*	nosZ2F/ nosZ2R	95ºC for 5 min 95ºC for 15 s / 65-60ºC for 60 s 95ºC for 15 s / 60ºC for 60 s / **80ºC for 30 s**	1 5 (TD) 40	95-97% soil = 95% All $r^2 > 0.99$%	Henry *et al*., 2006

Table I.1 Primer sets and conditions for genes analyzed with qPCR.

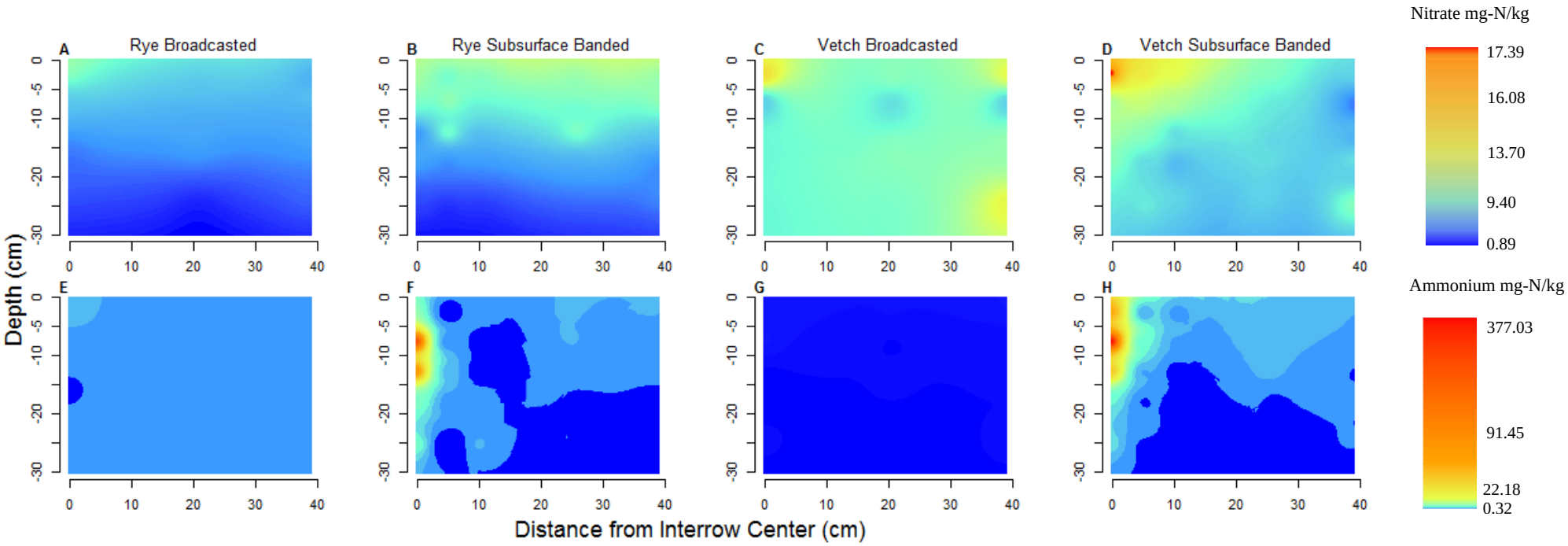

Figure I.1. Spatial distribution of ammonium-N (NH_4^+-N) concentrations (A-D) and nitrate-N (NO_3^--N) concentrations (E-H) during the corn V5 growth stage: A) NH_4^+ cereal rye with broadcast manure, B) NH_4^+ cereal rye with subsurface band, C) NH_4^+ hairy vetch with broadcast manure, D) NH_4^+ hairy vetch and subsurface band, E) NO_3^- cereal rye with broadcast manure, F) NO_3^- cereal rye with subsurface band, G) NO_3^- hairy vetch with broadcast manure, and G) NO_3^-hairy vetch and subsurface band. Within each panel, the inter-row center is on the left and the corn row is on the right. Figures are based on inverse distance weighted interpolation.

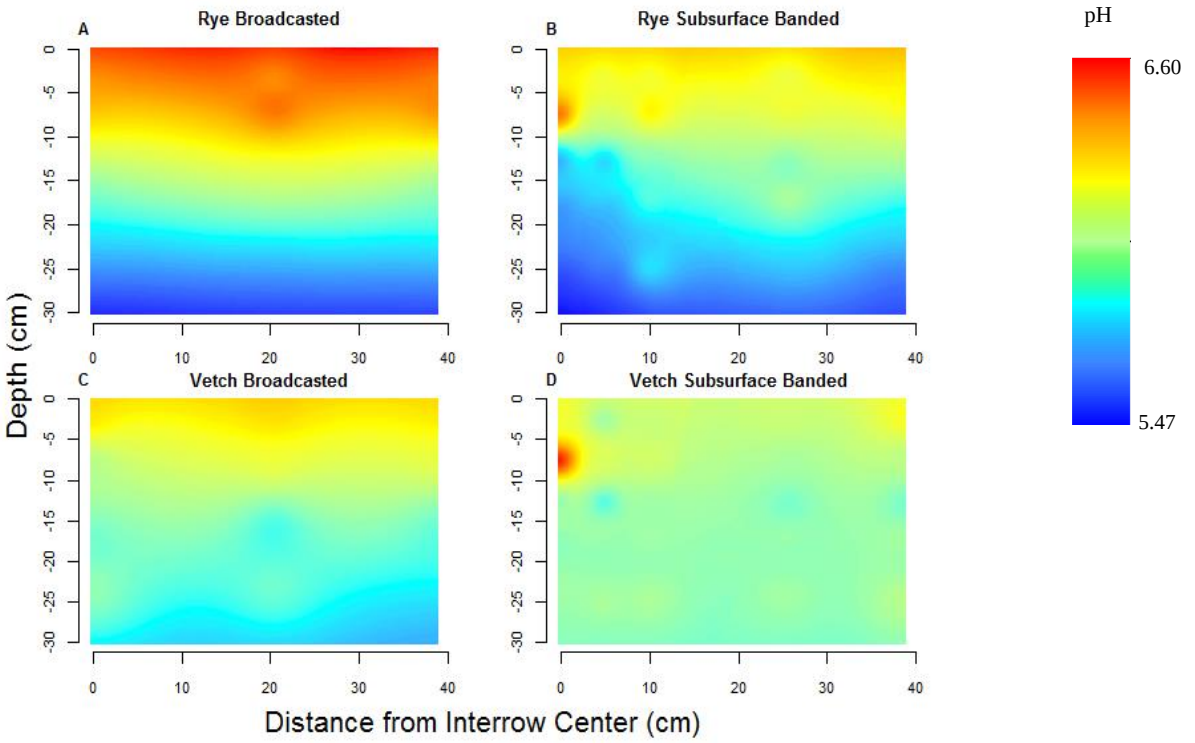

Figure I.2**.** Spatial distribution of soil moisture during the corn V5 growth stage: A) cereal rye with broadcast manure, B) cereal rye with subsurface band, C) hairy vetch with broadcast manure, and D) hairy vetch and subsurface band. Within each panel, the inter-row center is on the left and the corn row is on the right. Figures are based on inverse distance weighted interpolation.

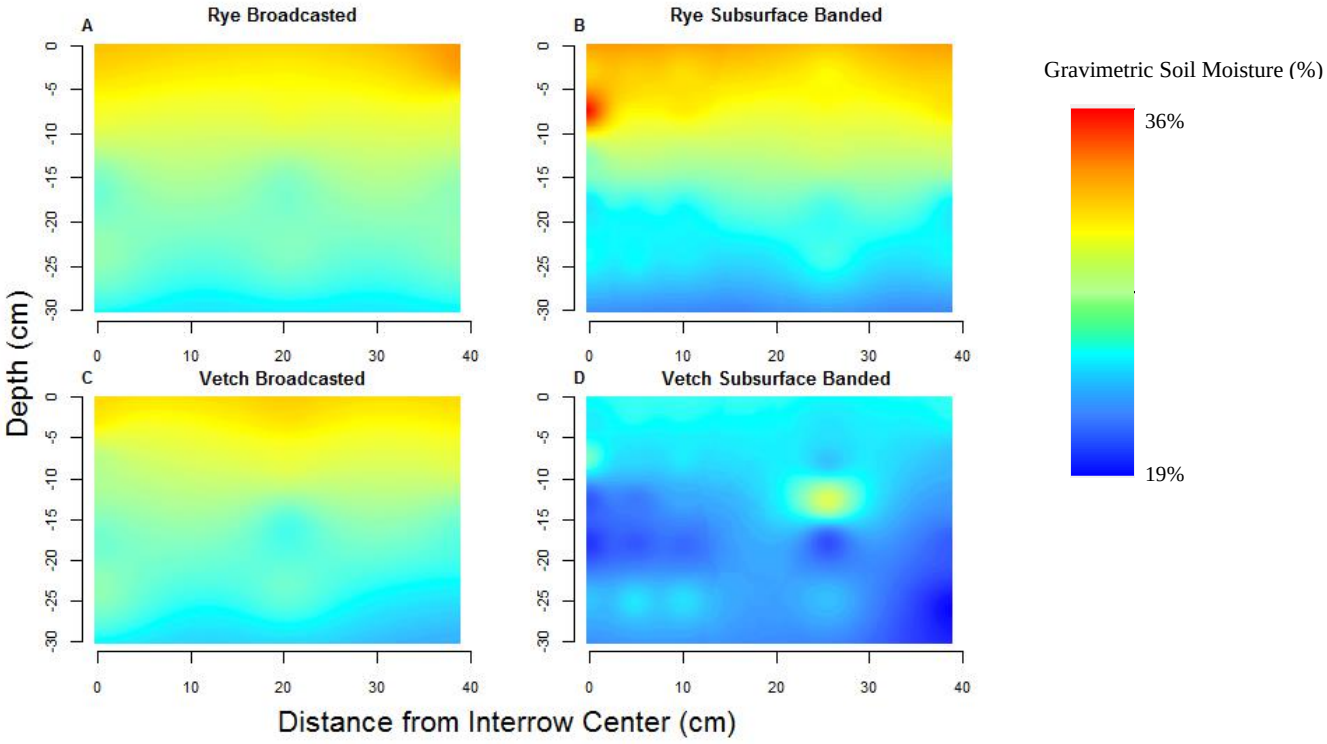

Figure I.3. Spatial distribution of pH during the corn V5 growth stage: A) cereal rye with broadcast manure, B) cereal rye with subsurface band, C) hairy vetch with broadcast manure, and D) hairy vetch and subsurface band. Within each panel, the inter-row center is on the left and the corn row is on the right. Figures are based on inverse distance weighted interpolation.

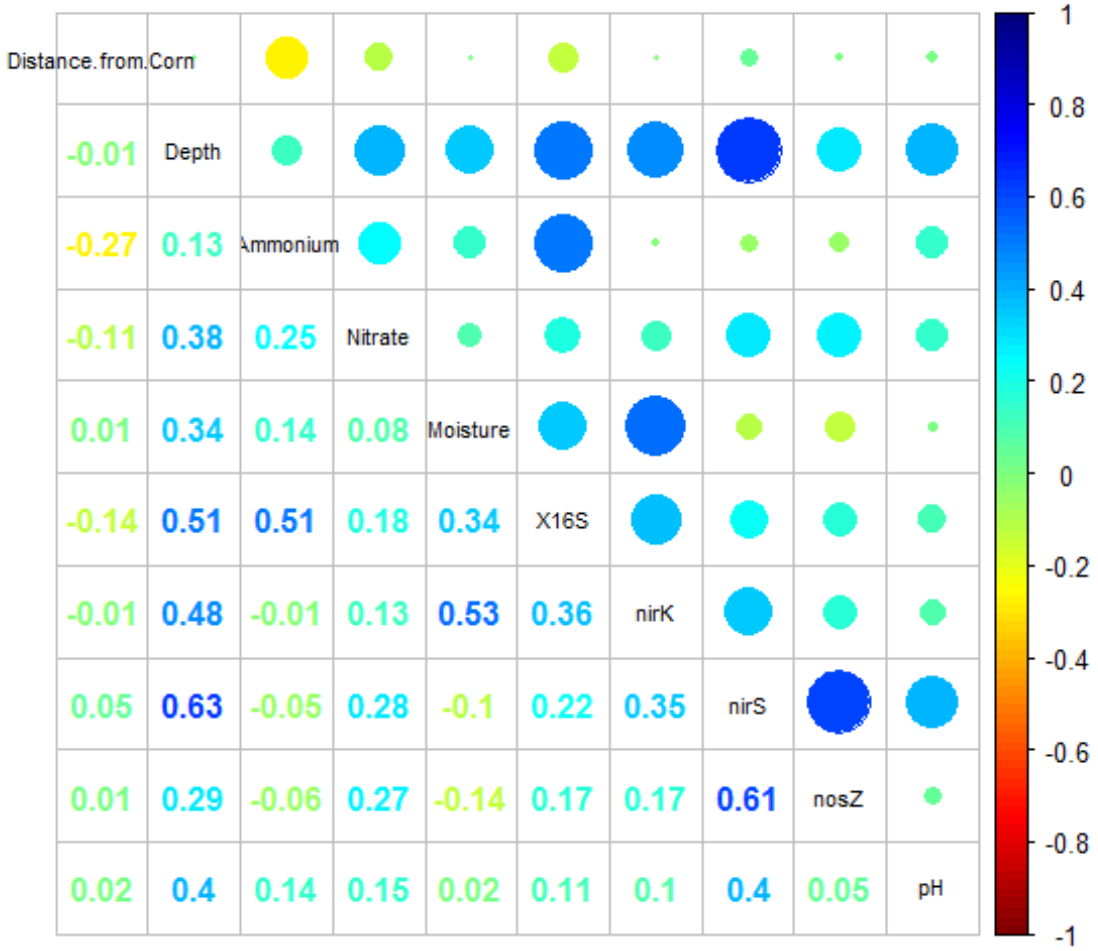

Figure I.4**.** Correlation matrix of measured soil variables including ammonium-N, nitrate-N, moisture, microbial genes 16S, ITS, *nirK*, *nirS*, and *nosZ*, depth and distance from the corn plant. The color and size of the circle indicate the direction and strength, respectively, of the correlation. r values are given in the lower matrix.

Appendix II: Chapter 3 Supplemental Information

Table II.1. Primer sets and conditions for genes analyzed with qPCR.

Gene	Function	Pure Culture	Primers	Thermocycler Conditions	Number of Cycles	Plasmid Standard and Soil Correction Efficiencies (%) (R^2 values)	Reference
16S	Bacterial biomass	*Escherichia coli*	Eub 338F/ Eub518R	95°C for 5 min 95ºC for 5 s / 55ºC for 15 s / **72 ºC for10 s**	1 40	98-106% soil = 89% All $r^2 > 0.99$%	Fierer et al., 2005
*nir*K	Nitrite reduction	*Sinorhizobium meliloti*	nirK876/ nirK1040	95ºC for 5 min 95ºC for 15 s / 63-58ºC for 60 s 95ºC for 15 s / **58ºC for 60 s**	1 5 (TD) 40	99-102% soil = NA All $r^2 > 0.99$%	Henry et al., 2004
*nir*S	Nitrite reduction	*Pseudomonas stutzeri*	nirSCD3aF/ nirSR3cd	95ºC for 5 min 95ºC for 15 s / 63-58ºC for 60 s 95ºC for 15 s / **58ºC for 60 s**	1 5 (TD) 40	98-100% soil = 100% All $r^2 > 0.99$%	Hristova and Six., 2006
*nos*Z2	Nitrous oxide reduction	*Pseudomonas stutzeri*	nosZ2F/ nosZ2R	95ºC for 5 min 95ºC for 15 s / 65-60ºC for 60 s 95ºC for 15 s / 60ºC for 60 s / **80ºC for 30 s**	1 5 (TD) 40	95-102% soil = 85% All $r^2 > 0.99$%	Henry et al., 2006
P450*nor*	Fungal denitrification	Environmental clone	p450nor-ppf/ p450nor-ppr	95ºC for 5 min 95ºC for 15 s / 58 ºC for 60 s / **72 ºC for 30 s**	1 40	93% soil = NA r^2=0.98%	Kim et al., 2009

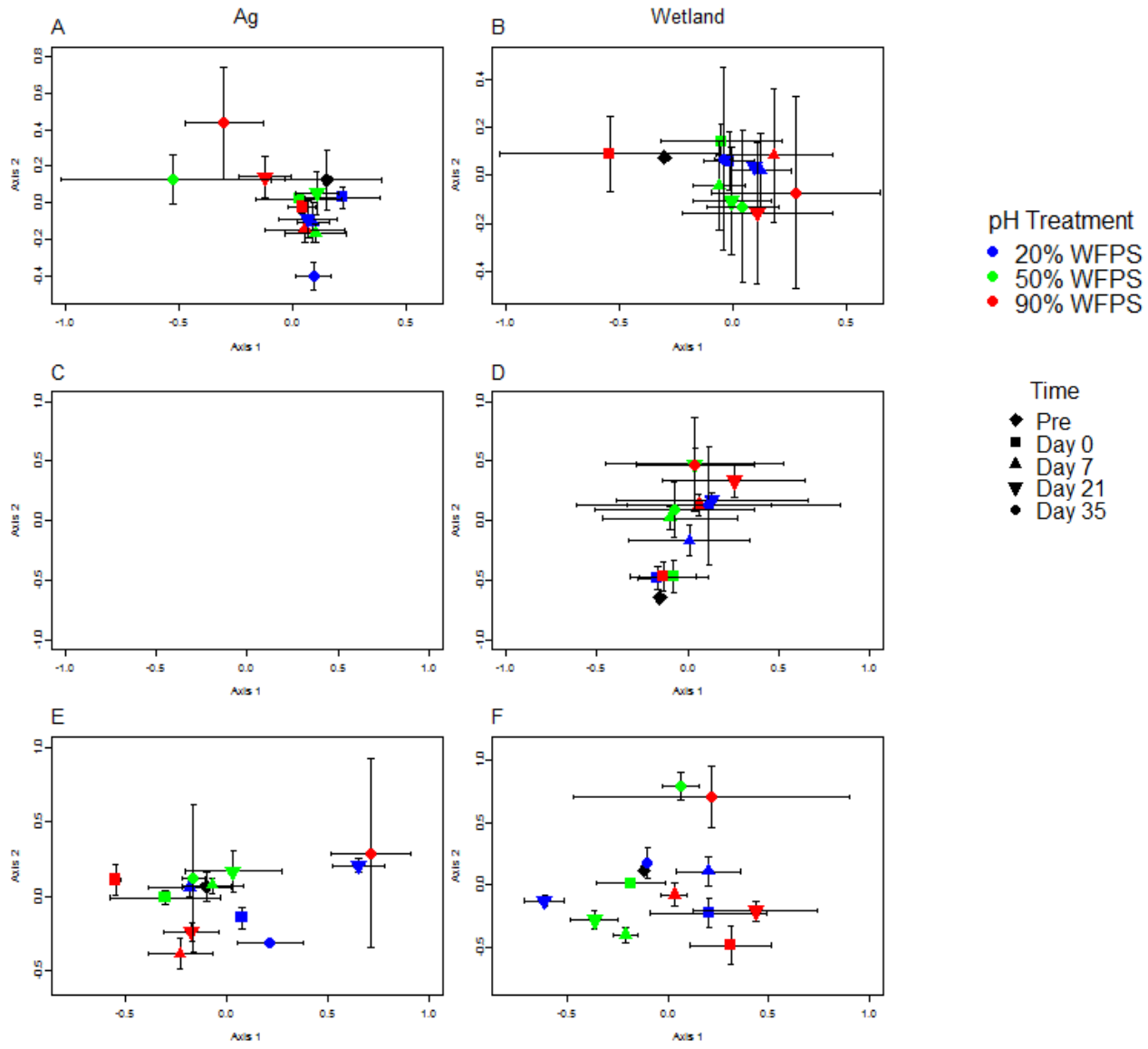

Figure II.1. NMS ordination of *nirK* (A-B), *nirS* (C-D), and *nosZ* (E-F) community composition based on TRLP profiles. Points represent the means of three replicated treatment jars with standard error bars. For *nirS*, ag soils (C), a solution could not be reached and no points are plotted.

Appendix III: Ammonia oxidizing archaea and bacteria

Table III.1. Primer sets and conditions for genes analyzed with qPCR.

Gene	Function	Pure Culture	Primers	Thermocycler Conditions	Number of Cycles	Plasmid Standard and Soil Correction Efficiencies (%) (R^2 values)	Reference
ureC	Urea hydrolysis	*Pseudomonas stutzeri*	ureC-F/ ureC-R	95°C for 5 min 95ºC for 5 s / 58ºC for 15 s / **72 ºC for10 s**	1 40	97-97% soil = 99% and 81% All $r^2 > 0.99$%	Burbank et al., 2011
AOA	Ammonia oxidation (archeal)	Environmental clone	CrenAmoAQ-F/ CrenAmoAQ-F	94ºC for 15 min 94ºC for 15 s / 52ºC for 45 s / 72ºC for 30 s / **78ºC for 10 s**	1 45	95-102% soil = 112% and 111% All $r^2 > 0.99$%	Miner et al., 2007
AOB	Ammonia oxidation (bacterial)	*Nitrosomonas europaea*	amoA-1F/ amoA-2R	95ºC for 5 min 95ºC for 15 s / 58ºC for 30 s / 7**2 for 60 s**	1 40	91-98% soil = 103% and 88% All $r^2 > 0.99$%	Aoi et al., 2004

Table III.2. P-values for ANOVA used to test the effects of cover crop and poultry litter placement on the *ureC*, archaeal *amoA* (AOA), and bacterial *amoA* (AOB) genes at each soil depth. Bold numbers indicate p-values <0.05.

Soil sampling depth	Response variable	Cover crop effect	Litter placement effect
All depths	*ureC*	0.52	0.71
	AOA	0.52	0.52
	AOB	**0.002**	**0.03**
0-5 cm	*ureC*	0.73	0.32
	AOA	0.97	0.63
	AOB	**0.0003**	**0.04**
5-10 cm	*ureC*	0.58	0.66
	AOA	0.59	0.89
	AOB	**0.002**	0.06
10-20 cm	*ureC*	0.56	0.92
	AOA	0.44	0.33
	AOB	**0.004**	**0.05**
20-30 cm	*ureC*	0.21	0.98
	AOA	0.45	0.13
	AOB	0.09	0.08

Table III.3. Correlation coefficients (r) for *ureC*, AOA, and AOB genes with soil covariates from Chapter 2. An asterisk indicates a p-value < 0.05. Gene copy numbers, ammonium-N concentrations, and nitrate-N concentrations were log transformed.

Gene	NH_4^+-N	NO_3^--N	Soil Moisture	pH	Distance from Interrow Center	Depth
ureC	0.10	0.40*	0.29*	0.39*	0.03	-0.85*
AOA	0.11	0.31*	0.21*	-0.01	0.10	-0.47*
AOB	0.09	0.46*	0.07	0.45*	0.1	-0.76*

Table III.4. Means (± standard errors) of log transformed gene copy numbers per gram of dry soil for the pH manipulation experiment.

Gene	Soil	Treatment				
		Pre	Control	pH 3	pH 7	pH9
ureC	Agricultural	9.20 ± 0.0003^{a}	8.95 ± 0.02^{ab}	8.21 ± 0.10^{c}	8.94 ± 0.03^{ab}	8.83 ± 0.03^{b}
	Wetland	9.73 ± 0.07^{a}	8.65 ± 0.06^{b}	8.06 ± 0.06^{c}	8.74 ± 0.08^{b}	8.69 ± 0.06^{b}
AOA	Agricultural	7.25 ± 0.16^{a}	7.35 ± 0.05^{a}	6.58 ± 0.14^{b}	7.28 ± 0.05^{a}	7.04 ± 0.11^{a}
	Wetland	7.54 ± 0.18^{a}	7.07 ± 0.06^{b}	6.78 ± 0.09^{c}	7.21 ± 0.04^{ab}	7.21 ± 0.05^{ab}
AOB	Agricultural	6.41 ± 0.03^{a}	6.25 ± 0.04^{a}	5.27 ± 0.21^{b}	6.21 ± 0.04^{a}	6.36 ± 0.08^{a}
	Wetland	5.95 ± 0.07^{a}	5.13 ± 0.18^{ab}	4.27 ± 0.10^{c}	5.07 ± 0.25^{ab}	4.52 ± 0.10^{bc}

Letters denote statistical differences among treatments for a given soil type based on Tukey post-hoc analysis for each gene. Means and standard errors were calculated from pre (undisturbed soil samples taken directly from the field), Day 7, Day 21, and Day 35 samples. Wetland and agricultural soils were statistically analyzed separately.

Table III.5. Means (± standard errors) of log transformed gene copy numbers per gram of dry soil for the soil moisture manipulation experiment.

Gene	Soil	Treatment			
		Pre	20% WFPS	50% WFPS	90% WFPS
ureC	Agricultural	9.20 ± 0.0003^{a}	8.87 ± 0.16^{b}	8.92 ± 0.02^{b}	8.95 ± 0.02^{b}
	Wetland	9.73 ± 0.07^{a}	8.86 ± 0.03^{c}	9.07 ± 0.02^{b}	8.65 ± 0.06^{d}
AOA	Agricultural	7.25 ± 0.16^{a}	7.32 ± 0.04^{a}	7.39 ± 0.05^{a}	7.35 ± 0.05^{a}
	Wetland	7.54 ± 0.18^{a}	7.13 ± 0.02^{b}	7.13 ± 0.05^{b}	7.07 ± 0.06^{b}
AOB	Agricultural	6.41 ± 0.03^{a}	6.29 ± 0.04^{a}	6.24 ± 0.03^{a}	6.25 ± 0.04^{a}
	Wetland	5.95 ± 0.07^{a}	5.07 ± 0.04^{b}	5.38 ± 0.13^{ab}	5.13 ± 0.18^{b}

Letters denote statistical differences among treatments for a given soil type based on Tukey post-hoc analysis for each gene. Means and standard errors were calculated from pre (undisturbed soil samples taken directly from the field), Day 7, Day 21, and Day 35 samples. Wetland and agricultural soils were statistically analyzed separately.

Table III.6. Pairwise correlation coefficients (r) between soil parameters and gene abundances and denitrification gas fluxes. All genes are log transformed.

	DEA	N2O	N2O:N2
Soil	-0.32[a]	0.26[b]	0.25[b]
pH	0.29[b]	-0.09	**-0.45[a]**
Grav. Soil Moisture	-0.16	0.23[c]	0.14
% WFPS	0.21[c]	-0.06	-0.11
Matric Potential	-0.15	-0.05	0.10
Ammonium-N	-0.36[a]	0.09	0.19[c]
Nitrate-N	-0.20[c]	-0.10	-0.01
Inorganic-N	-0.43[a]	0.07	0.21[c]
TN	-0.37[a]	-0.02	0.15
DOC	-0.24[b]	-0.16	-0.04
16S gene copy number	0.25[c]	0.36	-0.20[c]
ITS gene copy number	-0.05	-0.02	0.22[c]
ureC gene copy number	0.40[a]	0.32[b]	-0.33[a]
AOA gene copy number	0.42[a]	0.30[b]	-0.25[b]
AOB gene copy number	0.53[a]	0.17	-0.36[a]

*Soil type was coded as a dummy variables with the agricultural soil defined as 0 and the wetland soil defined as 1.

[a] $p<0.001$, [b] $P<0.01$, [c] $P<0.05$.

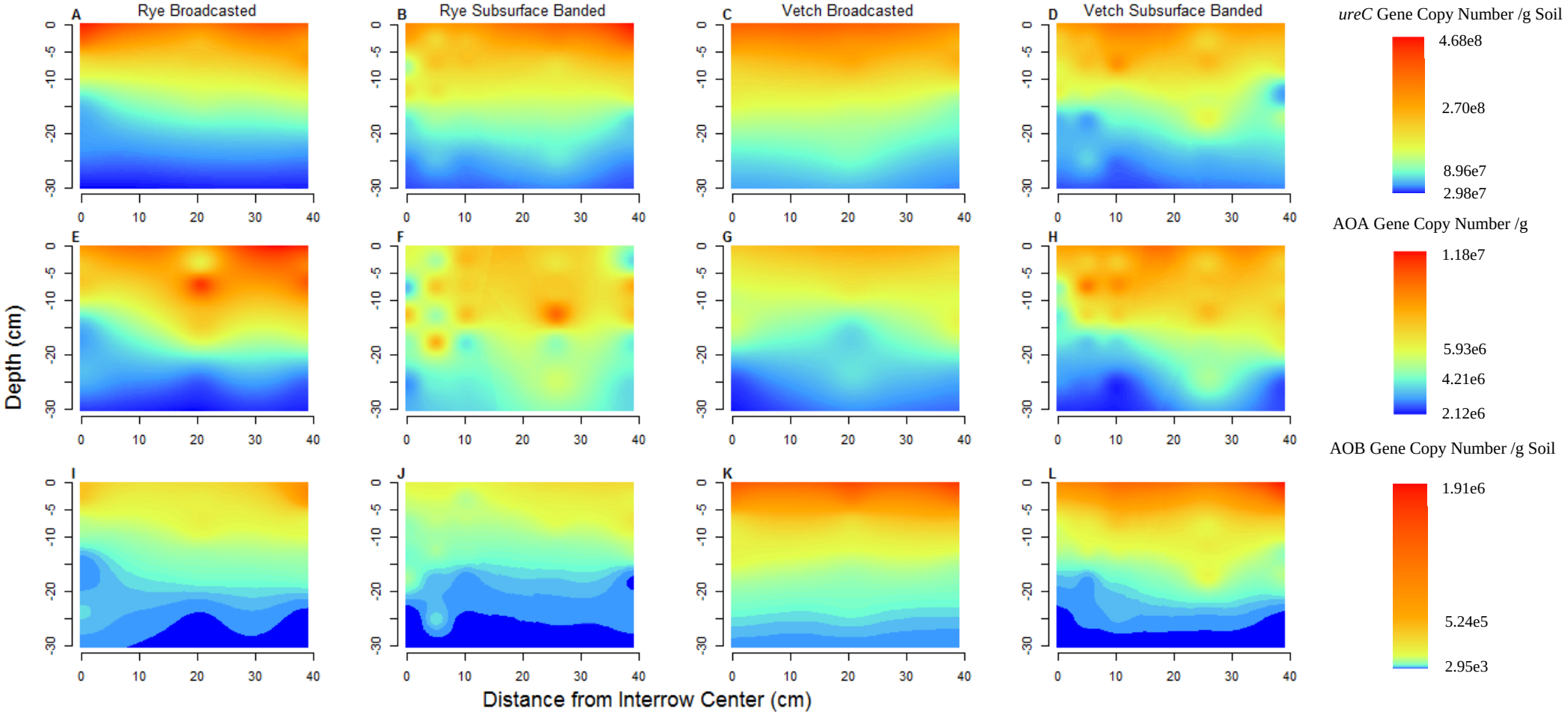

Figure III.1. Spatial distribution of *ureC* (A-D), ammonia oxidizing archaea (AOA) (E-H), and ammonia oxidizing bacteria (AOB) (I-L) genes at the corn V5 growth stage: A) *ureC* cereal rye with broadcast poultry litter, B) *ureC* cereal rye with subsurface banded poultry litter, C) *ureC* hairy vetch with broadcast poultry litter, D) *ureC* hairy vetch and subsurface banded poultry litter, E) AOA cereal rye with broadcast manure, F) AOA cereal rye with subsurface band, G) AOA hairy vetch with broadcast manure, H) AOA hairy vetch and subsurface band, I) AOB cereal rye with broadcast manure, J) AOB cereal rye with subsurface band, K) AOB hairy vetch with broadcast manure, and L) AOB hairy vetch and subsurface band. For each panel, the inter-row center is on the left and the corn row is on the right. Figures are based on inverse distance weighted interpolation.

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