LAND-USE, LANDFORM AND SEASONAL-DEPENDENT CHANGES IN MICROBIAL COMMUNITIES AND THEIR IMPACT ON NITROUS OXIDE EMISSION ACTIVITIES

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ABSTRACT

The greenhouse gas nitrous oxide (N_2O) is produced mainly by the microbial processes of nitrification and denitrification. I hypothesized that microbial community structure (composition and abundance) is linked to differences in soil N_2O emissions from these two processes. Microbial community composition (type and number of nitrifier and denitrifier genotypes), abundance and N_2O emission activity were determined and compared for soils from two landscapes characteristic of the North American "prairie pothole region" (cultivated vs. uncultivated wetlands). The landscape difference in composition of individual microbial communities was not predictive of soil N_2O emissions, indicating that there is redundancy in each microbial community in relation to N_2O emission activities. However, community factors influenced the pattern and distribution of N_2O emission from the soils of the study site. For example, nitrification was the dominant N_2O emitting process for soils of all landforms. However, neither nitrifier *amoA* abundance nor community composition had predictive relationships with nitrification associated N_2O emissions. This lack of relationship may be a consequence of using *amoA* as the gene target to characterize nitrifiers. For denitrifying bacteria, there was a temporal relationship between community composition and N_2O emissions. However, this may be related to the change in water-filled pore space over time. Alternatively, the presence of fungi can be linked directly to N_2O emissions from water accumulating landform elements. Under hypoxic conditions, there may be two fungal pathways contributing to N_2O release: fungal denitrification via P450nor and fungal heterotrophic nitrification. Results suggest that the relative importance of these two processes is linked to root exudates such as formate. It is the interaction between the seasonal fluctuations of the microbial and environmental factors that determine the level of N_2O emissions from soils.

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LIST OF ABBREVIATIONS

AMO	Ammonia monooxygenase
amoA	Gene encoding subunit A of ammonia monooxygenase
ANOVA	Analysis of variance
AOB	Ammonia oxidizing bacteria
BC	Basin centre
BLAST	Basic Local Alignment Search Tool
CTAB	Hexadecyltrimethyl ammonium bromide
CD	Cultivated depression centre
CV	Concave
CW	Cultivated wetland
CX	Convex
d'_D	Fraction of N_2O from denitrification
d'_N	Fraction of N_2O from nitrification
DEA	Denitrification enzyme activity
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate
EDTA	Ethylenediamine tetra-acetic acid
FAME	Fatty acid methyl ester
GC	Guanine and cytosine
HAW	Hours after wetting
N_2O_f	Rate of net N_2O formation
NAR	Nitrate reductase
NCBI	National Centre for Biotechnology Information
NIR	Nitrite reductase
NOB	Nitrite oxidizing bacteria
NOR	Nitrix oxide reductase
NOS	Nitrous oxide reductase
nosZ	Gene encoding a structural subunit of nitrous oxide reductase

OTU	Operational taxonomic unit
P450nor	Cytochrome P450 nitric oxide reductase
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
PVPP	Polyvinyl polypropylene
q-PCR	Quantitative polymerase chain reaction
rDNA	Ribosomal DNA
RG	Riparian grass
rN_2O	The ratio of N_2O_f to DEA
RNA	Ribose nucleic acid
rRNA	Ribosomal RNA
RT	Riparian tree
RFLP	Restriction fragment length polymorphism
T-RFLP	Terminal restriction fragment length polymorphism
TAE	Tris-acetic acid-EDTA
TE	Tris-HCl EDTA
UW	Uncultivated wetland

1 INTRODUCTION

Nitrification and denitrification are important microbial processes in global N cycling. Nitrification is the oxidation of NH_4^+ or NH_3 to NO_3^- via NO_2^- (Horz et al., 2004). Denitrification is the respiratory reduction of NO_3^- and NO_2^- to gaseous products, mainly N_2O and N_2 (Tiedje, 1994). Because N_2O, a greenhouse gas with 300 times the global warming potential of CO_2 (Jungkunst and Fiedler, 2007), can be produced by nitrification and denitrification (Wrage et al., 2005), the rates of these two processes and the controls on the rates of these two processes are important determinants on soil N_2O emissions.

The "prairie pothole region" encompassing the area from Alberta, Canada, to Iowa, USA, is the largest wetland habitat in North America, and positive N_2O fluxes from this area are characterized as event (i.e., rain, snow-melt) and topography (e.g., water accumulation in depressions) driven (Yates et al., 2006b). Under these conditions, denitrification processes dominate N_2O emissions (Corre et al., 1996; Pennock et al., 1992). However, N_2O from nitrification cannot be excluded because gas emissions were repeatedly observed from points where dry soil conditions prevailed (Yates et al., 2006a). Nitrous oxide emission from these landscapes might be a combination of nitrification and denitrification activities.

Available C, N (Avrahami et al., 2002; Svensson et al., 1991), and O_2 (Cavigelli and Robertson, 2000) are three proximal factors that influence the rates of N_2O emission from nitrification and denitrification. Land-use and landform (together referred to as landscape) are two important distal determinants on microbial community structure and proximal controls on rates of nitrification and denitrification. Land-use mainly influences nutrient availability (through fertilization and cropping) and soil disturbance (through tillage) (Bruns et al., 1999; Stres et al., 2004). Landform affects O_2 availability, nutrient distribution, and biological productivity through water redistribution (Hayashi et al., 1998; Yates et al., 2006a; Yates et al., 2006b). However, these factors act through the

microbial community whose composition and abundance reflects the long term climate, disturbance, and resource availability imposed on soils (Cavigelli and Robertson, 2000; Rich et al., 2003).

Bacteria have traditionally been the group of focus when considering the contributions of microbial activity to soil N_2O emissions. However, increasing evidence demonstrates the importance of fungal activity to the emission of N_2O from soils with specific characteristics (e.g., excess soil nitrogen). Laughlin and Stevens (2002) demonstrated that nearly 90% of soil N_2O emissions is attributable to fungal denitrification activity. Given that fungal biomass dominates in many ecosystems, their potential activity may be the dominant soil N_2O emitting process in a variety of soil systems. Fungal denitrifiers are ecologically significant because most fungal isolates capable of denitrification appear to lack nitrous oxide reductase – the enzyme that reduces N_2O to N_2 (Nakahara et al., 1993; Zhou et al., 2001). Therefore, unlike bacterial denitrification, the end-product of which is mostly N_2, the end-product of fungal denitrification is N_2O.

None of the current models used to predict N_2O emissions from soils account for variability in microbial community composition (Philippot and Hallin, 2005). This implies that the community composition of the microbes involved in N_2O emission is assumed to be the same regardless of environmental conditions; i.e., their involvement can be modeled as a constant. This suggests microbial community composition is unimportant for predicting N_2O emissions from soil. However, the importance of unique microbial communities responsible for N_2O emissions has been demonstrated in a number of soil systems (Holtan-Hartwig et al., 2000; Mintie et al., 2003; Rich et al., 2003; Webster et al., 2005). In these studies, rather than processes being controlled by environmental factors such as soil water content or substrate availability, populations within the microbial community were demonstrated to be uniquely adapted to existing conditions and could influence process rates independent of changes in the environment. In other ecosystems, however, differences in community composition were not related to differences in N_2O emission activity (Enwall et al., 2005; Rich and Myrold, 2004).

The primary objective of this study is to address the following question. Is there a relationship between soil microbial community composition and community function as

it pertains to soil N_2O emissions? In order to address this question, soil microbial community composition, abundance and N_2O emission activity were determined for two common landscapes characteristic of the North American "prairie pothole region": cultivated wetlands (CW) and uncultivated wetlands (UW). This question was answered by a series of studies designed to address the following hypotheses:

1. Land-use and landform (defined together as landscape) select for different N_2O producing microbial communities.
2. Landscape-selected microbial communities differ in N_2O emitting activity when soils are incubated under similar conditions.
3. Differences in soil microbial community composition are related to differences in soil N_2O emissions.

The research studies of this dissertation are presented in Chapters 3, 4 and 5. Chapter 3 explores the relationship between the spatial variability of soil microbial community composition and N_2O emissions. The relationship between seasonal variability in community composition and N_2O emitting activity is reported in Chapter 4. Chapter 5 represents an attempt to investigate alternate microbial pathways that could explain some of the observations made in the two preceding chapters. Each chapter was written as a self-contained research article addressing the primary objective of this study while focusing on a particular experiment. Chapter 6 is the concluding chapter of this dissertation. It integrates the findings of the three research chapters into a coherent discussion regarding the primary objective. A brief discussion of future perspectives is included in this chapter. The thesis contains some redundancy because of the research article format. In order to reduce this, the reference sections from all chapters were combined into a final list (Chapter 7).

2 LITERATURE REVIEW

2.1 Processes that Convert Nitrogen Oxides to Dinitrogen Gases

Biological and chemical processes leading to N_2O production are summarized in **Figure 2.1**. Nitrifying and denitrifying bacteria are the primary biological sources, and denitrifiers are the only biological sink, of N_2O (Conrad, 1996). Nitrous oxide is also produced through the abiotic process of chemodenitrification. Biotic denitrification can be subdivided into respiratory denitrification, non-respiratory denitrification, and two forms of nitrate reduction to ammonium, although the bulk of N_2O is produced by respiratory denitrification (Wrage et al., 2005).

2.1.1 Ammonia oxidation: Nitrification

Nitrification is the oxidation of ammonia (NH_3) and is a process carried out by chemoautotrophic bacteria for the purpose of obtaining energy (Kampschreur et al., 2006; Webster et al., 2005). Nitrification is a two-step process whereby NH_3 is oxidized to nitrite (NO_2^-) by ammonia oxidizing bacteria (AOB), and NO_2^- is oxidized to nitrate (NO_3^-) by nitrite oxidizing bacteria (NOB). Nitrification is an aerobic process, but when the supply of O_2 is limited by diffusional constraints AOB can use nitrite as an electron acceptor and reduce it to N_2O or N_2 by denitrification (Colliver and Stephenson, 2000). This process is known as nitrifier-denitrification. Hydroxylamine (NH_2OH) is the first intermediate formed in NH_3 oxidation and it may be oxidized and emitted as N_2O (Cantera and Stein, 2007; Wrage et al., 2005).

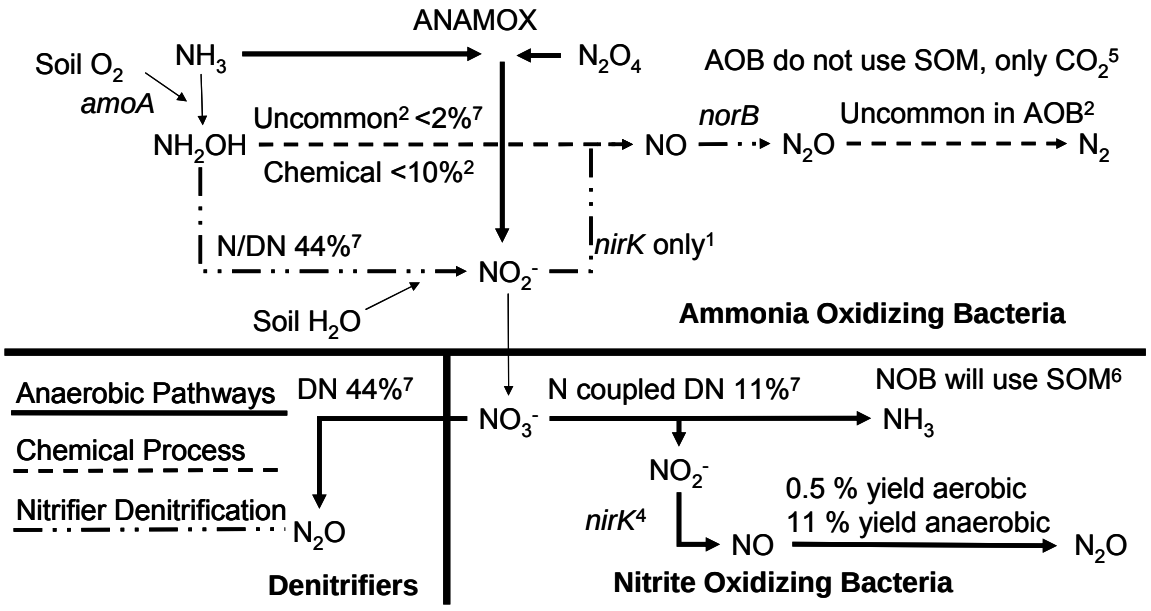

Figure 2.1 Processes leading to N_2O production. [1](Cantera and Stein, 2007); [2](Colliver and Stephenson, 2000); [3](Shaw et al., 2006); [4](Starkenburg et al., 2006); [5](Arp and Stein, 2003); [6](Freitag et al., 2005); [7](Wrage et al., 2005).

Nitrifier denitrification is often confused with coupled nitrification/denitrification and heterotrophic nitrification/aerobic denitrification (Wrage et al., 2001). The former is the process where denitrifiers use nitrate or nitrite produced by chemolithotrophic nitrifiers for respiratory denitrification (Wrage et al., 2005). In the latter process, heterotrophic organisms possess both the ability to nitrify and denitrify but energy derived from oxidative phosphorylation is by C oxidation rather than NH_4^+ oxidation (nitrification) and the substrate for denitrification can be nitrate or nitrite (whereas nitrite is the substrate for nitrifier denitrification) (Freitag et al., 2005; Wrage et al., 2005).

The supply of available C and O_2 are limiting factors to nitrifier growth. Available C is an indirect control of nitrification because of its importance to ammonification as opposed to nitrification (Freitag et al., 2005). Davidson and Verchot (2000) found that the amount of N_2O produced during nitrification was low until water content increased to 60% water-filled pore space (WFPS). This is because of a reduction in available O_2 as a result of restricted O_2 diffusion by the increase in pore water (Cantera et al., 2006; Cantera and Stein, 2007). Nitrous oxide produced from nitrification will increase with increasing water content until the soil becomes very wet or water logged, at which time denitrification will become the dominant N_2O producing process (Bateman and Baggs,

2005; Yates et al., 2006b; Zhang et al., 2005). Therefore, soil moisture content also control N_2O emission from nitrification.

2.1.2 Nitrate reduction: Denitrification

Denitrifiers are a diverse group that spans more than 50 genera of bacteria including all gram-negative bacteria and all gram positive bacteria excluding *Bacillus* (Cavigelli and Robertson, 2001; Zumft, 1997). The majority are facultative anaerobes that prefer oxygen over nitrogen oxides as electron acceptor. Hence, O_2 availability limits denitrification (Bateman and Baggs, 2005; Cavigelli and Robertson, 2000; Takaya et al., 2003). Carbon is an electron donor; hence, C source and availability are other important controls on denitrification (Kuwazaki et al., 2003; Uchimura et al., 2002). Soil NO_3^- availability also acts as a control and it is thought to be rate limiting with respect to overall denitrification (Yokoyama and Ohama, 2005). Low NO_3^- limits the availability of electron acceptors, encouraging denitrifiers to oxidize N_2O to N_2.

Denitrification is any process that reduces nitrogen oxides (NO_x^-) to N_2O or N_2 (Cavigelli and Robertson, 2000; Zumft, 1997). Respiratory denitrification is often the biological process referred to when the term denitrification is used. The nitrogen oxides in this reaction are electron acceptors in an energy conserving process, electron transport phosphorylation (Zumft, 1997). Therefore two features define respiratory denitrifiers: 1) nitrogen gases, principally N_2 and N_2O are products of nitrate and nitrite reduction and 2) this process is coupled with growth yield increase that is greater than if nitrogen oxide simply served as electron sinks (i.e., without electron transport phosphorylation) (Mahne and Tiedje, 1995).

The four enzymes that link electron transport phosphorylation to nitrogen reduction [nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS)] are usually induced sequentially under anaerobic conditions (Rich and Myrold, 2004; Wrage et al., 2001). Since N_2O is produced by NOR and consumed by NOS, N_2O accumulates under two sets of conditions: 1) after NOR but before NOS is induced and 2) following induction of the whole denitrification pathway when environmental conditions inhibit NOS activity to a greater extent than they inhibit NOR activity.

Non-respiratory denitrification is the reduction of NO_3^- or NO_2^-, to N_2O but not N_2, and it is not associated with enhanced growth (Freitag et al., 2005). A variety of organisms, including both aerobes and anaerobes, appear to possess this ability. This process is of potential significance to the atmospheric N_2O budget because it can occur under aerobic conditions in drier soils (Chen et al., 2003; Tiedje, 1994; Zhou et al., 2001). Eukaryotes have been known to catalyze dissimilatory reduction of nitrate to nitrite, but the fungus *Fusarium oxysporum* was the first eukaryote to demonstrate reduction of nitrate and nitrite to gaseous N-oxides (Shoun and Tanimoto, 1991).

Dissimilatory nitrate reduction to ammonium (DNRA), also referred to as nitrate ammonification, is similar to respiratory denitrification in that they are both regulated by the supply of O_2 (Strohm et al., 2007; Tiedje, 1994). Reduction of NO_3^- to NH_4^+ provides a higher capacity for electron acceptance per molecule than does the reduction of NO_3^- to N_2O in an environment that is poor in electron acceptors (low NO_3^-) (Strohm et al., 2007). Thus the process conserves soil N by minimizing the production of N_2O. Consequently, DNRA is most active in electron acceptor poor environments with high available organic C (Tiedje, 1994).

Assimilatory nitrate reduction to ammonium is regulated by the supply of NH_4^+ rather than O_2 (Tiedje, 1994). Both assimilatory and dissimilatory processes produce NH_4^+ at the expense of NO_3^-. Hence, interpretations where the loss of NO_3^- is assumed to be a result of the formation of N_2O or N_2, can be difficult to make. DNRA is distinguishable from assimilatory nitrate reduction in that the amount of N reduced during DNRA is more than what is needed for microbial growth (Strohm et al., 2007).

2.1.3 Chemodenitrification

Chemodenitrification is the generation of nitrogen gas products through reactions that are mediated non-biologically (Morkved et al., 2007). It involves the chemical decomposition of nitrous acid (HNO_2) or reaction of HNO_2 with amino acids, ammonia, urea and other soil constituents such as metal ions. The process occurs primarily, but not necessarily, at low pH values (5 or less). NO, N_2O and N_2 have been reported as products of chemodenitrification and of these NO is most abundant (Cantera et al., 2006; Venterea et al., 2005). Overall, chemodenitrification is not considered to be an important form of

denitrification (Wrage et al., 2005). However, heavy fertilization or urine inputs in grazed areas may result in decreased soil pH that could potentially cause significant chemodenitrification in the short term (Venterea et al., 2005).

2.2 Proximal Controls on the Production of N_2O

Factors that control the production of soil N_2O can be thought of as either proximal or distal (Beauchamp, 1997) (**Figure 2.2**). A proximal factor is an essential factor that controls N_2O production at a process level, e.g., O_2 controls expression and regulation of NOS enzyme in denitrification. Proximal factors are in turn regulated by factors that operate at a broader scale, e.g., denitrification is greater in water-accumulating landforms than water-shedding landforms because water accumulation limits O_2 diffusion. These are distal controls. Levels of available O_2, mineral N, and available organic C are strong proximal controls on the microbial processes responsible for soil N_2O emissions. Other proximal controls include soil moisture and soil temperature which are direct controls on microbial activity, and are indirect controls through their effect on O_2 supply and C and N cycling.

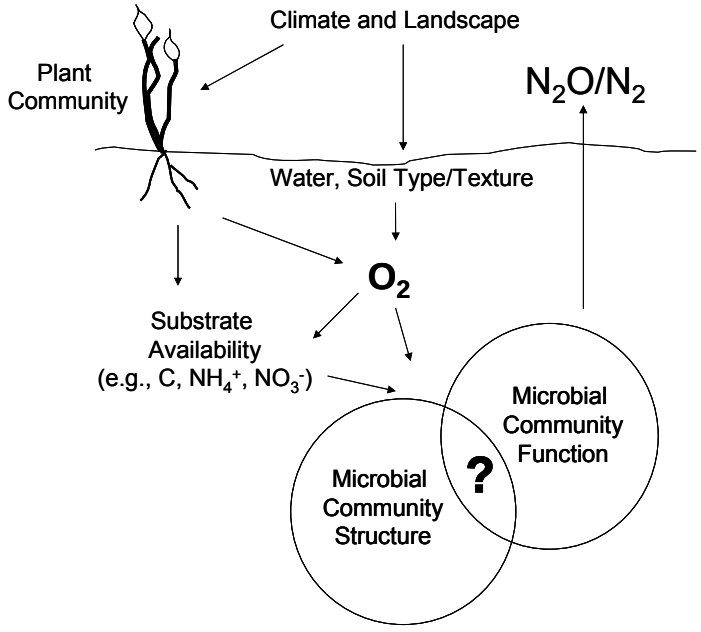

Figure 2.2 Relationships between controls over microbial activity and N_2O production in soils. ? = knowledge gap to be addressed by this thesis.

2.2.1 Soil water and oxygen

The availability of O_2 at a particular point in the soil is determined by the rate of O_2 diffusion to that point and the rate of O_2 consumption resulting from microbial activity (Bateman and Baggs, 2005; Cavigelli and Robertson, 2001). Oxygen is a master regulator on the synthesis and activity of reductive enzymes. Consequently, increases in soil O_2 result in a decline in total denitrification (Cavigelli and Robertson, 2000) whereas O_2 limitation corresponds to increased N_2O or denitrification activity (Takaya et al., 2003). Where O_2 is not limiting, denitrification is suppressed and any N_2O evolution that is produced is related to nitrification (Bateman and Baggs, 2005).

The O_2 status of a soil is difficult to measure in the field, thus O_2 availability has usually been assessed using surrogate measurements such as water-filled pore space (WFPS) (Maag and Vinther, 1999; Wolf and Russow, 2000). Presence of water in pore spaces or as films on soil aggregates slows diffusion of O_2 (Renault and Stengel, 1994; Wolf and Russow, 2000). Microbial activity will increase with soil water content until diffusion of O_2 is restricted and the environment becomes anaerobic (Bateman and Baggs, 2005; Wolf and Russow, 2000). Thus, soil water content is a control on both nitrification and denitrification. Nitrification occurs up to 60% WFPS (Davidson and Verchot, 2000). At WFPS greater than 60%, denitrification becomes dominant (Bateman and Baggs, 2005), and at WFPS >80%, O_2 diffusion is restricted to the point where the product of denitrification is primarily N_2 (Veldkamp et al., 1998; Wolf and Russow, 2000); i.e., N_2O is itself used as an electron acceptor and denitrified.

2.2.2 Available C and N

Available C and N control soil N_2O emissions because C is an electron donor and nitrogenous compounds, such as NO_3^-, are electron acceptors. Electron donors and acceptors are needed for microbial processes that supply energy for metabolic activities. The production of NO_3^- by nitrification is limited by the supply of NH_4^+ (Schmidt and Belser, 1994; Skiba et al., 1993). Nitrogen supply will have an effect on emissions in a soil that has a low concentration of NO_3^- (Myrold and Tiedje, 1985; Tiedje, 1988; Weier et al., 1993). That is to say, NO_3^- concentration exerts a control on N_2O emissions where it is limiting and less or no control where NO_3^- is not limiting. Corre et al. (1996) found

that C and N availability became important to N_2O emissions when soil moisture conditions were favorable for denitrification (i.e., availability of O_2 was restricted).

2.2.3 Rhizosphere effects on N_2O fluxes

The rhizosphere has been studied as a site of potential N_2O fluxes. Rhizosphere N_2O emissions are related to carbon and O_2 availability. Roots can 1) select microbial populations based on available C substrate; 2) reduce oxygen tension in soil by both root respiration and bacterial respiration stimulated by root exudates; 3) create a more aerated rhizosphere through water consumption; 4) consume nitrate, making it unavailable for denitrifiers; and 5) in wetland plants, transport O_2 down the stem and into the rhizosphere which can stimulate nitrification in nitrate-limited anoxic sediment (Gutknecht et al., 2006; Tiedje, 1988). Variability in rates of these rhizosphere processes results in fluxes of N_2O from the rhizosphere (Martin et al., 1999).

In the context of nitrogen respiration, the low molecular weight organic acid formate is an especially important root exudate (Jones, 1998) and intermediate and by-product of anaerobic carbon metabolism (Bott, 1997). The amount of formate in aerobic soils is reported to range from 6 to 26% of the total extractable low molecular weight organic acids (van Hees et al., 2005). Reported rhizosphere formate concentrations range from below detection limit for clover (*Trifolium repens*) (Bolan et al., 1994), 117 μ*M* for Norway spruce (*Picea abies*) (van Hees et al., 1996) and 563 μ*M* for quackgrass (*Elytrigia repens*) (Baziramakenga et al., 1995). Formate (together with acetate) is the end-product of the fermentation of citrate, oxaloacetate and pyruvate. It is also produced from H_2 and CO_2 by a variety of anaerobic microorganisms (e.g., acetagens, sulfate reducers, and methanogens) (Horn et al., 2003).

2.2.4 Soil temperature

A positive correlation between soil temperature and N_2O evolution has been observed (Kliewer and Gilliam, 1995; Maag and Vinther, 1999). Nitrification is limited at soil temperatures <4 °C (Anderson and Boswell, 1964; Gödde and Conrad, 1999). Soil temperature controls denitrification directly through its control on the activity of

denitrifiers. It also acts indirectly on denitrification; i.e., an increase in soil temperature is often accompanied by an increase in heterotrophic microbial activity that increases the consumption of O_2. This creates anaerobic micro-sites that encourage denitrification (Maag and Vinther, 1999). Temperature also influences the solubility of O_2 in water and the diffusion of O_2 to micro-sites of denitrification activity (Renault and Stengel, 1994).

2.3 Distal Controls on the Production of N_2O

2.3.1 Landform

A landform pattern of N_2O emissions has been observed by several authors (Ambus, 1998; Corre et al., 1996; Pennock et al., 1992). Ambus (1998) observed increased production of N_2O in depressions, which was attributed to the presence of wetter soils because of slower drainage. Pennock et al. (1992) noted that topography indirectly influenced the spatial distribution of denitrification by its direct control on the redistribution of water and its concentration in low areas. Corre et al. (1996) observed that foot-slope elements had a proportionally higher (72%) number of N_2O producing micro-sites (hotspots) compared to shoulder elements (28%), and they concluded that the spatial distribution of N_2O emissions was closely related to landform and its control on the micro-scale distribution of moisture.

2.3.2 Soil texture and structure

The percentage of WFPS that is required to restrict diffusion of O_2 and trigger denitrification is strongly influenced by soil texture. It was found that an intact clay soil became anoxic at lower percent WFPS than an intact loam soil (Del Grosso et al., 2000). Soil texture and structure determine the pore system, which is important for water movement, i.e., changes in WFPS. Therefore, size and tortuosity of pores control occurrence and duration of soil saturation and, in turn, this controls periods of restricted O_2 diffusion.

Aggregate size also affects the aeration status of sites of microbial activity. Renault and Stengel (1994) modeled aggregate size and found that for a given microbial

respiration rate, the aerobic volume fraction of an aggregate decreases as the radius of the aggregate increases. This is also exacerbated by the presence of water. Furthermore, they found small aggregates remained aerobic until saturated, but large unsaturated aggregates tended to have an anaerobic center.

2.3.3 Climate and weather

Seasonal changes in precipitation and temperature control the input of water into a landform and its loss through evapotranspiration. In this manner, climate controls soil moisture status, and C and N availability (Groffman et al., 2000). An example of this would be the increase in soil organic matter across the prairie region as mean annual precipitation increases. Episodes of precipitation can cause short-term changes in N_2O flux. Yates et al. (2006a) observed increased N_2O flux after precipitation events that cause short term increases in soil water content. Thus the magnitude of a precipitation event is a determining factor in what process will produce N_2O and the magnitude of the flux.

2.4 Soil Microbial Communities and N_2O-Related Activity

The advent of rRNA gene-based techniques in the late 1980s paved the way for a flood of publications on bacterial and fungal diversity. The DNA sequence of the rRNA gene provides species specific information. It contains both conserved and variable regions with sufficient information for accurate statistical analysis, lacks horizontal gene transfer artifacts and has a high copy number rendering it a major component of cellular mass (Moter and Göbel, 2000; Stackebrandt and Goebel, 1994). The application of these methods provided new insights into the composition and structure of microbial communities in various environments; however, the time has come to address the significance of this microbial diversity. Issues such as the consequences of the loss or introduction of microbial populations for ecosystem functioning, or whether some of the microorganisms involved in the same process are redundant, remain unresolved (see **Figure 2.2**). Analysis of functional communities may be the key to a better understanding of these issues.

Molecular methods used to quantify and identify environmental microorganisms involve a group of techniques that aim to answer different questions about the community found in a sample. The techniques used in this study include: amplifying the genetic information to workable concentrations using the polymerase chain reaction (PCR), quantifying the level of amplicons with fluorescence intensity using quantitative PCR (q-PCR), and processing DNA fingerprints of different communities and individual species with denaturing gradient gel electrophoresis (DGGE) or cloning and restriction fragment length polymorphisms (RFLP).

2.4.1 Polymerase chain reaction

Amplification of specific targets of DNA utilizes natural replication mechanisms to produce a high number of DNA copies which then can be used for further analysis. The PCR is one of the most widely used techniques in molecular biology, and has proven to be a highly sensitive process. There are three main steps to each PCR reaction: denaturation, annealing and extension. These are achieved by changing the temperature of the reaction mixture. The reaction mixture must contain the template DNA, reverse primers, forward primers, DNA deoxynucleotides and a heat stable DNA polymerase. Additional reaction components may be required in order to maximize efficiency and specificity.

The primers are single strands of DNA, measuring approximately 20 nucleotides in length. Upon denaturing the double stranded DNA, the single stranded primers can then anneal to their complementary region. The forward primer anneals to the negative DNA template strand and the reverse primer anneals to the positive strand, both primers will polymerize towards each other from 5′- to 3′-end (Marcheesi, 2001). The sensitivity of this process is dependent on the primer selection, reaction component concentrations, and temperature controls.

Studies involving the extraction of RNA, instead of DNA from samples of interest, are useful to detect active enzymes or microorganisms. RNA molecules are relatively unstable in the environment, as they are constantly under attack by RNases. This makes RNA techniques more useful at providing information on viable microorganisms, as the molecule is protected by the cellular membranes of live cells, but it also makes RNA

extractions more complex requiring the pretreatment of every object which comes in contact with the sample (Nogva et al., 2003; Rudi et al., 2005b). In order to PCR amplify the molecules using standard practices, it is necessary to first convert the RNA into a more laboratory stable DNA form. This is achieved using the viral enzyme, reverse transcriptase. For this reason, investigation of alternative methods that can be used to identify viable populations is of interest. In this study, the ethidium monoazide bromide (EMA) technique was optimized and used.

The chemical structure of EMA enables it to intercalate double stranded DNA by structural changes induced by high intensity light. The EMA dye can enter cells with damaged membranes and link to the DNA of those cells (Nogva et al., 2003; Rudi et al., 2005b). It is excluded from live cells by a passive process through diffusion barriers. Experiments performed in the dark showed that both ethidium bromide and ethidium monoazide have identical activities, but upon light exposure the azide derivative caused enhanced mutation rates (Sternglanz et al., 1978). Nogva et al. (2004) found that EMA crosslinking decreased the maximum PCR signal by 4.5 log units. The use of EMA with the viable and non-cultivable food pathogen *Campylobacter jejuni* and *Listeria monocytogenes* has been successful with quantitative PCR methods (Rudi et al., 2005a; Rudi et al., 2005b). However, recent evidence suggests that EMA does not exclusively inhibit the amplification of DNA from dead or damaged cells of *C. jejuni* and *L. monocytogenes* (Flekna et al., 2007). Pisz et al. (2007) suggested that the effectiveness of EMA to suppress amplification of non-viable organism DNA is matrix dependent. They found EMA effectively suppressed amplification of non-viable organism DNA in soil samples, but there was poor or no suppression in sulfur-block and biofilm samples, respectively.

2.4.2 Quantitative polymerase chain reaction

Quantitative PCR (q-PCR) is a PCR-based molecular assay that proceeds on the assumption that each DNA molecule is duplicated once during one cycle of amplification, resulting in an exponential accumulation of product (Dorigo et al., 2005; Nogva and Rudi, 2004). The PCR reaction is monitored by fluorescence that is measured at each stage of the reaction, and compared to that of a standard curve generated by a dilution series of

known amounts of DNA targets (Nogva and Rudi, 2004; Rutledge and Cote, 2003). This method is advantageous over regular PCR because it is an automated method which detects DNA amplification during the exponential phase where there is an exact doubling of product, rather than at the end where degradation begins to take place and variability increases.

Both TaqMan and SYBR Green I q-PCR product detectors are equally rapid and sensitive, but they differ in optimization and price (Ponchel et al., 2003). The fluorescent dye, SYBR Green I green binds to the minor groove of double stranded DNA and emits fluorescence (Pfaffl, 2001). The TaqMan dual labeled fluorogenic probe system emits fluorescence after 5′ nuclease polymerase activity cleaves off the quencher allowing expression of the reporter signal. The TaqMan fluorogenic probe system is much more difficult to optimize than SYBR Green I, requiring specific buffer concentrations and reaction temperatures (Yin et al., 2001), but SYBR Green I non-specifically binds to all double stranded DNA products, including primer dimers and secondary structures. High primer concentrations can lead to increased fluorescent signals when using SYBR Green I (Ponchel et al., 2003). These potential errors in signal representation must be considered when designing an assay involving the inexpensive SYBR Green I. Three different types of products dominate in the first cycles of q-PCR, they include the original target, undefined long products, and PCR accumulated non-specific products (Nogva and Rudi, 2004). The standard curve is generated based on the threshold cycle (Ct; at which first detection of fluorescence occurs) and the concentration of target DNA. From this curve a slope and intercept is obtained using linear regression analysis (Nogva and Rudi, 2004; Rutledge and Cote, 2003). The slope obtained from the standard curve is used to calculate the PCR reaction efficiency using the formula shown in Equation 2.1 (Nogva and Rudi, 2004; Pfaffl, 2001).

$$\text{Efficiency} = 10^{-\text{Slope}} - 1 \qquad \text{[Equation 2.1]}$$

At 100% efficiency each cycle of PCR would theoretically produce a doubling of the DNA copy number (Larionov et al., 2005; Rutledge and Cote, 2003). The accuracy of efficiency calculations is often disputed, as the efficiency of the PCR reaction is not uniform throughout the different stages of the reaction, being highly efficient during the exponential phase and declining in efficiency through the stationary phase (Larionov et

al., 2005). The efficiencies of the q-PCR reaction are based on various reaction parameters, including temperature and concentration of reaction components, which control the primer binding and subsequent amplification (Nogva and Rudi, 2004). Reaction efficiencies are usually below 0.9 because of these factors.

2.4.3 DNA fingerprinting of microbial communities

Once amplified, there exists a variety of complimentary techniques to separate out the mixture of amplified DNA fragments based on denaturing characteristics of nucleotide composition, fragment length polymorphism analysis, and cloning (Rich and Myrold, 2004; Sharma et al., 2006; Stres et al., 2004). Analysis by denaturing characteristics of nucleotide composition and fragment length polymorphism are known as 'fingerprinting' techniques because of the characteristic banding patterns generated from electrophoresis of gene fragments. Denaturing gradient gel electrophoresis (DGGE) was first used for studying environmental bacterial communities in 1993 (Muyzer et al.) and fungal communities in 1997 (Kowalchuk et al., 1997a). This technique separates PCR products of the same size based on differences in chemical denaturation property. Because three hydrogen bonds are formed between guanine and cytosine base-pairing (versus two hydrogen bonds between adenine and thymine pairing), DNA fragments of similar length but different guanine and cytosine content and distribution will differ in denaturing behaviour in a chemical denaturant gradient.

The banding pattern generated from DGGE is considered a snapshot image of the whole microbial community. It is expected that PCR fragments generated from an individual population will have the same denaturation characteristics and electrophoretic mobility (Oros-Sichler et al., 2006; Seghers et al., 2004). Kowalchuk et al. (1997b) demonstrated that co-migrating bands generally were of identical sequence. However, rDNA fragments of closely related microbes may not be resolved as separate bands (Buchholz-Cleven et al., 1997). Alternatively, non-related rDNA fragments may co-migrate to an identical position (ben Omar and Ampe, 2000; Kowalchuk et al., 1997b).

Another fingerprinting approach involves restriction analysis of cloned fragments of PCR-amplified gene fragments. Restriction fragment length polymorphism (RFLP) is one such technique. This approach is premised on the theory that DNA fragments from

unrelated groups of organisms will have a different number and location of restriction sites for a given restriction endonuclease. The resultant restriction pattern with three restriction endonucleases (for example) would produce a pattern that is characteristic of a unique cloned DNA fragment. A variant on this method is terminal restriction fragment length polymorphism (T-RFLP) (Mintie et al., 2003; Rich and Myrold, 2004; Stres et al., 2004). In this variation, the 5′-end of the PCR primer is fluorescently labelled and only the length of a labelled DNA fragment is recorded. The benefit of T-RFLP is that the analysis is automated and high throughput is possible.

For comparative analysis, similarities between fingerprint profiles based on presence/absence of bands, when taken in pairs, can be expressed as a percentage value of a similarity coefficient such as a Jaccard coefficient (Pisz et al., 2007), or a distance coefficient such as Euclidean measure. Another similarity index, the Pearson correlation coefficient, takes into consideration the relative intensity of each band (Seghers et al., 2004). A matrix of pair-wise similarity or distance coefficients can be displayed through clustering techniques. Clustering techniques such as unweighted pair group method using arithmetic averages (UPGMA) or Ward's linkage are applied to the fingerprinting profile to identify those samples with similar patterns (Boon et al., 2002; Seghers et al., 2004)

Multivariate ordination methods are another approach for analysing community fingerprint profiles. The goal of these methods is to integrate complex data sets (e.g., bands in DGGE patterns) into new mathematical variables that can be projected as a single point in 2 or 3-dimensional perspective (Fromin et al., 2002). Common ordination methods include non-metric multidimensional scaling (NMS), principal component analysis (PCA), correspondence analysis (CA), and canonical correspondence analysis (CCA). McCune and Mefford (2002) provide a detailed explanation of the underlying theory, considerations for application, and cautions associated with each method. The greatest advantage that ordination methods offer over clustering is the opportunity to test the relationships between community fingerprint profiles with environmental and microbial activity data through joint analysis (Mintie et al., 2003; Rich and Myrold, 2004). This approach provides graphical answers to questions such as whether variations in fingerprint profile are associated with variations in measured environmental variables (e.g., WFPS, NH_4^+ concentration) or community functions (e.g., N_2O production,

nitrification and denitrification rates). Using this approach, Rich et al. (2003) found that N_2O emissions from a forest-to-meadow transect were driven by variations in denitrifier *nosZ* community rather than environmental factors such was soil water content and substrate availability.

2.4.4 Genes of interest

Ammonia-oxidizing bacteria produce N_2O either as a byproduct of nitrification or an intermediate of nitrifier denitrification (Arp and Stein, 2003). As a nitrification byproduct, N_2O is formed during the incomplete oxidation of hydroxylamine to nitrite. Hydroxylamine is the product of ammonia oxidation by the enzyme ammonia monooxygenase (AMO). The functional genes encoding for AMO (with *amoA* encoding for the commonly studied catalytic subunit) have greater variability but similar phylogeny as 16S rRNA gene of AOB (Purkhold et al., 2000). This makes *amoA* a good target when studying phylogenetic relationships between AOB communities.

To assess the diversity of denitrifying bacteria responsible for N_2O consumption, investigators have focused on the nitrous oxide reductase gene (*nosZ*) because 16S rRNA results may include a large number of non-denitrifiers since denitrifiers are phylogenetically divergent (Stres et al., 2004). For example, Rich *et al.* (2003) used the *nosZ* marker to correlate denitrifying community composition with denitrification activity and soil and vegetation type. Although not all denitrifiers have *nosZ* (as respiratory denitrification is defined as the sequential reduction of NO_3^- or NO_2^- to gaseous nitrogen oxides – N_xO_y), the level of *nosZ* expression in the soil relative to N_2O production determines whether N_2O is released into the atmosphere or is reduced to N_2. It is important to note that a nitrifying or denitrifying "community" in this study is defined as any organism that contain *amoA* or *nosZ* that is amplifiable by PCR. There is no phylogenetic relationship intended by the use of "community".

3 RELATIONSHIP BETWEEN NITRIFIER AND DENITRIFIER COMMUNITY COMPOSITION AND ABUNDANCE IN PREDICTING NITROUS OXIDE EMISSIONS FROM EPHEMERAL WETLAND SOILS[1]

3.1 Abstract

The link between differences in the community composition of nitrifiers and denitrifiers to differences in the emission of nitrous oxide (N_2O) from soils remains unclear. Nitrifier and denitrifier community composition, abundance and N_2O emission activity was determined for two common landscapes characteristic of the North American "prairie pothole region": cultivated wetlands (CW) vs. uncultivated wetlands (UW). The hypotheses of this study were: 1) landscape selects for different nitrifier and denitrifier communities, 2) denitrification was the dominant N_2O emitting process, and 3) a relationship exists between nitrifier and denitrifier community composition, their abundance, and N_2O emission. Comparisons were made among soils from three CW and three UW at the St. Denis National Wildlife Area. Denaturing gradient gel electrophoresis was used to compare community composition, and quantitative polymerase chain reaction was used to estimate community size. Incubation experiments on re-packed soil cores with ^{15}N-labeled nitrate were performed to assess the relative contributions of nitrification and denitrification to total N_2O emission. Results indicate: 1) nitrification was primary source of N_2O emission, 2) cultivation increased nitrifier abundance but decreased nitrifier richness, 3) denitrifier abundance was not affected by cultivation but richness was increased by cultivation, and 4) differences in nitrifier and denitrifier community composition and abundance between land-use and landform did not correspond to differences in N_2O emission.

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Key words: ^{15}N, ammonia monooxygenase, *amoA*, denitrification, DGGE, N_2O, nitrification, nitrous oxide, nitrous oxide reductase, *nosZ*, q-PCR, stable isotope

3.2 Introduction

The "prairie pothole region" spanning the area from north-western Alberta, Canada, to north-western Iowa, USA, is the largest wetland landscape in North America. Positive fluxes of the greenhouse gas nitrous oxide (N_2O) from this area are characterized as event (i.e., rain, snow-melt) and topography (e.g., water accumulation in depressions) driven (Yates et al., 2006a). This indicates that denitrification processes dominate N_2O emissions (Corre et al., 1996; Pennock et al., 1992). Nitrification as a source of N_2O cannot be excluded because gas emissions were repeatedly observed at specific points when dry soil conditions prevailed (Yates et al., 2006b). Nitrous oxide emission from these landscapes thus appears to be a combination of nitrification and denitrification. However, the link between differences in the community composition of nitrifiers and denitrifiers to differences in the emission of N_2O from soils is not clear.

Increases in soil O_2 typically result in a decline in total denitrification because O_2 is a master regulator of the synthesis and activity of reductive enzymes in the bacterial denitrification pathway (Cavigelli and Robertson, 2000; Takaya et al., 2003). Under these conditions, N_2O produced in soil ecosystems is related to bacterial nitrification (Parton et al., 1988; Robertson and Tiedje, 1987). Autotrophic ammonia-oxidizing bacteria (AOB) produce N_2O either as a byproduct of nitrification or an intermediate of nitrifier denitrification (Arp and Stein, 2003). As a nitrification byproduct, N_2O is formed during the incomplete oxidation of hydroxylamine to nitrite. Hydroxylamine is the product of ammonia oxidation by the enzyme ammonia monooxygenase (AMO). The functional genes encoding for AMO (with *amoA* encoding for the commonly studied catalytic subunit) have greater variability but similar phylogeny as 16S rRNA gene of AOB (Purkhold et al., 2000). This makes *amoA* a good target when studying phylogenetic relationships between AOB communities. Alternatively, in nitrifier denitrification, N_2O is an intermediate produced during the reduction of nitrite to N_2 under O_2 limitations – similar to heterotrophic denitrification (Poth and Focht, 1985; Wrage et al., 2001).

Soil O_2 is difficult to measure directly; therefore, changes in soil O_2 levels are usually described using a surrogate, such as water-filled pore space (WFPS) (Davidson and Verchot, 2000; Lemke et al., 1998). Nitrification occurs at up to 60% WFPS (Davidson and Verchot, 2000). Denitrification becomes dominant at WFPS >60% (Lemke et al., 1998), and at >80% WFPS, O_2 diffusion is restricted to the point where N_2O is used as an electron acceptor and reduced to N_2 (Veldkamp et al., 1998), However, these WFPS values are not exact limits for nitrification and denitrification because O_2 availability is a combination of O_2 diffusion rate and O_2 consumption by heterotrophic activity.

Land-use induced changes in denitrifier diversity have been related to differences in N_2O emissions (Cavigelli and Robertson, 2000; Cavigelli and Robertson, 2001; Stres et al., 2004). It appears that land-use selects for distinct populations by controlling the regulators of denitrification [i.e., O_2 and pH (Cavigelli and Robertson, 2000), C and N availability (Avrahami et al., 2002; Svensson et al., 1991), temperature (Gödde and Conrad, 1999)]. Changes to these soil properties can affect nitrifier diversity as well. To assess the diversity of denitrifying bacteria responsible for N_2O consumption, investigators have focused on the nitrous oxide reductase gene (*nosZ*) because 16S rRNA results may include a large number of non-denitrifiers since denitrifiers are phylogenetically divergent. For example, Rich *et al.* (2003) used the *nosZ* marker to correlate denitrifying community composition with denitrification activity and soil and vegetation type. Although not all denitrifiers have *nosZ* (as respiratory denitrification is defined as the sequential reduction of NO_3^- to N_2O or N_2), the level of *nosZ* expression in the soil relative to N_2O production determines whether N_2O is released into the atmosphere or is reduced to N_2.

In this study, we examined nitrifier and denitrifier communities and N_2O emitting activity from two landscape features characteristic of the North American "prairie pothole region", i.e., cultivated (CW) and uncultivated (UW) wetlands. The objectives of the study were to determine (1) the contributions of nitrification and denitrification to net N_2O emissions at 50 and 70% WFPS in soils from these two landscapes and (2) whether nitrifier and denitrifier community composition and abundance was related to the observed N_2O emissions from these soils.

3.3 Experimental Procedures

3.3.1 Study Site

The St. Denis National Wildlife Area (SDNWA) in central Saskatchewan, Canada (52°12'N, 106°5'W), is a typical landscape of the North American prairie pothole region. It contains 216 wetlands distributed over an area of 3.84 km^2 (Hogan and Conly, 2002). The SDNWA is in the Dark Brown soil zone with loamy unsorted glacial till (Weyburn Association) parent materials and slope classes ranging from 10 to 15% (Miller et al., 1985). Within the SDNWA, six ephemeral wetlands were selected: three cultivated and three uncultivated. Ephemeral wetlands are those depressions in hummocky terrains that contain standing water in the spring, but typically dry-out during the growing season (Hayashi et al., 1998). Runoff into the wetlands occurs primarily from snowmelt during spring thaw.

A detailed topographic survey of the site was completed and a digital elevation model was produced with a 5 × 5 m grid cell extent (Yates et al., 2006a). Relative elevation and visual inspection of the site was used to segment the site into a set of landscape elements defined by landform (profile curvature) and land-use (cultivated and uncultivated). Locations in cultivated wetlands (CW) were classified as either convex (CX), concave (CV), or cultivated depression centre (CD). Convex elements were topographically high positions with a positive profile curvature. Concave elements were positions with negative profile curvature. Cultivated depression centre elements were level positions, roughly circular in shape, which collect rain or snowmelt water. Cultivated wetlands were fertilized with anhydrous ammonia (79 kg ha^{-1}) on May 7, 2004, and seeded with canola (*Brassica napus*) on May 17, 2004.

Non-agricultural portions of the site included vegetated depressions and were classified as uncultivated wetlands (UW). Uncultivated wetlands were further subdivided into three landform elements. The basin center (BC) is a level area covered by a variety of 99 non-grasses such as *Mentha arvensis* L., *Cirsium arvense* (L) Scop., and *Urtica gracilis* Ait. The riparian grass (RG) is a non-level fringe area covered with grasses such as *Bromus inermis* Leyss. The riparian trees (RT) are a partial fringe of mixed trees and shrubs such as *Salix spp., Populus balsamifera* L., and *Populus tremuloides* Michx.

(Hogan and Conly, 2002). Based on profile curvature, BC elements are analogous to CD elements and RT elements are analogous to CV elements (Yates et al., 2006a). Riparian (RG) grass elements and CX elements have dissimilar profile curvatures but they represent the driest landforms within the respective wetland type.

3.3.2 Soil sampling and soil properties

Each landform element was replicated in space (Bedard-Haughn et al., 2006b). For example, each uncultivated wetland contained a basin centre (BC) landform element. Because three uncultivated wetlands were sampled, there were three replicates (n = 3) of the BC element. Eighteen samples (2 land-uses × 3 wetlands × 3 landform elements) were collected on September 14, 2004. Each sample was a composite of 5 cores (0–15 cm; 15 cm diameter). Samples were placed on ice in coolers and transported to the laboratory where sub-samples were used immediately for DNA extraction. The remainder was air dried just enough (<24 h) to pass through a 2-mm sieve without smearing and stored at -20°C. Soil N (2 *M* KCl extracts) (Maynard et al., 2007), pH (1:2 soil:water extraction) (Miller and Curtin, 2007), and bulk densities (Maynard and Curran, 2007) were determined by standard methods and are listed in **Table 3.1**.

A total of 72 repacked soil cores [18 samples (2 landuses × 3 wetlands × 3 landforms) × 2 WFPS (50 and 70%) × 2 destructive sampling times (0 and 24 h)] were prepared. Soils were thawed from storage and packed into a 10-mL volume of 55-mL glass culture tubes (approximately 22 mm inner diameter) to yield bulk densities similar to those observed in the field (**Table 3.1**). Soil water content was determined using standard procedures with an assumed particle density of 2.65 g cm^{-3} (Topp and Ferré, 2002). Emitted N_2O is expressed per gram of dry soil.

Table 3.1 Some soil properties of the cultivated and uncultivated wetlands at the research site.

Land-use	Landform element	Soil properties[†]			
		NH_4^+ (μg N g^{-1} soil)	NO_3^- (μg N g^{-1} soil)	pH	Bulk density (g soil cm^{-3})
Cultivated	Convex (CX)	0.8 (0.4)	1.7 (0.2)	6.7 (0.2)	1.3 (0.0)
	Concave (CV)	2.9 (0.5)	2.1 (0.3)	6.6 (0.2)	1.3 (0.0)
	Depression centre (CD)	2.4 (0.8)	4.7 (0.1)	6.9 (0.0)	1.2 (0.0)
Uncultivated	Riparian grass (RG)	4.0 (0.5)	4.6 (0.1)	6.9 (0.0)	0.8 (0.1)
	Riparian tree (RT)	7.0 (3.1)	4.7 (0.2)	6.9 (0.1)	0.8 (0.1)
	Basin centre (BC)	2.6 (0.7)	4.7 (0.2)	7.1 (0.1)	0.8 (0.1)

[†] Results are means (n = 3) with standard errors in parenthesis.

After repacking, tubes were capped with parafilm and pre-incubated in the dark at room temperature (~23°C) for five days. After this 5-day period, water (0.5 ml) was added to moisten cores. Tubes were recapped with parafilm for two additional days of pre-incubation prior to the start of the experiment. At the start, 1.0 ml of a solution containing 1 mg NH_4^+-N L^{-1} and 1 mg 98%-enriched ^{15}N-NO_3^- L^{-1} was added to each tube. Water was then added to bring the soils to 50% or 70% WFPS. At time = 0 after WFPS adjustment, 36 tubes (18 samples × 2 WFPS) were destructively sampled for ammonium and nitrate by 2 *M* KCl extraction.

The remaining 36 tubes (plus 3 blank tubes) were capped with rubber septa. After a 24-h incubation at ~23°C, a 20 ml gas sample was collected with a syringe from each tube and injected into pre-evacuated (flushed with He prior to evacuation) 12 ml Exetainer® vials (Labco Limited, UK). The tubes were then destructively sampled for ammonium and nitrate.

Samples were analyzed at the University of California at Davis Stable Isotope Facilities via gas chromatography and mass spectrometry (Europa Hydra 20/20; SerCon Ltd., Crewe, UK) for net N_2 and N_2O emitted in 24 h as well as the $^{15}N_2$ and $^{15}N_2O$ content for estimation of the relative contribution of nitrification and denitrification to N_2O emissions (Stevens et al., 1997). The emitted N_2O was attributed to either denitrification (d'_D) of the ^{15}N-enriched NO_3^- pool or nitrification (d'_N) of the natural abundance NH_4^+ pool (Arah, 1997; Laughlin and Stevens, 2002). The diffusion disk technique described by Stark and Hart (1996) and modified by Bedard-Haughn et al. (2004) was used to collect soil ammonium and nitrate for mass spectrometry to determine N-pool enrichment and whether the labeled nitrate had cycled into the ammonium pool.

3.3.3 DNA extraction

The method described by Griffiths et al. (2000) was used, except that the soil mass was 0.7 g (wet mass) and centrifugation was carried out at 14 000× *g* (all PCR results are expressed per gram of dry soil).

3.3.4 PCR and DGGE analysis

Primer sets amoA-1F/amoA-2R (Rotthauwe et al., 1997) and nosZ-F/nosZ-R (Rich et al., 2003) with a GC-clamp (CCGCCGCGCGGCGGGCGGGGCGGGGGC-ACGGGG) on the 5'-ends of the forward primers were used to amplify partial gene fragments of *amoA* (~490 bp) and *nosZ* (~700 bp), respectively. PCR was done in 20 µL volume with 2.0 µL template DNA using the QuantiTect™ SYBR® Green PCR Master Mix with HotStart Taq® DNA Polymerase (Qiagen; Hilden, Germany) for *amoA* and the *Taq* PCR Master Mix system (Qiagen; Hilden, Germany) for *nosZ* and with the manufacture's recommended buffer, enzyme, and nucleotide conditions (1× Qiagen PCR buffer contains 1.5 m*M* $MgCl_2$, 2.5 units DNA polymerase, and 200 µ*M* of each dNTP). Final primer concentration was 0.5 µ*M* each. Template was amplified on a Robocycler Gradient 96 (Stratagene; California, USA) using the following conditions: 1) for *amoA* – 97°C for 20 min; 42 x (94°C, 40 s; 60°C, 40 s; 72°C, 1 min.); 72°C, 5 min.; 2) for *nosZ* – 94°C for 5 min; 35 x (94°C, 40 s; 56°C, 40 s; 72°C, 1 min.); 72°C, 5 min. PCR product was visualized by agarose gel electrophoresis and ethidium bromide staining.

Denaturing gradient gel electrophoresis (DGGE) was performed using the Bio-Rad DCode system (Bio-Rad Laboratories, Hercules, USA) (Muyzer et al., 1993). Aliquots of PCR product (containing approximately 500 ng of DNA) were mixed with the required amount of loading buffer (6×) and loaded onto 6% (w/v) polyacrylamide gradient gels (37:1 acrylamide/bis-acrylamide; 1.5 mm thick) in 0.5× TAE buffer (40 m*M* Tris, 20 m*M* acetic acid and 1 m*M* EDTA) after preconditioning of the gel at 60°C and 70V. The linear gradient used was from 40 to 60% denaturant, where 100% denaturing acrylamide was defined as containing 7 *M* urea and 40% (v/v) formamide. A 10 ml stacking gel containing no denaturants was added. Gels were run for 15 min at 70V and then for 16 h at 40V in 0.5× TAE buffer at a constant temperature of 60°C. To visualize bands, gels were stained with SYBR® Green I (Sigma-Aldrich Chemie GmbH, Steinheim, Germany; diluted in 2 µL in 20 ml 0.5× TAE) for 12 min with gentle agitation prior to

ultraviolet (UV) transillumination. Gel images were photographed with a Nikon CoolPix 4500 camera equipped with a SYBR® Green filter.

The two *amoA* DGGE bands found in UW soils were excised and the DNA was purified from gel (freeze-thawed in 0.5× TAE buffer then centrifuged at 14 000× *g* at 4°C) and amplified by PCR. These PCR products were cloned using the TOPO TA Cloning® Kit (Invitrogen, Burlington, Canada). Clones were sequenced (Plant Biotechnology Institute, Saskatoon, Canada) using the amoA-1F primer. Sequences were aligned with ClustalX (version 1.81) and visualized and edited with GeneDoc (version 2.6). A phylogenetic tree was created using the programs DNADIST (Jukes-Canter model), NEIGHBOR (neighbour-joining method; out-group: *Nitrosomonas europaea,* accession L08050), and SEQBOOT available in the PHYLIP (version 3.5c) computer package (http://evolution.genetics.washington.edu/phylip/software.html).

3.3.5 DGGE pattern analysis

DGGE patterns were processed and clustered using Bionumerics® v. 2.5 (Applied Maths, Kortrijk, Belgium). A similarity matrix between densitometric curves of band patterns was calculated by Pearson correlation and a dendrogram was constructed by Ward linkage method (Seghers et al., 2004). The cluster cophenetic correlation function was used to calculate cophenetic correlation coefficients which are indicators of cluster consistency. Values less than or equal to 50 would indicate random cluster occurrence. The cluster cutoff function was used to determine the level of similarity to prune the dendrogram for significant clusters (Bionumerics® manual 2.5).

3.3.6 Quantitative PCR

The amount of *amoA* and *nosZ* present in the soil DNA extracts was measured by quantitative PCR (q-PCR) using the QuantiTect™ SYBR® Green PCR Master Mix real-time PCR kit and an ABI 7500 real-time PCR machine (Applied Biosystems). The primer set nosZ-F/nosZ-R was used for the *nosZ* assay and amoA-1F/amoA-2R was used for the *amoA* assay. Each 25 µL reaction contained 12.5 µL of master mix, 10 pmol of the appropriate forward and reverse primers and 2 µL template DNA (~22.5 ng DNA μL^{-1}). For both gene fragments, the thermal cycling program was as follows: 97°C for 15 min.,

45 × (94°C, 20 s; 54°C, 40 s; 72°C, 40 s), and 77°C for 45 seconds followed by a melt curve from 50 to 95°C. The data was collected during the 77°C, 45 second step.

A standard curve for *nosZ* was generated using DNA extracted from the reference strain *Pseudomonas stutzeri* (ATTC 14405). The amount of DNA in the extract was determined spectrophotometrically and the number of *nosZ* copies calculated assuming the genome size was 6 Mbp and one copy of *nosZ* gene per genome. The *amoA* standard was purified PCR product from one of the soil DNA extracts amplified with amoA-1F/amoA-2R and the copy number calculated based on a 490 bp fragment. Both standard curves were linear over five orders of magnitude. For both assays, the efficiency of the reaction was 96% (based on the slope of the standard curves). The r^2 value for the standard curves was 0.97 for *amoA* and 0.99 for *nosZ*. One sharp peak was observed in the melt curve for the *nosZ* standard. A peak with shoulders was observed for *amoA* standard's melt curve.

Because matrix effects on PCR reactions may differ between soils, an analysis of amplification efficiency (based on (Mena et al., 2002)) between soils was done to validate q-PCR results. Efficiency of the q-PCR reactions was assessed by creating a five-point curve (in duplicate) of different DNA concentrations for each soil. The slope of the curve was determined using the LINEST function of Microsoft Excel XP (Microsoft Corp., Redmond, USA) and compared by one-way ANOVA.

3.3.7 Statistical analysis

Nitrous oxide gas data were imported to Minitab® (v. 11.21) and analyzed by two-way ANOVA with GLM ($\alpha = 0.1$) after verification that log-transformed data met the ANOVA assumptions (using Anderson-Darling test for normality and Bartlett's and Levene's tests for homogeneity of variance). The classification variables were land-use (CW and UW), landform (CX, CV, CD, RG, RT and BC) and WFPS (50 and 70%).

The q-PCR data were not normally distributed (and could not be transformed to yield a normal distribution); hence the data were analyzed using the non-parametric Kruskal-Wallis test. The multiple comparison extension was used to test for differences ($\alpha = 0.1$) between landform elements within a land-use class (CW *vs*. UW).

3.4 Results

3.4.1 N_2O emission source

From the incubations with ^{15}N-labeled nitrate, nitrification was the dominant N_2O emitting process from soils of all landforms except BC. At 50% WFPS, nitrification accounted for 99% of the N_2O emitted from all soils except for BC (32% from nitrification) (**Figure 3.1**A). There was no landform difference (except for BC) in N_2O emitted at this WFPS. The fraction of N_2O from denitrification increased at 70% WFPS for soils of all landforms except for CX and RG (**Figure 3.1**B). Within each land-use, soil N_2O emissions and N_2O attributable to denitrification increased as the profile curvature of the landform changed from water-shedding (e.g., CX) to water accumulating (e.g., CD). Land-use differences in N_2O emitted between soils from comparative landforms were only detected between CD and BC soils although there was a trend for greater N_2O emissions from UW soils than the comparative CW counterparts. There was no cycling of the labeled nitrate into the ammonium pool (unpublished data); therefore, the labeled N_2O gas detected was not from nitrification.

3.4.2 *nosZ* and *amoA* PCR-DGGE and q-PCR

Differences in PCR amplifiable nitrifier and denitrifier community compositions were expressed at the land-use level (CW *vs*. UW), but differences were not detected at the landform level. Nitrifier communities in these landscapes were simple (**Figure 3.2**). Regardless of landform, CW yielded a single-band DGGE pattern. Uncultivated wetland soils exhibited the same band plus an additional band that was lower in GC-content. Direct sequencing of each purified DGGE band resulted in unusable sequences. This suggested each band consisted of two or more dissimilar sequences. Cloning of the DNA from these DGGE bands provided the necessary resolution. Cloned *amoA* DNA sequences grouped into *Nitrosospira* Cluster 2 or Cluster 3 (represented by *N. briensis, N. multiformis* and *N. tenius*) (**Figure 3.3**). Clones T1, T2, B1 and B3 (T = sequence from DGGE band found only in UW soils; B = from DGGE band found in all soils) had 93 to 95% DNA sequence similarity to *Nitrosospira* sp. Nsp62 (accession AY123837).

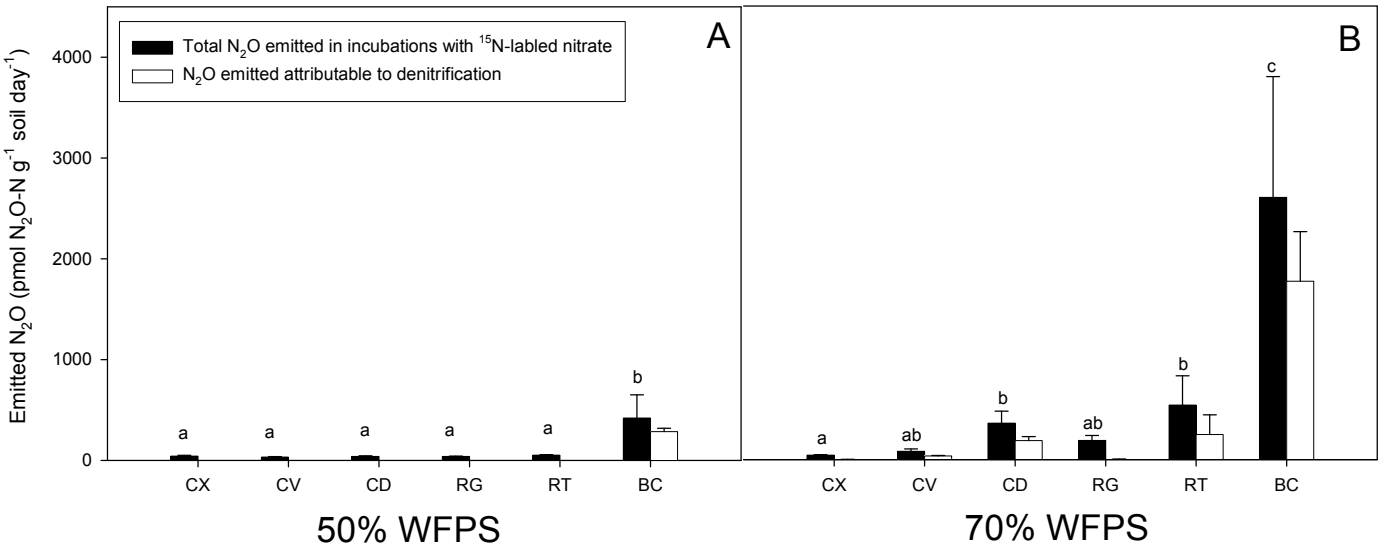

Figure 3.1 Total N_2O emissions and the portion of N_2O emissions attributable to denitrification in soils of cultivated and uncultivated wetlands as determined by ^{15}N-labeled NO_3^- isotope-ratio mass spectrometry. WFPS = water-filled pore space. Means labeled with the same letter are not significantly different ($\alpha = 0.1$). Reported N_2O emissions are means with standard error bar (n = 3). CX = Convex, CV = Concave, CD = Depression Centre, RG = Riparian Grass, RT = Riparian Tree, and BC = Basin Centre. Note: Results for N_2O attributable to denitrification from CV soils at 50% WFPS were not determined because of technical problems with mass spectrometer when the samples were analyzed.

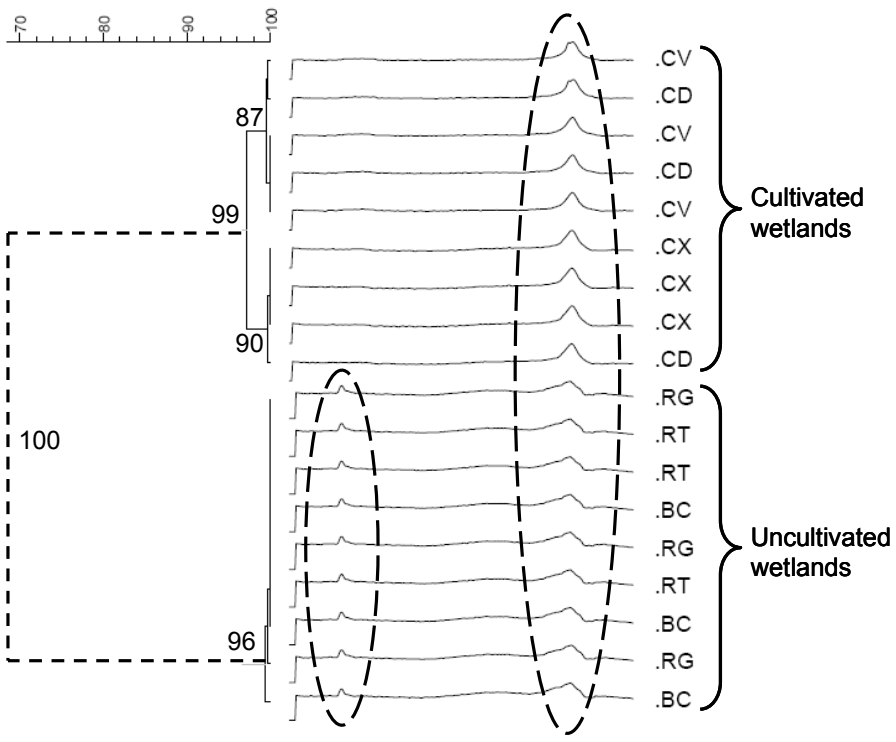

Figure 3.2 Cluster analysis of nitrifier *amoA* DGGE patterns based on densitometric curves. The scale above the dendrogram is percent similarity between DGGE patterns. The first three nodes are labeled with the cophenetic correlation coefficient. The dashed lines indicate the cluster cutoff level. Broken ovals highlight DGGE bands. CX = Convex, CV = Concave, CD = Depression Centre, RG = Riparian Grass, RT = Riparian Tree, and BC = Basin Centre.

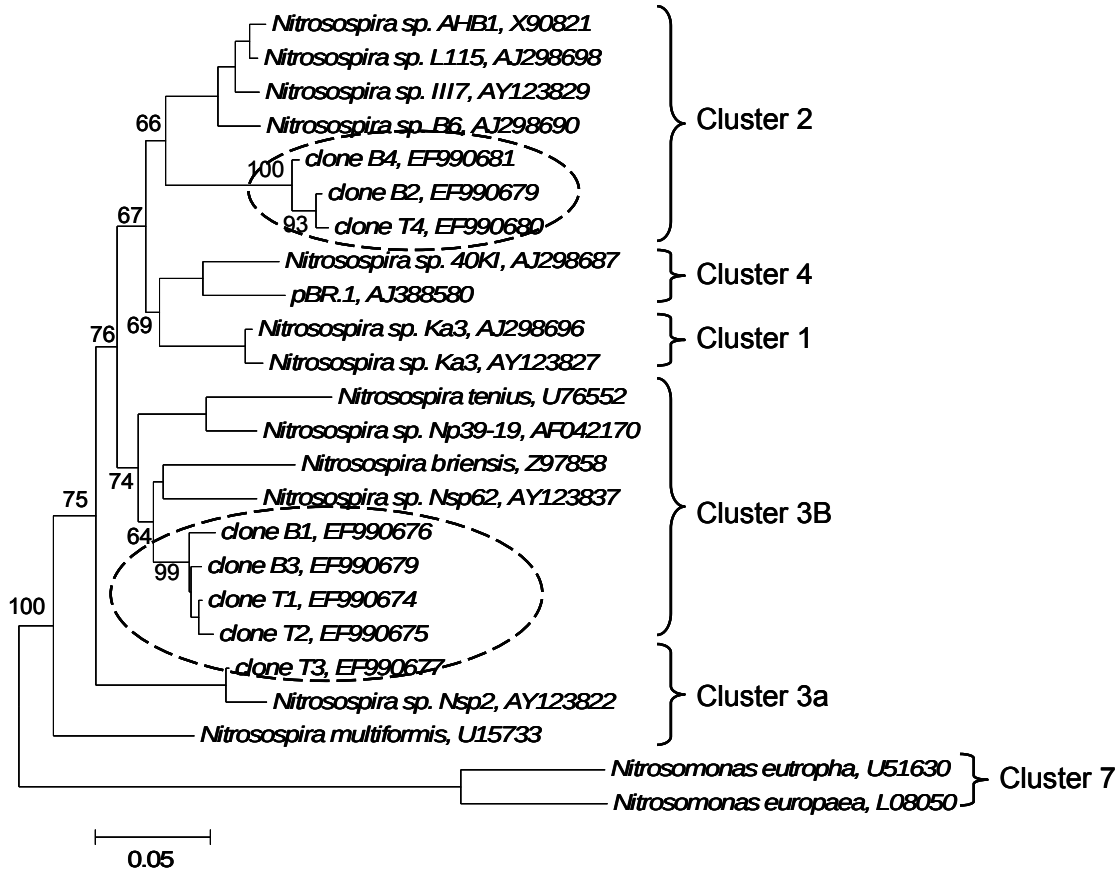

Figure 3.3 Phylogenetic tree of cloned ammonia monooxygenase subunit-A gene (*amoA*) fragments from bands of uncultivated wetland DGGE. Broken ovals highlight cloned sequences from this study. The label of the sequence used in the analysis is followed by their respective GenBank accession number. Branch nodes with bootstrap values greater than 60 are labeled. Scale bar indicates 5 changes per 100 nucleotide positions.

Although denitrifier DGGE patterns were more complex than nitrifier patterns, patterns for UW soils were visually distinguishable from those for CW (**Figure 3.4**). The land-use difference detected visually was confirmed by cluster analysis of the denitrifier DGGE patterns. There appeared to be landform differences in community composition because DGGE patterns clustered by landform; however, cluster cutoff analysis proved that significant clusters were based on land-use only. The abundance and distribution of nitrifier *amoA* copy number was affected by land-use (**Figure 3.5**), but denitrifier *nosZ* copy number was not (**Figure 3.6**). The median nitrifier *amoA* copy number was one order of magnitude larger ($P = 0.001$) in the CW soils (3.6×10^6 copies ng^{-1} DNA g^{-1} soil) than in the UW soils (3.7×10^5 copies ng^{-1} DNA g^{-1} soil). Unlike nitrifier *amoA*, denitrifier *nosZ* copy number was affected by landform in the uncultivated wetlands. There were 20% and 27% more (**Figure 3.6**; $P = 0.007$) *nosZ* copies in soils from the basin centre (BC) (1.5×10^5) than in soils from the riparian grassland (RG) (1.1×10^5) or riparian tree ring (RT) (1.2×10^5), respectively. There were no significant differences in *nosZ* copies between landforms in cultivated wetlands.

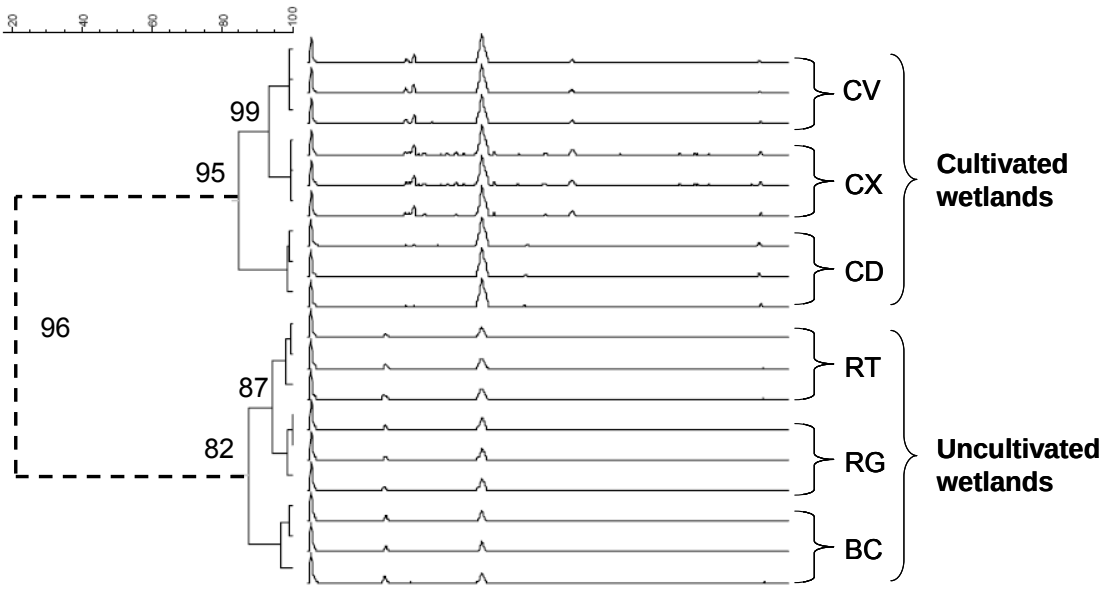

Figure 3.4 Cluster analysis of denitrifier *nosZ* DGGE patterns based on densitometric curves. The scale above the dendrogram is percent similarity between DGGE patterns. The first three nodes are labeled with the cophenetic correlation coefficient. The dashed lines indicate the cluster cutoff level. CX = Convex, CV = Concave, CD = Depression Centre, RG = Riparian Grass, RT = Riparian Tree, and BC = Basin Centre.

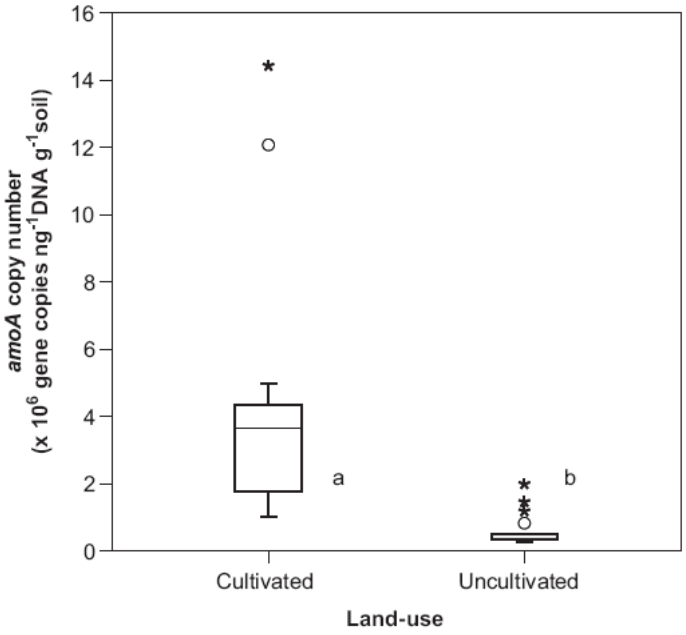

Figure 3.5 Boxplots of nitrifier *amoA* copy numbers as determined by q-PCR. Non-parametric Kruskal-Wallis test was used to determine difference between land-use ($\alpha = 0.1$; n = 27, i.e., 3 landforms × 3 replicate wetlands × 3 replicate q-PCR reactions per soil). Open circles denote outliers (1.5 to 3 × inter-quartile range) and stars denote extreme outliers (greater than 3 × inter-quartile range). Boxplots with the same letter are not significantly different.

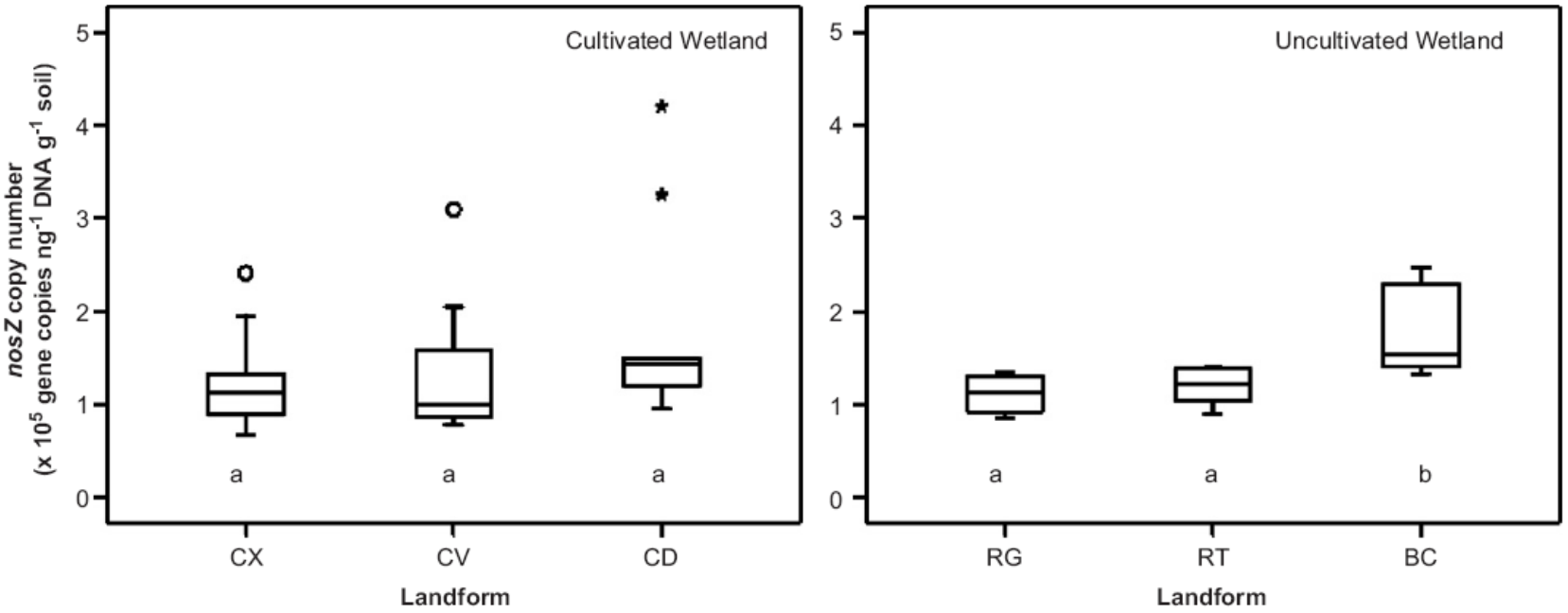

Figure 3.6 Denitrifier *nosZ* copy numbers in cultivated (Convex (CX), Concave (CV), Depression Centre (CD)) and uncultivated (Riparian Grass (RG), Riparian Tree (RT), Basin Centre (BC)) soils as determined by q-PCR. Non-parametric Kruskal-Wallis test with multiple comparison extension was used to determine difference between landform elements. Open circles denote outliers (1.5 to 3 × inter-quartile range) and stars denote extreme outliers (greater than 3 × inter-quartile range). Boxplots of landform elements ($\alpha = 0.1$; n = 9, i.e., 3 replicate wetlands × 3 replicate q-PCR reactions per soil) with the same letter are not significantly different.

3.5 Discussion

Nitrification was the dominant N_2O emitting process at 50% WFPS (the predominant moisture condition at the study site) for soils of all landforms except BC. Nitrifier activity at this WFPS results in a persistent background pattern of low N_2O emissions at the site (Yates et al., 2006b). The contribution of denitrification becomes more important at 70% WFPS for soils of all landforms except CX and RG. A WFPS of 70% is associated with event driven emissions such as those during spring thaw or precipitation (Yates et al., 2006a). We speculate that nitrifier denitrification may be an important pathway for N_2O emission from these soils. Nitrous oxide as a byproduct from nitrification (Arp and Stein, 2003) can explain the low emissions/high nitrification contribution pattern observed at 50% WFPS, as ammonia oxidation by AMO activity requires O_2. In contrast, at 70% WFPS, N_2O emission from nitrifiers likely occurs through nitrifier denitrification where nitrite (rather than labeled nitrate) produced during nitrification is reduced to N_2O when O_2 becomes limited (Poth and Focht, 1985). Alternatively, nitrogen dioxide (NO_2)-dependent ammonia oxidation by AMO may occur when O_2 is limited and NO_2 is used as the electron acceptor (Schmidt et al., 2002a; Schmidt et al., 2002b).

Nitrifier community composition and abundance was not predictive of N_2O emissions. If community composition and abundance was related to N_2O emission, differences in community composition and abundance should reflect differences in N_2O emission. While nitrifier communities (**Figure 3.2**) and abundance (**Figure 3.5**) differed by land-use, there was no difference in N_2O emissions between land-use at 50% WFPS except for soils from BC vs. CD landform (**Figure 3.1**A). If the N_2O attributed to denitrification is subtracted from the BC soil emission, there remains no emission difference between all landforms and land uses at 50% WFPS. Net emissions at 70% WFPS did not differ by land-use either except for CD vs. BC soil, but they differed by landform – a pattern not observed in nitrifier community composition or abundance. The landform difference in emission at 70% WFPS was the result of increased denitrification contributions.

The difference in nitrifier community composition and abundance between land-use types may be attributable to several of factors. Bruns *et al.* (1999) postulated that tillage increased soil habitat homogeneity which, in turn, decreased nitrifier richness. The application of anhydrous ammonia fertilizer in the cultivated depressions may have produced a shift in nitrifier community composition to more ammonia-tolerant nitrifiers (Horz et al., 2004). The recovered *amoA* sequences did cluster into two separate groups (**Figure 3.3**) – one belonging to *Nitrosospira* cluster 2 and the other belonging to *Nitrosospira* cluster 3. Isolates and 16S and *amoA* DNA sequences of *Nitrosospira* cluster 3 are usually associated with cultivated soils with high nitrogen input. Further work is needed to resolve whether either of these two DNA sequence groupings truly represent physiological differences to ammonia tolerance.

Greater abundance of *amoA* in the cultivated soils may reflect selective enrichment in response to the application of ammonia fertilizer (Bruns et al., 1999). Gross nitrogen mineralization and nitrification rates were previously found to be similar between the cultivated and uncultivated wetland soils throughout a growing season (Bedard-Haughn et al., 2006b), but the NH_4^+ pools were generally larger in uncultivated wetland soils (**Table 3.1**). This may be caused by ammonium assimilation into a larger nitrifier biomass, represented by greater *amoA* abundance, in the soils of cultivated wetlands. This may support the notion of r/K strategists amongst nitrifiers where r-strategists may quickly assimilate available ammonium for biomass and reproduction (Schramm et al., 1999) and may represent the previously discussed ammonia-tolerant nitrifiers (Horz et al., 2004).

Whether denitrifier community composition and abundance was predictive of N_2O emissions remains uncertain. At 50% WFPS, denitrifiers were irrelevant to N_2O emissions except in BC soils (**Table 3.1**A). Perhaps greater organic matter content in BC soils (Bedard-Haughn et al., 2006a) increased respiration creating anaerobic microsites with favourable conditions for denitrification to occur, i.e., heterotrophic O_2 consumption exceeding O_2 diffusion. At 70% WFPS, the pattern of denitrifier abundance (**Figure 3.6**) predicted the landform difference in N_2O emissions from soils of uncultivated wetlands (**Figure 3.1**B), i.e., greater abundance led to greater N_2O emission, but it was not predictive of the emissions from cultivated wetland soils. Denitrifier community

composition predicted the land-use difference in emissions between CD vs. BC soil. However, the pattern of landform difference in emissions was not detected for denitrifier communities.

Based on DGGE patterns, the composition of denitrifier communities appeared to increase in complexity in response to cultivation (**Figure 3.4**). Cultivation-induced increases in *nosZ* richness have been reported by other researchers (Rich and Myrold, 2004; Stres et al., 2004). Differences in land management at the St. Denis site have resulted in soils with differing soil properties (e.g., plant cover, soil organic carbon, inorganic nitrogen, tillage history, and bulk density), which, in turn, affect denitrifier community composition. We speculate that where cultivation decreased nitrifier community complexity (**Figure 3.2**) because of habitat homogenization, the same event redistributed organic carbon and nitrogen and increased aeration for heterotrophic activity. Therefore, a more complex denitrifier community may develop in cultivated soils.

The lack of denitrification contribution to N_2O emissions from CX and RG soils at 70% WFPS is puzzling (**Figure 3.1**B). The highly eroded nature of CX soils resulted in a soil with low organic matter (Pennock et al., 1994). This was reflected in the lowest amounts of ammonium and nitrate found in any landform (**Table 3.1**). However, the denitrifier community composition and abundance was not significantly different between CX and CD soils. Owing to the eroded nature of CX soils, perhaps the O_2 diffusion rate at 70% WFPS still exceeded the O_2 consumption rate by heterotrophic activity. This does not explain the lack of N_2O from denitrification in RG soils, where mineral N levels and denitrifier community (though lower abundance) were similar to those in the BC soils.

A direct link between nitrifier and denitrifier community composition and abundance to N_2O emissions remains elusive. Communities were clearly land-use specific but the difference was not related to land-use or landform differences in N_2O emission. This finding is similar to that of Rich and Myrold (2004) for denitrifiers in an agroecosystem. These authors suggested the relationship may be ecosystem specific because a tighter relationship between community composition and N_2O emission was found in meadow and forest soils (Rich et al., 2003). Soil properties may contribute more to activity than community composition and abundance. For example, in uncultivated

wetlands, higher organic matter content may promote heterotrophic activity while lower bulk density will lead to a larger volume of water at the same WFPS, which may restrict O_2 diffusion. Therefore, there is greater N_2O emission from denitrification for soils in uncultivated wetlands than from comparable landform elements from cultivated wetlands.

4 TEMPORAL DIFFERENCES IN MICROBIAL COMMUNITY COMPOSITION ARE CORRELATED TO CHANGES IN NITROUS OXIDE EMISSIONS FROM EPHEMERAL WETLAND SOILS

4.1 Abstract

Nitrous oxide (N_2O) is a greenhouse gas with 300 times the global warming potential of CO_2 and is a product of nitrification and denitrification processes. Consequently, the rates of these two microbially mediated processes and the controls on these rates are important determinants on soil N_2O emissions. We hypothesized that N_2O associated with nitrification and denitrification is linked to an increase or decrease in abundance of specific nitrifier or denitrifier genotypes over the course of a growing season. The denitrifying enzyme activity assay (DEA) and $^{15}NO_3^-$ pool dilution method were used to compare the rates of denitrification and nitrification and their associated N_2O emissions. Community composition was measured with restriction fragment length polymorphism (RFLP) profiles of nitrifier *amoA* and denitrifier *nosZ*. Community abundance was measured with quantitative polymerase chain reaction (q-PCR). The relationship between community composition and N_2O emitting processes was evaluated using a Non-metric Multidimensional Scaling (NMS) multivariate ordination technique. The change in denitrifier *nosZ* abundance and community composition during the course of a growing season was a good predictor of net soil N_2O emission. For the nitrifiers, neither *amoA* abundance nor community composition had predictive relationships with nitrification associated N_2O emissions.

Key words: *amoA*, denitrification, N_2O, nitrification, *nosZ*

4.2 Introduction

Nitrification and denitrification are important, microbially mediated processes in the global nitrogen (N) cycle. Nitrification is the oxidation of NH_4^+ or NH_3 to NO_3^- via

NO_2^- (Horz et al., 2004). Denitrification is the respiratory reduction of NO_3^- and NO_2^- to gaseous products, mainly N_2O and N_2 (Tiedje, 1994). Because N_2O, a greenhouse gas with 300 times the global warming potential of CO_2 (Jungkunst and Fiedler, 2007), can be produced by nitrification and denitrification (Wrage et al., 2005), the rates of these two processes and the controls on the rates of these two processes are important determinants on soil N_2O emissions.

Available C, N, and O_2 (Avrahami et al., 2002; Cavigelli and Robertson, 2000; Svensson et al., 1991) are three factors that control the rates of N_2O production/consumption via nitrification and denitrification. However, these factors act through the microbial community whose composition and abundance reflects the long-term climate, soil disturbance history, and resource availability imposed on soils (Cavigelli and Robertson, 2000; Rich et al., 2003). Traditionally, members of a microbial community are thought to be equivalent in function if they have a similar array of genes and enzymes (Cavigelli and Robertson, 2000). However, evidence indicates that differences in nitrifier and denitrifier community composition affects rates of nitrification and denitrification, which in turn influences N_2O emissions (Avrahami et al., 2002; Cavigelli and Robertson, 2000; Mintie et al., 2003; Rich et al., 2003; Webster et al., 2005). This suggests either whole community adaptation or a change in the relative importance of certain members of the community.

Land-use and landform (together referred to as landscape) are perhaps the two most important long-term determinants of microbial community structure and controls on nitrification and denitrification. The effects of land-use mainly influence nutrient availability (through fertilization and cropping) and soil disturbance (through tillage) (Bruns et al., 1999; Stres et al., 2004). Landform affects O_2 availability, nutrient distribution, and biological productivity through redistribution of water (Hayashi et al., 1998; Yates et al., 2006b).

The objective of this study was to examine the spatial and seasonal variation in nitrifier and denitrifier community composition and abundance and N cycling processes in soils from two landscapes common to the North American prairie pothole region – cultivated and uncultivated wetlands. Because soils of these two landscapes differed in available N (Ma et al., 2008a), soil organic carbon content (Bedard-Haughn et al., 2006a),

and water regime (Yates et al., 2006b), we hypothesized that N_2O associated with nitrification and denitrification is linked to changes in abundance of specific nitrifier or denitrifier genotypes over the course of a growing season. Accordingly, the composition of these microbial communities is expected to differ between the landscapes over time. The denitrifying enzyme activity assay (DEA) and $^{15}NO_3^-$ pool dilution method were used to compare rates of denitrification and nitrification and the associated N_2O emissions. Community composition was measured with restriction fragment length polymorphism (RFLP) profiles of nitrifier *amoA* and denitrifier *nosZ*. Community abundance was measured with quantitative polymerase chain reaction (q-PCR). The relationship between community composition and N_2O emitting processes was evaluated using a Non-metric Multidimensional Scaling (NMS) multivariate ordination technique.

4.3 Experimental Procedures

4.3.1 Study Site

The St. Denis National Wildlife Area (SDNWA) in central Saskatchewan, Canada (52°12'N, 106°5'W), is a typical landscape of the North American prairie pothole region. It contains 216 wetlands distributed over an area of 3.84 km^2 (Hogan and Conly, 2002). The SDNWA is in the Dark Brown soil zone with loamy unsorted glacial till (Weyburn Association) parent materials and slope classes ranging from 10 to 15% (Miller et al., 1985). Within the SDNWA, six ephemeral wetlands were selected: three cultivated and three uncultivated. Ephemeral wetlands are those depressions in hummocky terrains that contain standing water in the spring, but typically dry out during the growing season (Hayashi et al., 1998).

A detailed topographic survey of the site was completed and a digital elevation model was produced with a 5-m × 5-m grid cell extent (Yates et al., 2006b). Relative elevation and visual inspection of the site was used to segment the site into a set of landscape elements defined by landform (profile curvature) and land-use (cultivated and uncultivated). Locations in cultivated wetlands (CW) were classified as either convex (CX), concave (CV), or cultivated depression centre (CD). Convex elements were topographically high positions with a positive profile curvature. Concave elements were

positions with negative profile curvature. Cultivated depression centre elements were level positions, roughly circular in shape, which collect rain or snowmelt water.

Non-agricultural portions of the site included vegetated depressions and were classified as uncultivated wetlands (UW). Uncultivated wetlands were further sub-divided into three landform elements. The basin center (BC) is a level area covered by a variety of 99 non-grasses surrounded by a non-level fringe area covered with grasses such as *Bromus inermis* Leyss [riparian grass (RG)]. The outer region of these wetlands [riparian trees (RT)] consists of a partial fringe of mixed trees and shrubs such as *Salix spp., Populus balsamifera* L., and *Populus tremuloides* Michx. (Hogan and Conly, 2002). Based on profile curvature, where landform elements were classified as water-shedding or water-accumulating, BC elements are analogous to CD elements, and RT elements are analogous to CV elements (Yates et al., 2006b). Riparian grass (RG) elements and CX elements have dissimilar profile curvatures, but they represent the driest landforms within the respective wetland type and were therefore considered to be analogous. Soil types ranged from thin Chernozemic Rego Dark Brown (Typic Calciborolls) at CX elements through to thicker Chernozemic Orthic Dark Brown (Typic Haploborolls) in CV, RG and RG elements, to Chernozemic Eluviated Dark Brown (Albic Argiborolls) and Gleysolic Humic Luvic (Argic Cryaquolls) in CD and BC elements. Soil textures range from loam at topographically high positions to silt loam in depressions.

4.3.2 Soil sampling

Each landform element was replicated (n = 3) in space (Bedard-Haughn et al., 2006b). In all, 18 samples (2 land-uses × 3 wetlands × 3 landform elements) were collected on each of four sampling dates [June 1 (Ordinal day 152), July 13 (Ordinal day 194), August 16 (Ordinal day 228), and September 12, 2006 (Ordinal day 255)]. Each sample was a composite of 5 cores (0–15 cm; 15 cm diameter). Samples were placed on ice in coolers and transported to the laboratory where sub-samples were used immediately for denitrifying enzyme activity (DEA), gravimetric soil water content determination, and DNA extraction. The remainder was air dried just enough (<24 h) to pass through a 2-mm sieve without smearing and stored at -20°C.

4.3.3 Soil denitrifying enzyme activity (DEA) assay

Because N_2O is produced by nitric oxide reductase (NOR) and consumed by nitrous oxide reductase (NOS), N_2O accumulates in the headspace under two conditions: 1) after NOR but before NOS is induced, and 2) after the entire denitrification pathway is induced but environmental conditions inhibit NOS activity more than NOR activity (Cavigelli and Robertson, 2000). The DEA assay is designed to eliminate environmental constraints and measure the current status of denitrification enzyme expression in soil. It requires the measurement of N_2O accumulation in the headspace of vessels with or without C_2H_2. The denitrification rate as determined by the incubation with C_2H_2 (DEA) only confirms the activity of NOR (i.e., N_2O production from reduction of NO). Nitrous oxide formation/accumulation (N_2O_f) in the incubation without C_2H_2 allows for the assessment of N_2O consumption by NOS activity. The rate of N_2O accumulation (rN_2O) is the ratio of N_2O_f and DEA ($rN_2O = N_2O_f/DEA$). The value of rN_2O will range between 1 (N_2O_f = DEA) and zero. The value of rN_2O approaches zero when N_2O consumption by NOS activity exceeds N_2O production by NOR.

Each soil sample was assessed for DEA on the day of sampling. The assay involved measuring the N_2O formed after incubating anaerobic slurries for 3 h at ~23°C. Each DEA slurry contained 10 g soil (wet mass); 10 ml of a solution containing glucose (10 mM) and NO_3^- (5 mM); and C_2H_2 (10%, v/v) in a 70-ml crimp-sealed serum bottle (Rich and Myrold, 2004). The N_2O_f was also was measured in anaerobic slurries that received the same treatment as DEA but without the C_2H_2, and the ratio of N_2O_f to DEA calculated (Cavigelli and Robertson, 2000). A 20-ml gas sample was withdrawn from the headspace of the slurry using a 20 cc disposable syringe equipped with a 25-gauge needle and injected into a pre-evacuated 12-ml Exetainer® vial (Labco Ltd., UK). Concentrations of N_2O in the headspace gas were determined using a gas chromatograph equipped with an electron capture detector (Yates et al., 2006b). All values are expressed per gram of oven-dried soil.

4.3.4 ^{15}N stable isotope incubation

Soil cores were prepared by packing the processed field soils into a 10 ml volume in 55-ml glass culture tubes (22 mm inner diameter) to yield bulk densities similar to

those observed in the field (Ma et al., 2008a). Gravimetric soil water content was determined using standard procedures, and an assumed particle density of 2.65 g cm^{-3} was used for the calculation of water-filled pore space (Topp and Ferré, 2002). After packing, tubes were capped with parafilm and pre-incubated in the dark at room temperature (~23°C) for five days. After this pre-incubation period, deionized water (0.5 ml) was added to moisten cores and the tubes were recapped with parafilm and stored for an additional two days prior to introduction of the ^{15}N-labeled NO_3^-. The soils were labeled by adding 1.0 ml of a solution containing 2 mg 98%-enriched ^{15}N-NO_3^- L^{-1} to each tube. The soils were then brought to 70% WFPS with deionized water. At time = 0 (i.e., immediately after WFPS adjustment), half the repacked cores were destructively sampled for ammonium and nitrate (**Table 4.1**) using a 2 M KCl extraction (Maynard et al., 2007). The remaining tubes (plus 3 blank tubes) were capped with rubber septa and incubated for 24-h at ~23°C. At t = 24 h, a 20 ml gas sample from each tube was collected with a syringe and injected into pre-evacuated (flushed with He prior to evacuation), 12-ml Exetainer® vials (Labco Limited, UK). The cores were then destructively sampled for ammonium and nitrate.

Gas and 2 M KCl extractable N samples were analyzed at the University of California at Davis Stable Isotope Facility using gas chromatography coupled with isotope ratio mass spectrometry (Europa Hydra 20/20; SerCon Ltd., Crewe, UK). Total N_2 and N_2O produced in 24 h together with the $^{15}N_2$ and $^{15}N_2O$ produced were used to estimate the relative contribution of nitrification and denitrification to N_2O emissions (Stevens et al., 1997). The emitted N_2O was attributed to either denitrification (d'_D) of the ^{15}N-enriched NO_3^- pool or nitrification (d'_N) of the natural abundance NH_4^+ pool (Arah, 1997; Laughlin and Stevens, 2002). The diffusion disk technique (Stark and Hart, 1996) as modified by Bedard-Haughn et al. (2004) was used to collect soil ammonium and nitrate from KCl extracts. Total NH_4^+/NO_3^- and $^{15}NH_4^+/^{15}NO_3^-$ was used to determine nitrification rates by the pool dilution method and to check whether cycling of labeled N into the ammonium pool had occurred (Bedard-Haughn et al., 2006b).

Table 4.1 2 M KCl extractable ammonium and nitrate concentrations during sampling period in 2006.

	Ammonium (μg N g^{-1} soil)†				Nitrate(μg N g^{-1} soil)†			
Landform	Jun 01	Jul 13	Aug 16	Sep 12	Jun 01	Jul 13	Aug 16	Sep 12
Convex (CX)	1.5 (0.2)	1.1 (0.2)	0.9 (0.2)	0.9 (0.2)	2.1 (0.1)	1.8 (0.3)	2.1 (0.2)	1.8 (0.2)
Concave(CV)	3.3 (0.1)	3.1 (0.2)	2.7 (0.1)	2.6 (0.2)	2.0 (0.3)	2.1 (0.2)	1.7 (0.2)	2.1 (0.1)
Cultivated Depression (CD)	3.0 (0.2)	3.0 (0.2)	2.6 (0.2)	2.7 (0.3)	4.2 (0.3)	4.8 (0.4)	4.1 (0.4)	4.6(0.1)
Riparian Grass (RG)	5.1 (0.2)	4.8 (0.1)	4.4 (0.3)	4.5 (0.5)	4.7 (0.3)	4.4 (0.1)	4.2 (0.3)	4.4 (0.1)
Riparian Tree (RT)	6.3 (0.3)	5.5 (0.2)	4.7 (0.4)	4.0 (0.4)	4.5 (0.2)	5.0 (0.4)	4.4 (0.2)	4.6 (0.1)
Basin Centre (BC)	3.6 (0.2)	3.0 (0.3)	3.0 (0.1)	2.6 (0.7)	4.6 (0.4)	4.3 (0.4)	4.8 (0.3)	4.4 (0.2)

† Results are means (n = 3) with standard errors in parenthesis.

4.3.5 DNA extraction from soils treated with ethidium monoazide bromide (EMA)

Prior to DNA extraction, soil samples were treated with EMA to differentiate between DNA from viable versus non-viable microorganisms (Nogva et al., 2003). Ethidium monoazide bromide can intercalate double-stranded DNA and prevent its replication during PCR. Because EMA cannot enter intact cells, it can only bind to extracellular DNA or DNA in cells with compromised membranes. The EMA treatment of soils was used as described by Pisz et al. (2007). Soil DNA was extracted using the method described by Griffiths et al. (2000) except the soil mass was 1.0 g (wet mass) and centrifugation was at 14 000× *g*. Results were expressed g^{-1} dry soil.

4.3.6 Quantitative PCR

Quantitative PCR (q-PCR) was performed on all samples to determine the abundance of *amoA* and *nosZ*, using the procedures and conditions reported by Ma et al. (2008a). Briefly, the primer sets amoA-1F/amoA-2R (Rotthauwe et al., 1997) and nosZ-F/nosZ-R (Rich et al., 2003) were used to amplify *amoA* and *nosZ*, respectively. Prior to q-PCR, all DNA extracts were diluted to the same concentration. Amplification was carried out using the QuantiTect™ SYBR® Green PCR Master Mix real-time PCR kit (Qiagen). Thermal cycling and quantification was carried out using an ABI 7500 real-time PCR machine (Applied Biosystems). For *nosZ*, the standard curve was generated with DNA from *Pseudomonas stutzuri* (ATCC 14405). The standard for *amoA* was the amoA-1F/amoA-2R amplified PCR product from one of the soil extracts. Differences in amplification efficiency between samples were tested according to Mena et al. (2002).

4.3.7 Cloning, RFLP, and phylogenetic analysis of PCR products

Previous work failed to demonstrate a detectible landform difference in *amoA* and *nosZ* community composition (Ma et al., 2008a). Therefore, we limited the community composition analysis here to soil samples from the water accumulating landforms (i.e., the CD and BC elements). The analysis also was limited to samples from the start (June 1)

and end (September 12) of the sampling season because the greatest difference in gene abundance and measured activity occurred between these two dates.

Fragments of *amoA* and *nosZ* were amplified, cloned, and analyzed for restriction fragment length polymorphism (RFLP). Procedures and conditions for amplifiying *amoA* and *nosZ* fragments were described previously (Ma et al., 2008a). PCR products of the expected size (490 bp for *amoA* and 700 bp for *nosZ*) were excised after agarose gel electrophoresis, purified using QIAquick® Gel Extraction Kit (Qiagen), and cloned using TOPO® TA Cloning Kit (Invitrogen). Forty-eight clones were selected for each sample and gene combination. Clones were screened for the proper inserted fragment by PCR product size. The PCR product for each clone was then used in three separate reactions with the endonucleases *Alu*I, *Hha*I, and *Rsa*I (Invitrogen) and visualized by agarose gel electrophoresis (3% w/v gel; 80V for 90 min). Clones were classified into operational taxonomic units (OTUs) based on the combination of the three separate RFLP patterns.

Based on Indicator Species Analysis (see section 4.3.8), five clones from each indicator OTU – clones that differed significantly between land-use and time for each gene – were sequenced at the National Research Council Plant Biotechnology Institute (Saskatoon, Canada) using the amoA-1F or nosZ-F primer. A consensus sequence for each OTU was generated by alignment in ClustalX (v1.81) and edited with GeneDoc (v2.6). Phylogenetic trees using the consensus sequences were created using the programs DNADIST (Jukes-Canter model), NEIGHBOR (neighbor-joining method; out-group = *Nitrosomonas europaea* accession L08050 for *amoA* and *Ralstonia eutropha* accession X65278 for *nosZ*), and SEQBOOT available in the PHYLIP (v3.5c) computer package. Nucleotide sequences of the indicator *amoA* OTUs were assigned GenBank accessions EU395816 to EU395820 and the indicator *nosZ* OTUs were assigned accessions EU395821 to EU395825.

4.3.8 Statistical analyses

Data were imported into SPSS 14.0 and transformed where necessary to meet ANOVA assumptions (using the Anderson-Darling test for normality and Bartlett's and Levene's tests for homogeneity of variance). Pearson correlations were used to examine

potential temporal relationships between *amoA* and *nosZ* abundance with the corresponding N_2O emitting functions.

Land-use and seasonal differences in the community composition based on OTUs were graphically examined by Non-metric Multidimensional Scaling (NMS) using the autopilot program with the slow and thorough analysis option and the default settings in PC-ORD v4.0 (McCune and Mefford, 1999). Non-metric Multidimensional Scaling is a non-parametric ordination method suited to community data because it avoids the assumptions about the underlying structure of the data made by other ordination methods (Clarke, 1993; Kenkel and Orlóci, 1986). Activity/functional variables (e.g., nitrification rate for *amoA* and DEA for *nosZ*) were correlated to NMS axes to evaluate the relationship between community composition and measured functions. Coefficients of determination (r^2) between functional variables and NMS axes were displayed as vectors radiating from the centroid of the NMS plot. The vector is the hypotenuse of a right triangle whose sides represent the r^2 of the function to the individual NMS axes (McCune and Mefford, 2002). A Multi-Response Permutation Procedure (MRPP) (Zimmerman et al., 1985) with Sørensen's distance was used to test the hypothesis of no difference in community composition between land-use and time. The MRPP *T*-statistic describes the separation between groups (the more negative the *T*-value, the stronger the separation); the *A*-statistic describes within-group relatedness relative to that expected by chance alone (if $A = 1$, all items in a group are homogeneous; if $A = 0$, there is no similarity between items in a group) (McCune and Mefford, 2002). Indicator Species Analysis was used to identify OTUs (note that an OTU is functionally defined as a "species" in this analysis) that differentiated communities by land-use and time (Rich et al., 2003; Rich and Myrold, 2004). The significance ($\alpha = 0.1$) of the indicator values were tested using a Monte Carlo simulation of 1000 runs where samples were randomly reassigned to groups and indicator values recalculated.

4.4 Results

4.4.1 Nitrifier and denitrifier abundance and activity

Regardless of land-use or landform, nitrifier abundance increased up to 10-fold during the course of the sampling season (**Figure 4.1**A and D). Nitrification rates (**Figure 4.1**B and E) and nitrification-associated N_2O emissions (**Figure 4.1**C and F), in contrast, declined up to three-fold during the same period. Negative correlations (**Table 4.2**) between nitrifier abundance and nitrification rate (r = -0.466) and nitrification-associated N_2O (r = -0.267) were significant (α = 0.01). Nitrification rate and nitrification-associated N_2O emission were positively correlated (r = 0.344).

Contrary to nitrifier *amoA* abundance, denitrifier *nosZ* abundance declined during the sampling season in all soils (**Figure 4.2**A and E). The gross potential N_2O production activity as described by the DEA results did not change during the time-course (**Figure 4.2**B and F). However, the $r N_2O$ ratio increased over time (**Figure 4.2**C and G) and was negatively correlated to *nosZ* abundance (**Table 4.2**). Denitrifier abundance and DEA was positively correlated to percent water-filled pore space (WFPS) at the time of soil sampling while $r N_2O$ was negatively correlated to WFPS (**Table 4.2**). The greatest differences in community abundance and activity occurred between the first and last sampling dates and similar temporal patterns were observed for each landform. Thus additional analyses to relate community composition to N_2O emitting activity were conducted using only the CD and BC samples (i.e., water accumulating landforms) collected on June 1 and Sep.12.

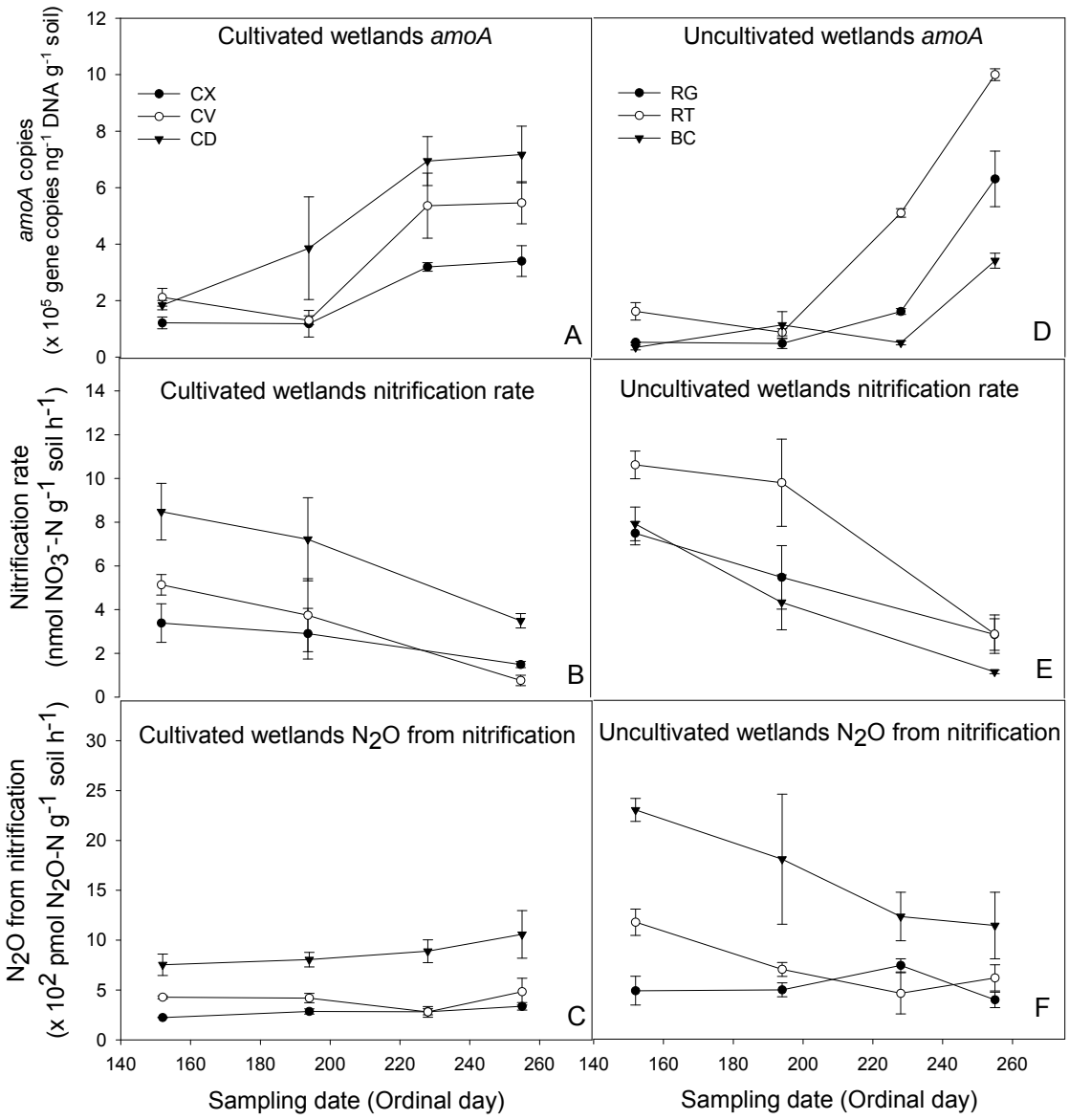

Figure 4.1 Abundance of nitrifier *amoA* copies (panels A and D), nitrification rate (panels B and E), and N_2O emission attributable to nitrification (panels C and F) for cultivated (panels A, B and C) and uncultivated (panels D, E and F) wetland soils. Locations in cultivated wetlands (CW) were classified as either convex (CX), concave (CV), or cultivated depression centre (CD). Uncultivated wetlands were divided into three landform elements, basin center (BC), surrounded by a non-level fringe area covered with grasses (RG) and an outer region consisting of trees of shrubs (RT). Based on profile curvature, BC elements are analogous to CD elements, and RT elements are analogous to CV elements. Riparian grass (RG) elements and CX elements represent the driest landforms within the respective wetland type. Reported values are means (n = 3) with standard error bars.

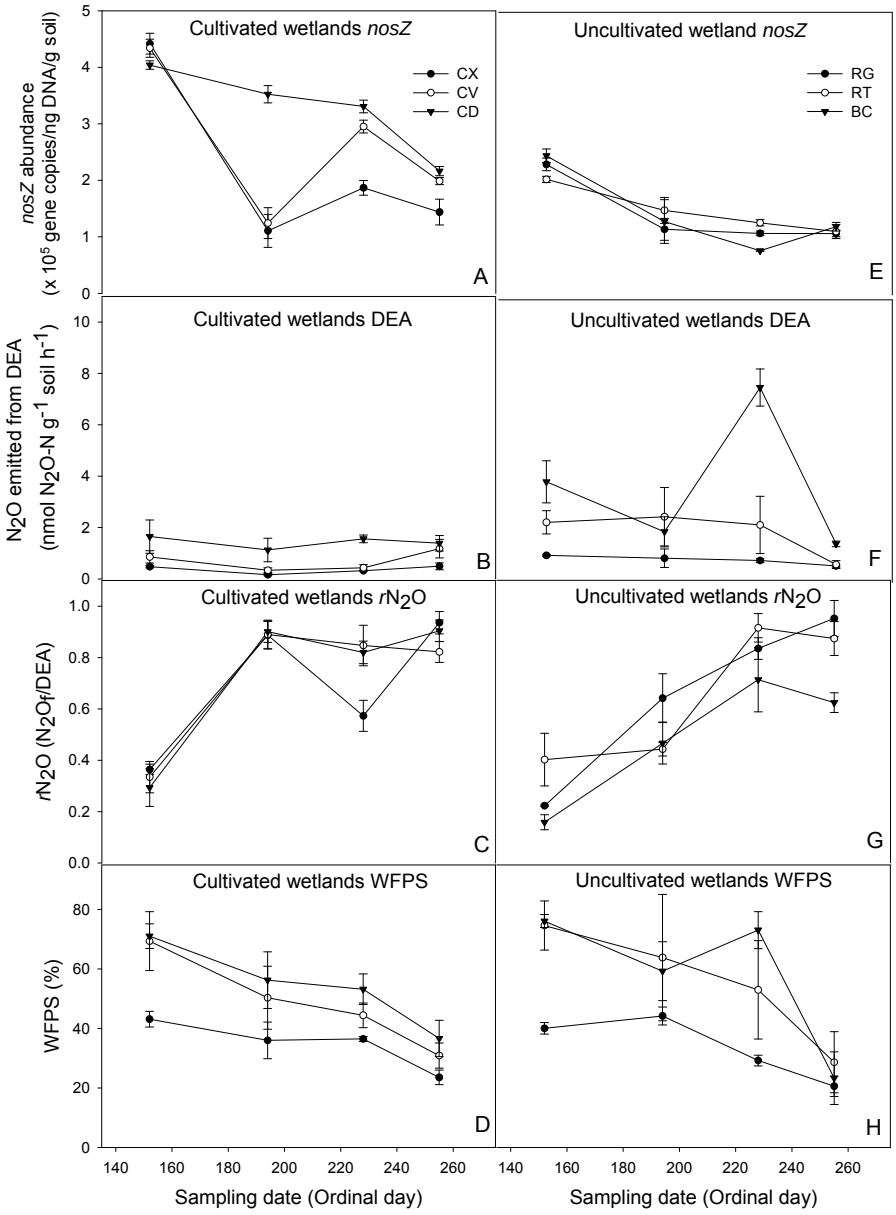

Figure 4.2 Abundance of denitrifier *nosZ* copies (panels A and E), N_2O emitted from denitrification enzyme activity assay (DEA; panels B and F), rN_2O (N_2O_f/DEA; panels C and G), and % water-filled pore space (WFPS; panels D and H) for cultivated (panels A, B, C and D) and uncultivated (panels E, F, G and H) wetland soils. Locations in cultivated wetlands (CW) were classified as either convex (CX), concave (CV), or cultivated depression centre (CD). Uncultivated wetlands were divided into three landform elements, basin center (BC), surrounded by a non-level fringe area covered with grasses (RG) and an outer region consisting of trees of shrubs (RT). Based on profile curvature, BC elements are analogous to CD elements, and RT elements are analogous to CV elements. Riparian grass (RG) elements and CX elements represent the driest landforms within the respective wetland type. Reported values are means (n = 3) with standard error bars.

Table 4.2 Pearson correlation coefficients between *amoA* and *nosZ* abundance with ^{15}N-N_2O emission for nitrification experiment and denitrification enzyme assay, respectively.

	amoA	Nit.†	N_2O‡	$[NH_4^+]$		*nosZ*	DEA	*r*N_2O	WFPS
amoA	1	-0.466**	-0.267**	-0.282**	*nosZ*	1	0.317**	-0.234*	0.387**
Nit.		1	0.344**	0.378**	DEA		1	-0.113	0.575**
N_2O			1	-0.231*	*r*N_2O			1	-0.361**

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

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

† Nit. = Nitrification; as determined by the pool dilution method from incubations with $^{15}NO_3^-$.

‡ Portion of N_2O attributable to nitrification in ^{15}N stable isotope incubations.

4.4.2 Relating nitrifier and denitrifier community composition, abundance and activity

Denitrifier community composition differed by land-use and time within soils of water-accumulating landforms (**Figure 4.3**). The NMS identified a two-dimensional solution for the differences in denitrifier community composition. The coefficient of determination (r^2) between denitrifier operational taxonomic units (OTUs) with each dimension (axis) was 0.40 and 0.29 for the first and second axes, respectively. Therefore, 69% (sum of r^2-values for the two axes) of the true variance structuring the denitrifier communities was accounted for by this ordination solution. Axis 1 separated the denitrifier communities by land-use while Axis 2 separated them by time.

The Multi-Response Permutation Procedure (MRPP) confirmed that there was significant difference between communities as the result of land-use and time (**Table 4.3**). The joint plot of *nosZ* abundance, DEA, rN_2O, and WFPS indicated these parameters correlated strongly with the time gradient (Axis 2) of the NMS plot. That is, the difference in denitrifier community composition over time was correlated to difference in denitrifier abundance and activity and soil moisture at sampling time. Note, only functions with $r^2 \geq 40\%$ to either NMS axes are shown in joint plots.

Table 4.3 Results of the Multi-Response Permutation Procedure (MRPP) testing of the null hypothesis of no significant difference in denitrifier *nosZ* community composition between land-use and time (Date).

Land-use	Date	Average distance	*N*	MRPP statistics
Cultivated wetlands	Jun 01	0.9560	3	Observed delta = 0.1367
	Sep 12	0.1740	3	Expected delta = 0.7009
Uncultivated wetlands	Jun 01	0.1884	3	T = -7.2334, A = 0.8050
	Sep 12	0.8877	3	$P < 0.01$

Average distance is the mean Euclidean distance between each pairwise combination of land-use and sampling date: *N* is the number of replicate wetlands sampled. The observed delta is calculated from the data while the expected delta is derived from a null distribution: *T* is the MRPP test statistic, and *A* is the chance corrected within-group agreement.

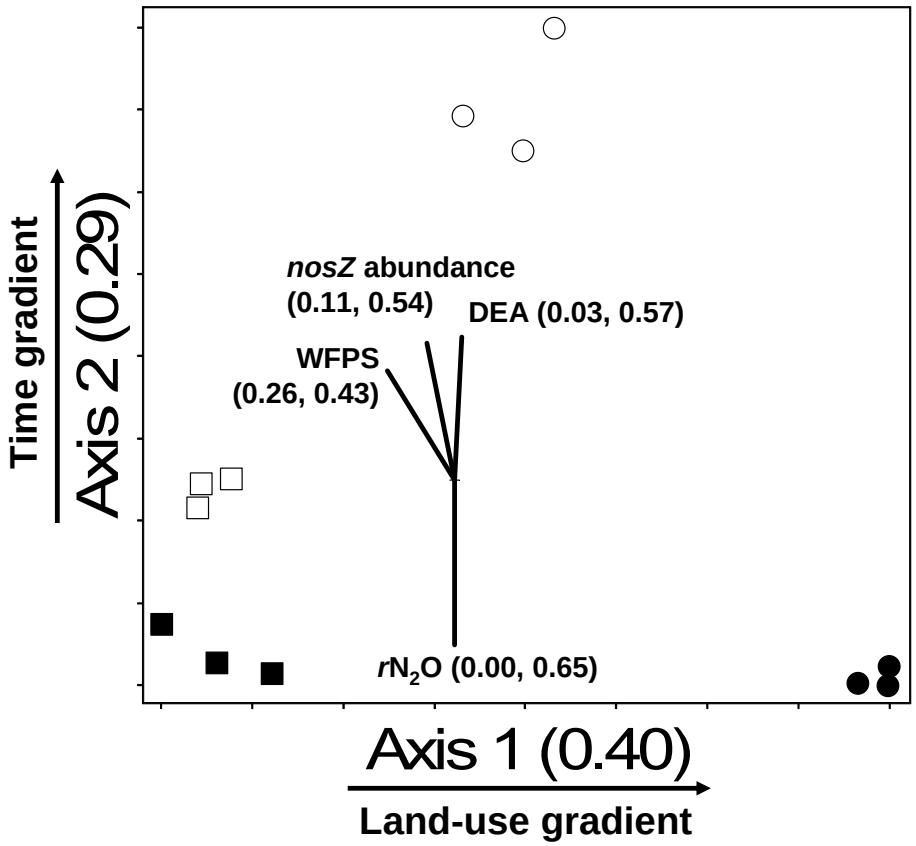

Figure 4.3 Non-metric Multidimensional Scaling (NMS) ordination of denitrifying communities based on presence/absence of operational taxonomic units (OTUs) defined by *nosZ* RFLP patterns. □ = Jun 01; cultivated wetlands. ■ = Sep 12; cultivated wetlands. ○ = Jun 01; uncultivated wetlands. ● = Sep 12; uncultivated wetlands. Vectors show the direction and magnitudes of the coefficient of determination (r^2) between NMS ordination axes and functional variables. The r^2 for the correlation between functional variable and axis 1 and axis 2 are in parentheses (in the order r^2 to axis 1, r^2 to axis 2).

Indicator species analysis was used to identify *nosZ* OTUs that differentiated denitrifier communities based on land-use and time. Five *nosZ* OTUs were identified to significantly differentiate ($P < 0.1$) denitrifier communities. There was no discernable pattern in proportional abundance for these OTUs (**Figure 4.4**). However, OTU 7 was the only *nosZ* genotype exclusive to cultivated wetland soils, and OTU 24 was the only genotype exclusive to the Sep 12 sampling date for both land-uses. The majority of these OTUs clustered with Rhizobiaceae of the α-Proteobacteria (**Figure 4.5**). Only OTU 24 had greater than 80% sequence similarity to a *nosZ* sequence from a previously cultured and identified bacteria (100% coverage and 86% identity with *Bradyrhizobium japonicum*, accession AJ002531).

Similar to denitrifiers, the nitrifier *amoA* NMS produced a 2-dimensional solution with $r^2 = 0.42$ and 0.54 for Axis 1 and 2, respectively (**Figure 4.6**). Therefore, nearly all (96%) of the true variance structuring the difference in nitrifier *amoA* community composition was a function of time and land-use as represented in this ordination space. The correlation coefficients (r) for nitrification rate and nitrification associated N_2O emission were -0.86 and 0.10, respectively. Therefore, 75% of the variation (r^2) in nitrification rate, but only 1% of the variation in N_2O associated with nitrification, can be related to the seasonal difference in *amoA* community composition. In comparison, land-use related difference in *amoA* community composition can account for 11% and 20% of the variability in nitrification rate and N_2O from nitrification, respectively. Water-filled pore space at the time of soil sampling in the field also had high negative correlation to change in *amoA* community composition over time.

Five *amoA* OTUs were identified as indicator species. Four of the five OTUs grouped within Cluster 3 of ammonia oxidizing bacteria (**Figure 4.7**). These four OTUs increased in proportional abundance over time in one or both land-uses (**Figure 4.4**). Only *amoA* OTU 17 grouped within Cluster 2, and its proportional abundance declined over time.

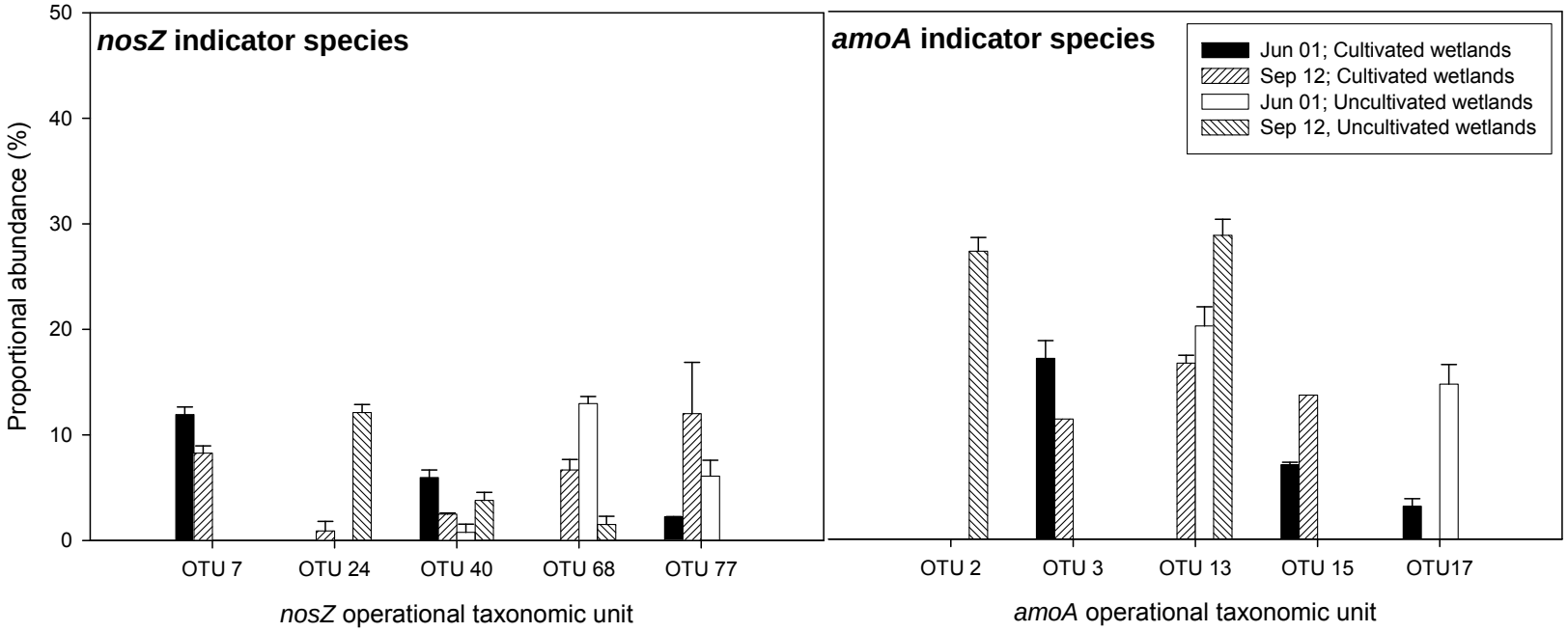

Figure 4.4 *nosZ* and *amoA* operational taxonomic units (as defined by RFLP) that differed significantly between land-use and time based on Indicator Species Analysis ($P < 0.1$). Results are means with standard errors (n = 3). Proportional abundance is the percentage of recovered clones with the defined RFLP. Forty-eight clones were screened for each sample.

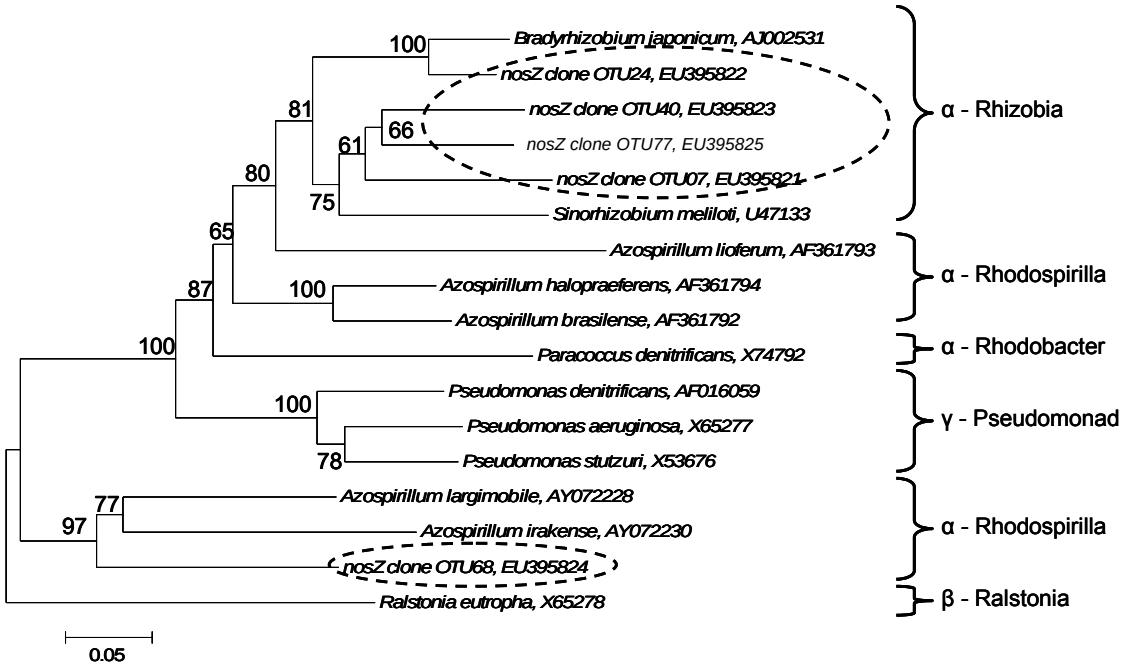

Figure 4.5 Phylogenetic tree of cloned *nosZ* operational taxonomic units that significantly delineated land-use and time as determined by Indicator Species Analysis. Broken ovals highlight cloned sequences from this study. The label of the sequence used in the analysis is followed by their respective GenBank accession number. Branch nodes with bootstrap values greater than 60 are labeled. Scale bar indicates 5 changes per 100 nucleotide positions.

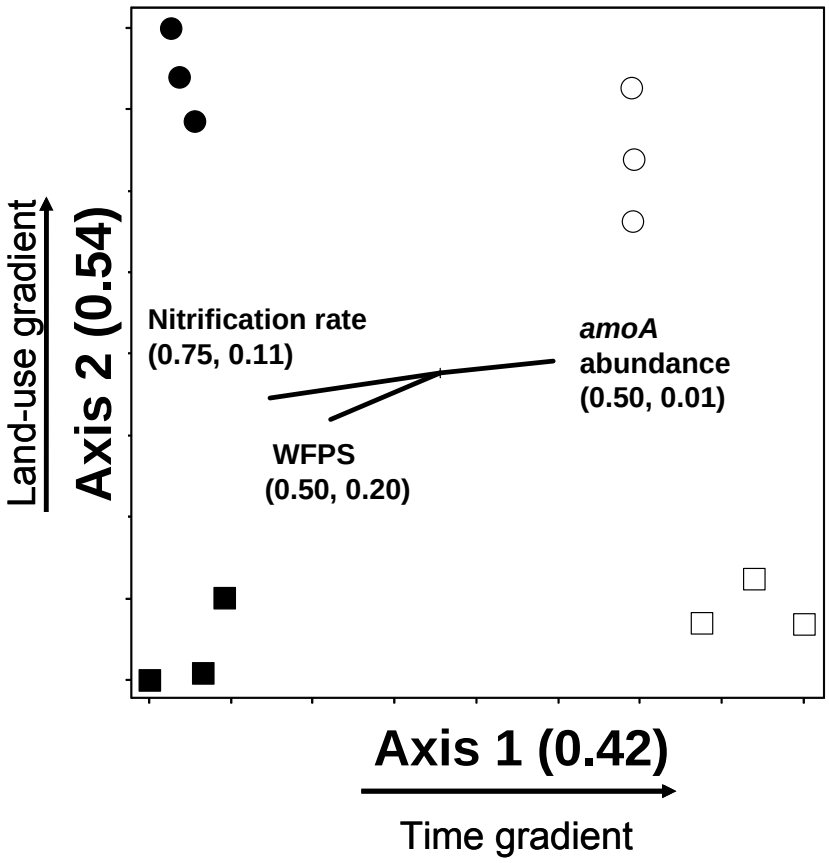

Figure 4.6 Non-metric Multidimensional Scaling ordination of nitrifying communities based on presence/absence of operational taxonomic units (OTUs) defined by *amoA* RFLP patterns. □ = Jun 01; cultivated wetlands. ■ = Sep 12; cultivated wetlands. ○ = Jun 01; uncultivated wetlands. ● = Sep 12; uncultivated wetlands. Vectors show the direction and magnitudes of the correlation coefficient (r^2) between NMS ordination axes and functional variables. The r^2 for the correlation between functional variable and axis 1 and axis 2 are in parentheses (in the order r^2 to axis 1, r^2 to axis 2).

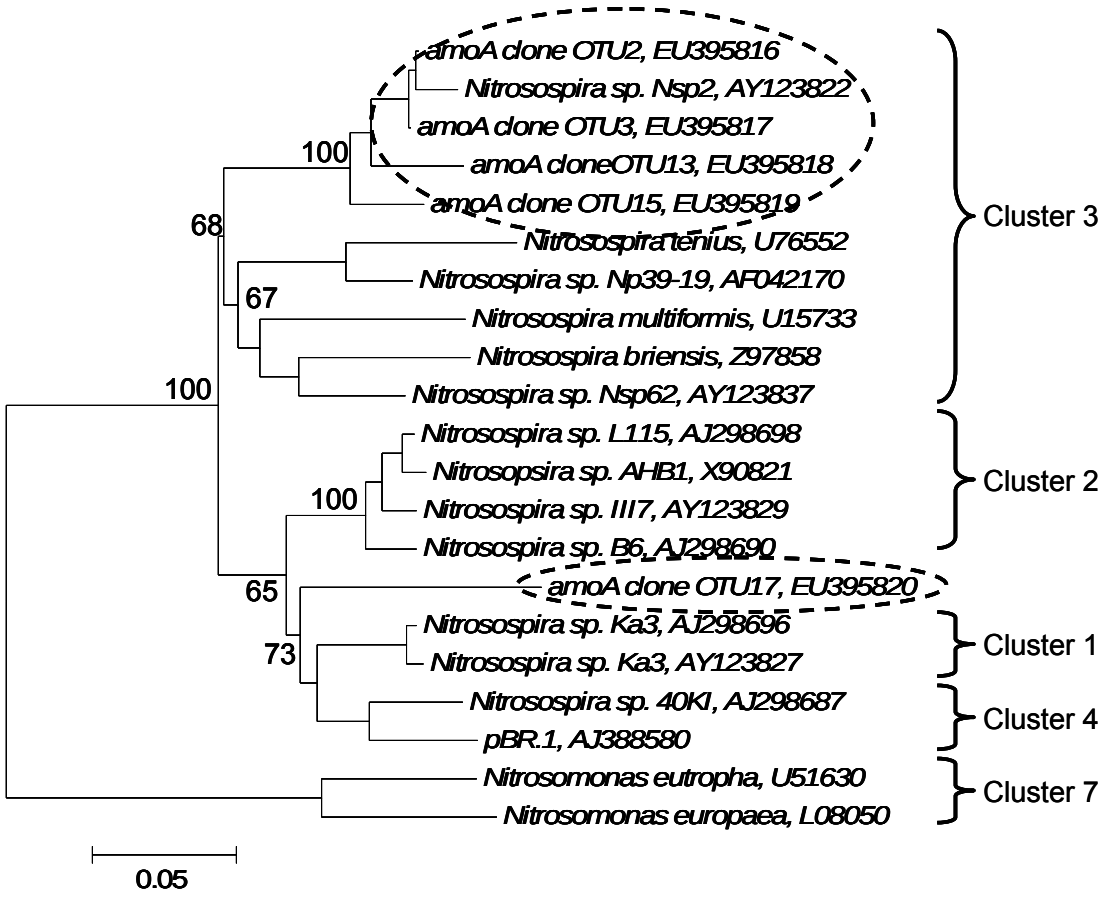

Figure 4.7 Phylogenetic tree of cloned *amoA* operational taxonomic units that significantly delineated land-use as determined by Indicator Species Analysis. Broken ovals highlight cloned sequences from this study. The label of the sequence used in the analysis is followed by their respective GenBank accession. Branch nodes with bootstrap values greater than 60 are labeled. Cluster labels based on Horz et al. (2004). OTU17 could not exlusively be included with Cluster 1 or 4, but it has common lineage with both clusters. Scale bar indicates 5 changes per 100 nucleotide positions.

4.5 Discussion

4.5.1 Links between nitrifier *amoA* community structure and N_2O emitting activity

Despite the nitrification rates declining over the summer, the *amoA* community abundance/density increased. Nitrification rates were negatively correlated to *amoA* abundance over time (**Table 4.2**). This might be caused by soil drying and competition for available ammonia. As soil moisture (represented by WFPS) declines over time (**Figure 4.2** D and H), the abundance of nitrifiers increase because rate of oxygen supply to oxidize available ammonia would also increase. When ammonia gets limiting, their activity may decline. This was observed as nitrification activity (**Figure 4.1**B and E) and the available ammonia (**Table 4.1**) declined over time, and a positive correlation (r = 0.378) existed between these two measurements (**Table 4.2**). This means that the ammonia oxidizers loose activity but maintain biomass. The observed decline in ammonia over time might be caused by declining rates of mineralization relative as the soil dries or growing plant biomass developing over a growing season might be a competitive sink for available ammonia.

The abundance of *amoA* for AOB is about 10% of some recently qPCR-determined values reported for soils (Jia and Conrad, 2009; Offre et al., 2009). However, the abundance reported here would suggest that nitrification activity per AOB cell is at least 2-times greater than values previously reported (Jia and Conrad, 2009). Isolation and physiological characterization will be required to confirm whether the nitrifiers in these soils are indeed hyperactive. Alternatively, another microbial population is responsible for the elevated nitrification rates per cell in these soils. Although debatable (Di et al, 2009; Jia and Conrad, 2009), ammonia oxidizing archaea (AOA) are found to be an important group of microorganisms responsible for ammonia oxidation (the first step in nitrification) in a variety of terrestrial (Leininger et al., 2006,) and marine (Francis et al., 2005) environments. Further investigation is needed to differentiate the nitrification rates of AOB and AOA in soils from the study site.

The rate of N_2O production is about 10% of the nitrification rate. This rate of N_2O production from nitrification is higher than normally cited for N_2O as a percentage of nitrification (i.e., 2%; Wrage et al., 2005). However, this value is similar to previously

reported results from Bedard-Haughn et al. (2006) for the same study site. Those authors suggested other nitrifier-related N_2O emitting processes might be responsible for higher than expected N_2O related to nitrification as determined by the ^{15}N-pool dilution method.

In comparison to its relationship with seasonal differences in *amoA* community composition, N_2O from nitrification or, more accurately, N_2O from processes that oxidize unlabeled mineral nitrogen sources was poorly related to the land-use difference in *amoA* community composition. This result is similar to that reported previously where land-use difference in *amoA* community composition was not related to N_2O emissions attributable to nitrification (Ma et al., 2008a). It was postulated that processes that oxidize unlabeled mineral nitrogen sources may be more dependent on aeration, as controlled by WFPS, rather than biotic factors. For example, if nitrifier denitrification is the process for N_2O production where nitrite (rather than labeled nitrate) produced during nitrification is reduced to N_2O when O_2 becomes limited (Arp and Stein, 2003), then N_2O production may not be directly relatable to *amoA* activity, abundance and community composition as a function of land-use.

4.5.2 Links between denitrifier *nosZ* community structure and N_2O emitting activity

Our results indicated that a combination of reduced *nosZ* abundance and nitrous oxide consumptive activity were acting in concert to reduce net N_2O emissions. Because the sequential reduction of nitrogen oxides by denitrification has increasing sensitivity to O_2, decreased WFPS would increase aeration and cause a decline in the expression of *nosZ*, the last catalytic enzyme in the denitrification pathway (Cavigelli and Robertson, 2000). Further, the drying field conditions and increasing competition with plants for resources over the course of the season can cause the observed decline in abundance of *nosZ*-containing denitrifiers (Bardgett et al., 1999; Wang and Bakken, 1997). If this was true, denitrifiers with *nosZ* may be more transient in abundance and activity (declined abundance/increased rN_2O) than those without *nosZ* (steady DEA). This hypothesis will require future determination of abundance and activity of other genes (*nar/nir/nor*) in the denitrification pathway in these soils.

The effects of soil drying on rN$_2$O in the Cultivated and Uncultivated soils were highly contrasting. In the Cultivated soils, rN$_2$O sharply changed to approach 1 with marginal soil drying between Julian Day 150 and 190. In the Uncultivated soils, rN$_2$O steadily approached 1 over the course of the study period with the change in WFPS. This suggests the pore size distribution in these soils differ, and this might influence denitrification and denitrification-related N$_2$O emissions from these soils. For example, a WFPS of 70% in a soil with 50% (by volume) pore space (Uncultivated soils) would have 100% more water than a soil with 25% pore space (e.g., Cultivated soils). Hence, drying might have a threshold effect on N$_2$O consumption by nitrous oxide reductase activity, as exemplified by the sharp increase of rN$_2$O towards 1 (i.e., no consumption) with minor amount of drying, in the Cultivated soils.

The abundance of active denitrifiers based on *nosZ* abundance reported here is approximately 10 to 100-times lower reported for cultivation-based enumeration from other soils (McCarty et al., 2007). However, *nosZ* abundance in these soils is comparable to recent qPCR-based reports (Baudoin et al., 2009). Furthermore, the rate of N$_2$O production per *nosZ* gene copy is similar to Baudoin et al. (2009).

In soils from the water-shedding landforms, a *nosZ* community difference among times, but not between land-use was linked to N$_2$O emitting activity. The NMS analysis found that 57% and 65% of the variation in DEA and rN$_2$O, respectively, were explained by the differences in the *nosZ* community composition over time (**Figure 4.3** joint plot). This is greater than what is reportedly explained by environmental factors (e.g., soil water content) alone (Rich et al., 2003). We found WFPS explained 33% and 13% of the variation in DEA and rN$_2$O, respectively (**Table 4.2**). In contrast, less than 5% of the variation in DEA and rN$_2$O was correlated to the land-use differences in the *nosZ* community composition (**Figure 4.3**). This corresponds to the previously reported absence of relationship between *nosZ* community composition and denitrification associated N$_2$O emission as a function of land-use and landform based on cluster analysis (Ma et al., 2008a). The absence of a land-use relationship may be ecosystem specific. For example, while Rich and Myrold (2004) and Enwall et al. (2005) did not find a link in agricultural systems, Rich et al. (2003) did find a relationship in meadow and forest soils.

The seasonal abundance of denitrifier *nosZ* was related to potential soil N_2O emission because *nosZ* abundance directly affected nitrous oxide reductase activity (NOS); i.e., NOS activity declined (rN_2O increased) as abundance of *nosZ* declined. Similarly, seasonal differences in *nosZ* community composition were related to *nosZ* abundance, DEA and rN_2O. Because the majority of the indicator species were from the family *Rhizobiaceae*, shifts in the abundance of individual members this family of the α-*Proteobacteria* during the course of a growing season might be relatable to changes in potential soil denitrification activity and N_2O emissions. However, land-use differences in *nosZ* community composition did not account for much of the variations in potential denitrification activity. This indicated a level of redundancy in *nosZ* communities in this agroecosystem, i.e., community composition may differ between land-use, but the potential level of denitrification activity was similar for any given time in the growing season.

The dominance of rhizobial *nosZ* sequences recovered may have interesting implications. A number of isolates from the family of Rhizobiaceae are reportedly capable of denitrification and contain genes for part or complete denitrification pathway (Mesa et al., 2004; Monza et al., 2006). *Rhizobium* and *Rhizobium*-legume symbiosis may contribute to N_2O emissions in several ways: 1) provide N-rich residue for decomposition; 2) atmospheric N_2 fixed by legumes can be nitrified or denitrified in the same manner as fertilizer N; and 3) localized reduction of available O_2 during N_2 fixation could stimulate N_2O emission from rhizobia, either free-living or living symbiotically in root nodules, capable of denitrification. Alternatively, H_2 gas produced by the *Rhizobium*-legume symbiosis has shown to improve soil fertility and plant growth through selection of plant growth promoting, H_2-utilizing microorganisms in soil (Dong et al., 2003; Maimaiti et al., 2007). However, these benefits may be countered by stimulating N_2O emissions from formate-dependent fungal denitrification (Ma et al., 2008b) because formate can be produced from H_2 and CO_2 (Horn et al., 2003).

The $^{15}NO_3^-$ incubations showed the majority of the potential N_2O produced in these soils at 70% WFPS were not related to denitrification processes. This is similar to the findings in Ma et al. (2008) and Bedard-Haughn et al. (2006) where majority of potential N_2O produced from the same tested soils were not related to the reduction of the

labelled $^{15}NO_3^-$. This appears contradictory to the finding in this report that temporal changes in denitrifier, rather than ammonia oxidizer, community composition and abundance is correlated to potential N_2O production. Both observations are valid. First, nitrification is predominantly from processes that do not reduce $^{15}NO_3^-$ - an observation common to this and the previously published works. However, the work reported here suggests the fraction of potential N_2O produced from non-denitrification processes are not correlated to AOB *amoA* abundance or community composition. This does not preclude the observation that potential N_2O attributable to denitirifaction can temporally be correlated to *nosZ* abundance and community composition.

In summary, both *amoA* and *nosZ* changed dramatically over the course of the season but only in the case of *nosZ* was this change correlated to differences in potential N_2O emissions. We postulate that this may be because of (a) competition for ammonia amongst ammonia oxidizers and with plant which altered the nitrifiers *amoA* community composition and abundance in a non-predictive manner based on the parameters we measured or (b) that the majority of N_2O emissions from nitrifiers are arising from the *nirK* pathway of AOB and thus, *amoA* is a poor surrogate for N_2O production from autotrophic AOB. Our results indicate that *nosZ* may be an effective tool to monitor denitrifier contributions to N_2O emissions in a field setting, but a more refined genetic target is needed to characterize N_2O emissions from nitrifiers.

4.6 Acknowledgements

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5 SOIL FORMATE REGULATES THE FUNGAL NITROUS OXIDE EMISSION PATHWAY[2]

5.1 Abstract

Fungal activity is a major driver in the global nitrogen cycle, and mounting evidence suggests fungal denitrification activity contributes significantly to soil emissions of the greenhouse gas nitrous oxide (N_2O). The metabolic pathway and oxygen requirement for fungal denitrification is different from bacterial denitrification. We hypothesized that soil N_2O emission from fungi is formate and O_2-dependent and that land-use and landforms would influence the proportion of N_2O coming from fungi. Using substrate-induced respiration inhibition (SIRIN) under anaerobic and aerobic conditions in combination with ^{15}N gas analysis, we found that formate and hypoxia (vs. anaerobiosis) was essential for fungal reduction of ^{15}N-labeled nitrate to $^{15}N_2O$. As much as 65% of soil emitted N_2O was attributable to fungi; however, this was found only in soils from water-accumulating landforms. From these results, we hypothesize that plants could affect N_2O production from fungi via the proposed fungal pathway through root exudates.

Key words: denitrification, formate, fungi, N_2O, nitrous oxide

5.2 Introduction

The importance of fungal denitrification to the emission of nitrous oxide (N_2O; an important greenhouse gas) from soils has been demonstrated in a number of systems. Up to 89% of soil N_2O emissions could be attributed to fungal activity (Laughlin and Stevens, 2002). Given that fungal biomass dominates in many ecosystems, their potential activity may be the dominant soil N_2O emitting process. Fungal denitrifiers are ecologically significant because most fungal isolates capable of denitrification appear to lack nitrous

[2] A modified version of this chapter was published published in Ma, W.K., R.E. Farrell, and S.D. Siciliano. 2008. Applied & Environmental Microbiology 74:6690-6696.

oxide reductase – the enzyme that reduces N_2O to N_2 (Nakahara et al., 1993; Zhou et al., 2001). Therefore, unlike bacterial denitrification, the end-product of which is mostly N_2, the end-product of fungal denitrification is N_2O.

Accumulated evidence from work with fungal isolates indicates that the fungal pathway for respiratory reduction of nitrogen oxides to nitric oxide (NO) and N_2O is different from that of bacteria. Fungal nitric oxide reductase (P450nor) is a cytochrome p450-containing enzyme that receives electrons directly from NADH for the reduction of NO to N_2O (Nakahara et al., 1993; Zhou et al., 2001). Consequently, small amounts of O_2 (hypoxia) are required to generate NADH from the oxidation of citrate in the tricarboxylic acid cycle (**Figure 5.1**). This is contrary to bacterial denitrification where successive enzymes in the pathway are increasingly sensitive to O_2 inhibition (Tiedje, 1994; Zumft, 1997). The O_2 requirement for fungal denitrification, however, has not been tested explicitly in soil.

An interesting feature in some fungal denitrification pathways is the coupling of nitrate or nitrite reduction with formate ($HCOO^-$) oxidation (**Figure 5.1**) (Kuwazaki et al., 2003; Uchimura et al., 2002). Low molecular weight organic acids such as formate are important root exudates (Jones, 1998) and are intermediates and by-products of anaerobic carbon metabolism (Bott, 1997). Formate (together with acetate) is the end-product of the fermentation of citrate, oxaloacetate and pyruvate. It is also produced from H_2 and CO_2 by a variety of anaerobic microorganisms (e.g., acetagens, sulfate reducers, and methanogens) (Horn et al., 2003). The amount of formate in aerobic soils is reported to range from 6 to 26% of the total extractable low molecular weight organic acids (van Hees et al., 2005). Reported rhizosphere formate concentrations range from below detection limit for clover (*Trifolium repens*) (Bolan et al., 1994), 117 μ*M* for Norway spruce (*Picea abies*) (van Hees et al., 1996) and 563 μ*M* for quackgrass (*Elytrigia repens*) (Baziramakenga et al., 1995). In addition to external sources, fungi can produce formate. Under O_2-limited conditions, formate is produced from the fermentation of pyruvate (Zhou et al., 2002). Because of its various sources and relative ubiquity in soils, formate-dependent respiratory reduction of nitrogen oxides to N_2O by fungi may be an important contributor to net N_2O emissions from soils.

We hypothesized that soil N_2O production by fungi is formate and O_2-dependent and that land-use and landform influence the proportion of N_2O attributable to fungi. Land-use factors such as tillage influence fungi by physically disturbing the soil (Frey et al., 1999), while fertilizer applications can have an inhibitory effect on fungi (Bardgett and McAlister, 1999; Donnison et al., 2000). Land-use and landform also affect water distribution (Hayashi et al., 1998; Mentzer et al., 2006) and the quantity and quality of soil organic matter (van der Wal et al., 2006), which in turn, can affect soil fungi. Using substrate-induced respiration inhibition (SIRIN) under anaerobic and aerobic conditions, in combination with ^{15}N gas analysis, we evaluated the importance of formate and O_2 to N_2O production by fungi in cultivated and uncultivated soils at the St. Denis National Wildlife Area in Saskatchewan, Canada.

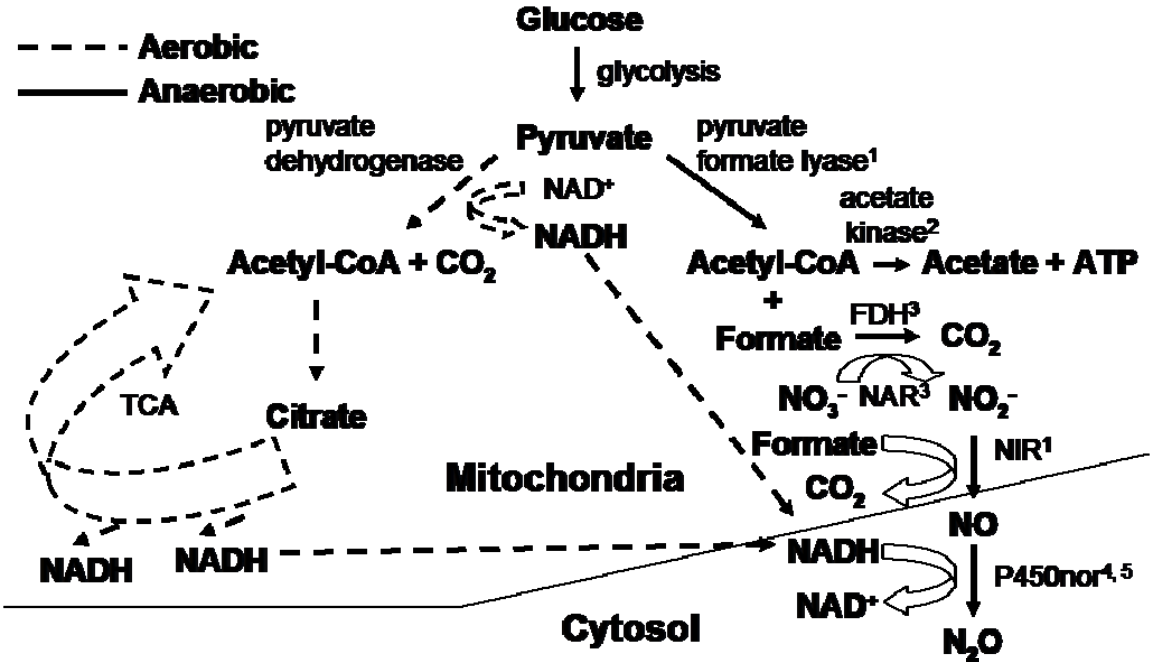

Figure 5.1 Proposed O_2- and formate-dependent fungal denitrification pathway developed from the cited works. [1] Kuwazaki et al., 2003; [2] Zhou et al., 2002; [3] Uchimura et al., 2002; [4] Nakahara et al., 1993; [5] Zhou et al., 2001. TCA = tricarboxylic acid cycle; FDH = formate dehydrogenase; NAR = nitrate reductase; NIR = nitrite reductase; P450nor = cytochrome P450 nitric oxide reductase.

5.3 Experimental Procedures

5.3.1 Study Site

The St. Denis National Wildlife Area in central Saskatchewan, Canada (52°12'N, 106°5'W) is a typical landscape of the North American prairie pothole region. It contains 216 wetlands within an area of 3.84 km^2 (Hogan and Conly, 2002) in the Dark Brown soil zone. Soil types range from thin Typic Calciborolls and thick Typic Haploborolls for water-shedding landform elements to Albic Argiborolls and Argic Cryaquolls in water-accumulating elements (Yates et al., 2006a). Soils (Weyburn Association) developed on loamy unsorted glacial till parent materials in hummocky terrain with slope classes ranging from 10 to 15% (Miller et al., 1985). Six ephemeral wetlands (three cultivated and three uncultivated) were selected for study. Ephemeral wetlands are those depressions in hummocky landscapes that contain standing water in the spring, but typically dry-out during the growing season (Hayashi et al., 1998).

A digital elevation model was used to sub-divide the cultivated wetlands (CW) into convex (CX; topographically high positions with a positive profile curvature that sheds water) and cultivated depression centre (CD; level positions, roughly circular in shape, which temporarily collect rain or snowmelt water) landform elements (Yates et al., 2006b). These two landforms represent the extremes in terms of N_2O emission, soil moisture conditions, and biological productivity within the cultivated landscape (Hogan and Conly, 2002; Yates et al., 2006a; Yates et al., 2006b). Uncultivated wetlands (UW) were found in non-agricultural portions of the site. These were divided into two landform elements roughly equivalent to those in the cultivated wetlands. Basin centres (BC) are level areas covered by 99 non-grass plant species, collect rain and snowmelt water, and are analogous to the CD elements. Uncultivated wetlands also included non-level fringe areas covered with *Bromus inermis* – termed the riparian grass area (RG). The RG elements represent the driest areas within uncultivated wetlands and, in this sense, are analogous to CX elements in the cultivated wetlands.

5.3.2 Soil sampling and soil carbon and nitrogen determination

Three cultivated and three uncultivated wetlands [each constituting a land-use replicate (Bedard-Haughn et al., 2006b)] were sampled on 12 September 2006. Five soil cores (0–15 cm; 15 cm i.d.) were collected from the individual landform elements in each of the six wetlands. The cores from each individual landform element were bulked together to form a composite sample for that location, yielding a total of 12 composite samples (2 land-uses × 2 landforms × 3 replicates). Samples were transported on ice and sub-samples were collected for soil formate extraction and lyophilization (for PLFA extraction). The remaining soil was air dried just enough (<24 h) to pass through a 2-mm sieve without smearing and stored at -20°C. Inorganic nitrogen (2 *M* KCl extracts) (Maynard et al., 2007), soil organic carbon (dry combustion) (Skjemstad and Baldock, 2007) and total nitrogen (Dumas method) (Rutherford et al., 2007) were determined by standard methods and are listed in **Table 5.1**.

Table 5.1 Organic carbon and mineral and total nitrogen in soils of the St. Denis National Wildlife Area, Saskatchewan, Canada. The C:N ratio is SOC/Total N.

Land-use	Landform	Mean (SE)[†]				
		SOC (%)	NH_4^+	NO_3^-	Total N (%)	C:N[‡]
			---μg N g^{-1} soil---			
Cultivated	Convex (CX)	2.3 (0.1)	1.8 (0.1)	1.7 (0.2)	0.2 (0.0)	9 (0)
	Depression centre (CD)	3.2 (0.2)	2.7 (0.1)	2.9 (0.4)	0.4 (0.0)	9 (0)
Uncultivated	Riparian grass (RG)	2.4 (0.1)	3.4 (0.4)	1.6 (0.2)	0.3(0.0)	9 (0)
	Basin centre (BC)	3.5 (0.1)	4.7 (0.8)	6.0 (1.6)	0.4 (0.0)	9 (0)

[†] Reported values are means (n = 3) with standard error in parentheses.
[‡] The C:N ratio is SOC/Total N.

5.3.3 SIRIN incubations to determine contribution of fungi to N_2O emissions

Substrate induced respiration inhibition studies involved the following treatments: control soil (no added microbial inhibitor), cycloheximide-amended soil, and streptomycin-amended soil. Whereas cycloheximide was chosen for targeted suppression of fungal activities, streptomycin was chosen as a broad-spectrum bacterial activity inhibitor (Badalucco et al., 1994). Soils were removed from cold storage, thawed at room temperature and packed into a 10-ml volume at the bottom of a 55-ml glass culture tube (22-mm i.d.) to yield bulk densities similar to those observed in the field. Soil water content was determined using standard procedures with an assumed particle density of 2.65 g cm^{-3} (Topp and Ferré, 2002). Preliminary experiments determined that when repacked cores are initially wetted to 50% or 70% water-filled pre space (WFPS), a burst of N_2O was observed at 24 hours after wetting, declined to background levels at 48 h after wetting, and remained at background levels for several days thereafter. Thus, soil water content was first adjusted to 50% WFPS, capped with parafilm and stored at 4°C for 48 h prior to treatment.

Preliminary experiments also were conducted to determine the optimal cycloheximide and streptomycin concentrations for use with the CD and BC soils. Optimum concentrations of the inhibitors were determined using a modification of the method described by Laughlin and Stevens (2002). The modifications included bringing the soils to 70% WFPS and adding potassium formate (2 mg formate-C g^{-1} soil; equivalent to about 2% of the maximum soil organic carbon found in the St. Denis soils) and potassium nitrate (80 µg NO_3-N g^{-1} soil; this gave C:N ratio of 24:1). The optimum concentration of both cycloheximide and streptomycin was found to be 4 mg inhibitor g^{-1} soil for the CD soils and 8 mg inhibitor g^{-1} soil for the BC soils. These levels of inhibitor also were applied to the respective upland soils. Because cycloheximide is only soluble in methanol, the cycloheximide stock solution consisted of 4 g of cycloheximde dissolved in 10 ml of methanol and dispersed in deionized water to a final volume of 50 ml. Streptomycin and control (no biocides) solutions were made with similar volume and methnol:water ratio. Addition of the inhibitors resulted in an increase in soil moisture content to approximately 60% WFPS. Thus, the tubes were recapped with parafilm and incubated in the dark at 23°C for an additional 24 h prior to addition of the formate and nitrate (Castaldi and Smith, 1998). After adjustment of the soils to a final moisture content of

70% WFPS, the tubes were sealed with butyl-rubber caps and incubated in the dark at 23ºC for 24-h incubation. After 24-h, a 20-ml gas sample was withdrawn from the headspace using a 20-cc disposable syringe equipped with a 25-guage needle and injected into a pre-evacuated 12-ml Exetainer® vial (Labco Ltd., UK). Headspace N_2O concentrations were determined using a gas chromatograph equipped with an electron capture detector (Yates et al., 2006b).

5.3.4 ^{15}N stable isotope incubation with N_2O, ammonium and nitrate analyses

Modified SIRIN incubations were repeated for the CD and BC soils in which the N-substrate was enriched with ^{15}N. That is, the added KNO_3 consisted of 78-µg KNO_3-N g^{-1} soil and 2-µg $K^{15}NO_3$-^{15}N g^{-1} soil (at 98% ^{15}N enrichment). The ^{15}N studies were conducted under both aerobic and anaerobic conditions. Anaerobic systems were prepared by replacing the headspace air in the culture tubes with ultra high purity N_2 (i.e., working in an anaerobic chamber under a N_2 atmosphere). Gas samples were collected as described for SIRIN incubations, after which the soils were destructively sampled for ammonium and nitrate by extraction with 2 *M* KCl.

^{15}N-labeled N_2O was analyzed by isotope ratio mass spectrometry (IRMS) at the University of California–Davis Stable Isotope Facility (Davis, USA). Fractionation of ^{15}N-labeled N_2O was calculated as described by Arah (1997) and Stevens et al. (1997). The diffusion disk technique described by Stark and Hart (1996) and modified by Bedard-Haughn et al. (2004) was used to concentrate soil ammonium and nitrate for ^{15}N analysis.

5.3.5 Soil N_2O emissions in response to increasing concentration of formate

Soils were removed from cold storage, thawed at room temperature and packed to a 10-ml volume at the bottom of a 55-ml glass culture tube to yield bulk densities similar to those observed in the field. The soil water content was adjusted to 50% WFPS and the tubes capped with parafilm and stored at 4°C for 48 h. The soils were then amended with formate (at concentrations of 0.0, 0.1, 0.2, 0.4, 0.8 and 1.6 nmol formate-C g^{-1} soil) and adjusted to 70% WFPS; the tubes were then sealed with butyl-rubber caps and incubated in the dark at 23ºC for 24-h. Gas sampling and analysis were carried out as described for the SIRIN incubations. The effect of formate additions on N_2O emissions was modeled using the

Enzyme Kinetics Module (single-substrate model) in SigmaPlot® version 9.0 (Systat Software Inc., San Jose, USA).

5.3.6 Soil formate extraction and analysis

Soil formate was extracted using the centrifugation drainage technique described by van Hees et al. (2002). Extracts were stored at -20°C prior to analysis, and were analyzed using the capillary electrophoresis method for anion detection described by Swallow and Low (1994). The analyses were carried out using a Waters Quanta 4000 capillary electrophoresis system (Waters Corporation, Millford, USA) equipped with a 60-cm × 75-µm (i.d.) fused silica column. The electrolyte buffer was 5 m*M* sodium chromate–0.4 m*M* OFM-BT© (Waters Corporation) adjusted to pH 8 with lactic acid and filtered through a 0.45 µm Millipore membrane filter (Millipore, Billerica, USA). Anions were detected at 254 nm using indirect UV detection. Samples and standards were analyzed according to the following sequence: (1) 30-s hydrostatic injection, (2) 5-min run time at 25 kV, (3) rinse for 1.5-min with 0.1 *M* NaOH, (4) rinse for 4-min with nanopure deionized water, and (5) purge for 5-min with electrolyte buffer.

5.3.7 Phospholipid fatty acid extraction and analysis

Phospholipid fatty acids (PLFAs) were extracted from lyophilized soil samples (4.0 g) using the method described by White et al. (1979). Briefly, lipids were extracted from soils in a mixture of methanol:chloroform:phosphate-buffer (2:1:0.8 v/v/v). Lipids were separated on silica gel columns (Bond Elut®, Varian Inc., Mississauga, Canada) by sequential applications of chloroform, acetone, and methanol (with the methanol fraction containing the phospholipids) (Högberg, 2006). Phospholipids were methylated and separated on a Hewlett Packard 5890 Series II gas chromatograph equipped with a 25-m × 0.2-mm (i.d.) Ultra 2 column (J&W Scientific; MIDI Inc., Newark, USA) and a flame ionization detector. Lipid peaks were identified by comparison of retention times to the TSBA version 4.1 and CLIN version 4.0 lipid libraries (MIDI Inc.). Methylnonadecanoate (19:0) fatty acid (1 µg) was added into each sample as the internal standard before the methylation step (Högberg, 2006). The PLFA 18:2ω6,9 was used as the fungal biomass marker (Bääth, 2003).

5.3.8 Statistical analysis

The SIRIN and ^{15}N gas data were imported into Minitab® (v. 11.21, State College, PA, USA) and analyzed using a two-way ANOVA with GLM (α=0.1) after verification that data met the ANOVA assumptions (using the Anderson-Darling test for normality and Bartlett's and Levene's tests for homogeneity of variance). Classification variables were landform and treatment. Because there were significant landform (P=0.001) and landform × treatment (P<0.001) differences, Tukey's pair-wise comparison was used to assess the significance of treatment differences within a landform. PLFA data were compared using a one-way ANOVA and Tukey's pair-wise comparisons with landform as the classification variable.

5.4 Results

Nitrous oxide emissions attributable to fungal activity were found (p<0.1) in both the cultivated depression (CD) and basin center (BC) soils (**Figure 5.2**). Indeed, CD and BC soils incubated with cycloheximide produced 46% and 65% less N_2O, respectively, than the control soil. The wide spectrum bacterial inhibitor, streptomycin, also decreased N_2O emissions in the CD (-51%) and BC (-47%) soils. The inhibitors had no significant effect on N_2O emissions from the riparian grass (RG) soils, but produced increased emissions in soils from the convex (CX) landform elements. These water-shedding landforms, RG and CX, either do not have significant fungal N_2O emission or the inhibitors were ineffective in these landforms. Thus, because our focus was on fungal denitrification, subsequent incubation studies designed to probe the regulation of fungal denitrification with ^{15}N-labeled nitrate and inhibitors was restricted to the CD and BC soils.

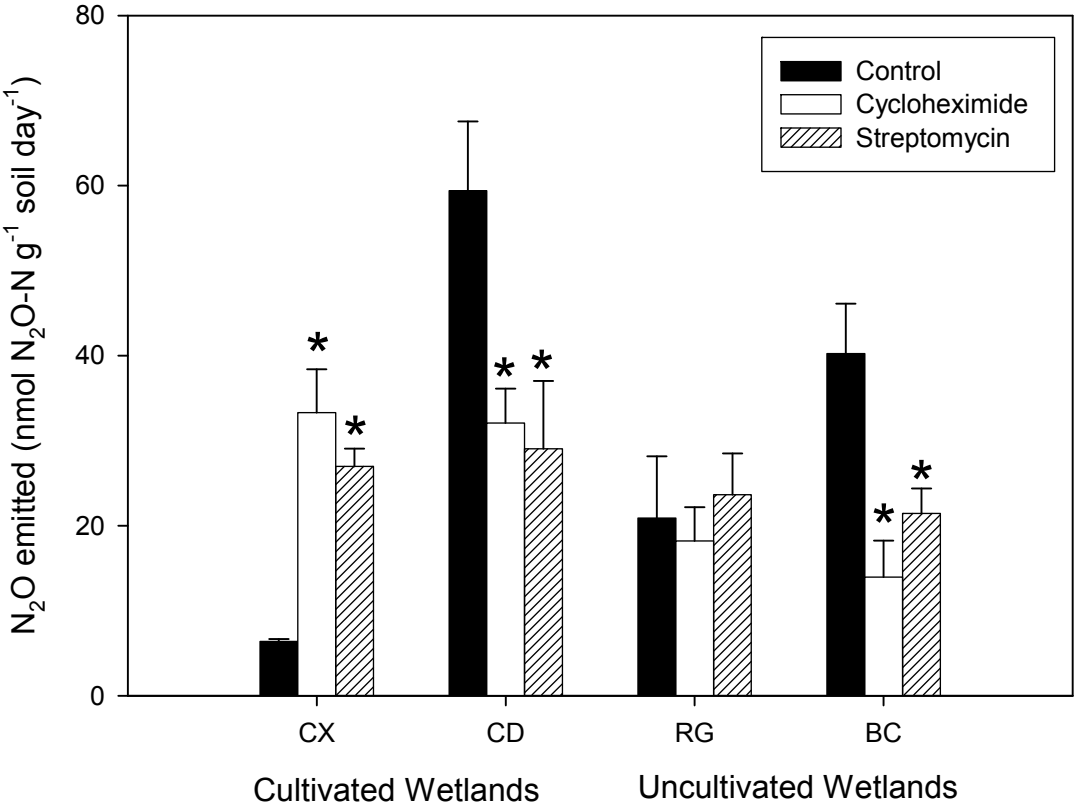

Figure 5.2 Nitrous oxide emission from substrate induced respiration inhibition (SIRIN) assay incubated at 70% WFPS for 24 hours. CX = convex; CD = depression centre; RG = riparian grass; BC = basin centre. Reported values are means with standard error bars (n = 3). Asterisk (*) above error bar denotes N_2O emission from treatment incubation that was significantly different than control incubation ($P < 0.1$).

Relative to the appropriate controls, N_2O emissions from the CD and BC soils exhibited a 30-fold increase when incubated with formate (**Figure 5.3**A and B). Furthermore, this formate effect was most pronounced under hypoxic conditions (i.e., soils incubated at 70% WFPS with an aerobic headspace). In the presence of cycloheximide, N_2O emissions from CD and BC soils decreased by about 30% when incubated in a low oxygen environment. Conversely, when incubated under anaerobic conditions (i.e., soils at 70% WFPS with a nitrogen atmosphere), there was no difference between incubations with or without the inhibitor – suggesting a bacterial source for this N_2O. In the presence of formate, nearly all of the N_2O emitted was derived from the labeled $^{15}NO_3^-$ pool (**Figure 5.3**C and D). In the

absence of formate, the majority of the N_2O was derived from the unlabelled soil-N pool. Nitrogen-15 enrichment of the ammonium pool was not observed during the incubation period (data not shown).

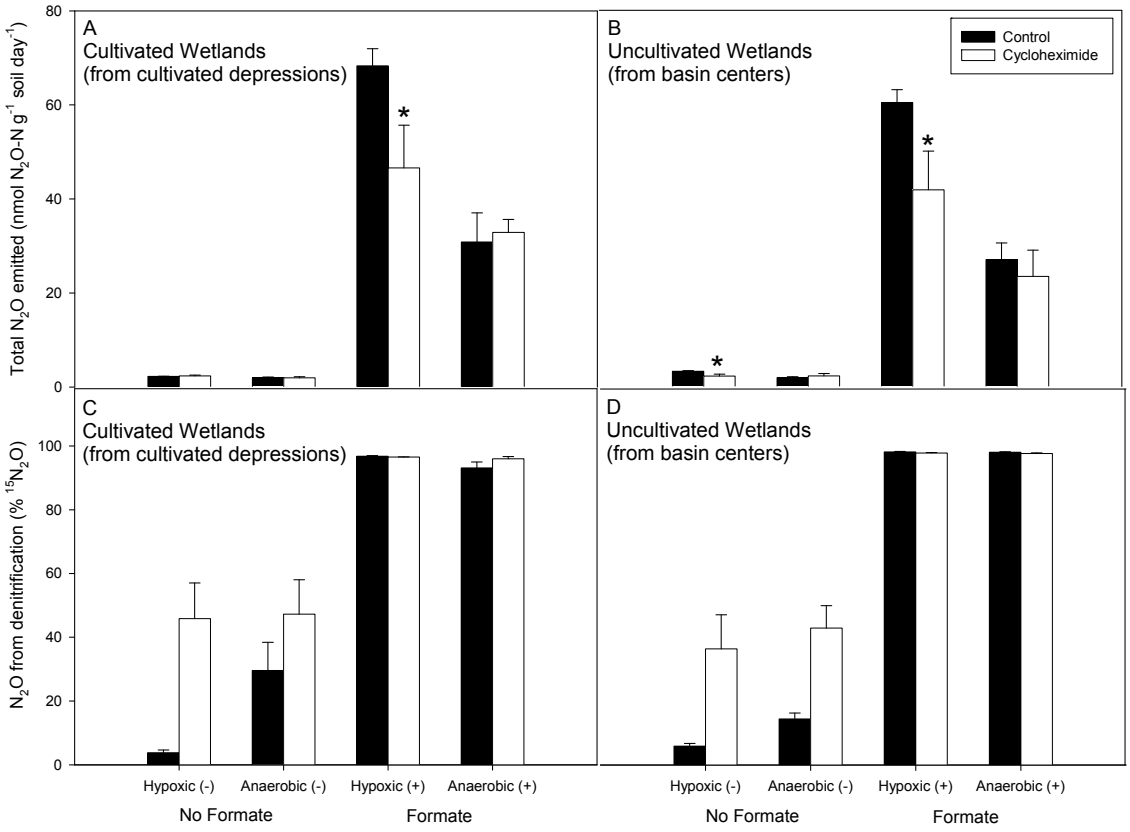

Figure 5.3 SIRIN assay with ^{15}N-labeled nitrate incubated at 70% WFPS for 24 hours. Reported values are means with standard error bars (n = 3). Panels A and B are total N_2O emissions. Asterisk (*) above error bar in panels A and B denotes N_2O emission from treatment incubation that was significantly different than control incubation ($P < 0.1$). Panels C and D are percentage of emitted N_2O attributable to denitrification or processes that oxidized $^{15}NO_3^-$ to $^{15}N_2O$.

In general, N_2O emissions increased as the amount of formate added to the soils increased (**Figure 5.4**). Moreover, soil N_2O emissions demonstrated a hyperbolic dependence on formate concentration. As such, Michaelis-Menten (M-M) kinetics was used to describe the formate-dependent N_2O emissions from all soils (r^2 values ranged from 0.94 to 0.99). Soils from water-accumulating (CD and BC) landforms had greater affinity (lower K_m values) for formate than soils from drier landforms (CX and RG). Not surprisingly then, there was less extractable formate in soils from water-

shedding or drier landforms. Nitrous oxide emissions attributable to fungal activity were greatest in the water-accumulating landforms (**Figure 5.2**). However, the water-accumulating landforms yielded the least extractible fungal PLFA (**Figure 5.5**A) and exhibited the lowest fungi:bacteria PLFA ratios (**Figure 5.5**B).

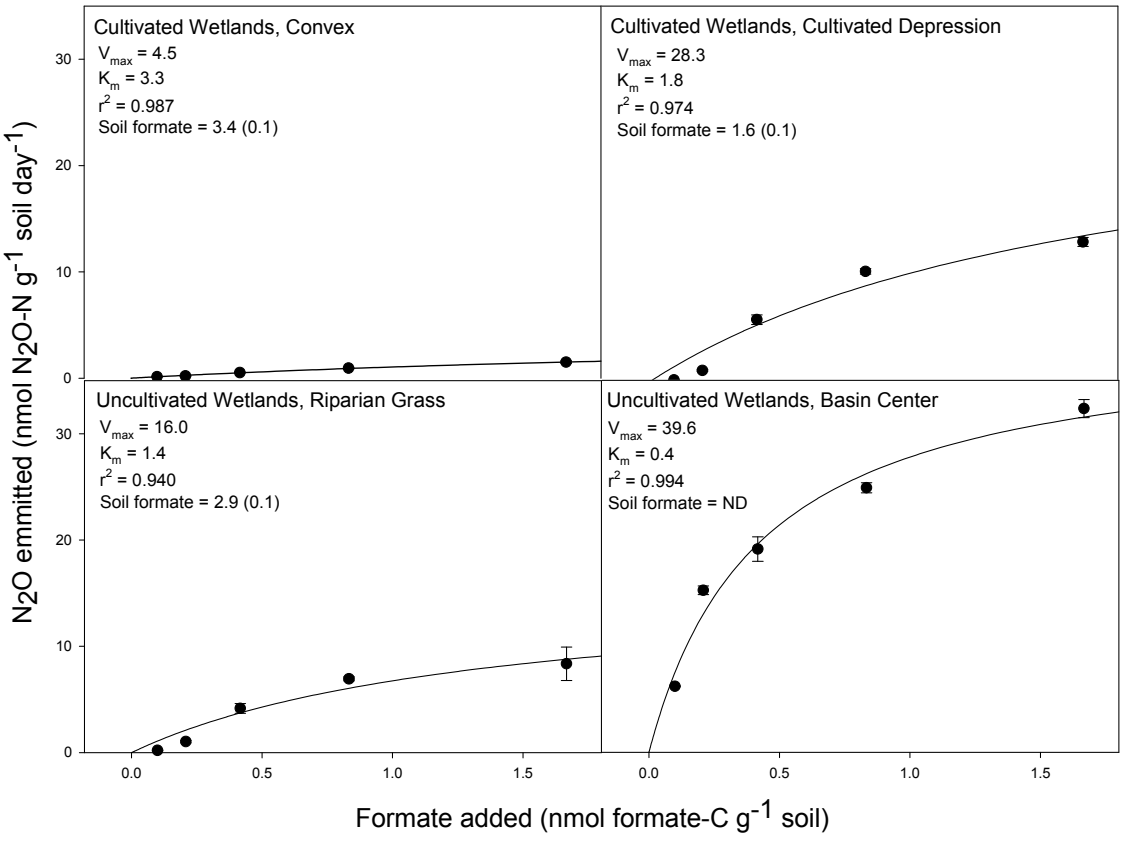

Figure 5.4 N_2O emitted as function of added formate concentration. Closed circle symbols (•) are experimental means of N_2O emitted with standard error bars (n = 3); solid line is the modeled response. V_{max} has the units of nmol N_2O-N $nmol^{-1}$ formate-C day^{-1}. K_m has the same units as the x-axis. The r^2 is the goodness-of-fit for the non-linear regression line determined by the Enzyme Kinetics Module for SigmaPlot 9.0 using the single-substrate (formate) model. Reported soil formate concentrations are means with standard error in bracket and the units of nmol formate-C g^{-1} soil. ND = not detected.

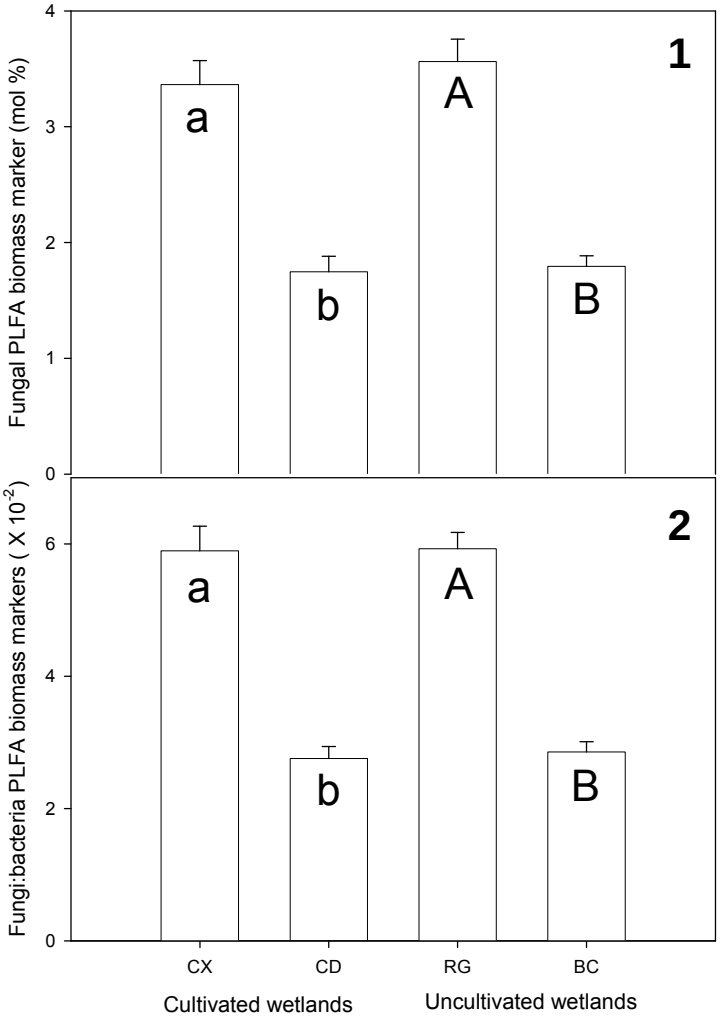

Figure 5.5 Fungal biomass in soil as determined by phospholipid fatty acid (PLFA) extraction. Panel 1 = mol % of fungal PLFA biomass marker. Panel 2 = fungi:bacteria PLFA biomass markers. Reported values are means with standard error bars (n = 3). Different letters below error bars denote significant difference ($\alpha = 0.1$); lower case letters for comparison between soils of cultivated wetlands; capitalized letters for comparison between soils of uncultivated wetlands. CX = convex; CD = depression centre; RG = riparian grass; BC = basin centre.

5.5 Discussion

The O_2 and formate dependence of N_2O emissions associated with fungi can be explained by the proposed fungal denitrification pathway (**Figure 5.1**). To date, fungal nitric oxide reductase activity and the P450nor genotype has been reported for only a few fungi isolated from soil and wastewater reactors (Watsuji et al., 2003; Zhang et al., 2001; Zhou et al., 2001). In all cases, expression of the P450nor gene was essential for the proposed fungal denitrification pathway. Thus, it was not surprising to find that fungal denitrification was not directly linked to fungal biomass. Rather, it is likely linked to the presence and expression of a specific functional genotype – P450nor.

The hyperbolic dependence of N_2O emission on formate concentration, as modeled by M-M kinetics, supports our contention outlined in **Figure 5.1** that a formate dehydrogenase/nitrate reductase couple and/or a formate-dependent nitrite reductase is directly linked to N_2O emissions. The enzymatic characteristics of the formate-dependent N_2O emissions differed between soils of water-accumulating landforms (CD and BC) and the soils from the corresponding water-shedding or drier landforms (CX and RG). For example, the higher affinity (lower K_m) for formate in CD and BC soils would impart a competitive advantage to those fungi in soils with low soil formate concentrations. Also, the potential maximum formate-dependent N_2O emission per unit formate-C (V_{max}) was 2.5 to 6 times greater in soils from water-accumulating versus upland landforms. The reported enzymatic parameters and magnitude of the formate-dependent N_2O emissions may be underestimated because experimental additions of formate can encourage nitrogen immobilization by increasing the C:N ratio (McLain and Martens, 2006).

Formate stimulated non-fungal N_2O emissions. In the bacterial pathway, formate mediates the reduction of nitrate to ammonia (nitrate ammonification) (Berks et al., 1995) with N_2O being released as a result of the non-specific action of dissimilatory nitrate reductase. Although energetically less favourable than denitrification (Strohm et al., 2007), nitrate ammonification is reported to be the more important process in respiratory nitrate reduction under anaerobic conditions and when nitrate is limiting (Tiedje, 1988; Tiedje, 1994). However, this did not appear to be the case; i.e., we did not observe any ^{15}N-enrichment of the ammonium pool as would have been expected. Alternatively, bacteria

capable of mixotrophy (e.g., *Paracoccus denitrificans*) can denitrify nitrate using formate/H_2 as an electron donor (Smith et al., 1994; Smith et al., 2001).

Under hypoxic conditions, heterotrophic nitrification may also contribute to N_2O release. Evidence suggests this is an important N-transformation process for organic N and NH_4^+ in a variety of soil systems (Castaldi and Smith, 1998; Laughlin et al., 2008; Schimel et al., 1984). Heterotrophic nitrification derives energy from organic C oxidation rather than NH_3 oxidation (Kuenen and Robertson, 1994), with N_2O produced as a product of the incomplete oxidation of hydroxylamine. Castaldi and Smith (Castaldi and Smith, 1998) demonstrated that fungal heterotrophic nitrification can be the dominant N_2O emitting process in woodland and arable soils. In addition, heterotrophic nitrifiers produce N_2O from denitrification of nitrification products (i.e., fungal nitrifier denitrification) (Crenshaw et al., 2008). Fungal heterotrophic nitrification would produce primarily $^{14}N_2O$. Under hypoxic conditions with formate, $^{15}N_2O$ dominated whereas without formate only 10% of the N_2O was $^{15}N_2O$. Thus, under hypoxic conditions, there may be two fungal pathways contributing to N_2O release: fungal denitrification via P450nor and fungal heterotrophic nitrification. Our results suggest that the relative importance of these two processes is linked to soil formate concentrations.

Nitrous oxide emissions associated with fungi were landform, but not land-use, dependent. It is often reported that fungal biomass is greater in native (uncultivated) soils than in adjacent cultivated soils and that fungal biomass increases with decreasing land-use intensity (e.g., in no-tillage vs. conventional tillage soils) (de Vries et al., 2007; de Vries et al., 2006; Frey et al., 1999). Such was not the case in the present study; instead, differences in the fungal community appeared to be linked to landscape position (i.e., landform) through its influence on water redistribution. For example, fungal biomass (mycorrhizal and saprotrophic) has been shown to decline in soils that experience periods of flooding or high soil water content (Mentzer et al., 2006). This presumably reflects decreased mycorrhizal associations (Rickerl et al., 1994) or decreased saprotrophic activity under conditions of decreased O_2 availability imposed during these periods.

We did not observe fungal-linked N_2O emissions in the CX and RG landforms on the basis of our cycloheximide inhibition experiment. The cycloheximide concentrations were optimized for the CD and BC landforms and then applied to the CX and RG landforms. We

hypothesized that since soils of CD and BC landforms have the highest organic matter (**Table 5.1**), the levels of cycloheximide that inhibit fungi in these soils would be effective in the soils of water-shedding landforms, CX and RG. If this was incorrect, we may have underestimated the importance of fungal-linked N_2O emissions in the water-shedding landform elements.

Technical limitations and questions associated with the specificity of the inhibitors and substrate (formate) would dictate that future investigations will require novel ideas to validate the findings of this work. To validate the specific activity of the inhibitors, quantification of 18S and 16S rRNA gene fragments or transcripts (Sharma et al., 2006) could shed light on how the 18S or 16S pool is affected by the different inhibitor treatments. Isolation and characterization of the P450nor and formate dehydrogenase enzymes by mass spectrometry (Nakahara et al., 1993; Uchimura et al., 2002) from soil would provide credence to our hypothesis as described by **Figure 5.1**. As well, inoculation with and activity monitoring of fungi with wild-type and mutant genotypes of these two enzymes into soil may also support our arguments.

For the first time, soil N_2O emissions related to fungi is linked to soil formate concentration and O_2 availability. This finding poses interesting management considerations. One, selection of crop varieties that exude lower amounts of formate (Bolan et al., 1994) could mitigate N_2O production from fungi in areas where fungal denitrification is prevalent. Two, the benefits of improved soil fertility and plant growth through H_2 release from nodules of legumes (Dong et al., 2003; Maimaiti et al., 2007) could be countered by increased N_2O production from soil fungi because of formate production from H_2 and CO_2 (Horn et al., 2003).

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6 SUMMARY, CONCLUSIONS AND FUTURE CONSIDERATIONS

6.1 Key Findings

Whether it is linked to nitrogen fertilizer usage in crop production, reclamation of nitrogen-contaminated fresh water supplies or waste water treatment, or greenhouse gas reduction and accounting strategies, research in the areas of soil nitrogen transformation and N_2O emissions has important implications on our everyday life. However, predictive models of N_2O emissions from soil have traditionally ignored the impact of variations in the primary drivers of N_2O production – the composition and abundance of nitrifying and denitrifying microbial communities. To date, the importance of the microbial community parameters of abundance and composition to N_2O emissions is conflicting. This uncertainty is caused by the variety of microorganisms that can perform a variety of nitrogen transformations that lead to N_2O production. Thus, the primary goal of this study was to determine whether microbial community composition and abundance can be used to predict N_2O emissions from soils of two landscapes common to the North American "prairie pothole region". Did landscape select for different N_2O producing microbial communities, and if microbial communities differed by landscape, did it relate to differences in N_2O emitting activity? Were environmental properties more predictive of N_2O emitting activity than microbial community parameters? Although the contributions of bacteria (Chapters 3 and 4) and fungi (Chapter 5) to N_2O emissions were dealt with in separate sections, the study's primary goal and corollary questions were consistently addressed in each experimental chapter. Bear in mind the reported results of this thesis are within the context of laboratory-based experiments and are intended to generate mechanisms and hypothesis for future field scale testing.

To summarize, the landscape difference in composition of individual microbial communities was not predictive of soil N_2O emissions. There is redundancy in each microbial community in relation to N_2O emissions activity. Although the communities may differ by landscape and over time, there is no change in N_2O emissions that can be

related to the change in microbial community composition. The presence of specific groups of microorganisms in the soil may be more useful in predicting N_2O emissions. Autotrophic aerobic nitrification and anaerobic denitrification were considered to be the main microbial processes in the nitrogen cycle and associated N_2O emission. However, fungi were demonstrated to be an important group in N_2O production in the soils of St. Denis. In fact, presence of fungi can be linked directly to N_2O emissions from water accumulating landform elements.

Biotic factors influenced the pattern and distribution of N_2O emission from the soils of the study site. That is, the landform difference in N_2O emissions remained even when soils from the various landforms were amended with unlimiting C and N substrate and incubated at similar WFPS (**Figure 5.2**). However, this is not to say that abiotic soil properties were irrelevant. Water-filled pore space was directly linked to the change in abundance (**Figure 4.2** and **Table 4.2**) and community composition (**Figure 4.3**) of denitrifiers, and WFPS was the dominant parameter controlling N_2O consumption through its control on O_2 (**Figure 3.1**). The availability of C, formate in particular, may directly stimulate the fungal denitrification pathway (**Figure 5.3**). This observation may have management implications for selecting crops depending on formate exudation or crop rotation with legumes because H_2 produced during N-fixation may be used to reduce CO_2 to formate (Horn et al., 2003). Finally, NH_4^+/NH_3 fertilization directly impacted nitrifier community composition (**Figure 3.5**). Therefore, abiotic factors, in essence, produced the conditions under which the microbial nitrifier and denitrifier community developed and acted.

Nitrification was the dominant N_2O emitting process for soils of all landforms (**Figure 3.1**). Two types of N_2O emission patterns may be described. One was a persistent background pattern of low N_2O emissions when conditions were relatively dry (e.g., 50% WFPS). Under these conditions, 99% of emitted N_2O was from nitrification. During event driven emissions such as those during spring thaw or precipitation, where WFPS may be 70% or greater, nitrifier denitrification is an important pathway for N_2O emission from these soils. In the nitrifier denitrification pathway, nitrite (rather than ^{15}N-labeled nitrate) produced during nitrification is reduced to N_2O when O_2 becomes limited.

Neither nitrifer *amoA* abundance nor community composition had predictive relationships with nitrification associated N_2O emissions. This was observed for both the single-sampling date (Chapter 3) and time-course (Chapter 4) experiments. The lack of predictability between nitrifier *amoA* and nitrification associated N_2O emissions may reflect the potential importance of heterotrophic nitrification or nitrifier denitrification pathways (Arp and Stein, 2003; Wrage et al., 2005). Both community parameters, *amoA* abundance and composition, were negative correlation to nitrification rates. This may be caused by proliferation of ammonia assimilating nitrifiers (**Figure 3.1**) or increased assimilatory, rather than nitrification, activity to better compete with plants and other soil organisms (Horz et al., 2004). This suggests potential saprophytic or heterotrophic tendencies by nitrifiers. Incubation experiments with isotopically labelled amino acids versus labelled ammonium could test this hypothesis.

The change in denitrifier *nosZ* abundance and community composition during the time course experiment showed these two parameters were good predictors of net soil N_2O emission regardless of land-use and landform. This relationship is likely linked to soil water content as it will control N_2O consumption by nitrous oxide reductase activity. Further work is needed to determine whether the change in abundance of specific *nosZ* genotypes over time is related to differences in N_2O emitting activity during all sampling times.

The O_2 and formate dependence of N_2O emissions associated with fungi can be explained by the proposed fungal denitrification pathway (**Figure 5.1**). That a cycloheximide-induced decrease in fungal N_2O emissions was observed when the CD and BC soils were incubated under hypoxic conditions, but not when they were incubated under a N_2 atmosphere, suggests that hypoxia is required for fungal denitrification. Under hypoxic conditions heterotrophic nitrification also may contribute to N_2O release. Heterotrophic nitrification derives energy from organic C oxidation rather than NH_3 oxidation, with N_2O produced as a product of the incomplete oxidation of hydroxylamine (Kuenen and Robertson, 1994). In addition, heterotrophic nitrifiers produce N_2O from the denitrification of nitrification products (nitrite and nitrate) (Zhou et al., 2001). Thus, under hypoxic conditions, there may be two fungal pathways contributing to N_2O release: fungal denitrification via P450nor and fungal heterotrophic nitrification. These results

suggest that the relative importance of these two processes is linked to root exudates such as formate.

The mechanism formate-dependent fungal denitrification was demonstrated using soils from only the water-accumulating landform (CD and BC). The possibility of this mechanism occurring in soils from water-shedding areas (CX and RG) cannot be dismissed because this particular set of incubations was not performed. However, given the lower affinity for and higher accumulation of formate in the soil from water-shedding landforms when compared to their water-accumulating counterpart (**Figure 5.4**), formate-dependent fungal denitrification as proposed in Chapter 5 is likely insignificant in soils from the water-shedding elements.

Seasonal changes in denitrifier community composition were relatable to N_2O emission from soil (**Figure 4.3**). However, seasonal changes in WFPS determined the possible development and activity of denitrifiers over time. It is the interaction between the seasonal fluctuations of the biotic and abiotic factors that determine the level of N_2O production and consumption that lead to the resultant net emission of N_2O from soils. My research has identified the key parameters that control net emission and explained their mechanistic relevance. Future work needs to occur at the field level to explore how the contributions of these key abiotic and biotic parameters interact temporally and spatially.

6.2 Room for Improvement

A number of experimental issues should be considered when interpreting the results reported herein. These include (i) a possible dilution effect during sampling of the incubation system and (ii) the effects of sample processing on soil microbial community composition and abundance. The headspace volume of the incubation vessels is small (45 ml), thus sampling the vessels may create a vacuum that draws in ambient air and dilutes the N_2O sample. Although the magnitude of any sampling error was not determined, this type of systematic error was not considered problematic as it would be unlikely to affect the relative treatment differences. Nevertheless, if the sampling error was not systematic (i.e., sample dilution differs between vessels), it could result in higher variability in the reported N_2O emissions which, in turn, could affect the statistical analyses. Thus, future studies may want to examine this source of potential error by sampling tubes containing

one or more N_2O standards and measuring recovery concentrations to determine a possible dilution factor.

The use of microbial community composition and abundance data obtained from fresh field soils to predict N_2O emissions from processed and lab-incubated soils is not without drawbacks. Arguably, incubating soils in the lab disconnects the soil from environmental and hydrological conditions that could interact with the microbial community. However, this is an inherent problem in any study that incubates soils in vitro (e.g., Laughlin and Stevens, 2002; Stevens et al., 1997). One might extract microbial biomarkers (DNA, RNA, phospholipids) post incubation. This approach could not be reconciled with this study's goal of examining the relationship between field microbial community and net soil nitrous oxide emissions with post-incubation community determination would relate the in vitro-adapted (rather than field) microbial community to gas produced in the microcosm.

The data presented in this thesis represent an important step towards linking field microbial community composition to nitrous oxide production in soil from a hummocky landscape. It requires validation with field nitrous oxide emission measurements. For example, an approach combining measurement of microbial community composition and abundance using EMA treatment (Chapter 4) and intact core $^{15}N\text{-}NO_3^-$ pool dilution incubation in the field (Bedard-Haughn et al., 2006) could be part of a future program to validate the results presented in this thesis.

6.3 Linking Community Composition and Activity: A Proteomics Future

In hindsight, the nucleic acid based-techniques used to assess microbial community composition and abundance, though powerful, may ultimately be inadequate to address the relationship between community composition and ecosystem function (i.e., soil N_2O emissions). Mounting evidence based on community gene composition and abundance suggests the relationship between community composition and N_2O emissions is system specific. That is, the presence or absence of the relationship must be empirically determined through direct measurement. Therefore, community composition has no predictive value on N_2O emissions. The problem with the nucleic acid approach to assess community structure is that the presence of a specific genotype, even when dealing with

RNA or EMA-based techniques, does not imply a functioning gene product or phenotype. Alternatively, various isolates from soils may differ in N_2O emission activity under similar, substrate unlimited, incubation conditions. However, this is unrealistic because conditions in soils are rarely non-limiting and populations rarely function in absence of competition in a community.

Proteomics could bring us one step closer to relating community structure to community function. Unlike RNA, enzymes are the end product of a complicated regulatory cascade, and their presence accounts for any regulation in the transcription, translation and post-translational steps imposed by the cellular machinery in response to environmental conditions. Metz et al. (2003) used an immunological approach to study denitrifying populations that expressed copper nitrite reductase *in situ*. They were able to link phylogenetic relationships with enzyme expression within denitrifier populations by using flow cytometry to count cells with antibody-labeled reductase in combination with 16S rRNA probes. Furthermore, they were able to correlate N_2O production rates and expression of the nitrite reductase in batch culture after switches between aerobic and anaerobic conditions. However, it is unlikely that any one technique will provide the definitive answer for determining the relationship between microbial community structure and microbial community function. The range of nucleic acid and protein assays along with isolate models and *in situ* monitoring systems will have to be combined to further our understanding of community structure and function in regards to soil N_2O emissions.

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