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Nitrous oxide emissions from ephemeral wetland soils are correlated with microbial community composition

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Nitrous oxide (N_2O) is a greenhouse gas with a global warming potential far exceeding that of CO_2. Soil N_2O emissions are a product of two microbially mediated processes: nitrification and denitrification. Understanding the effects of landscape on microbial communities, and the subsequent influences of microbial abundance and composition on the processes of nitrification and denitrification are key to predicting future N_2O emissions. The objective of this study was to examine microbial abundance and community composition in relation to N_2O associated with nitrification and denitrification processes over the course of a growing season in soils from cultivated and uncultivated wetlands. The denitrifying enzyme assay and $^{15}NO_3^-$ pool dilution methods were used to compare the rates of denitrification and nitrification and their associated N_2O emissions. Functional gene composition was measured with restriction fragment length polymorphism profiles and abundance was measured with quantitative polymerase chain reaction. The change in denitrifier nitrous oxide reductase gene (*nosZ*) abundance and community composition was a good predictor of net soil N_2O emission. However, neither ammonia oxidizing bacteria ammonia monooxygenase (bacterial *amoA*) gene abundance nor composition predicted nitrification-associated-N_2O emissions. Alternative strategies might be necessary if bacterial *amoA* are to be used as predictive *in situ* indicators of nitrification rate and nitrification-associated-N_2O emission.

Keywords: bacterial *amoA*, denitrification, nitrous oxide emissions, nitrification, *nosZ*, agriculture

INTRODUCTION

Nitrous oxide (N_2O) is a greenhouse gas with 300 times the global warming potential of CO_2 (Jungkunst and Fiedler, 2007) and can be produced by nitrification (Avrahami et al., 2002; Mintie et al., 2003) and denitrification (Cavigelli and Robertson, 2000; Rich et al., 2003). Nitrification and denitrification are important processes in the global nitrogen (N) cycle. Nitrification is the oxidation of NH_3 to NO_3^- via NO_2^- (Horz et al., 2004) and denitrification is the respiratory reduction of NO_3^- and NO_2^- to gaseous products, mainly N_2O and N_2 (Tiedje, 1994). Consequently, the rates of these two microbially mediated processes, and the controls on the rates of these two processes, are important determinants of soil N_2O emissions.

Available C, N, and O_2 are three proximal factors that control the rates of N_2O production/consumption via nitrification and denitrification (Svensson et al., 1991; Cavigelli and Robertson, 2000; Avrahami et al., 2002). Land-use and landform (together referred to as landscape) are two long-term determinants of these proximal factors. 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., 2006). Furthermore, seasonal changes in precipitation and temperature control the input of water into a landscape and its loss via evapotranspiration (Groffman et al., 2000).

The composition and abundance of the microbial community reflect the long-term climate, soil disturbance history, and resource availability imposed on soils (Cavigelli and Robertson, 2000; Rich et al., 2003) by landscape factors. Understanding the effects of landscape on microbial communities, and the subsequent influences of microbial abundance and composition on the processes of nitrification and denitrification are key to predicting future N_2O emissions.

Traditionally, members of a microbial community were thought to be equivalent in function if they have a similar array of genes and enzymes (Cavigelli and Robertson, 2000). Evidence indicates that differences in ammonia oxidizing bacteria (AOB) and denitrifier community composition affect rates of nitrification and denitrification, which in turn may influence N_2O emissions (Cavigelli and Robertson, 2000; Avrahami et al., 2002; Mintie et al., 2003; Rich et al., 2003; Webster et al., 2005). These studies indicate that N_2O emissions can be altered through whole community adaptation or a change in the relative importance of certain members of the microbial community.

In a previous study, we determined that there was no difference in ammonia oxidizing bacterial *amoA* and denitrifier *nosZ* community composition between landforms in cultivated and uncultivated wetlands of a North American prairie pothole region (Ma et al., 2008). However, available soil N (Ma et al., 2008), soil organic carbon content (Bedard-Haughn et al., 2006a), and soil water regime (Yates et al., 2006) are different between land-uses

(i.e., cultivated and uncultivated) in these wetland landscapes. We did not include AOA ammonia monooxygenase (archaeal *amoA*) since the role of AOA in nitrification in agricultural soils is still debatable (Di et al., 2009; Jia and Conrad, 2009) and AOA activity is not linked to nitrous oxide emissions (Di et al., 2010).

The objective of this study was to examine microbial abundance and community composition in relation to N_2O associated with nitrification and denitrification processes over the course of a growing season in soils from cultivated and uncultivated wetlands. The abundance and community composition of AOB ammonia monooxygenase (bacterial *amoA*) and denitrifier nitrous oxide reductase (*nosZ*) genotypes were observed to (i) determine how these communities respond to different land-use and environmental conditions over time; and (ii) understand the possible correlations with N_2O emissions via nitrification and denitrification processes.

MATERIALS AND METHODS

STUDY SITE

The St. Denis National Wildlife Area (SDNWA) in central Saskatchewan, Canada (52°12′N, 106°5′W), is a typical example of the North American prairie pothole region. The wildlife area contains 216 wetlands distributed over an area of 3.84 km^2 (Hogan and Conly, 2002). Slope classes range from 10 to 15% (Miller et al., 1985) and soils are in the Dark Brown soil zone with loamy unsorted glacial till parent materials (Weyburn association). 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., 2006). Locations in cultivated wetlands were classified as convex (CX), concave (CV), or cultivated depression (CD) center. Convex elements were topographically high positions with a positive profile curvature. Concave elements were positions with negative profile curvature. CD elements were level positions that collected rain or snowmelt water.

Uncultivated wetlands were non-agricultural portions of the site and included vegetated depressions, which were further classified as basin center (BC), riparian grass (RG), or riparian trees (RT). BC elements were level areas covered by a variety of 99 non-grasses. RG elements were a non-level fringe area surrounding the BC and covered with grasses such as *Bromus inermis* Leyss. RT were the outer region of these wetlands and consisted of a partial fringe of mixed trees and shrubs, such as *Salix spp., Populus balsamifera* L., and *P. 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., 2006). 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 SAMPLING

Each landform element was replicated ($n = 3$) in space (Bedard-Haughn et al., 2006b). A total of 18 samples (2 land-uses × 3 wetlands × 3 landform elements) were collected on each of four sampling dates (June 1, July 13, August 16, and September 12, 2006). Each sample was a composite of five cores (0–15 cm depth; 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 (<24 h) to allow passage through a 2 mm sieve without smearing and stored at −20°C.

SOIL DENITRIFYING ENZYME ACTIVITY ASSAY

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 (field moist), 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). Nitrous oxide formation (N_2O_f) was also measured in anaerobic slurries that received the same treatment as DEA but without the C_2H_2. The ratio of N_2O_f to DEA (abbreviated as rN_2O) was 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 gage 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., 2006). All values are expressed per gram of oven-dried soil (dried at 105°C for 24 h).

^{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., 2008). Gravimetric soil water content was determined using standard procedures with an assumed particle density of 2.65 g cm^{-3} (Topp and Ferré, 2002). After packing, tubes were capped with parafilm and pre-incubated in the dark at room temperature (∼23°C) for 5 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 2 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} (0.2 μg $N{-}NO_3^-$ g^{-1} soil) to each tube. The soils were then brought to 70% water-filled pore space (WFPS) with deionized water. At time = 0 (i.e., immediately after WFPS adjustment), half the repacked cores were destructively sampled for ammonium and nitrate using a 2 M KCl extraction (Maynard et al., 2007). The remaining tubes (plus three 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 Ltd., UK). The cores were then destructively sampled for ammonium and nitrate by using 2 M KCl extraction.

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_2O produced in 24 h, together with the $^{15}N_2O$ produced, was 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). $^{15}N_2O$ denitrification rates are not reported. 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 (i.e., dissimilatory nitrate reduction to ammonia) had occurred (Bedard-Haughn et al., 2006b).

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; Cenciarini-Borde et al., 2009; Delgado-Viscogliosi et al., 2009). Ethidium monoazide bromide can intercalate double-stranded DNA, but because EMA cannot enter intact cells, it can only bind to extracellular DNA or DNA in cells with compromised membranes. Therefore, EMA can prevent the replication of DNA from non-viable organisms during polymerase chain reaction (PCR). The EMA treatment of soils followed Pisz et al. (2007). Soil DNA was extracted using the method described by Griffiths et al. (2000), except that soil mass was 1.0 g (field moist), centrifugation was at 14,000 × *g* and DNA were precipitated in PEG overnight at 20°C.

QUANTITATIVE PCR

Quantitative PCR (QPCR) was performed on all samples to determine the abundance of bacterial *amoA* and denitrifier *nosZ*, using the procedures and conditions reported by Ma et al. (2008). 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 QPCR, 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 stutzeri* (ATCC 14405). The standard for *amoA* was the *amoA*-1F/*amoA*-2R amplified PCR product from one of the soil extracts. All standards derived from soil were cloned and sequenced to confirm primer specificity. QPCR were performed in duplicate and amplification efficiencies were between 95 and 99%. Melting curve analysis was performed on each well and only those samples demonstrating melting curves similar to the control samples were used. QPCR results were expressed as number of gene copies per gram oven-dried soil (dried at 105°C for 24 h).

CLONING AND RFLP ANALYSIS OF PCR PRODUCTS

At our study site there is no landform difference in bacterial *amoA* and *nosZ* community composition (Ma et al., 2008). Therefore, we only evaluated community composition in water-accumulating landforms (i.e., the CD and BC elements). Community composition analysis was also 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 amplifying *amoA* and *nosZ* fragments follow Ma et al., 2008. PCR products of the expected size (490 bp for bacterial *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; 80 V 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 (McCune and Mefford, 2002), 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, SK, 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 cloned sequences were created using the programs DNADIST (Jukes–Cantor 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 (Felsenstein, 1997).

STATISTICAL ANALYSES

Data were imported into SPSS 14.0 and log transformed 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 the potential temporal relationships between *amoA* and *nosZ* abundance with the corresponding N_2O emitting functions.

Land-use and temporal differences in the community composition based on the presence/absence of 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). NMS 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 (Kenkel and Orlóci, 1986; Clarke, 1993). 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 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.

RESULTS

AMMONIA OXIDIZING BACTERIA AND DENITRIFIER ABUNDANCE AND ACTIVITY

Regardless of land-use or landform, AOB abundance increased up to 10-fold during the course of the sampling season (**Figures 1A,D**). Nitrification rates (**Figures 1B,E**) and nitrification-associated-N_2O emissions (**Figures 1C,F**), in

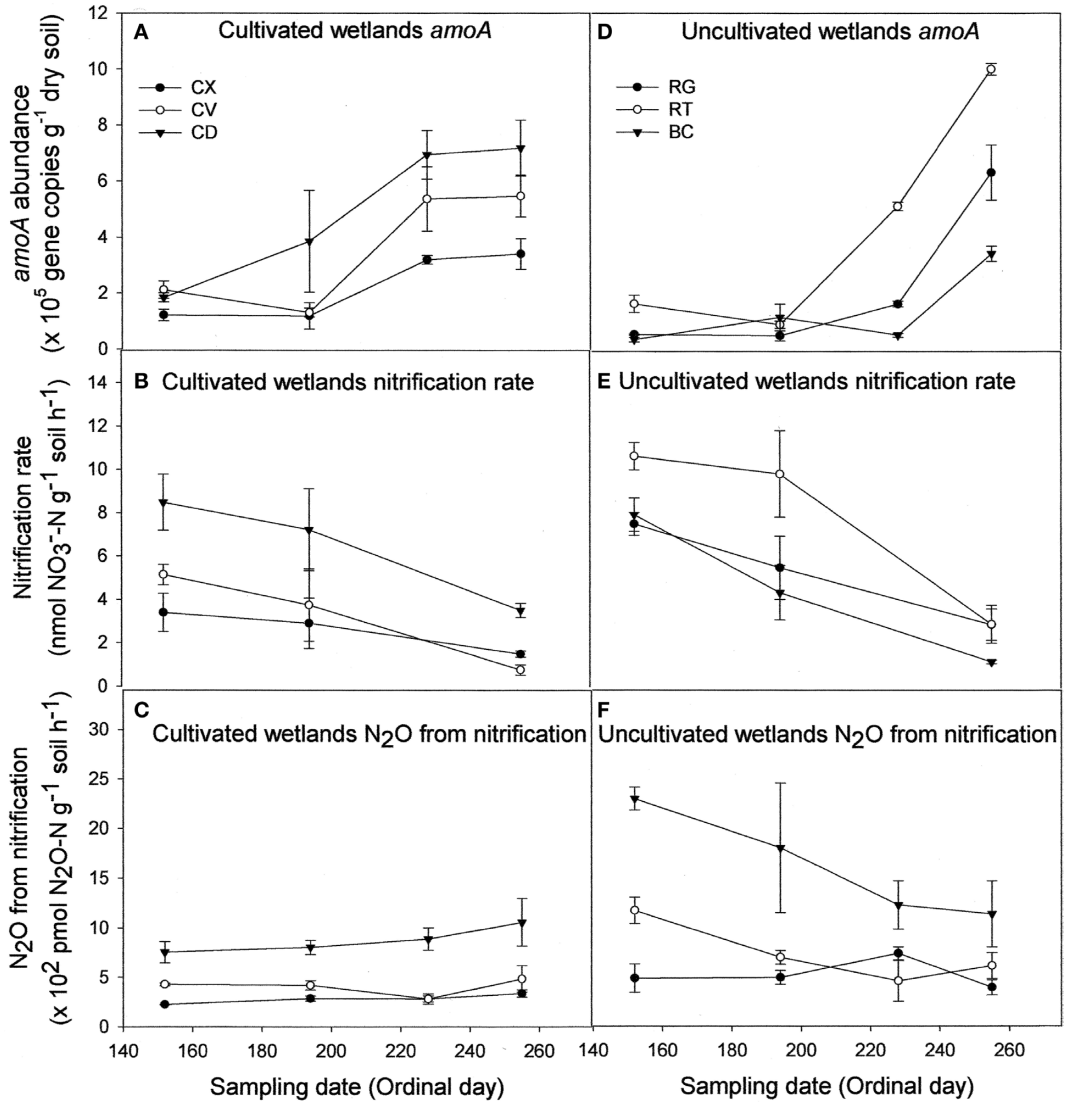

FIGURE 1 | Abundance of nitrifier *amoA* copies (A,D), nitrification rate (B,E), and N_2O emission attributable to nitrification (C,F) for cultivated (A–C) and uncultivated (D–F) wetland soils. Locations in cultivated wetlands (CW) were classified as either convex (CX), concave (CV), or cultivated depression (CD) center. 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 SE bars.

contrast, declined up to three-fold during the same period. Negative correlations between AOB abundance and nitrification rate ($r = -0.466$) and nitrification-associated-N_2O ($r = -0.267$) were significant (**Table 1**). Nitrification rate and nitrification-associated-N_2O emission were positively correlated ($r = 0.344$).

Contrary to bacterial *amoA* abundance, denitrifier *nosZ* abundance declined during the sampling season in all soils (**Figures 2A,E**). The gross potential N_2O production activity as described by the DEA results did not change during the season (**Figures 2B,F**). However, the rN_2O ratio increased over time (**Figures 2C,G**) and was negatively correlated to *nosZ* abundance (**Table 1**). Denitrifier abundance and DEA were positively correlated to percent WFPS at the time of soil sampling, while rN_2O was negatively correlated to WFPS (**Figures 2D,H**; **Table 1**).

AMMONIA OXIDIZING BACTERIA AND DENITRIFIER COMMUNITY COMPOSITION AND ACTIVITY

The *amoA* NMS produced a two-dimensional solution, with $r^2 = 0.42$ and 0.54 for Axis 1 (time) and 2 (land-use), respectively (**Figure 3**).Therefore, nearly all (96%) of the true variance structuring the difference in *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 temporal difference in *amoA* community composition. WFPS at the time of soil sampling in the field also had a negative correlation to change in *amoA* community composition over time. 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.

Five *amoA* OTUs were identified as indicator species. Four of the five OTUs grouped within Cluster 3 of AOB (**Figure 4**). These four OTUs increased in proportional abundance over time in one or both land-uses (**Figure 5**). Only *amoA* OTU 17 grouped within Cluster 2, and its proportional abundance declined.

The NMS identified a two-dimensional solution for the differences in denitrifier community composition, with $r^2 = 0.40$ and 0.29 for Axis 1 (land-use) and 2 (time), respectively (**Figure 6**). Therefore, 69% of the true variance structuring the differences in denitrifier community composition was a function of land-use and time as represented in this ordination solution. The MRPP confirmed that communities differed as the result of land-use and time (**Table 2**). The joint plot of *nosZ* abundance, DEA, rN_2O, and WFPS indicated these parameters correlated strongly with the time gradient (54, 57, 65, and 43% respectively; **Figure 6**). The difference in denitrifier community composition over time was correlated to difference in denitrifier abundance and activity and soil moisture at the time of sampling.

Indicator species analysis was used to identify *nosZ* OTUs that differentiated denitrifier communities based on land-use and time. Five *nosZ* OTUs were identified that significantly differentiated denitrifier communities ($P < 0.1$). There was no discernable pattern in proportional abundance for these OTUs (**Figure 5**). However, OTU 7 was the only *nosZ* genotype exclusive to cultivated wetland soils, and OTU 24 was the only genotype exclusive to the September 12 sampling date for both land-uses. The majority of these OTUs clustered with Rhizobiaceae of the α-Proteobacteria (**Figure 7**). 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).

DISCUSSION

We found abundance and community composition of bacterial *amoA* and denitrifier *nosZ* to be structured by both time and land-use. While temporal abundance of denitrifier *nosZ* appears to be related to potential soil N_2O emissions, temporal abundance of bacterial *amoA* abundance was negatively correlated with nitrification and nitrification-associated-N_2O. Environmental conditions, seasonal shifts in community composition and nitrification activity, as well as, methodological limitations may account for this trend in *amoA* abundance. While differences in community composition between land-use were observed for both bacterial *amoA* and denitrifier *nosZ*, these differences were not clearly linked to N_2O emitting activity. However, differences in denitrifier *nosZ* community composition over time were correlated with differences in denitrifier abundance, denitrifier activity and soil moisture at the time of sampling.

The temporal abundance of denitrifier *nosZ* is related to potential soil N_2O emission because *nosZ* abundance directly affects nitrous oxide reductase activity (NOS). Accordingly, we found NOS activity declined (rN_2O increased) as abundance of *nosZ* declined. The abundance of active denitrifiers based on *nosZ* abundance reported here is similar to that observed using qPCR-based reports (Baudoin et al., 2009) but approximately 10 to 100 times lower than that reported for cultivation-based enumeration from other soils (McCarty et al., 2007).

Table 1 | Correlations between *amoA* and *nosZ* abundance with ^{15}N-N_2O produced from nitrification and denitrification enzyme assay and water-filled pore space, 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 (two-tailed).*

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

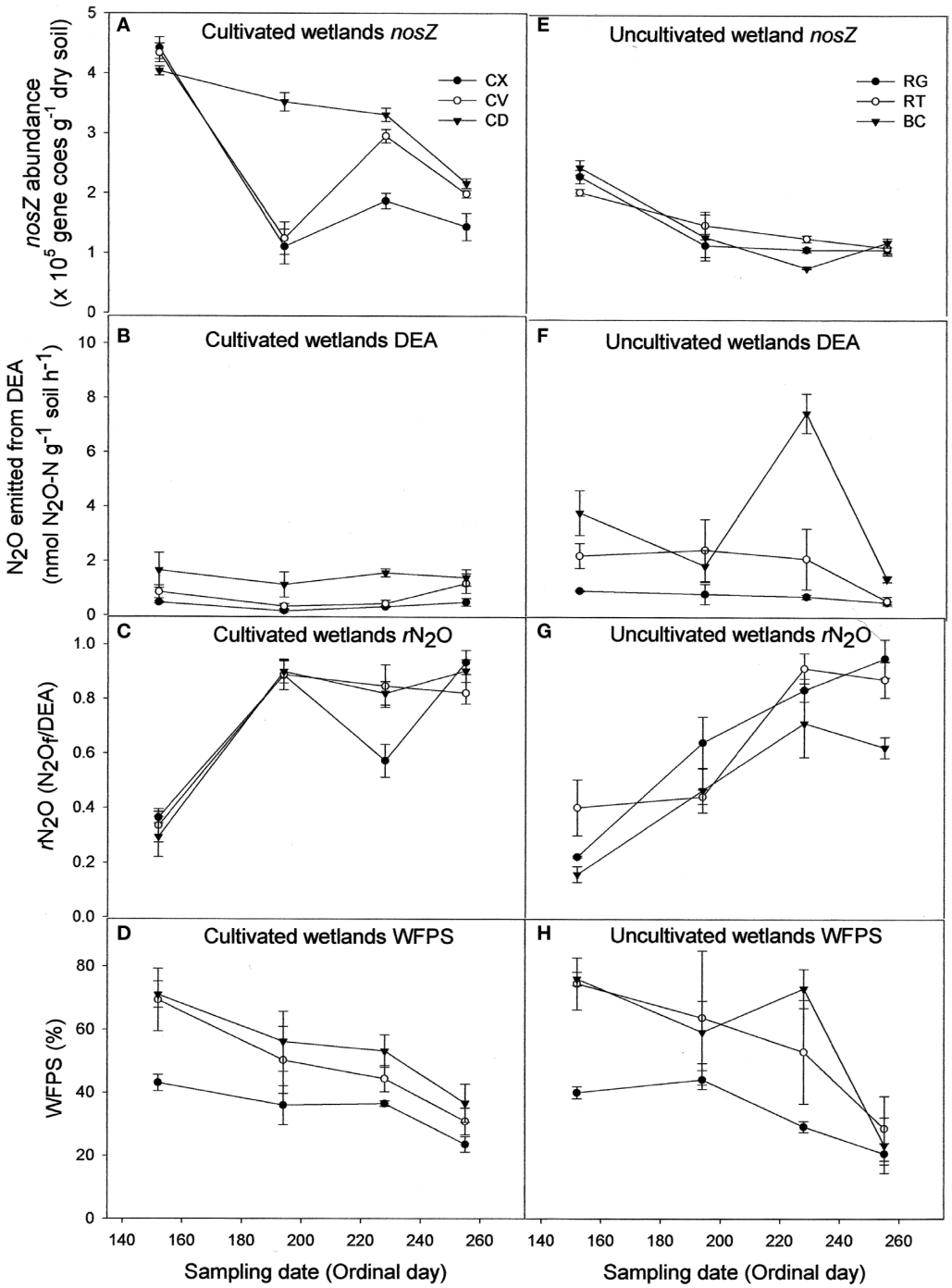

FIGURE 2 | Abundance of denitrifier *nosZ* copies (A,E), N_2O emitted from denitrification enzyme assay [DEA; (B,F)], rN_2O [N_2O_f/DEA; (C,G)], and percentage water-filled pore space [WFPS; (D,H)] for cultivated (A–D) and uncultivated (E–H) wetland soils. Locations in cultivated wetlands (CW) were classified as either convex (CX), concave (CV), or cultivated depression (CD) center. 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 or 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 SE bars.

A negative correlation between bacterial *amoA* abundance and nitrification activity is counter-intuitive. Varying environmental conditions, community composition and nitrification activity, as well as, methodological limitations may account for this discrepancy. Changes in environmental conditions (i.e., available water, N, and O_2) could cause an increase in abundance of

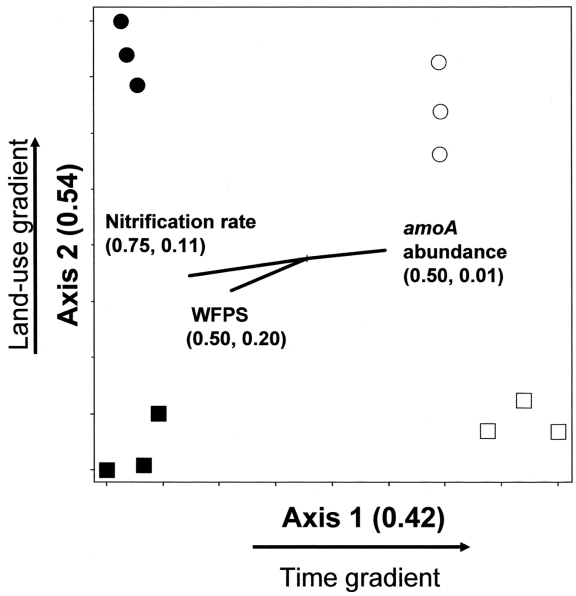

FIGURE 3 | Non-metric multidimensional scaling ordination of nitrifying communities based on presence/absence of operational taxonomic units (OTUs) defined by *amoA* RFLP patterns. □ = Jun 01; cultivated wetlands. ■ = Sep 12; cultivated wetlands. ○ = Jun 01; uncultivated wetlands. ● = Sep 12; uncultivated 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).

certain populations, which may alter AOB community composition (**Table 3**). For example, Webster et al. (2005) demonstrated members of *Nitrosospira* cluster 3a were present in soils at low levels until ammonia concentrations were reduced. Therefore, community composition and abundance of specific AOBs must be considered in combination to assess the rates of different processes, such as nitrification. Within the context of our observations, the resultant AOB communities might shift from a community with populations that are highly active to a community with populations that are less active, over the course of a growing season. Thus, in the latter part of the growing season nitrifier abundance is not directly proportional to activity, because early season growth, results in nitrifier biomass that is no longer active late in the season. Alternatively, another microbial population may be oxidizing ammonia to nitrate, but this nitrification activity is erroneously assigned to AOB due to our inability to separate their independent contributions.

Gene copy numbers alone cannot account for the myriad of transcriptional responses a dynamic AOB community might have in response to changes in environmental conditions. Freitag and Prosser (2009) observed an incongruity between methyl coenzyme M reductase sub-unit A gene (*mcrA*) abundance for methanogens and methanogenesis. However, they found a significant predictive relationship between the ratio of *mcrA* transcripts to gene copies and the rate of methanogenesis. This ratio might be an improved indicator of process rates because it normalizes the response of each population to environmental condition (i.e., transcripts) and to their relative abundance (i.e., gene abundance). A similar approach might be necessary to better understand the

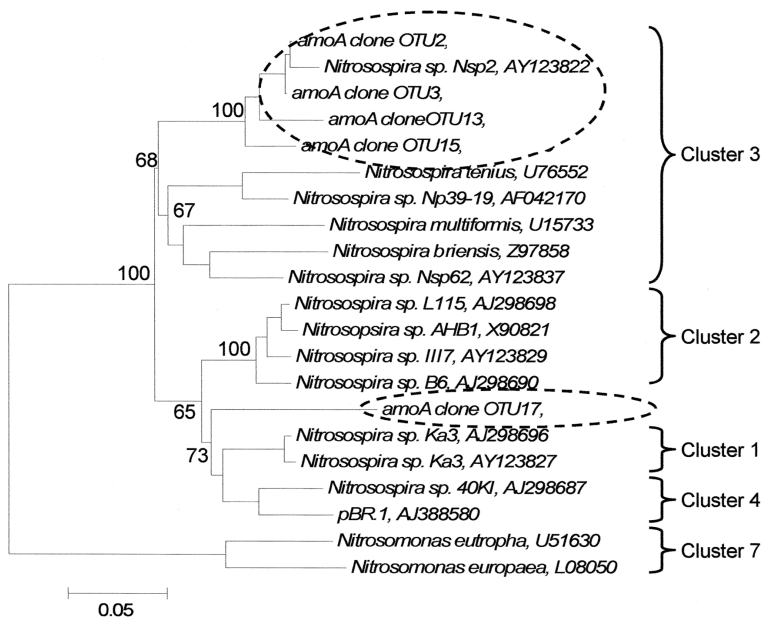

FIGURE 4 | Phylogenetic tree of cloned *amoA* operational taxonomic units that significantly delineated land-use as determined by indicator species analysis. Broken ovals highlight cloned sequences from this study. The label of the sequence used in the analysis is followed by their respective GenBank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

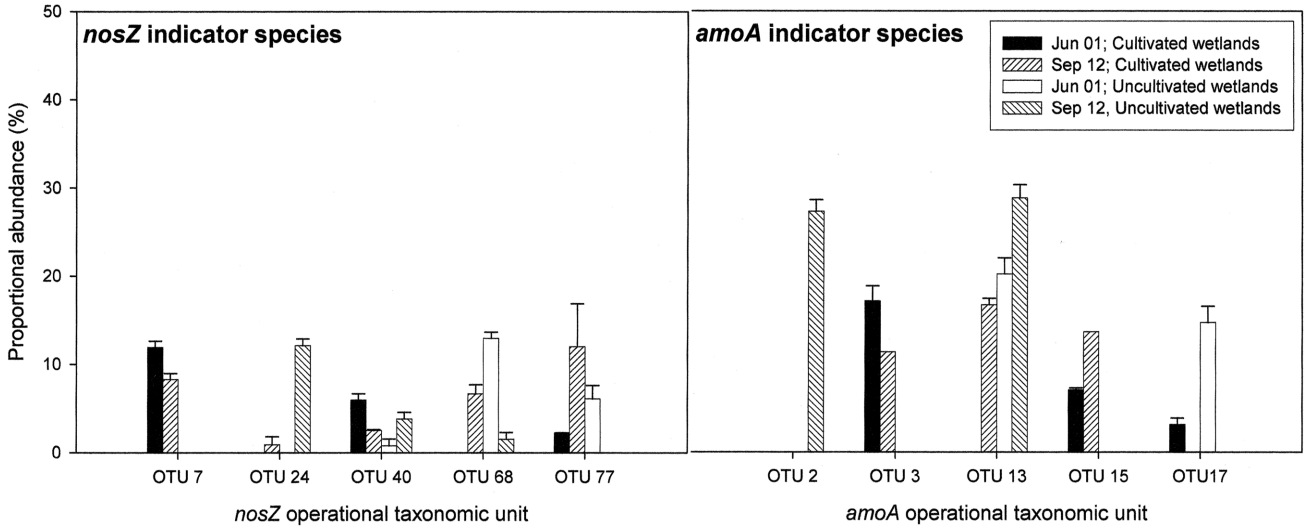

FIGURE 5 | *nosZ* and *amoA* operational taxonomic units (as defined by RFLP) that differed significantly between land-use and time based on indicator species analysis ($P < 0.1$). Results are means with SE ($n = 3$). Proportional abundance is the percentage of recovered clones with the defined RFLP.

relationship between bacterial *amoA* abundance, nitrification rate, and nitrification-associated-N_2O emissions.

In addition, our ^{15}N isotope labeling methodology has limitations. By using only ^{15}N-labeled ammonium and nitrate, cross feeding of nitrifier produced NO_2^- and NO_3^- to denitrification is masked. Wrage et al., 2005 present a dual labeling approach that employs an ^{18}O–^{15}N-enrichment method allowing for distinction between nitrous oxide (N_2O) from nitrification, nitrifier denitrification and denitrification. Use of a dual labeling approach may have improved our ability to discriminate between the contributions of nitrifier and conventional denitrification to total N_2O production.

In our study, *amoA* abundance is only about 10% of that reported by other investigators (Jia and Conrad, 2009; Offre et al., 2009). Higher *amoA* abundance reported in other studies, likely reflects measurement of whole community DNA, including non-viable copies of *amoA*. We measured only the viable organisms because we had pre-treated the soil samples with EMA to isolate these organisms. We previously evaluated EMA efficiency

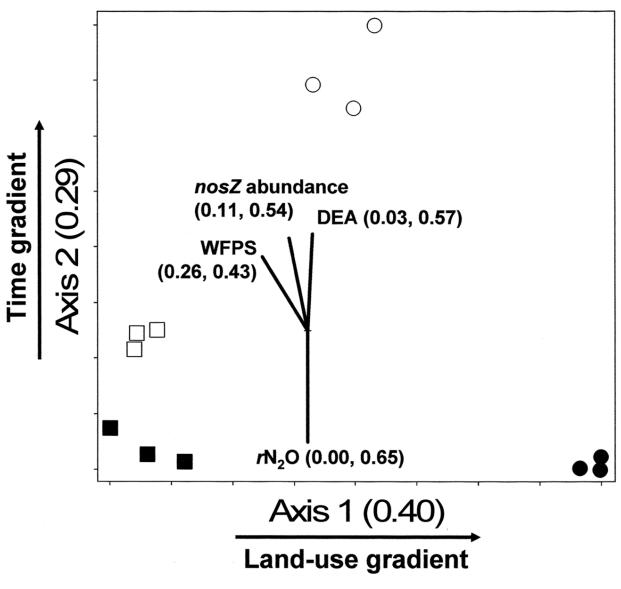

FIGURE 6 | Non-metric multidimensional scaling (NMS) ordination of denitrifying communities based on presence/absence of operational taxonomic units (OTUs) defined by *nosZ* RFLP patterns. □ = Jun 01; cultivated wetlands (CW). ■ = Sep 12; cultivated wetlands (BC). ○ = Jun 01; uncultivated wetlands. ● = 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). Note: only functions with $r^2 \geq 40\%$ to either NMS axes are shown in joint plots.

Table 2 | 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	

Average distance is the mean Euclidean distance between each pair-wise 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. The MRPP was significant (P (0.01).

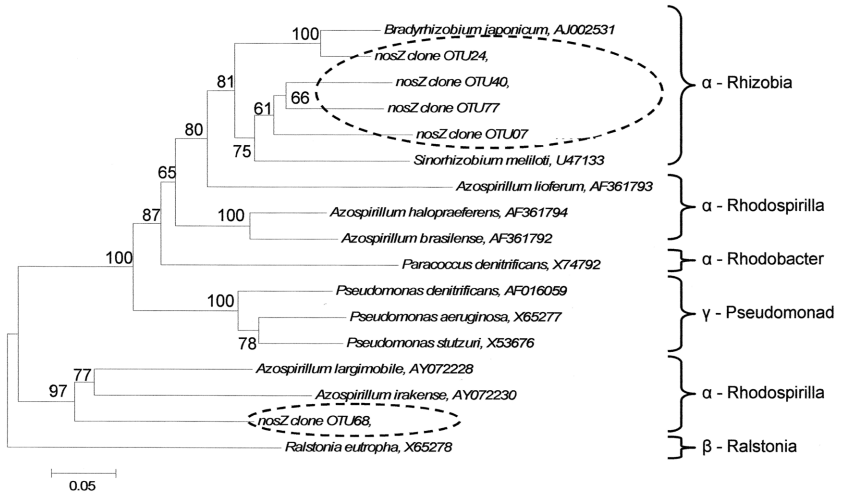

FIGURE 7 | Phylogenetic tree of cloned *nosZ* operational taxonomic units that significantly delineated land-use and time as determined by indicator species analysis. Broken ovals highlight cloned sequences from this study. The label of the sequence used in the analysis is followed by their respective GenBank accession. Branch nodes with bootstrap values greater than 60 are labeled. Scale bar indicates 5 changes per 100 nucleotide positions.

Table 3 | Two molar KCl extractable ammonium and nitrate concentrations in soils from each landform class in the St. Denis National Wildlife Area collected June 1st–September 12th, 2006.

Landform	Ammonium (μg N g^{-1} soil)†				Nitrate (μg N g^{-1} soil)†			
	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 center (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 SE in parenthesis.*

on nitrifiers and found that it worked well, however this evaluation was limited to polar soils (Pisz et al., 2007). It is possible that the EMA technique is under-reporting *amoA* abundance in these agriculture soils. While all inhibitors have significant drawbacks, an alternative inhibitor, such as propidium monoazide (PMA) may reduce the possibility of under-reporting due to EMA penetrating intact cells and removing target DNA (Nocker et al., 2006). Intact cell membranes are impermeable to PMA and several studies have used PMA to differentiate naked DNA and dead cells from bacteria with an intact cell membrane (Nocker et al., 2007; Yergeau et al., 2010).

In soils from the water-accumulating landforms, differences in *nosZ* community composition over time were linked to N_2O emitting activity. However, differences in community composition between land-use were not linked to N_2O emitting activity. The effects of *nosZ* are greater than what is explained by environmental factors (e.g., soil water content) alone (Rich et al., 2003). We found WFPS accounts for 33 and 13% of the variation in DEA and $r$$N_2O$, respectively. In contrast, less than 5% of the variation in DEA and $r$$N_2O$ is correlated to the land-use differences in the *nosZ* community composition (**Figure 6**). These findings are in agreement with those of Ma et al. (2008), who found no relationship between *nosZ* community composition and denitrification-associated-N_2O emission as a function of landscape. In agricultural systems others have also found no link between *nosZ* community composition and N_2O emission (Rich and Myrold, 2004; Enwall et al., 2005), however, *nosZ* and N_2O have been linked in meadow and forest soils (Rich et al., 2003).

CONCLUSION

In our study, both *amoA* and *nosZ* changed dramatically over the course of the season, but only *nosZ* was related to differences in potential N_2O emissions. Differences in community composition between land-use (i.e., cultivated and uncultivated) were not clearly linked to the N_2O emitting activity of *amoA* or of *nosZ*. The negative correlation between bacterial *amoA* abundance and nitrification and nitrification-associated-N_2O may be in response to several factors. Changing environmental conditions, seasonal shifts in community composition and nitrification activity, as well as, methodological limitations may act in concert to result in a

negative correlation. Regardless, our study indicates that *nosZ* may be an effective tool to monitor denitrifier contributions to N_2O emissions in a field setting. To characterize N_2O emissions from ammonia oxidation processes, however, a more refined genetic target or approach is clearly needed.

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REFERENCES

Arah, J. R. M. (1997). Apportioning nitrous oxide fluxes between nitrification and denitrification using gas-phase mass spectrometry. *Soil Biol. Biochem.* 29, 1295–1299.

Avrahami, S., Conrad, R., and Braker, G. (2002). Effects of soil ammonium concentrations on N_2O release and on the community structure of ammonia oxidizers and denitrifiers. *Appl. Environ. Microbiol.* 68, 5685–5692.

Baudoin, E., Philippot, L., Cheneby, D., Chapui-Lardy, L., Fromin, N. Bru, D., Rabary, and B., and Brauman, A. (2009). Direct seeding mulch-based cropping increases both the activity and the abundance of denitrifier communities in a tropical soil. *Soil Biol. Biochem.* 41, 1703–1709.

Bedard-Haughn, A., Jongbloed, F., Akkerman, J., Uijl, A., de Jong, E., Yates, T., and Pennock, D. J. (2006a). The effects of erosional and management history on soil organic carbon stores in ephemeral wetlands of hummocky agricultural landscapes. *Geoderma* 135, 296–306.

Bedard-Haughn, A., Matson, A. L., and Pennock, D. J. (2006b). Land use effects on gross nitrogen mineralization, nitrification, and N_2O emissions in ephemeral wetlands. *Soil Biol. Biochem.* 38, 3398–3406.

Bedard-Haughn, A., Tate, K. W., and van Kessel, C. (2004). Using nitrogen-15 to quantify vegetative buffer effectiveness for sequestering nitrogen in runoff. *J. Environ. Qual.* 33, 2252–2262.

Bruns, M. A., Stephen, J. R., Kowalchuk, G. A., Prosser, J. I., and Paul, E. A. (1999). Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. *Appl. Environ. Microbiol.* 65, 2994–3000.

Cavigelli, M. A., and Robertson, G. P. (2000). The functional significance of denitrifier community composition in a terrestrial ecosystem. *Ecology* 81, 1402–1414.

Cenciarini-Borde, C., Courtois, S., and La Scola, B. (2009). Nucleic acids as viability markers for bacterial detection using molecular tools. *Future Microbiol.* 4, 45–64.

Clarke, K. R. (1993). Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* 18, 117–143.

Delgado-Viscogliosi, P., Solignac, L., and Delattre, J. M. (2009). Viability PCR, a culture-independent method for rapid and selection quantification of viable *Legionella pneumophila* cells in environmental water samples. *Appl. Environ. Microbiol.* 75, 3502–3512.

Di, H. J., Cameron, K. C., Shen, J. P., Winefield, C. S., O'Callaghan, M., Bowatte, S., and He, J. Z. (2009). Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nat. Geosci.* 2, 621–624.

Di, H. J., Cameron, K. C., Sherlock, R. R., Shen, J. P., He, J. Z., and Winefield, C. S. (2010). Nitrous oxide emissions from grazed grassland as affected by a nitrification inhibitor, dicyandiamide, and relationships with ammonia-oxidizing bacteria and archaea. *J. Soil Sediments* 10, 943–954.

Enwall, K., Philippot, L., and Hallin, S. (2005). Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. *Appl. Environ. Microbiol.* 71, 8335–8343.

Felsenstein, J. (1997). An alternating least squares approach to inferring phylogenies from pairwise distances. *System. Biol.* 46, 101–111.

Freitag, T. E., and Prosser, J. I. (2009). Correlation of methane production and functional gene transcriptional activity in a peat soil. *Appl. Environ. Microbiol.* 75, 6679–6687.

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., and Bailey, M. J. (2000). Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl. Environ. Microbiol.* 66, 5488–5491.

Groffman, P. M., Brumme, R., Butterbach-Bahl, K., Dobbie, K. E., Mosier, A. R., Ojima, D. S., Papen, H., Partin, W. J., Smith, K. A., and Wagner-Riddle, C. (2000). Evaluating annual nitrous oxide fluxes at the ecosystem scale. *Global Biogeochem. Cycles* 14, 1061–1070.

Hayashi, M., Van der Kamp, G., and Rudolph, D. L. (1998). Water and solute transfer between a prairie wetland and adjacent uplands, 1. Water balance. *J. Contam. Hydrol.* 207, 42–55.

Hogan, J. M., and Conly, F. M. (2002). *St. Denis National Wildlife Area Land Cover Classification: 1997.* Technical Report Series No. 384. Canadian Wildlife Service, Prairie and Northern Region, Saskatoon.

Horz, H. P., Barbrook, A., Field, C. B., and Bohannan, B. J. M. (2004). Ammonia-oxidizing bacteria respond to multifactorial global change. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15136–15141.

Jia, Z., and Conrad, R. (2009). Bacteria rather than archaea dominate microbial ammonia oxidation in an agricultural soil. *Environ. Microbiol.* 11, 1658–1671.

Jungkunst, H. F., and Fiedler, S. (2007). Latitudinal differentiated water table control of carbon dioxide, methane and nitrous oxide fluxes from hydromorphic soils: feedbacks to climate change. *Global Change Biol.* 13, 2668–2683.

Kenkel, N. C., and Orlóci, L. (1986). Applying metric and nonmetric multidimensional-scaling to ecological studies: some new results. *Ecology* 67, 919–928.

Laughlin, R. J., and Stevens, R. J. (2002). Evidence for fungal dominance of denitrification and codenitrification in a grassland soil. *Soil Sci. Soc. Am. J.* 66, 1540–1548.

Ma, W. K., Bedard-Haughn, A., Siciliano, S. D., and Farrell, R. E. (2008). Relationship between nitrifier and denitrifier community composition and abundance in predicting nitrous oxide emissions from ephemeral wetland soils. *Soil Biol. Biochem.* 40, 1114–1123.

Maynard, D. G., Kalra, Y. P., and Crumbaugh, J. A. (2007). "Nitrate and exchangeable ammonium nitrogen," in *Soil Sampling and Methods of Analysis*, 2nd Edn, eds M. R. Carter and E. D. Gregorich (Boca Raton: CRC Press), 71–80.

McCarty, G. W., Mookherji, S., and Angier, J. T. (2007). Characterization of denitrification activity in zones exfiltration within a riparian wetland ecosystem. *Biol. Fertil. Soils* 43, 691–698.

McCune, B., and Mefford, M. J. (1999). *PC-ORD: Multivariate Analysis of Ecological Data.* Gleneden Beach, OR: MjM Software Design.

McCune, B., and Mefford, M. J. (2002). *Analysis of Ecological Communities.* Gleneden Beach, OR: MjM Software Design.

Miller, J. J., Acton, D. F., and St. Arnaud, R. J. (1985). The effect of groundwater on soil formation in a morainal landscape in Saskatchewan. *Can. J. Soil Sci.* 65, 293–307.

Mintie, A. T., Heichen, R. S., Cromack, K., Myrold, D. D., and Bottomley, P. J. (2003). Ammonia-oxidizing bacteria along meadow-to-forest transects in the Oregon Cascade Mountains. *Appl. Environ. Microbiol.* 69, 3129–3136.

Nocker, A., Cheung, C. Y., and Camper, A. K. (2006). Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J. Microbiol. Methods* 67, 310–320.

Nocker, A., Sossa-Fernandez, P., Burr, M. D., and Camper, A. K. (2007). Use of propidium monoazide for live/dead distinction in microbial ecology. *Appl. Environ. Microbiol.* 73, 5111–5117.

Nogva, H. K., Drømtorp, S. M., Nissen, H., and Rudi K. (2003). Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5′-nuclease PCR. *BioTechniques* 34, 804–813.

Offre, P., Prosser, J. I., and Nicol, G. W. (2009). Growth of ammonia-oxidizing archaea in soil microcosms is inhibited by acetylene. *FEMS Microbiol. Ecol.* 70, 99–108.

Pisz, J. M., Lawrence, J. R., Schafer, A. N., and Siciliano, S. D. (2007). Differentiation of genes extracted from non-viable versus viable microorganisms in environmental samples using ethidium monoazide bromide. *J. Microbiol. Methods* 71, 312–318.

Rich, J. J., Heichen, R. S., Bottomley, P. J., Cromack, K., and Myrold, D. D. (2003). Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. *Appl. Environ. Microbiol.* 69, 5974–5982.

Rich, J. J., and Myrold, D. D. (2004). Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. *Soil Biol. Biochem.* 36, 1431–1441.

Rotthauwe, J. H., Witzel, K. P., and Liesack, W. (1997). The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* 63, 4704–4712.

Stark, J. M., and Hart, S. C. (1996). Diffusion technique for preparing salt solutions, Kjeldahl digests, and persulfate digests for nitrogen-15 analysis. *Soil Sci. Soc. Am. J.* 60, 1846–1855.

Stevens, R. J., Laughlin, R. J., Burns, L. C., Arah, J. R. M„ and Hood, R. C. (1997). Measuring the contributions of nitrification and denitrification to the flux of nitrous oxide from soil. *Soil Biol. Biochem.* 29, 139–151.

Stres, B., Avgustin, G., and Tiedje, J. M. (2004). Nitrous oxide reductase gene fragments differ between native and cultivated Michigan soils. *Appl. Environ. Microbiol.* 70, 301–309.

Svensson, B. H., Klemedtsson, L., Simkins, S., Paustian, K., and Rosswall, T. (1991). Soil denitrification in three cropping systems characterized by differences in nitrogen and carbon supply.1. Rate-distribution frequencies, comparison between systems and temporal N-losses. *Plant Soil* 138, 257–271.

Tiedje, J. M. (1994). "Denitrifiers," in *Microbiological and Biochemical Properties*, Vol. 5, *Methods of Soil Analysis*, Part 2, eds R. W., Weaver, J. S., Angle, and P. Bottomley (Madison, WI: Soil Science Society of America), 245–267.

Topp, G. C., and Ferré, P. A. (2002). "Water Content," in *Methods of Soil Analysis*, Part 1, *Physical and Mineralogical Properties*, eds J. Dane and C. Topp (Madison, WI: Soil Science Society of America), 417–545.

Webster, G., Embley, T. M., Freitag, T. E., Smith, Z., and Prosser, J. I. (2005). Links between ammonia oxidizer species composition, functional diversity and nitrification kinetics in grassland soils. *Environ. Microbiol.* 7, 676–684.

Wrage, N., van Groenigen, J. W., Oenema, O., and Baggs, E. M. (2005). A novel dual-isotope labelling method for distinguishing between soil sources of N2O. *Rapid Commun. Mass Spectrom.* 19, 3298–3306.

Yates, T. T., Si, B. C., Farrell, R. E., and Pennock, D. J. (2006). Wavelet spectra of nitrous oxide emission from hummocky terrain during spring snowmelt. *Soil Sci. Soc. Am. J.* 70, 1110–1120.

Yergeau, E., Hogues, H., Whyte, L. G., and Greer, C. W. (2010). The functional potential of high Arctic permafrost revealed by metagenomic sequencing, qPCR and microarray analyses. *ISME J.* 4, 1206–1214.

Zimmerman, G. M., Goetz, H., and Mielke, P. W. (1985). Use of an improved statistical method for group comparisons to study effects of prairie fire. *Ecology* 66, 606–611.

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