Experimental Nitrogen Deposition Influences Microbial Denitrifying Communities and Increases Denitrification Rates in a Northern Hardwood Forest

By

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Abstract

With more biologically reactive nitrogen (N) becoming available in terrestrial ecosystems due to atmospheric N deposition, it is important to understand its impact on forest biogeochemistry. Long-term experimental N deposition in a northern hardwood ecosystem increased the assimilation of N by plants (Zak et al., 2004) as well as dramatically increased the export of NO_3^- via leaching (Pregitzer et al., 2004). This study aimed to quantify changes in denitrification, another possible fate of N, and changes in the bacterial denitrifier community after more than 16 years of experimental N deposition. We hypothesized that the increased NO_3^- availability would increase denitrification rates and N_2O production due to an increase in gene abundance and changes in composition of bacterial denitrifiers. Using laboratory assays, we found that denitrification rates and N_2O production were significantly higher under experimental N deposition. However, contrary to our hypothesis, five denitrification genes (*norB*, *narG*, *nirK*, *nirS*, and *nosZ*) decreased under experimental N deposition. Additionally, no relationship occurred between the abundance of *nosZ* and N_2O production. This apparent uncoupling of functional gene abundance and function may be due to differences in denitrifier community membership. These results indicate that the observed increases in denitrification rates and N_2O production, shift in denitrifier composition, and decrease in functional gene abundance demonstrate that chronic atmospheric N deposition has altered denitrification on both a molecular and ecosystem-level scale, albeit these responses are small at an ecosystem level.

Keywords: atmospheric N deposition, denitrification, bacterial denitrifiers, functional gene abundance, N saturation

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Introduction

The global nitrogen (N) cycle has been modified by human food and energy production, specifically the production of N fertilizers, planting of leguminous crops, and burning fossil fuels. These anthropogenic processes have doubled the quantities of biologically reactive N in terrestrial and aquatic ecosystems (Vitousek et al., 1997). Nitrogen deposition is projected to increase substantially due to the growing demand for energy and food, as human populations increase into the future (Galloway et al., 2004). Terrestrial deposition of NO_y alone has increased from 6.6 Tg N yr^{-1} in 1860, to 24.8 Tg N yr^{-1} in the 1990's and is projected to reach 42.2 Tg N yr^{-1} by 2050 (Galloway et al., 2004). The deposition of NH_x has even higher projections reaching 83 Tg N yr^{-1} by 2050 in terrestrial environments (Galloway et al., 2004). Moreover, atmospheric N deposition is predicted to be more highly concentrated in certain geographic locations, such as southeastern Asia, Europe, and the eastern US (Galloway et al., 2004; Galloway et al., 2008). Consequently, it is imperative to understand this modification of the global N cycle because it has implications for many organisms and contributes to eutrophication, smog, climate change, acid deposition, and stratospheric ozone depletion (Galloway et al., 2004).

Nitrogen deposition is the input of biologically reactive N into the biosphere from the atmosphere, which can occur as cloud, dry, or wet deposition (Fowler, 1980; Lovett and Kinsman, 1990; Lovett, 1994). Cloud deposition is the input of small, condensed water droplets on plant surfaces and is mainly restricted to coastal or mountainous regions that are often immersed in fog (Lovett, 1994). Dry deposition is the direct deposition of particles and gases on surfaces such as soil, vegetation, and water (Lovett, 1994). Wet deposition occurs

when N gases, such as NO_x, dissolve in precipitation, causing it to become more acidic (Lovett, 1994). Both dry and wet deposition are ubiquitous, but vary in importance geographically (Lovett, 1994). For example, anthropogenic emissions of N in the Midwest, Southeast, and Northeast US result in elevated deposition of NO_3^- over the Eastern US, whereas there is greater deposition of NH_4^+ in the Midwest and eastern Great Plaints due to intensive agriculture (Lovett, 1994). Ammonia is deposited locally because its residence time in the atmosphere is only 1-2 hours; however, NO_2 can be transported and deposited much further from its source because its residence time in the atmosphere is ~ 1-2 days (Langan, 1999).

The consequences of atmospheric N deposition are global and widespread within terrestrial and aquatic systems. The anthropogenic increase of available N can increase ecosystem productivity, and it can also decrease biodiversity due to acidification and eutrophication (Matson et al., 2002; Rabalais, 2002). Nitrogen deposition in forests can alter plant community composition and plant-soil interactions, in addition to creating an "open" N cycle, rather than the naturally occurring "closed loop" retaining N in terrestrial ecosystems (Nadelhoffer, 2008). There are human health concerns as well, because NO_3^- is soluble and can leach to ground and surface waters. Evidence suggests that elevated levels of NO_3^- in drinking water can increase the risk of certain cancers, adverse reproductive outcomes, and other chronic health effects (Ward et al., 2005).

Excess N tends to "cascade" through the environment, meaning that one single molecule of reactive N can participate in many biogeochemical processes. This complicates the notion of an ultimate fate for the excess reactive anthropogenic N. In the 1990's, the fate of only 35% of reactive N inputs to terrestrial ecosystems were known and included

denitrification in marine and terrestrial ecosystems and deposition in the ocean (Galloway et al., 2008). Therefore, the fate of 65% of reactive N remains unknown, and it is assumed to have accumulated in soils, vegetation, or is lost to groundwater or denitrification (Galloway et al., 2008). In an 8-year study, chronic NO_3^- deposition significantly increased the production and leaching of NO_3^- and organic N compounds, which has implications in regulating food webs of aquatic ecosystems (Pregitzer et al., 2004). Aside from plant uptake and chelation with soil organic matter, an ideal fate for excess reactive N in the biosphere is to be returned to the atmosphere as N_2 via the process of denitrification.

Denitrification is a microbially mediated N transformation of nitrate (NO_3^-) to either N_2 or nitrous oxide (N_2O). The ability to denitrify is widespread among bacteria, encompassing more than 60 genera, and to a lesser extent among Archaea and fungi (Philippot et al., 2007). Denitrifiers use NO_3^- and nitrite (NO_2^-) as electron acceptors during the stepwise reduction to N_2 under anaerobic conditions.

There are several main factors that control denitrification. Because denitrifying bacteria are heterotrophs, the availability of organic substrates directly influences their physiological activity. Anaerobic conditions are also necessary, because NO_3^- is respired via denitrification when O_2 partial pressures are very low (Tiedje, 1982). In soil, anaerobic conditions are often induced by soil texture and drainage, which could account for 86% of observed denitrification variability (Groffman and Tiedje, 1989). Additionally, denitrification has been found to significantly increase with NO_3^- availability (Merrill and Zak, 1992). Generally, most denitrifying bacteria are found in the upper 5 cm of soil and their abundance decreases with depth (Mergel et al., 2001), indicating this biogeochemical process is restricted to surface soil horizons.

The transformations reducing NO_3^- to N_2 are enzymatically mediated steps; therefore, there is a specialized enzyme responsible for each reduction. Consequently, the genes that code these enzymes are often targets in molecular approaches to characterize the denitrifying community (Wallenstein et al., 2006). Comparisons of culturable denitrifying bacteria to those revealed by molecular analyses indicate that DNA-based techniques are adequate in assessing seasonal changes in denitrifying populations (Mergel et al., 2001). However, molecular methods involving 16S rRNA do not appropriately detect this physiological group in the environment, because denitrifiers do not have a close phylogenetic relationship (Braker et al., 1998). Nitrite reductase (*nir*) genes were the first to be used in denitrifier diversity studies and have continued to be the most common molecular marker for denitrifier communities (Wallenstein et al., 2006). There are two types of nitrite reductase enzymes: copper (Cu) *nir*K genes and a cytochrome cd_1-nitrite reductase encoded by *nir*S genes. *nir*S in more widespread but less conserved among bacteria than is *nir*K (Bothe, 2000; Coyne, 1989); *nir*K is present in a wider range of phylogenetically disparate bacteria (Coyne, 1989). This concept is exemplified in Priemé et al (2002), wherein they could amplify *nir*K genes from both marsh and upland Michigan soil, but *nir*S could only be amplified from the marsh soil. Real-time PCR has revealed *nir*K densities of 9.7×10^4 to 3.9×10^6 copies per gram of soil (Henry et al., 2004). These quantitative measures of gene copy number can be used to infer cell numbers, because there is only one copy of the *nirK* gene in denitrifying bacteria (Philippot, 2002).

Other genes used during the denitrification pathway have also been studied. The dissimilatory reduction of nitrate has two homologous enzymes (Nar and Nap), but since these nitrate reductases can be found in bacteria that do not denitrify, the genes *narG* and

napA are not widely used for denitrification studies (reviewed in Wallenstein et al., 2006). Also, the nitric oxide reductase gene (*norB*) has been studied for its unique ability to form a N-N bond (Braker and Tiedje, 2003).

Nitric oxide reductase (*nos*) genes have also been studied, because N_2O is the final product for organisms lacking *nos*Z and N_2O is a potent greenhouse gas. Approximately 30% of denitrifying bacteria lack the *nos*Z gene and therefore contribute to global N_2O emissions, especially from soils under agricultural management (Philippot et al., 2011). The abundance of *nosZ* genes have been reported to be 10^8 to 10^9 per gram of dry soil (Henry et al., 2006). To date, denitrifying bacteria are believed to have one copy of *nosZ*, making it possible to also estimate the size of their population in soil (Philippot, 2002). Because soils are the largest source of N_2O in the atmosphere (IPCC, 2001), it is imperative to understand the effects of atmospheric N deposition on N_2O and N_2 emission from soil. For example, higher NO_3^- concentrations in cultivated soil have led to higher fluxes of N_2O from soil when compared to native soil, a response driven by the use of N-based fertilizers in cultivated soil (Stres et al. 2004). Therefore, it is important to further understand how unmanaged forests will respond as atmospheric N deposition continues to increase over the next century. It is also important to note that soils can function as N_2O "sinks" through the consumption of N_2O by organisms that have *nos*Z (reviewed by Chapuis-Lardy et al., 2007). Denitrifying bacteria community composition also has an effect on N_2O emissions due to differences in *Nos* activity (Cavigelli and Robertson, 2000).

The objectives of this study are to determine if greater soil NO_3^- availability, caused by over a decade of experimental atmospheric N deposition, has increased denitrification rates and if greater soil NO_3^- availability has increased the abundance of denitrifying bacteria

or changed community composition. In other words, has greater substrate availability (i.e., NO_3^-), due to N deposition, altered the abundance or composition of denitrifying bacteria thereby subsequently increasing rates of denitrification?

Materials and Methods

Study site and field sampling

Denitrifying bacteria community composition, abundance, and denitrification rates were quantified at four locations spanning a 500-km climatic and atmospheric N deposition gradient in Michigan (Fig. 1; Table 1). The locations are sugar maple (*Acer saccharum* Marsh.) dominated hardwood forest stands that have been receiving experimental N deposition since 1994. The soils are well-drained, sandy, isotic, frigid Typic Haplorthods in the Kalkaska series.

Each location has six 30-m by 30-m plots of which three receive ambient plus 30 kg NO_3^- N ha^{-1} yr^{-1} and three receive ambient N deposition alone; each plot is surrounded by a 10-m buffer that also receives the experimental treatments. Plots receiving experimental N deposition are treated with solid $NaNO_3$ granules (5 kg N ha^{-1}) six times per growing season (April – September); the dry granules are broadcast over the forest floor. The experimental N deposition treatment was designed to simulate rates of atmospheric N deposition that are projected to occur in eastern North America and Europe by 2050 (Galloway et al., 2008).

In October 2009, forest floor samples composed of the Oe and Oa horizons were collected. Ten random samples, measuring 10-cm by 10-cm, were collected in each plot, homogenized, and then composited within each plot. Subsamples were placed in sterile 50

mL polypropylene tubes and flash frozen using liquid N_2 for transport to the University of Michigan for storage at -80°C. Genomic DNA was then extracted from 2.5 g of forest floor using PowerMax TM Soil Extraction Kits (Mo Bio Laboratories, Solana Beach, CA). Sample preparation and GeoChip analysis was conducted at the Zhou lab (University of Oklahoma).

In May 2011, soil samples were collected using stratified random sampling methods to quantify *nosZ* abundance and denitrification rates. Ten soil cores were extracted from each plot by driving a 5-cm-diameter steel core to a depth of 5 cm, beginning at the surface of the Oe and A horizons. The 10 samples in each plot were homogenized into one composite sample. Because the soil texture is single-grained sand and there is no visible aggregation, sample homogenization produced a representative sample from each plot, while not destroying sample integrity because no soil structure exists. Samples were stored on dry-ice during transit to the laboratory to prevent degradation of DNA. Water content of field moist samples was measured by weight loss upon drying at 105 ^{o}C for 48 hours. Soil organic C and total N content was determined by a Carlo Erba CN analyzer.

Soil temperature and gravimetric water content data for each plot were used as covariates in the biogeochemical and molecular statistical analysis of denitrification. Data is publically available at: http://forest.mtu.edu/research/michigangradient/data.htm.

Denitrification rates and N_2O production

Denitrification and N_2O production for the soil samples (May 2011 samples) were estimated using assays with and without C_2H_2. Total denitrification was estimated as the amount of N_2O produced in presence of C_2H_2, whereas N_2O production was assessed in the absence of C_2H_2.

Denitrification potential was measured by using the denitrification enzyme activity (DEA) assay (Groffman et al., 1999). In a 20-mL glass vial, 5 g of field moist soil, 40 mg kg^{-1} dextrose, 100 mg N kg^{-1}as KNO_3, and 10 mg kg^{-1} chloramphenicol were added and the vial was sealed air tight with butyl rubber septa (Groffman et al., 1999). Anaerobic conditions in the flask were created by flushing the media and headspace with N_2 for 3 min each. Purified acetylene was then added to the flask to achieve a final concentration of 10% (10kPa) in the gas phase (Tiedje, 1994). Soil was incubated for 90 min at 22 °C before the headspace was sampled.

A modified version of the static core method was used to measure relative denitrification rates; C_2H_2 was injected into the headspace of soil cores and then N_2O accumulated over an incubation period (Groffman et al., 1999). This procedure was also repeated on a separate set of soil subsamples without C_2H_2 to quantify N_2O production. A 5-g soil sample was placed in a 20-mL glass vial and sealed air tight with butyl rubber septa. Anaerobic conditions in the flask were created by flushing the headspace with N_2 for 3 min. Soil was incubated at 22 °C for 24 hrs before the headspace was sampled and N_2O was quantified.

Gas samples were analyzed using a HP 5890 series gas chromatograph equipped with a PoraPLOT-Q column (0.53 mm I.D. x 25 m) and electron capture detector (Agilent Technologies, Santa Clara, CA). The oven, inlet, and detector temperatures were -10 °C, 125 °C, and 275 °C, respectively, and a gas flow rate of 56 ml min^{-1} (protocol Lee et al., 2009; Im et al., 2011). N_2 was used as both the carrier and makeup gas.

For the static core method, headspace volume and mass of dry soil were used to calculate µg N_2O-N g^{-1}. The DEA assay required that Bunsen's coefficient also be applied in order to account for the amount of N_2O dissolved in the media.

Denitrification functional gene abundance and composition using GeoChip

The functional gene composition and abundance of the bacterial denitrifier community (October 2009 samples) was characterized with the GeoChip 4.0 microarray using the following genes: *norB*, *narG*, *nirK*, *nirS*, and *nosZ*. Oligonucleotide design, synthesis, and fabrication, sample labeling, GeoChip processing, and data normalization were completed via the specifications in He et al. (2007). The sum of the normalized signal intensity for each gene was then standardized for the number of probes per gene. The number of probes per gene was: 59 for *norB*, 660 for *narG*, 263 for *nirK*, 276 for *nirS*, and 167 for *nosZ.*

Abundance of nosZ using qPCR

Using the composite samples from each plot that were also used in the denitrification assays, genomic DNA was extracted from 5-g soil samples using MoBio PowerMax Soil DNA isolation kits (MoBio Laboratories, Carlsbad, CA) and then further purified using Power Clean DNA Clean-Up kit (MoBio Laboratories, Carlsbad, CA). The quantity and quality of DNA after extraction and after clean-up was measured at spectrophotometrically at 260 nm using a Nano Drop 8000 (Thermo Scientific, Wilmington, DE). The concentration of DNA upon initial extraction and after the clean-up procedure was compared in order to correct for the change in concentration of DNA.

A 259-bp DNA fragment was amplified using the nosZ1F (5'-WCS-YTG-TTC-MTC-GAC-AGC-CAG -3') and nosZR1 (5'-ATG-TCG-ATC-ARC-TGV-KCR-TTY-TC-3') (Henry et al., 2006). Amplification of qPCR products were carried out with a Strategene Mx3000P using Brilliant SYBR Green as a detection system in a 25µl volume containing: 12.5 µl SYBR Green PCR Master Mix, 1µM of each primer for *nosZ*, 2µl of template DNA ranging from 13 to 60 ng μl^{-1} of DNA, 0.03µM ROX reference dye, and Rnase-free water to complete the 25µl volume. Thermal cycling conditions for the nosZ1 primers were as follows: 95°C for 15 min; 6 cycles of 94°C for 30s, 65°C for 45s with a touchdown of -1°C per cycle, and 72°C for 30s; 35 cycles of 94°C for 30s, 60°C for 45s, 72°C for 30s, and 80°C for 15s (data acquisition step); and 1 cycle 95°C for 45s and 60°C for 45s to 95°C for 30s. Each sample was run twice in duplicate and two no-template controls (NTCs) were run with each assay.

Genomic DNA from *Pseudomonas stutzeri* (B-775 USDA culture collection) was used to generate a standard curve containing dilutions between 50-500,000 copies of the gene of interest.

Statistical Analyses

Statistical differences between denitrification rates and denitrification potential in experimental N deposition and ambient treatments were assessed with a two-way ANCOVA using site and treatment as factors and soil temperature and soil matric potential as covariates. Statistical differences between *nosZ* copy number in experimental N deposition and ambient treatments were assessed with an ANOVA using site and treatment as factors ($\alpha = 0.05$). The concentration of genomic DNA after extraction and after the clean-up step was

quantified in order to calculate the change in concentration. The *nosZ* copy number was normalized for the change in concentration. The concentration of DNA between sites and treatments was tested using a two-way ANOVA. A linear regression was used to examine the correlation between denitrification rates and potentials with the copy number of *nosZ*. Differences in functional gene abundance and composition between treatments were tested using a two-way ANOVA. Similarities and differences of denitrification functional genes were visualized using the nonmetric multidimensional scaling (nMDS) of a Bray-Curtis distance matrix.

All statistical analyses were conducted using the GLM procedure of SAS version 9.2 (SAS Institute, Cary, NC) except the nMDS was produced using the computer software Primer 6 (Primer-E, Ivybridge, Devon, UK).

Results

Denitrification rates and N_2O production

Denitrification Enzyme Assay

Experimental N deposition had no significant effect on total denitrification rates, as measured by the DEA assay in the presence of C_2H_2 ($P = 0.5481$). The average denitrification rate increased from 13.1 μg N g^{-1} under ambient N deposition to 15.6 μg N g^{-1} in the experimental N deposition treatment, representing an 18% increase. However, denitrification rates were significantly different among sites (Fig. 2A). The denitrification potential was significantly higher in site B than in sites C and D ($P = 0.007$). Moreover, denitrification rates responded differently among replicate sites, wherein all sites except site C experienced

an increase in denitrification rates under experimental N deposition treatment. No significant interaction between site and treatment was found ($P = 0.064$).

Experimental N deposition had no significant effect on DEA in the absence of C_2H_2, which provided insight into N_2O production potential ($P = 0.161$). The average N_2O production increased from 4.9 μg N g^{-1} under ambient N deposition to 8.5 μg N g^{-1} in the experimental N deposition treatment, resulting in a 75% increase in N_2O production. There also was significant variation in DEA among replicate sites (Fig. 2A). N_2O production was significantly higher in site B compared to all other sites ($P < 0.001$), wherein site B was the only site to increase N_2O production potential with experimental N deposition. There was no significant interaction between site and treatment ($P = 0.1186$).

Static Core Assay

Denitrification rates were significantly higher under experimental N deposition when measured using the static core method in the presence of C_2H_2 ($P = 0.021$; Fig. 2B). The mean denitrification rate was 79.9 μg N g^{-1} under ambient N deposition and increased to 129.0 μg N g^{-1} in the experimental N deposition; this represents a 68% increase in denitrification. Denitrification rates were also significantly different among sites. For example, site B had a significantly greater rate of denitrification than site D ($P = 0.006$; Fig. 2B). No significant interaction between site and treatment was found ($P = 0.065$). The covariates of soil temperature and soil matric potential were also not significant ($P = 0.287$ and 0.262, respectively).

N_2O production was significantly higher under experimental N deposition as measured by the static core assay in the absence of C_2H_2 ($P < 0.001$; Fig. 2B). The mean N_2O

production increased from 50.5 μg N g^{-1} to 114.3 μg N g^{-1} under experiment N deposition resulting in a 127% increase, relative to rates under the ambient treatment. N_2O production was also significantly higher in site B compared to sites C and D (P < 0.001; Fig. 2B). No significant interaction between site and treatment was found (P = 0.065). The covariates of soil temperature and soil matric potential were also not significant (P = 0.287 and 0.262, respectively).

In summary, the significant increases in total denitrification and N_2O production under experiment N deposition as measured by the static core method supports the hypothesis that an increase in available soil NO_3^- will increase rates of denitrification. However, no significant increase in denitrification or N_2O production was found using the DEA method and the replicate sites had inconsistent responses. Therefore, the DEA assay did not support the hypothesis that increased soil NO_3^- availability will increase potential denitrification rates and N_2O production.

Molecular Assays

Functional gene abundance and composition

The Geochip analysis indicated that there was a lower abundance of genes found mediating denitrification under experimental N deposition (P = 0.039 to 0.012); moreover, the abundance of denitrification genes was significantly different among sites (P = 0.0004 to <0.0001). However, there were significant interactions between site and treatment (P = 0.057 to 0.022) for all 5 of the functional genes (*norB*, *narG*, *nirK*, *nirS*, and *nosZ*; Fig. 3A). The decreases in gene abundance under experimental N deposition treatment were driven by the ~30% decrease occurring in the northern sites (A and B), whereas the southern sites (C and

D) were not affected by the N deposition treatment (Fig. 3A). The collective abundance of genes involved with denitrification decreased by 12% under experimental N deposition. Individually, the five genes experienced a 10-14% decrease in the experiment N deposition treatment. Therefore, the total functional gene abundance decreased under experimental N deposition, while the relative proportion of each denitrification gene was unchanged (Fig. 3B).

The nMDS visualization revealed that the denitrification functional gene composition within the southern sites (C and D) is very similar despite the N deposition treatment (Fig. 4). However, the northern sites (A and B) have a functional gene composition, which clearly separates ambient and experimental N deposition treatments (Fig. 4). Therefore, the trends visualized in the nMDS confirms the ANOVA results that the northern sites experienced a treatment effect on the abundance of functional genes while the southern sites had similar functional gene abundance despite the experimental N deposition treatment.

nosZ abundance

The *nosZ* copy number had a significant site by treatment interaction ($P = 0.0434$); however, the main factors of site and experimental N deposition treatment did not exert a significant influence on *nosZ* abundance ($P = 0.1690$ and 0.2033, respectively; Fig. 5A). The significant interaction can be explained by the differing responses under experimental N deposition among sites; sites A, B, and D, which experienced a decrease in *nosZ* abundance, whereas, site C had a substantial increase. Overall, the abundance of *nosZ* decreased by 6% under experimental N deposition; average copy number decreased from 11,371 copies g^{-1} under ambient N deposition to 10,740 copies g^{-1} under experimental N deposition. The

efficiencies of all qPCR assays ranged between 92-116% and the r^2 value ranged from 0.906-0.996.

DNA concentration extracted from soil did not differ between N deposition treatments ($P = 0.3354$), but concentrations did significantly differ between sites A and C and sites C and D ($P = 0.0032$ and 0.0215, respectively). In order to assess the proportional change in *nosZ* genes, the ratio of *nosZ* to DNA was calculated. The ratio of *nosZ* to total DNA decreased under experimental N deposition in all sites except site C, where the ratio of *nosZ* to DNA more than doubled (Fig. 5B).

If the abundance of *nosZ* directly affects the amount of N_2O produced, then we expected to observe an inverse relationship between *nosZ* copy number and N_2O production. However, a weak positive relationship occurred between *nosZ* abundance and N_2O production in the ambient treatment using the static core method ($r^2 = 0.51$; $P = 0.009$). No relationship was found for N_2O production under ambient conditions when measured using the DEA method ($r^2 = 0.04$; $P = 0.493$) and no relationship was found for N_2O production under experimental N deposition using either method ($r^2 = 0.005$ and 0.044; $P = 0.814$ and 0.512). Overall, we found that the copy number of *nosZ* was not a good indicator for N_2O production.

In combination, the analysis of the GeoChip and qPCR revealed inconsistent responses in functional gene abundance under experimental N deposition. The GeoChip functional gene analysis indicated decreases in all five denitrification functional genes (*norB*, *narG*, *nirK*, *nirS*, and *nosZ*) in the northern sites (A and B), but revealed no change in the southern sites (C and D). The *nosZ* abundance measured using qPCR demonstrated that sites

A, B, and D experienced a decrease in *nosZ* copy number under experimental N deposition, whereas it increased in site C. GeoChip analysis indicated that while there may have been changes in functional gene abundance, the relative abundance of functional genes did not change under experimental N deposition. Additionally, Geochip analysis revealed a shift in composition in the northern sites (A and B) under experimental N deposition. These results provide some evidence to support the hypothesis that denitrification bacterial functional gene abundance and composition change under experimental N deposition, albeit the relative proportion of genes was unchanged.

Discussion

Atmospheric N deposition is a global phenomenon that is increasing the amount of biologically reactive N entering terrestrial ecosystems (Galloway et al., 2004). The excess N can be assimilated by plants, leached to ground or surface waters, or denitrified to the atmosphere as N_2 or N_2O. In our long-term study, we observed that experimental N deposition, at a rate expected in the near future, increased denitrification rates and N_2O production, as measured under laboratory conditions, while decreasing the abundance of bacterial genes encoding key enzymes in the denitrification pathway. Despite the decrease in gene abundance, the relative proportion of denitrification genes was unchanged under experimental N deposition. Although experimental N deposition did increase denitrification rates and N_2O production, this response was small relative to the large amounts of NO_3^- that are leaching or assimilated by plants (Pregitzer et al., 2004; Zak et al., 2004). Even though denitrification is a minor fate of biologically reactive N in these northern hardwood forests, the observed increases in denitrification rates and N_2O production, shift in denitrifier

composition, and decrease in functional gene abundance demonstrate that chronic atmospheric N deposition has altered denitrification on both a molecular and ecosystem-level scale, albeit these responses are small at an ecosystem level.

Our hypothesis that a decade of experimental N deposition would increase denitrification was supported by our observations; however, despite this increase, actual denitrification losses are likely to be minor, relative to the large amount of N assimilated by plants and exported by leaching. An ecosystem inventory of N conducted in 2004 revealed 30 g N m^2, applied over a decade in our experimental treatment, could be accounted for by increases in overstory N (3.1 g N m^2) and mineral soil/forest floor N (49.6 ± 22.6 g N m^2; Zak et al., 2008). Additionally, export of DON and NO_3^- has greatly increased under experimental N deposition, wherein ~10 g N m^2 was lost to leaching over same 10 year period (Pregitzer et al., 2004). If the average ambient field denitrification rate of 24 µg N_2O-N m^2 (Merrill and Zak, 1992) has increased by 68%, as estimated in the present study, then denitrification losses are ~ 0.17 g N m^2 for the same decade of experiment N deposition. The large leaching losses of N is evidence that N saturation has occurred, which is the result of the finite ability for an ecosystem to assimilate and retain N, leading to NO_3^- leaching, increased N_2O emissions, and changes in ecosystem structure and chemistry (Aber et al., 1989; Aber et al., 1998). In combination, these studies indicate that increased atmospheric N deposition has resulted in N saturation for these northern hardwood ecosystems causing increased leaching and denitrification, albeit the increase in denitrification is not substantial relative to the magnitude of losses attributed to plant assimilation and leaching.

The increase in N_2O production under experimental N deposition provides evidence that the release of N_2O from soils may be greater as atmospheric N deposition continues into

the future. We found that N_2O composed 10-98% of denitrification, which is similar to the 25-90% range observed in similar forests in northern Michigan (Merrill and Zak, 1992). We observed that the average proportion of denitrification composed of N_2O increased from 78% to 89% under experimental N deposition. Previous studies have found that the proportion of denitrification that is N_2O increases with increasing organic C, NO_3^-, NO_2^-, O_2, and pH (e.g. Firestone et al., 1980, Weier et al., 1993; Szukics et al., 2009). Our experimental N deposition treatment has not affected pH or O_2 concentrations (Patterson et al., 2012); however, both organic C and N content have increased under experimental N deposition (Zak et al., 2004; Zak et al., 2008). Soil solution concentrations of NO_3^--N were 20 times higher under experimental N deposition than under ambient N deposition (Pregitzer et al. 2004). In the forest floor (Oe/a) and mineral soil, organic matter has increased by 12% and N has increased by 9% (Zak et al., 2008). In combination, our results indicate that an increase in organic C and NO_3^- could be one of the factors that increase N_2O production under experimental N deposition.

Our prediction that an increase in denitrification could be accompanied by a shift in the relative proportion of denitrification genes was not supported by our analyses. For example, the relative proportion of denitrification genes did not differ between treatments, even though some replicate sites experienced a decrease in gene abundance (i.e. *norB*, *narG*, *nirK*, *nirS*, and *nosZ*). This similarity in relative proportion between N deposition treatments is interesting when considering the nitrite reductase genes (*nirK* and *nirS*). For example, *nir* genes are mutually exclusive and not associated with specific taxonomic groups, but are dispersed across 60 genera mainly in the *proteobacteria* (α, β, γ, ε) but also found in other genera such as *Firmicutes* and *Aquificaceae* (Braker and Conrad, 2011; Philippot et al.,

2007). Because both *nirK* and *nirS* decreased by a similar magnitude, we infer that chronic N deposition did not favor or inhibit the abundance of organisms whose genomes contain one of these genes. Furthermore, organisms containing *nirK* or *nirS* genes in their genome have different tolerances to pH, dissolved organic matter, and nutrients (Bárta et al., 2010; Priemé et al., 2002). Nonetheless, we found no difference in the relative proportion of these denitrification genes under experimental N deposition, further indicating that organisms containing *nirK* or *nirS* in their genome responded similarly to chronic N deposition.

Differences in denitrifier bacterial community composition may account for some of the variability of denitrification rates and N_2O production between replicate sites, as well as account for the inconsistent responses of *nosZ* abundance among sites (Enwall et al., 2005). We assumed that since we observed a decrease in functional genes and an increase in denitrification rates that the physiological functioning of denitrifiers increased and/or denitrifier community membership changed in order to explain this seemingly uncoupled response. Cultured isolates have been found to respond differently to environmental regulators, such as O_2, influencing the activity of enzymes in the denitrification pathway; therefore, taxonomic diversity could plausibly influence ecosystem-level function (Cavigelli, 1998). Our study measured the abundance and relative proportion of denitrification functional genes; however, understanding the identity of denitrifiers present and active would have provided greater insight into community composition and function. The GeoChip analysis provides some insight into the lineage of denitrifiers, indicating that the denitrifier communities under experimental N deposition in sites A and B are different from the ambient treatment. Additional information on community membership is needed to understand how community composition, membership, or diversity affects denitrification rates, N_2O

production, or *nosZ* abundance. Despite this limitation, we did not see an ecologically significant shift in denitrification genes or function after 16 years of N additions.

Our hypothesis that over a decade of experimental N deposition would increase the abundance of denitrification functional genes was not supported by our observations; instead we found a decreasing trend in gene abundance and a significant site by treatment interaction, which hinders our ability to make confident predictions about the effect of N deposition on northern hardwood ecosystems. For example, the abundance of *nosZ* responded differently in replicate sites, but had an overall 6% decrease under experimental N deposition. A reduction in *nosZ* abundance could have implications for the proportion of denitrification that is N_2O because *nosZ* encodes for the enzyme that reduces N_2O to N_2. Copy numbers of *nosZ* ranged from 5 x 10^3 to 2 x 10^4 copies g^{-1}, which is low compared to studies that found between 2 x 10^5 and 1. 5x 10^8 copies g^{-1} (Henry et al., 2006; Dandie et al., 2011). When taking into account the well-drained and well-aerated soil of our study sites, these lower copy numbers are not unexpected. The abundance of five denitrification genes (*norB*, *narG*, *nirK*, *nirS*, and *nosZ*) quantified using GeoChip displayed similar trends as *nosZ* with variable responses to experimental N deposition among sites, but an overall 12% decrease under experimental N deposition. Contrary to our hypothesis, greater NO_3^- availability, the result of experimental N deposition, did not positively influence the abundance of genes performing key biochemical steps during the denitrification process.

It appears that gene abundance and function are uncoupled, because we observed no relationship between the abundance of *nosZ* and N_2O production. Similarly, other studies have also found no correlation between denitrifier community structure, abundance, or denitrification gene expression and N_2O fluxes, denitrification rates, or denitrification

potential (Dandie et al, 2008, 2011; Wallenstein, 2004). Additionally, environmental conditions such as soil organic C, water-filled pore space, and NO_3^- were found to explain more variance in potential denitrification than denitrifier abundance (Attard et al., 2010). However, some studies found significant correlations between the abundance of *nosZ* and *nir* genes and the potential denitrification rate or N_2O emissions (Petersen et al., 2012; Morales et al., 2010). Again, denitrifier community composition may be the underlying factor that can account for the differences in physiological functioning under different environmental conditions that would allow us to predict function. Because chronic N deposition did not yield an ecologically-significant response in gene abundance or denitrification rates, it is not unexpected that no relationship was found between the abundance and function.

Our hypothesis that increased NO_3^- availability would increase the abundance of denitrification genes and thereby increase denitrification rates was not supported by our analysis. Rather, we observed that denitrification rates increased despite the apparent negative impact that N deposition had on functional gene abundance. This uncoupling between functional gene abundance and function may be due to a shift in community membership as indicated by our analyses or an increase in physiological functioning of denitrifiers under experimental N deposition. Our study indicates that increased NO_3^- availability may influence denitrifier communities over the long-term, which is contrary other studies indicating that NO_3^- primarily affects instantaneous denitrification rates and has only indirect effects on denitrifier communities (reviewed in Wallenstein et al., 2006). Additional research is needed to characterize the membership of these denitrifier communities in order to support or refute this hypothesis. Collectively, this study demonstrates that chronic atmospheric N deposition may suppress functional denitrification

genes, while concomitantly increasing denitrification rates and N_2O production; albeit denitrification is still only a minor fate of N when compared to the large losses due to leaching in these northern hardwood ecosystems and therefore the increase in denitrification is not ecologically-significant.

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Table 1. Location and soil characteristics of Michigan Gradient study sites.

Characteristic	Site A	Site B	Site C	Site D
Location				
Latitude (N)	46°52'	45°33	44°23	43°40
Longitude (W)	88°53'	84°52'	85°50'	86°09'
Soil Characteristics				
Temperature (°C)*	8.3	9.6	10.5	11.2
Moisture (MPa)*	-0.07	-0.09	-0.09	-0.08
pH†	4.63	4.92	4.40	4.60
Carbon (%)	2.16	5.18	2.29	3.20
Nitrogen (%)	0.17	0.34	0.17	0.21

*May 2011 data; soil matric and soil temperature measured at 15cm depth
†D.R. Zak unpublished data; pH measured at 10cm depth

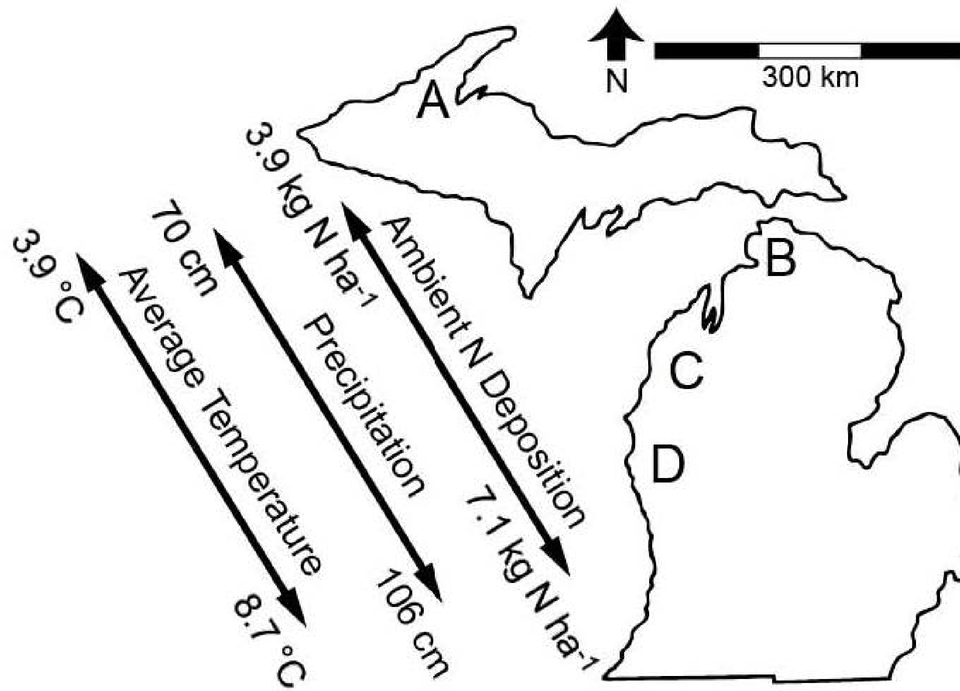

Figure 1. The Michigan Gradient study sites, spanning a climatic and ambient N deposition gradient, are represented by letters A-D. At each site there are three ambient and three experimental N deposition plots.

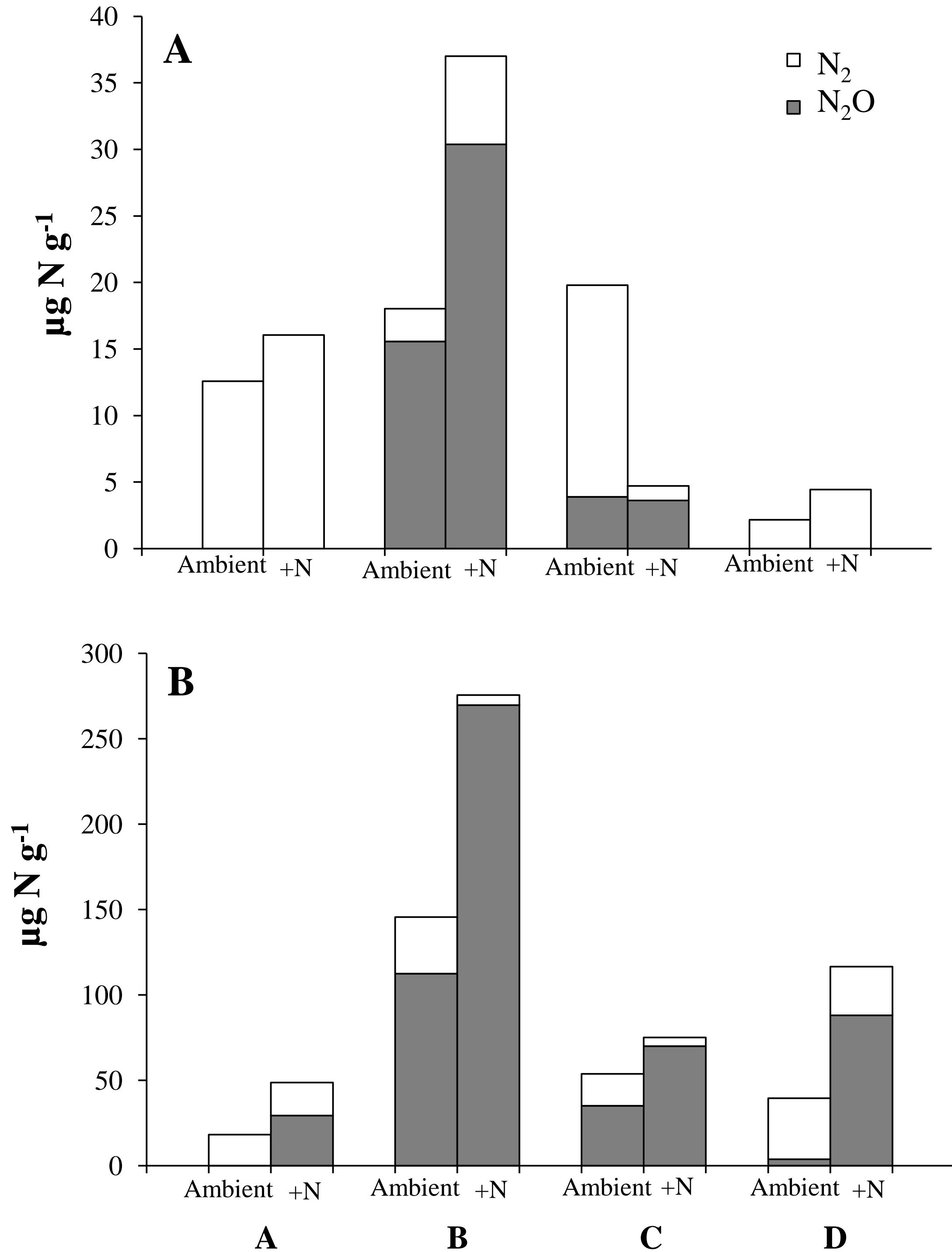

Figure 2. Amount of nitrogen gas produced per gram of soil for DEA assay (A) and static core method (B). Entire bar represents total denitrification while the proportions of N_2 and N_2O gas is represented as white and grey bars, respectively. Site was statistically significant for the DEA assay (A), while both treatment and site were significant for the static core method (B). [CV range for DEA = 11 – 173%; static core = 12 – 147%]

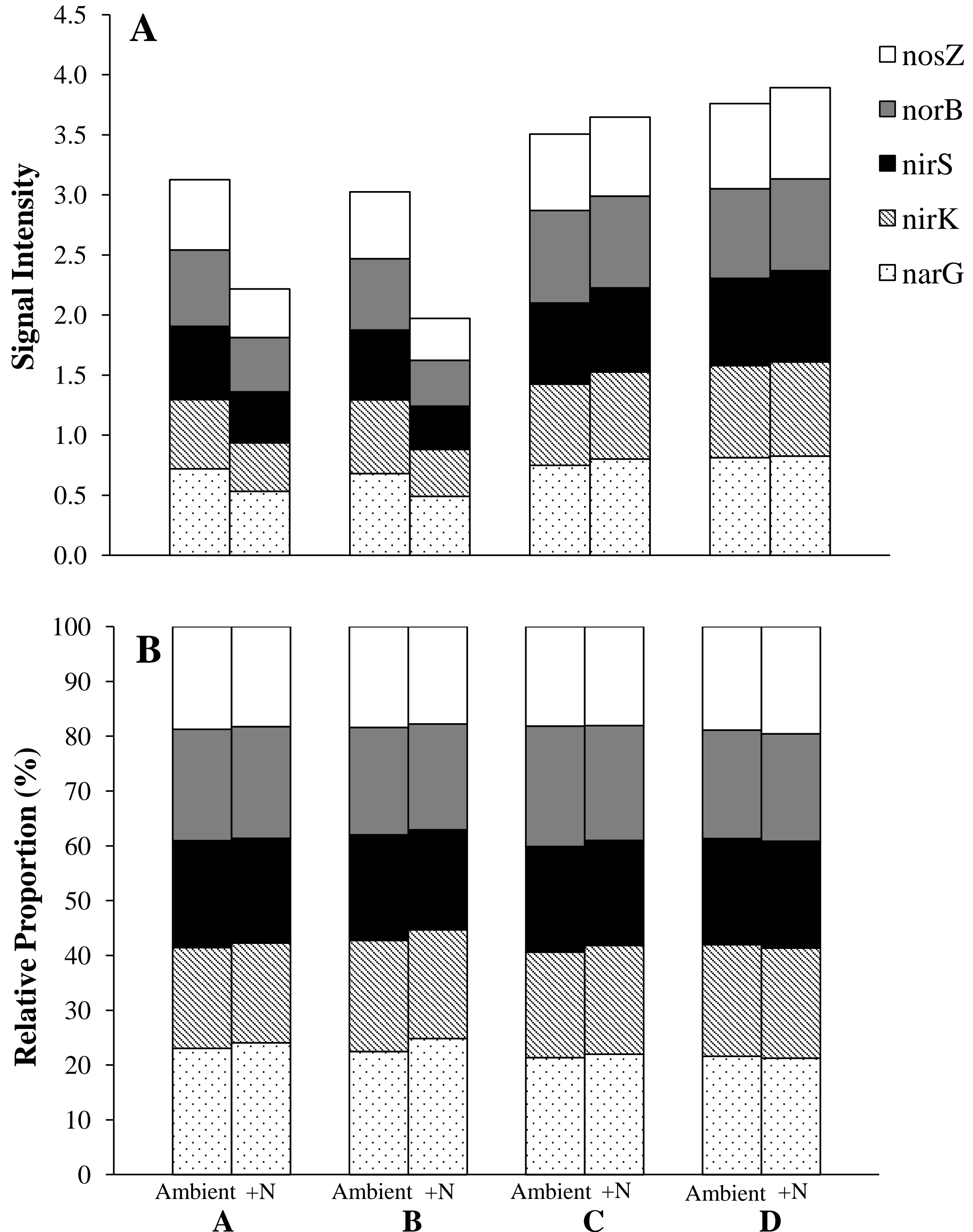

Figure 3. The corrected signal intensity (A) and relative proportion (B) of the denitrification functional genes using normalized signal intensity from the GeoChip. The signal intensity was corrected by dividing the sum of the normalized signal intensity for each gene by the number of probes per gene, which represents the abundance of each gene. The abundance of denitrification functional genes was significantly different between site ($P < 0.0004$), treatment ($P < 0.0387$), and the interaction ($P < 0.0574$) for all 5 of the functional genes. [CV range = 1.6 – 37.8%]

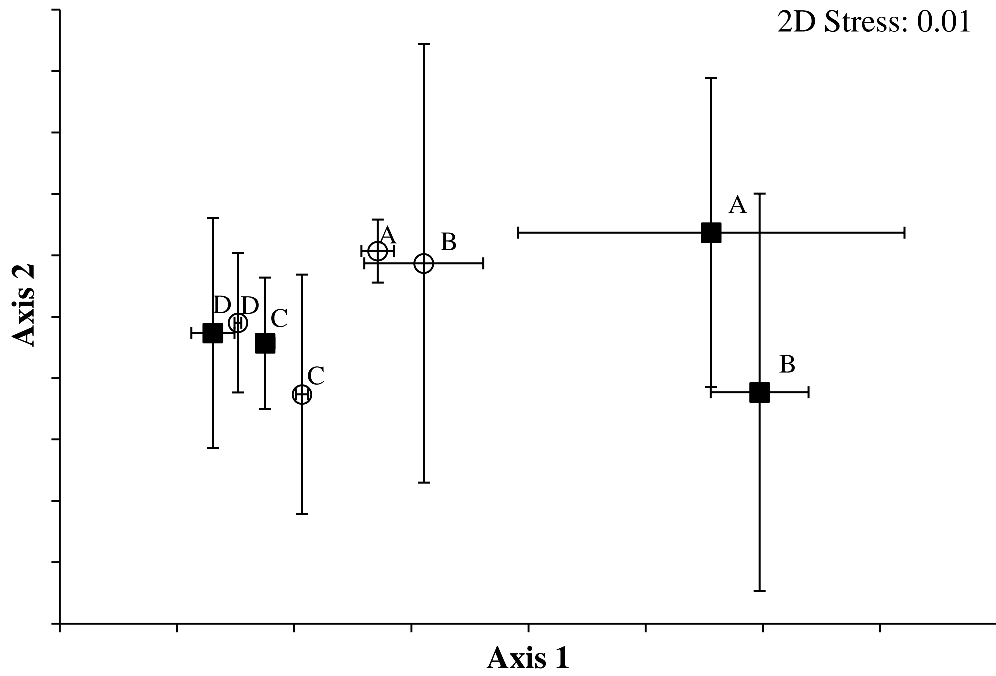

Figure 4. Nonmetric multidimensional scaling (nMDS) of the denitrification functional genes in the ambient and experimental N deposition treatment groups from sites A, B, C, and D using the GeoChip microarray. The experimental N deposition treatment is shown as black squares and the ambient treatment is shown as open circles.

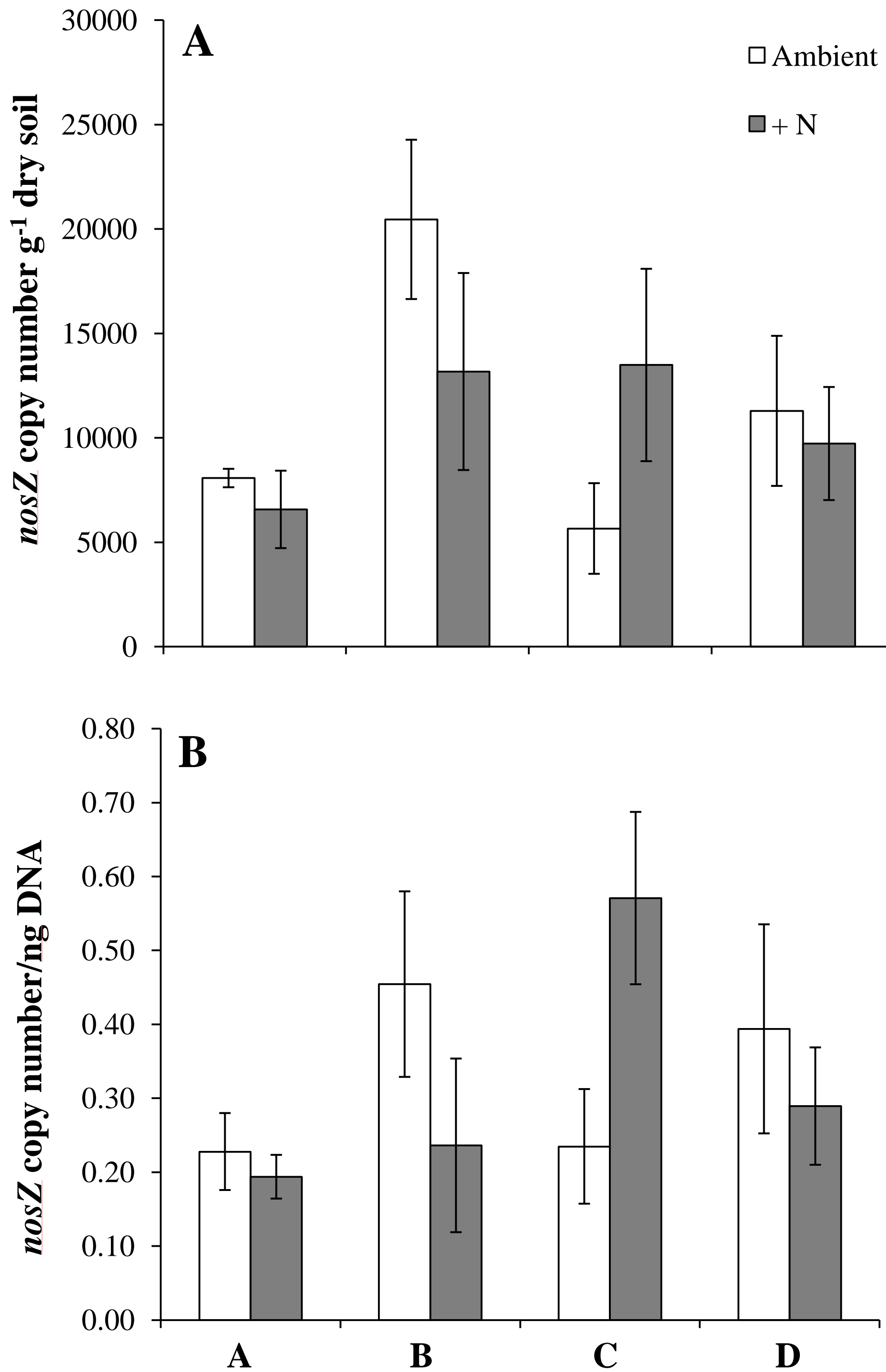

Figure 5. Copy number of *nosZ* g^{-1} dry soil (A) and ratio of *nosZ* copy number to ng DNA extracted from soil (B) under ambient and experimental N depsotion. Values are treatment means within sites A-D and error bars represent standard error (n = 3). The main factors were not found to be significant, however there was a significant site by treatment interaction (P = 0.0434).