



Towards a molecular understanding of N cycling in northern hardwood forests under future rates of N deposition



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ABSTRACT

The combustion of fossil fuels and fertilizer use has increased the amount of biologically available N over the last 150 years. Future rates of atmospheric N deposition may slow organic matter decay and alter microbial community composition and function. However, our understanding of how anthropogenic N enrichment may alter the physiological mechanisms by which soil microorganisms assimilate and cycle N in soil are largely unknown. Since 1994, we have experimentally increased NO₃ deposition to replicate ($n = 4$) northern hardwood forest stands across a 500-km climatic gradient in the Great Lakes region of North America. Our goal was to examine how functional genes mediating N-cycle processes in soil microbial communities have responded to experimental N deposition using the functional gene microarray, GeoChip 4.0. Experimental N deposition decreased the abundance and richness of key protein-coding genes in Archaea and Bacteria responsible for N fixation, ammonification, denitrification and assimilatory nitrate reduction; the same was true for bacterial genes mediating nitrification and dissimilatory nitrate reduction. However, the extent to which experimental N deposition decreased abundance and richness was site-specific, which was revealed by a significant site by treatment interaction. Experimental N deposition also caused a community composition shift via dispersion (increased β -diversity) in archaeal and bacterial gene assemblages. In combination, our observations suggest future rates of atmospheric N deposition could fundamentally alter the physiological potential of soil microbial communities.

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1. Introduction

Atmospheric deposition of biologically available nitrogen (N) has increased ten-fold across much of the Northern Hemisphere over the last 150 years (e.g., from 0.5 to 1 to 15–20 kg N ha⁻¹ y⁻¹) and is projected to more than double in the next century (Galloway et al., 2004). At present, anthropogenic creation of reactive N surpasses that of all natural terrestrial sources combined (Vitousek et al., 1997). Increased N availability in terrestrial ecosystems, such as temperate forests in which plant growth is often N limited (Vitousek et al., 2002), may lead to a phenomenon called N saturation (Aber et al., 1998, 1989) which models a series of plant and soil responses as N limitation is alleviated. Sugar maple (*Acer saccharum*

Marsh.) dominated forests in the upper Great Lakes region, U. S. A., are especially prone to N saturation (Zak et al., 2006) due to their high rates of net N mineralization (80–120 kg N ha⁻¹ y⁻¹; Zak and Pregitzer, 1990), and moderate rates of atmospheric deposition (7–12 kg N ha⁻¹ y⁻¹; MacDonald et al., 1991).

Since 1994, replicate forest stands have experimentally received experimental NO₃⁻ deposition across a 500-km climatic gradient spanning the north-south geographic range of the sugar maple dominated northern hardwood forest in the Great Lakes region of North America (Braun, 1950). We have demonstrated that experimental N deposition at a rate expected by 2050 across North America and other regions (Galloway et al., 2004) has increased woody biomass production, as well as organic matter stored in the forest floor and surface mineral soil (Pregitzer et al., 2008). Surprisingly, the observed increase in soil C sequestration has occurred despite no change in above- or belowground litter production (Burton et al., 2004), indicating organic matter has accumulated due to a reduction in plant litter decay (Zak et al., 2008).

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It is well established that litter decay rates are correlated with the initial ratios of C:N, lignin:N (Aerts, 1997; Hobbie, 2008; Prescott, 2010), and interactions among N, lignin, and cellulose (Talbot and Treseder, 2012). Thus, our understanding of how microbial communities directly involved in N cycling respond to anthropogenic N enrichment is key to understanding the fate of C and N under environmental change. Nitrogen enrichment can decrease microbial respiration (Burton et al., 2004; Janssens et al., 2010) and biomass (Wallenstein et al., 2006) and alter archaeal (Nemergut et al., 2008), bacterial (Eisenlord and Zak, 2010; Fierer et al., 2011; Nemergut et al., 2008), and fungal (Allison et al., 2007; Edwards et al., 2011) community composition. Our knowledge regarding the function of archaeal communities in the microbial N-cycle is both emerging and essential, as Archaea exhibit a common presence in soils (Bates et al., 2011) and the ability to mediate both assimilatory and dissimilatory processes of the N cycle (Cabello et al., 2004). For example, Archaea are dominant among ammonia-oxidizing prokaryotes across a variety of soil types (Leininger et al., 2006). Although most studies focus on how anthropogenic N enrichment may affect microbial populations, impacts on specific microbial communities responsible for soil N cycling processes is poorly understood.

We hypothesized that the experimental N deposition has altered the functional potential of forest floor microbial assemblages. From this hypothesis, we predict a decline in abundance and diversity of archaeal and bacterial functional genes related to N-cycling, as well a shift in community composition of functional gene assemblages mediating N cycling processes in response to chronic N deposition. To test our hypothesis, we used GeoChip 4.0, a PCR-independent microarray encompassing 73 archaeal and 2803 bacterial genes mediating N cycling processes in soil.

2. Materials and methods

2.1. Site description

The influence of simulated N deposition on soil microbial communities was investigated in four sugar maple (*A. saccharum* Marsh.) dominated northern hardwood forest stands in lower and upper Michigan, USA (Fig. 1). These locations span the north-south geographic range of the northern hardwood forests in the Great Lakes region of North America (Braun, 1950). The sites are



Fig. 1. The geographic distribution of the study sites A–D in Lower and Upper Michigan. In each stand beginning in 1994, three plots received ambient atmospheric N deposition and three plots received ambient plus 30 kg NO₃-N ha⁻¹ yr⁻¹.

floristically and edaphically similar, but span a climatic and atmospheric N deposition gradient (Table 1; MacDonald et al., 1991; Zak et al., 2008). The forest floor is composed of a thin Oi horizon dominated by sugar maple leaf litter, and a thicker Oe horizon interpenetrated by a dense root mat. At each study site, six 30-m by 30-m plots were established in 1994. Three plots in each stand receive ambient N deposition, and three receive experimental N deposition which consists of six equal applications of NaNO₃ pellets delivered to the forest floor over the growing season (30 kg N ha⁻¹ yr⁻¹). In our study sites, NO₃⁻ comprises ~60% of wet plus dry atmospheric N deposition (MacDonald et al., 1992).

2.2. Forest floor collection and DNA extraction

Forest floor sampling was performed in early October 2009, a time in which ample moisture favors high rates of microbial activity. In each 30-m by 30-m plot, 10 random 0.1-m by 0.1-m forest floor samples (Oe/Oa horizons) were collected after manually removing the freshly fallen Oi horizon. Following manual removal of large organic debris, samples were composited, homogenized by hand, and were immediately frozen in the field using liquid N₂.

Genomic DNA was extracted from 2.5 g of forest floor samples using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following manufacturer's instructions. Extracted DNA was quantified by the Quant-iT PicoGreen (Invitrogen, Carlsbad, CA, USA) assay according to manufacturer's instructions and stored at -80 °C until further analysis.

2.3. Target preparation, GeoChip hybridization, and data pre-processing

GeoChip 4.0 was applied in this study as described previously (Lu et al., 2011). A full list of functional genes and the number of associated probes (gene variants) mediating N cycling processes are included in the supplementary information (Table S1). Briefly, 1 µg of genomic DNA from each replicate sample was purified by the Genomic DNA Clean & Concentrator kit (Zymo Research, Irvine, CA, USA) and labeled with fluorescent dye Cy-3 using random primers (Wu et al., 2006). The labeled gDNA was dried and rehydrated with 2.7 µl of sample tracking control to confirm sample identity, followed by incubation at 50 °C for 5 min. This DNA solution was mixed with 7.3 µL of hybridization buffer containing the universal

Table 1

Site, climatic, overstory, and ambient nitrogen deposition rates of four study sites receiving experimental NO₃⁻ additions.

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°9"
Climate				
Mean annual temperature	4.7	6.0	6.9	7.6
Mean annual precipitation	873	871	888	812
Wet + dry NO ₃ -N deposition, g N m ⁻² yr ⁻¹	0.38	0.58	0.78	0.76
Wet + dry total N deposition, g N m ⁻² yr ⁻¹	0.68	0.91	1.17	1.18
Vegetation				
Overstory biomass, Mg ha ⁻¹	261	261	274	234
<i>Acer saccharum</i> biomass, Mg ha ⁻¹	237	224	216	201
Soil (0–10 cm)				
Sand, %	85	89	89	87
pH (1:1 soil/H ₂ O)	4.8	5.0	4.5	4.7
Cation exchange capacity, mmolc kg ⁻¹	3.4	3.8	2.6	3.0
Base saturation, %	71	96	73	80

Table adapted from Eisenlord and Zak (2010).

standard DNA labeled with Cy-5 dye; it was then denatured at 95 °C for 5 min and then maintained at 42 °C until it was loaded onto GeoChip arrays (NimbleGen, Madison, WI, USA). The hybridization was performed on a Hybridization Station (MAUI, Roche, CA, USA) at 42 °C for 16 h with agitation. After three washings, the arrays were scanned using a MS 200 Microarray Scanner (NimbleGen) at laser power of 100% PMT (photomultiplier tube). The signal intensities of spots on GeoChip were normalized across samples by the mean of Cy-5 labeled universal standard signal intensities and the sum of Cy-3 labeled signal intensities of hybridized spots within samples. After this two-step normalization, the unreliable or “noise” spots were removed when their original signal intensities were <2000, signal-to-noise ratio (SNR) was ≤ 2.0 , or the coefficient of variation (CV) of the background was >0.8 . Probes that appeared in only one of three replicates in each treatment group were also excluded. Natural-log transformations were then applied to all normalized signal intensities, and the final value of each spot was expressed as the ratio of finalized signal intensity to the average finalized signal intensity of all positive spots within that sample (mean ratio). The mean ratio data were subjected to statistical analyses using the pipeline developed at University of Oklahoma (<http://ieeg.ou.edu>).

2.4. Statistical analyses

Previous studies have established that GeoChip probe signal intensities are linearly correlated to target DNA or RNA concentrations of pure cultures to environmental samples (He et al., 2007; Rhee et al., 2004; Wu et al., 2001, 2004). For this reason, resulting data matrices from microarray image analysis were considered as either: i) gene variant abundance as indicated by signal intensity of each probe or ii) binary presence/absence matrices. Additionally, relative abundance data were calculated by dividing the signal intensities of each individual probe by the sum of signal intensities for a given plot, effectively accounting for absolute differences in DNA abundance between plots.

The effects of site, simulated N deposition, and their interaction on archaeal and bacterial functional genes was initially determined by two-way analysis of variance (ANOVA), combined with a protected Fisher's LSD test of means (SPSS Statistics, Version 20, IBM Corp., Armonk, NY, USA); significance was accepted at $\alpha = 0.05$. To test the hypothesis that simulated N deposition caused a decline in abundance of functional genes mediating N cycling processes, the sum of signal intensities of gene probes were corrected, such that each GeoChip “gene category” (e.g. N fixation, denitrification) had equal influence. For example, because the number of probes designed for gene variants within each gene category were unequal, greater weight would be placed on categories with a greater number of probes on the microarray (Table S1). Presence–absence data was used to determine if experimental N deposition decreased functional richness, defined as the proportion of gene variants detected (Liu and Greaver, 2010; Van Nostrand et al., 2009), among genes mediating N cycling processes, and were similarly corrected such that each gene category had an equal weight. All proportion data were arcsine transformed prior to statistical analysis. To test the hypothesis that simulated N deposition caused a similar decline in the diversity of functional genes, the normalized signal intensity of all relevant gene variants was used to calculate the Shannon Index (H' ; Shannon and Weaver, 1963). The full abundance matrix was used in the calculation of the response ratio (Luo et al., 2006) to determine if functional genes of bacterial taxa respond differently to simulated N deposition. Taxonomic information for archaeal probes lacked sufficient resolution for such analysis.

Effects of experimental N deposition on archaeal and bacterial functional gene composition, as observed in microarray presence–

absence data, were further assayed based on Bray–Curtis dissimilarity coefficients (Legendre and Legendre, 1998). Calculation of dissimilarity coefficients and all downstream analysis were performed in Primer (version 6, Primer-E Ltd., Plymouth, UK). A dissimilarity matrix based on arcsine transformed proportion of gene variants detected within each functional gene category was generated for all possible pairs of samples and was used for all remaining analyses. Ordinations were obtained from principal coordinate analysis to test the hypothesis that simulated N deposition altered the composition of archaeal and bacterial gene assemblages. To determine whether specific functional genes were primarily responsible for any observed compositional changes, Spearman correlation coefficients were calculated to ascertain the degree of correlation of each gene variant to the primary principal coordinate. The significance of compositional differences between ambient and experimental N deposition assemblages were confirmed by analysis of similarity (ANOSIM; Clarke et al., 1993), and permutational multivariate analysis of variance (PerMANOVA; Anderson, 2001). The ANOSIM test provides an R -statistic, ranging between -1 and 1 based on rank dissimilarities, with an R -statistic of zero indicating a completely random grouping. PerMANOVA allows multivariate information to be partitioned according to the experimental design (with interaction terms), and determines significance by random permutation, but cannot determine if observed shifts are due to location or dispersion. Therefore, a distance-based test for homogeneity of multivariate dispersions (PERMDISP; Anderson, 2004), a β -diversity measure, was used to discern the directional nature of observed assemblage shifts. Finally, contributions of each functional gene to the total dissimilarity between the two groups (ambient, simulated N deposition) were determined using Similarity Percentage (SIMPER; Clarke et al., 1993).

3. Results

3.1. Functional gene abundance

If not presented in text, all relevant means and standard errors can be found in Table S2. Two-way ANOVA of GeoChip signal intensities revealed a significant decrease in abundance of functional genes mediating N cycle processes under experimental N deposition in both archaeal ($P = 0.03$) and bacterial ($P = 0.02$) gene assemblages (Fig. 2). A significant site by treatment interaction was observed in both archaeal ($P = 0.01$) and bacterial ($P = 0.03$) assemblages; wherein, pairwise protected Fisher's LSD revealed significant effects of experimental N deposition in sites A (Archaea $P < 0.01$; Bacteria $P = 0.01$) and B (Archaea $P < 0.01$; Bacteria $P < 0.01$), with no effect in C or D ($P > 0.15$).

Archaeal gene assemblages declined in abundance evenly across each N cycle process represented on the microarray. However, when absolute differences were taken into account, the relative abundance of bacterial genes encoding assimilatory NO_3^- reduction increased ($P < 0.03$; ambient mean = $3.7 \pm 0.05 \times 10^{-3}$; N deposition mean = $3.9 \pm 0.1 \times 10^{-3}$), and those encoding nitrification marginally declined ($P = 0.06$; ambient mean = $3.6 \pm 0.1 \times 10^{-3}$; N deposition mean = $3.3 \pm 0.1 \times 10^{-3}$) disproportionately to other bacterial genes.

3.2. Diversity of functional genes

The effect of experimental N deposition on the functional richness within each gene category was investigated using the binary presence-absence data matrix. We observed that experimental N deposition caused a decline in the functional richness of archaeal ($P = 0.04$) and bacterial ($P = 0.02$) assemblages (Fig. 3), as shown by

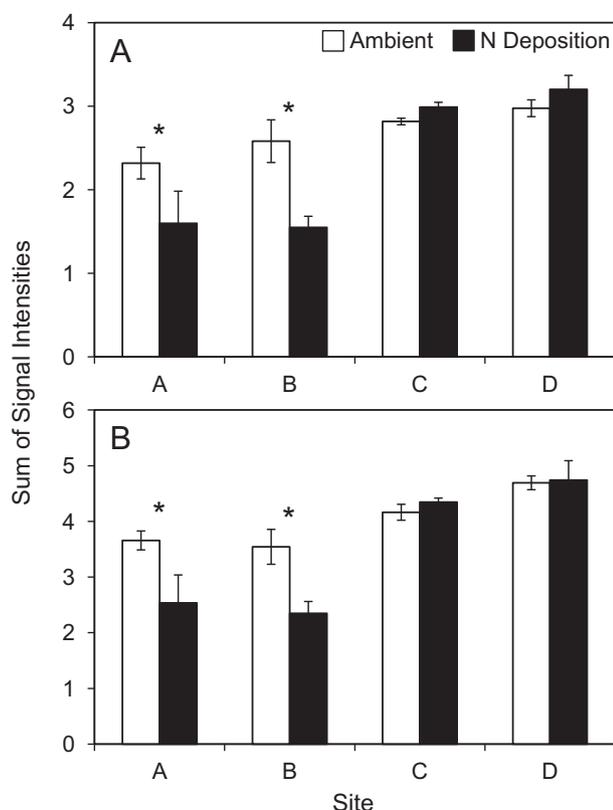


Fig. 2. Normalized signal intensities of archaeal (A) and bacterial (B) probes within the nitrogen cycle gene category across sites A–D. Error bars indicate SE, asterisk indicates significance ($P < 0.05$) by Fisher's Least Significant Difference. All means and SEs are reported in Table S2.

two-way ANOVA. The response was site-specific as indicated by a significant site by treatment interaction (Archaea $P = 0.04$; Bacteria $P = 0.02$), wherein experimental N deposition elicited a negative response in archaeal and bacterial genes in site A and B, and no response in sites C and D. Archaeal genes slightly increased in functional richness in site D ($P = 0.05$) in response to experimental N deposition.

The decline in functional richness occurred evenly across each gene in archaeal gene assemblages. In contrast, the relative proportion of probes detected encoding assimilatory NO_3^- reduction increased ($P = 0.03$; ambient mean = 0.43 ± 0.003 ; N deposition mean = 0.45 ± 0.01), and those encoding nitrification declined ($P = 0.05$; ambient mean = 0.43 ± 0.01 ; N deposition mean = 0.41 ± 0.01) disproportionately as compared to others.

The effect of experimental N deposition on assemblage diversity, as indicated by the Shannon index (H'), was determined based on normalized signal intensity values for all probes associated with archaeal and bacterial genes. Experimental N deposition reduced archaeal ($P = 0.04$) and bacterial ($P = 0.02$) gene diversity; a significant site by treatment interaction term indicated a site-specific response (Archaea $P = 0.02$; Bacteria $P = 0.05$). Again, experimental N deposition caused declines in diversity at sites A and B and no responses in sites C and D (Fig. 3).

3.3. Community composition of functional genes

Ordinations obtained from principal coordinate analysis (PCoA) revealed compositional similarities among ambient plots and disparate composition among experimental N deposition plots across study sites (Fig. 4). ANOSIM detected a clear divergence of

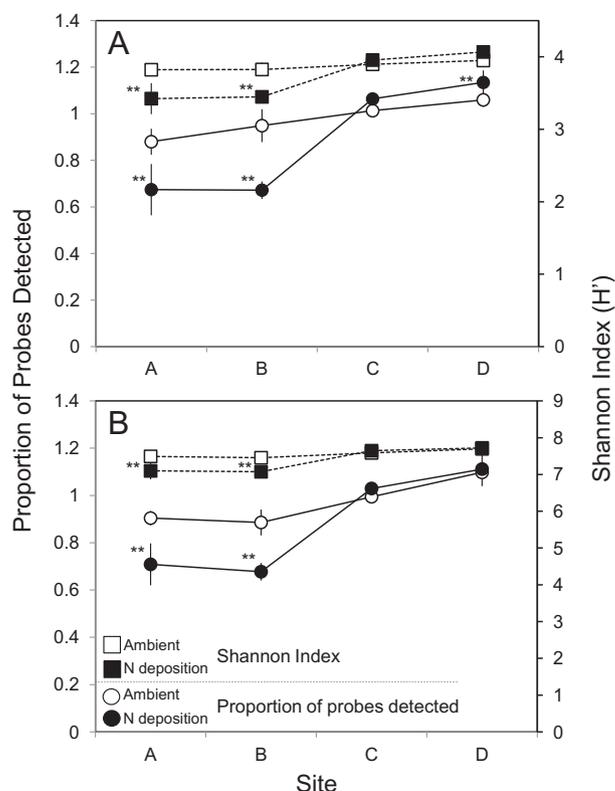


Fig. 3. Shannon index and functional richness (proportion of probes detected) of archaeal (A) and bacterial (B) probes within the nitrogen cycle gene category across sites A–D. Error bars indicate SE, asterisk indicates significance ($P < 0.05$) by Fisher's Least Significant Difference. All means and SEs are reported in Table S2.

gene assemblages between ambient and experimental N deposition treatments for both archaeal ($R = 0.44$, $P < 0.01$) and bacterial ($R = 0.50$, $P < 0.01$) genes mediating N cycling processes.

PerMANOVA revealed a significant treatment effect on both archaeal ($P = 0.04$) and bacterial ($P = 0.01$) functional gene composition, although site-specific responses occurred, indicated by a significant site by treatment interaction (Archaea $P = 0.01$; Bacteria $P = 0.05$). Pairwise PerMANOVA of archaeal gene assemblages indicated a significant effect of experimental N deposition in site B ($P = 0.01$), with a marginal treatment effect in sites A ($P = 0.10$) and C ($P = 0.10$); no effect occurred in site D ($P = 0.32$). Among bacterial gene assemblages, experimental N deposition caused a significant composition shift in site B ($P = 0.03$), with a slight effect in site A ($P = 0.10$), and no effect in sites C and D ($P > 0.15$). Significant PERMDISP results indicated experimental N deposition increased β -diversity of functional genes in both archaeal ($F = 6.3$, $P = 0.04$) and bacterial ($F = 14.2$, $P < 0.01$) assemblages.

Similarity Percentage (SIMPER; Clarke et al., 1993) was used to further distinguish which genes contributed to the compositional dissimilarity between ambient and experimental N deposition treatments (Table 2). Archaeal gene assemblages exhibited a mean dissimilarity of 21.2%, wherein genes mediating denitrification contributed most to assemblage dissimilarity between ambient and experimental N deposition treatments ($14.2 \pm 3.4\%$). This trend was driven by *norB* and *nosZ*, which together amount to 41.5% of the total dissimilarity. Among bacterial genes, the mean dissimilarity was 12% between ambient and experimental N deposition treatments. Contributions to assemblage dissimilarity ranged from 9 to 5%, with genes mediating nitrification (8%), N fixation (7%), and assimilatory N reduction (7%), on average, exhibiting the most difference between groups.

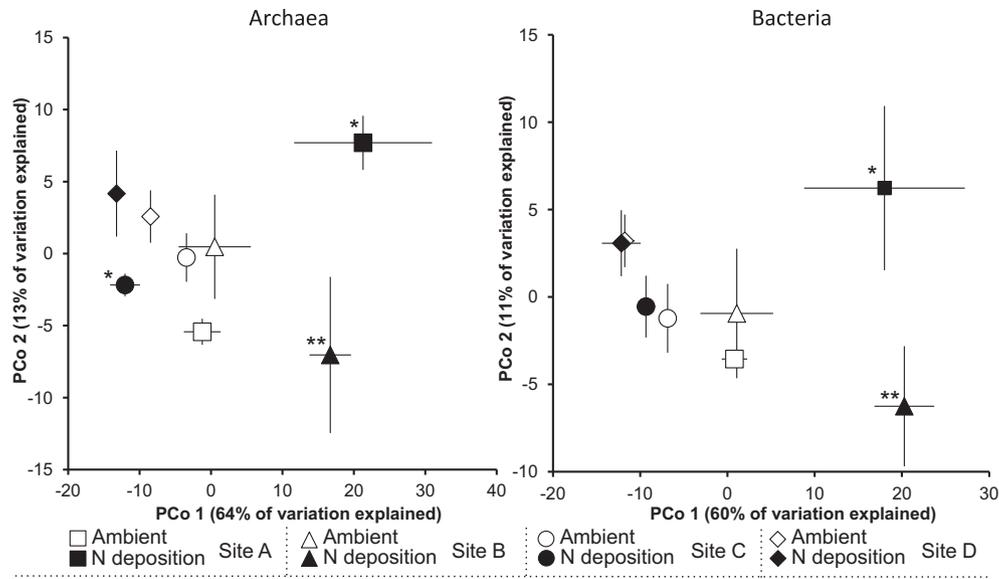


Fig. 4. Principle coordinate analysis of archaeal and bacterial N-cycle gene assemblage composition. Open symbols represent sites receiving ambient N deposition, closed symbols represent sites receiving ambient plus simulated N deposition. Data represent Bray–Curtis dissimilarity of the full presence/absence matrix. ** $p \leq 0.05$; * $p \leq 0.10$ for each site by pairwise PerMANOVA.

Table 2
Contributions of N-cycle functional genes to compositional dissimilarity as indicated by SIMPER analysis.

	Average dissimilarity	Diss./SD	% Contribution
Archaea			
Denitrification	3.02 ± 0.73	0.98 ± 0.05	14.21 ± 3.42
<i>norB</i>	4.56	0.98	21.45
<i>nosZ</i>	4.25	0.95	20.03
<i>nirK</i>	1.92	1.13	9.04
<i>narG</i>	1.34	0.84	6.33
Ammonification	1.82 ± 0.71	1.00 ± 0.06	8.57 ± 3.34
<i>gdh</i>	2.53	1.06	11.91
<i>ureC</i>	1.11	0.94	5.23
Assimilatory N reduction	1.52 ± 0.39	0.99 ± 0.20	7.17 ± 1.84
<i>nirA</i>	2.39	1.25	11.27
<i>nirB</i>	1.29	0.54	6.08
<i>nasA</i>	0.88	1.18	4.17
N Fixation	0.95	1.50	4.49
<i>nifH</i>	0.95	1.50	4.49
Bacteria			
Nitrification	0.94	1.31	8.01
<i>hao</i>	0.94	1.31	8.01
N Fixation	–	–	–
<i>nifH</i>	0.83	1.44	7.08
Assimilatory N reduction	0.79 ± 0.06	1.45 ± 0.07	6.75 ± 0.47
<i>nirA</i>	0.92	1.66	7.83
<i>nirB</i>	0.84	1.39	7.15
<i>nasA</i>	0.74	1.40	6.35
<i>NiR</i>	0.66	1.36	5.66
Dissimilatory N reduction	0.77 ± 0.01	1.32 ± 0.04	6.58 ± 0.12
<i>napA</i>	0.78	1.35	6.70
<i>nrfA</i>	0.76	1.28	6.46
Denitrification	0.77 ± 0.06	1.26 ± 0.04	6.58 ± 0.55
<i>nirS</i>	1.01	1.37	8.67
<i>nirK</i>	0.77	1.30	6.62
<i>nosZ</i>	0.71	1.31	6.06
<i>norB</i>	0.71	1.15	6.04
<i>narG</i>	0.64	1.17	5.50
Ammonification	0.70 ± 0.13	1.20 ± 0.07	5.94 ± 1.04
<i>gdh</i>	0.82	1.13	6.98
<i>ureC</i>	0.57	1.27	4.90

Average dissimilarity ± SE are shown for each N-cycle process. One gene per process was present on GeoChip where no SE is included.

In both archaeal and bacterial principal coordinate ordinations, the majority of the variation in assemblage composition was explained by principal coordinate 1 (Archaea, 64%; Bacteria, 60%). Among archaeal assemblages, genes mediating denitrification (*norB*; $\rho = -0.75$) and assimilatory N reduction (*nirA*; $\rho = -0.52 \pm 0.23$) were most highly correlated to PCo1. Among bacterial assemblages, genes mediating nitrification (*hao*, $\rho = -0.50 \pm 0.05$) and N fixation (*nifH*, $\rho = -0.49 \pm 0.01$) were more highly correlated with PCo1 than other N cycle genes.

Response ratios (Luo et al., 2006) were calculated to determine if functional genes from bacterial phyla represented on GeoChip 4.0 responded differently to experimental N deposition (Fig. S1). Functional genes representative of Bacteroidetes responded most negatively (-0.75 ± 0.51), followed by probes representing uncultured bacteria (-0.20 ± 0.04) and γ -proteobacteria (-0.17 ± 0.11), all of which are significantly less than zero based on >90% confidence intervals. Functional gene abundance of Firmicutes responded positively to experimental N deposition (1.49 ± 0.19) and was greater than zero at the 99% confidence interval. Archaeal probe taxonomic resolution was not sufficient to perform these analyses.

4. Discussion

4.1. Mixed responses of soil microbial communities under future rates of atmospheric NO_3^- deposition

In our long-term field study, experimental N deposition led to a decrease in abundance, richness (proportion of gene variants detected), and diversity (H') of a suite of genes mediating N cycle processes, with a concomitant shift in community composition of both archaeal and bacterial functional gene assemblages. This response occurred with fidelity in the northernmost two sites, A and B, but was not evident in the southernmost sites (C and D). In addition to the response in sites A and B, chronic N deposition caused a shift in archaeal community composition in site C ($P = 0.10$), and increased the β -diversity of gene assemblages at all sites. This is not a surprising result, as a recent study demonstrated a consistent decline in microbial respiration and biomass under N enrichment across a variety of soil types (Ramirez et al., 2012);

others have demonstrated that N additions can decrease the diversity (Allison et al., 2008; Campbell et al., 2010) and richness (Shen et al., 2010) of soil microbial communities. However, unlike previous studies which focus on general microbial community composition (Campbell et al., 2010; Edwards et al., 2011; Fierer et al., 2011; Ramirez et al., 2012), this study employed a functional gene microarray, providing a unique opportunity to gain insight into the physiological potential of microbial communities in our experiment to mediate soil N cycling processes.

The significant site by treatment interactions we observed suggest that soil microbial assemblages responsible for assimilating and cycling N may be somewhat idiosyncratic in their responses to atmospheric NO_3^- deposition. Historically, microbial responses to anthropogenic N enrichment have been variable; some have reported stimulation (Neff et al., 2002; Newell et al., 1996; Tietema, 1998), or suppression (Edwards et al., 2011; Eisenlord and Zak, 2010; Gallo et al., 2004; Ramirez et al., 2012; Wallenstein et al., 2006) of microbial activities, whereas others have found no effect (Hobbie and Vitousek, 2000). Here, we observe a consistent and robust response of microbial assemblages to experimental N deposition in the northernmost two sites, A and B, with a moderate to non-significant response in sites C and D. The four experimental forest stands were chosen to span the north–south range of the northern hardwood forests in the Great Lakes region of North America; if we observed no interaction between site and treatment, it would infer a uniform response to future rates of atmospheric N deposition across an expansive and diverse ecosystem. As this was not the case, it provides an opportunity to further explore the mechanisms that underlie our observed response. Over the last decade, ambient N deposition has ranged from ~ 4 (site A) to ~ 12 (site D) $\text{kg N ha}^{-1} \text{y}^{-1}$ across our study sites (Burton et al., 1993; National Atmospheric Deposition Program, 2005). Because our N deposition treatment ($30 \text{ kg N ha}^{-1} \text{y}^{-1}$) is consistent across sites, site A experiences a 10-fold increase in N deposition, whereas site D experiences a 3-fold increase. It is possible that microorganisms previously exposed to relatively high concentrations of ambient N deposition may not have been affected by our treatment in a way we would be able to detect using GeoChip. It is also plausible that microbial communities in our southernmost sites (C & D), which experience relatively high levels of ambient N deposition, have already acclimated to this anthropogenic perturbation, whereas those in the northernmost sites have not.

Within the context of previous results obtained from our long-term field study, experimental N deposition more broadly suppressed microbial activities than was previously established. For example, long-term experimental N deposition resulted in significant declines in soil respiration (-15% ; Burton et al., 2004), active microbial biomass (-23% ; DeForest et al., 2004a), and phenol oxidase (-33%), and peroxidase (-30%) activity (DeForest et al., 2004b, 2005), which reduced decay, increased soil organic matter (SOM) content ($+18\%$; Zak et al., 2008) as well as increased DOC ($+26\%$) and NO_3^- ($+680\%$) leaching (Pregitzer et al., 2004). One possible alternative explanation for an overall decline in gene abundance under experimental N deposition could be Na^+ toxicity, resulting from long-term NaNO_3 addition. However, Na^+ concentrations in soil water ($0.45 \pm 0.00 \text{ mmol}$; W. E. Holmes, unpublished data) are two orders of magnitude below those known to decrease microbial biomass, respiration, and enzyme activity ($\sim 40 \text{ mmol Na}^+$ as NaCl ; Bernardet and Nakagawa, 2003; Garcia and Hernandez, 1996), indicating the observed response cannot be due to Na^+ toxicity. Refer to Zak et al. (2008) for more discussion on this matter.

For this study, the use of the functional gene microarray, GeoChip 4.0, provided the luxury to examine the abundance and composition of 2876 gene variants encompassing 15 genes mediating N-cycle processes while not requiring PCR amplification of

each gene. However, given that probe design is limited by gene sequence availability from public databases (e.g. Genbank), the problem still remains that microarrays can only detect the limited number of sequences covered by the probes included on the array. For a more detailed discussion of advantages and disadvantages of microarrays such as GeoChip, see Van Nostrand et al. (2012). In future studies, the use of shotgun metagenomics may prove helpful in elucidating more nuanced responses of microbial assemblages to experimental N deposition. Our results emphasize the assertion that, in light of new and emerging metagenomic technologies, more highly replicated experimental designs are needed to accurately assess microbial community dynamics (Knight et al., 2012).

4.2. Towards a molecular understanding of community and ecosystem-level responses to NO_3^- deposition

Taken together, our results infer communities are diverging under experimental N deposition, but these changes are not consistent among sites. Overall, experimental N deposition caused a site-dependent decrease in α -diversity, but an increase in β -diversity of functional genes, suggesting that future rates of N deposition may cause soil microbial communities to become more functionally heterogeneous. In this study, bacterial gene variants mediating nitrification were strongly affected by our N deposition treatment; these assemblages decreased in abundance and richness disproportionately relative to other gene categories and were responsible for the majority of the variation observed in PcoA (Fig. 4; Pco1 $\rho = -0.50 \pm 0.05$) and SIMPER analysis (Table 2). This observed molecular response to our N deposition treatment has clear functional implications, as Zak et al. (2006) noted a significant increase of annual net nitrification in experimental N deposition treatments ($+27\%$), a finding consistent with other studies (Jefts et al., 2004; Lamontagne and Schiff, 2000). It is possible that composition shifts in nitrifying assemblages may favor those organisms which are more efficient in their nitrification pathways, leading to ultimately greater rates of net nitrification despite these assemblages being less abundant.

NO_3^- assimilation and denitrification may also prove to be significant transformations as N-cycling assemblages acclimate to future rates of N deposition. Previous results from our long-term field experiment report a significant microbial assimilation of $^{15}\text{NO}_3^-$ occurred within hours of addition in experimental N deposition plots (Zogg et al., 2000); similar findings have also been reported in lab incubations (Zak et al., 2006) and agricultural soils (Burger and Jackson, 2003). In this study, gene variants mediating assimilatory NO_3^- reduction disproportionately increased in abundance and richness, and contributed highly to total gene dissimilarity among treatments. The combination of field, laboratory, and molecular analyses together suggest microbial assimilation of N to be an important process by which anthropogenic N initially cycles in soil. Among archaeal assemblages, genes encoding denitrification were most affected by experimental N deposition. These assemblages were responsible for the majority of the variation in PcoA (Fig. 4; Pco1 $\rho = -0.44 \pm 0.12$) and were found to greatly contribute to assemblage dissimilarity in SIMPER analysis (Table 2). Nitrogen enrichment can increase rates of denitrification in forest soils (Buckley et al., 1998; He et al., 2007; Nicol and Schleper, 2006); however, process rates were unavailable at this time for our experiment. A robust analysis of microbial N transformations in the forest floor is needed to more clearly determine if the observed compositional shifts may elicit a functional change in response to experimental N deposition.

Several recent large-scale sequencing efforts have revealed significant and consistent phylum-level shifts in soil bacterial populations in response to N fertilization (Campbell et al., 2010;

Nemergut et al., 2008; Ramirez et al., 2012, 2010). The copiotrophic hypothesis (Fierer et al., 2007) predicts that N enrichment may induce a shift from slower growing oligotrophic taxa to more fast growing copiotrophic taxa (Fierer et al., 2007), and this has been empirically supported (Cleveland et al., 2007; Fierer et al., 2011; Nemergut et al., 2010; Ramirez et al., 2012, 2010). Our results were both consistent, as well as contrary to the copiotrophic hypothesis. Functional genes of the Firmicutes, a copiotrophic group (Cleveland et al., 2007) increased in abundance under experimental N deposition (Fig. S1); similarly, Firmicutes increased in abundance (+3.04%) under N fertilization across a variety of soil types in lab incubations (Ramirez et al., 2012). Functional genes attributable to another copiotrophic group, the Bacteroidetes, exhibited the most negative response to experimental N deposition in our study, conflicting with predictions of the copiotrophic hypothesis. Conversely, Bacteroidetes increased in abundance under N amendments in alpine tundra soils (Nemergut et al., 2008), as well as in agricultural and grassland soils (Fierer et al., 2011); however, these responses occurred at substantially greater rates of N deposition than those in our experiment ($>100 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ vs. $30 \text{ kg N ha}^{-1} \text{ yr}^{-1}$). Bacteroidetes are capable of utilizing macromolecules such as proteins and polysaccharides, namely cellulose (Bernardet and Nakagawa, 2003), which is often encased by lignin within plant cell walls. Experimental N deposition decreased activities of lignolytic enzymes peroxidase and phenol oxidase (DeForest et al., 2004b) in our study sites, as well as the expression of fungal laccase (*lcc*; Edwards et al., 2011). It is possible that a reduction in lignin metabolism resulted in fewer complex polysaccharides, which may adversely affect the growth, and therefore proliferation of protein-coding genes of these soil Bacteria.

4.3. The emerging appreciation of the role of Archaea in the soil N cycle

A paucity of knowledge exists regarding archaeal community responses to N enrichment. In our study, archaeal and bacterial gene abundance and composition responded similarly to experimental N deposition, namely site-dependent decreases in the abundance, richness, and diversity (H') of functional genes mediating N cycling processes, as well as shifts in gene composition. A similar response of ecological coherence between archaeal and bacterial assemblages has been previously observed over several fertilization regimes (Wessen et al., 2010), whereas another study provided no evidence for archaeal community shifts in N-fertilized agricultural soils in which shifts in bacterial communities were observed (Taylor et al., 2012). It has been hypothesized that Archaea play an important role in soil nitrification (Bates et al., 2011; Leininger et al., 2006; Nicol and Schleper, 2006). Furthermore, possible inhibition of soil archaeal communities by high levels of available N was observed in N amended grassland soils, possibly due to competitive interactions with bacterial nitrifiers (Bates et al., 2011). Recent studies have attempted to resolve possible competitive interactions between ammonia-oxidizing Archaea (AOA) and Bacteria (AOB), with some proposing bacterial dominance in nitrification (Di et al., 2009; Long et al., 2012), whereas some suggest the opposite (Leininger et al., 2006) and others suggest a soil-dependent response (Taylor et al., 2010). Unfortunately, the probe composition of GeoChip 4.0 does not allow for such comparisons in our study. Though PCR-based molecular techniques have greatly improved existing knowledge of this domain since its inception nearly 20 years ago (Woese et al., 1990), we still have an inadequate understanding of terrestrial Archaea (Bates et al., 2011), and even less is known regarding effects of anthropogenic N deposition on these soil organisms. Genome sequencing has revealed putative NO_3^- transporters, and genes encoding enzymes involved in NO_3^-

reduction and denitrification in both Crenarchaeota and Euryarchaeota, two families within the Archaea (Cabello et al., 2004). Furthermore, archaeal assimilatory NO_3^- and NO_2^- reductases have been purified from pure cultures (Martinez-Espinosa et al., 2001). Undoubtedly, information obtained through the rise in large-scale sequencing efforts will allow for more robust analyses of the archaeal response to anthropogenic N enrichment.

5. Conclusions

The results we present here support the hypothesis that chronic N deposition can decrease the abundance and diversity of functional genes mediating N cycling processes as well as altering their composition, albeit this response was site-dependent. In the northernmost two sites, the overall decline in the abundance of functional genes encoding N-cycling processes occurred as part of a broad overall suppression of microbial activities in response to anthropogenic N deposition which results in reduced decay, greater soil C storage, and increased DOC and NO_3^- leaching. Evidence from this study suggests denitrification, nitrification, and NO_3^- assimilation to be key transformations as N-cycling microbial assemblages respond to future rates of atmospheric N deposition. Our results support the assertion that ecosystem-level microbial responses to anthropogenic N deposition may fundamentally alter the physiological potential of soil microbial communities and soil N cycling dynamics within the northern hardwood forest ecosystem.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2013.07.010>.

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