# Simulated Atmospheric Nitrogen Deposition Alters Actinobacterial Community Composition in Forest Soils

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Anthropogenic N deposition can slow the decay of plant detritus, leading to an accumulation of soil organic matter and the production of phenolic dissolved organic C (DOC), which can leach from soil to ground and surface waters. *Actinobacteria* are one of the few groups of saprotrophic microorganisms that oxidatively depolymerize lignin, producing substantial soluble polyphenolics in the process. In combination, these observations present the possibility that lignolytic *Actinobacteria* may become more important agents of lignin decay as atmospheric N deposition continues to increase during the next decade. To test this idea, we quantified actinobacterial abundance and community composition in a well-replicated field study in which atmospheric N deposition has been experimentally increased since 1994. Actinobacterial abundance was assessed using quantitative polymerase chain reaction of 16S rRNA and community composition was evaluated using clone libraries and phylogenetic community analyses (i.e., LIBSHUFF and UniFrac). Contrary to our expectation, experimental atmospheric N deposition had no effect on actinobacterial abundance in the forest floor (~$10^{10}$ gene copies $kg^{-1}$); however, it significantly decreased actinobacterial abundance by 47% and total DNA by 31% in surface soil. Our analyses revealed that experimental N deposition further elicited a significant membership change in forest floor and surface soil communities, as well as significant differences in the phylogenetic diversity of forest floor *Actinobacteria*. This shift in community composition occurred in concert with a slowing of plant litter decay, accumulation of soil organic matter, and a greater production of phenolic DOC. These observations are consistent with the idea that changes in actinobacterial community composition may underlie biogeochemical responses to experimental N deposition.

**Abbreviations:** DOC, dissolved organic carbon; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction.

Human activity has globally increased the amount of N entering terrestrial ecosystems (Galloway et al., 2004), which could foster higher rates of C sequestration in the N-limited forests of the northern hemisphere (Magnani et al., 2007). Presently, these ecosystems are a large, global sink for atmospheric $CO_2$, the magnitude of which could be influenced by the input of anthropogenic N. Empirical studies and simulation models have indicated, however, that anthropogenic N deposition could have either a minor or a substantial impact on the amount of C sequestered by these ecosystems, a set of observations that continues to fuel scientific discourse on this matter (Magnani et al., 2007; Reay et al., 2008). The majority of scientific attention has focused on understanding how plant growth may or may not respond to chronic atmospheric N deposition. This agent of global change also has the potential to negatively affect microbial communities in the soil, a response that can slow litter decay (−38%) and increase the amount of C stored in the forest floor and surface soil (10%; Pregitzer et al., 2008; Zak et al., 2008).

Fungi and *Actinobacteria* are key mediators of plant litter decay in forests, and their activity could be altered by the chronic deposition of anthropogenic N. For some basidiomycete fungi, high inorganic N concentrations under laboratory conditions repress the transcription of genes encoding enzymes that depolymerize lignin and cellulose in plant detritus (Tien and Tu, 1987; Li et al., 1994). In contrast, laboratory studies have demonstrated that some soil *Actinobacteria*

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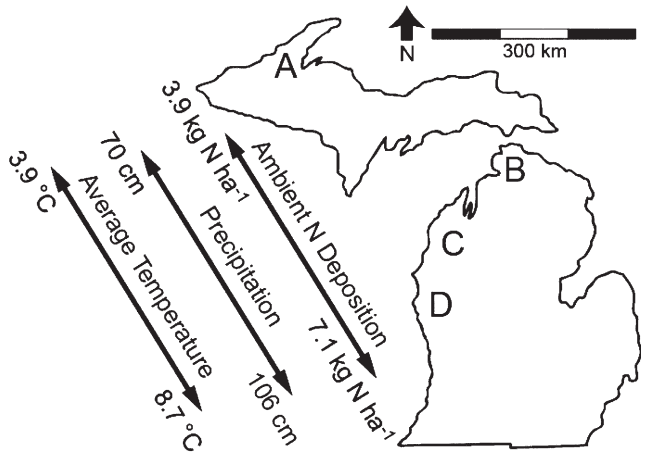


**Fig. 1. The geographic distribution of the study sites in Lower and Upper Michigan. In each stand, three plots received ambient atmospheric N deposition and three plots received ambient plus 30 kg $NO_3^-$–N $ha^{-1}$ $yr^{-1}$. These treatments have been in place since 1994 in the four sugar maple dominated northern hardwood stands.**

upregulate lignocellulolytic enzyme activity as inorganic N availability increases (Bardner and Crawford, 1981; Giroux et al., 1988; Vidal et al., 1989). Although lignolytic basidiomycetes can mineralize lignin to $CO_2$, the process of lignin metabolism by *Actinobacteria* is characterized by the production of soluble polyphenols (~60% of products), with only minor amounts of $CO_2$ originating from lignin (<4%; Mason et al., 1988; Godden et al., 1992; Berrocal et al., 1997). These contrasting responses to high inorganic N availability suggest that chronic atmospheric N deposition could suppress lignin mineralization by lignolytic basidiomycetes, thereby shifting this process to *Actinobacteria*, some of which can solubilize lignin into polyphenols. Such a response should decrease the activity of lignolytic enzymes as well as rates of litter decay because some lignolytic *Actinobacteria* produce smaller amounts of these extracellular enzymes than do basidiomycetes (Ramachandra et al., 1987; D'Souza et al., 1996). These observations present the possibility that anthropogenic N deposition could elicit a similar response under field conditions by increasing inorganic N concentrations in the soil solution; however, such a response has gone largely unexplored.

Since 1994, we have experimentally manipulated atmospheric N deposition in four sugar maple (*Acer saccharum* Marshall) dominated northern hardwood forest stands that span the geographic distribution of this forest type in the Upper Lake States region (Fig. 1). In this long-term experiment, we have observed a series of biogeochemical responses that are consistent with the idea that soil *Actinobacteria* have become more prominent agents of litter decay. For example, experimental N deposition, at a rate expected by the middle of this century (30 kg N $ha^{-1}$ $yr^{-1}$; Galloway et al., 2004), has significantly slowed plant litter decay (−38%), thereby increasing (10%) organic matter accumulation in the surface soil (Zak et al., 2008). Soil respiration has simultaneously declined (−15%) under experimental N deposition, despite no change in the fine root biomass or respiration (Burton et al., 2004); these observations indicate that experimental N deposition has decreased the microbial activity in the forest floor, surface soil, or both.

Additionally, extracellular phenol oxidase and peroxidase activity have declined (approximately −35%) under experimental N deposition (DeForest et al., 2004), evidence that lignolytic activity has slowed in response to higher inorganic N concentrations (280%; Zak et al., 2008). These observations led us initially to hypothesize that experimental N deposition had decreased the abundance of lignolytic basidiomycetes, which are the dominant agents of lignocellulose metabolism in forests (Osono, 2007). Contrary to our expectations, however, experimental N deposition has not altered the abundance of basidiomycete laccase genes in either the forest floor or the surface soil, suggesting that it has not altered the abundance of lignolytic basidiomycetes in our experiment (Hassett et al., 2009). In addition to the biogeochemical responses summarized above, experimental N deposition has elicited a threefold increase in DOC production and a 1.5-fold increase in the phenolic content of this material (Pregitzer et al., 2004; Smemo et al., 2006, 2007). Taken together, these observations suggest that lignolytic *Actinobacteria* may have become more dominant agents of litter decay under experimental N deposition.

Our objective was to determine if experimental N deposition had increased the actinobacterial abundance, altered the actinobacterial community composition, or both in the forest floor and surface soil. To address our objective, we amplified the actinobacterial 16S rRNA gene using real-time quantitative polymerase chain reaction (qPCR) to estimate the actinobacterial abundance under ambient and experimental N deposition. We used phylogenetic community analyses of amplified, cloned, and sequenced actinobacterial 16S rRNA genes to determine whether experimental N deposition had altered the composition of actinobacterial communities in our experiment.

## MATERIALS AND METHODS

### Study Sites

We quantified the effect of chronic atmospheric N deposition on actinobacterial community abundance and composition in a series of sugar maple dominated northern hardwood forest stands that have continuously received experimental N deposition beginning in 1994. These four study sites lie along a 500-km climatic and atmospheric N deposition gradient in the Great Lakes region (MacDonald et al., 1991; Fig. 1). Our study sites are of similar overstory age, plant composition, and soil development. The soils are well-drained, sandy, isotic, frigid Typic Haplorthods of the Kalkaska series. The sites range in temperature, growing season length, and ambient N deposition (Table 1; Zak et al., 2008). The soil pH does not significantly differ among sites or treatments (Table 1; under ambient conditions = 4.5 ± 0.25; under N deposition = 4.7 ± 0.32).

At each of the four sites (Fig. 1), there are six 30- by 30-m plots; three receive ambient N deposition and three receive ambient plus 30 kg $NO_3^-$–N $ha^{-1}$ $yr^{-1}$. The experimental N deposition treatment was designed to simulate rates of atmospheric N deposition anticipated to occur in the northeastern United States and Europe by 2050 (Galloway et al., 2008). Plots receiving experimental N deposition are treated with solid $NaNO_3$ granules, which are broadcast over the forest floor in six increments (5 kg N $ha^{-1}$) throughout the growing season (April–September); $NO_3^-$ composes ~60% of the atmospheric N deposition in the region.

Each plot is surrounded by a 10-m buffer, which also receives ambient or experimental N deposition.

At each study site, forest floor (Oi horizon) and surface soil samples (Oe, Oa, and A horizons) were separately collected from all plots receiving ambient ($n$ = 3) and experimental ($n$ = 3) N deposition; sampling occurred during mid-May 2007. Previously, we documented that microbial decay had slowed in the Oe, Oa, and upper A horizons (Zak et al., 2008), and we purposely designed our sampling to distinguish organic matter in these horizons from litter deposited the previous autumn (Oi horizon). The forest floor (Oi horizon) was collected from 10 randomly placed 10- by 10-cm areas within each plot. While in the field, the forest floor samples were composited within each plot and homogenized with a sterilized hand-held food processor. The surface soil was collected from the center of each 10- by 10-cm area using a 2.5-cm-diameter soil corer, which extended to a depth of 5 cm to capture the Oe, Oa, and A horizons. The surface soil samples were composited by plot and homogenized by passing through a 2-mm sieve in the field. The samples were stored on ice in DNA extraction vials and transport to the University of Michigan, where they were held at −20°C until DNA extraction.

**Table 1. Climatic, floristic and edaphic characteristics of four northern hardwood study sites (A–D) receiving simulated atmospheric N deposition. Sites are located in Lower and Upper Michigan and have been receiving experimental N deposition since 1994. Stands are similar in age (92–97 yr), plant composition, and soil development but differ in temperature, growing season length, and ambient N deposition.**

| Characteristic | Site A | Site B | Site C | Site D |
|---|---|---|---|---|
| Location | | | | |
| Latitude | 46°52′ N | 45°33′ N | 44°23′ N | 43°40′ N |
| Longitude | 88°53′ W | 84°52′ W | 85°50′ W | 86°9′ W |
| Climate | | | | |
| Mean annual temperature, °C | 4.7 | 6.0 | 6.9 | 7.6 |
| Mean annual precipitation, mm | 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 |
| ***Acer saccharum*** biomass, Mg $ha^{-1}$ | 237 | 224 | 216 | 201 |
| Soil (0–10 cm) | | | | |
| Sand, % | 85 | 89 | 89 | 87 |
| pH (1:1 soil/$H_2O$) | 4.8 | 5.0 | 4.5 | 4.7 |
| Cation exchange capacity, $mmol_c$ $kg^{-1}$ | 3.4 | 3.8 | 2.6 | 3.0 |
| Base saturation, % | 71 | 96 | 73 | 80 |

## *Actinobacteria* rRNA Amplification

Genomic DNA was extracted from all forest floor and surface soil samples using MoBio PowerMax Soil DNA isolation kits (MoBio Laboratories, Carlsbad, CA). Extractions followed the manufacturer's instructions, starting with approximately 1.5 g of field-moist forest floor or 5.0 g of field-moist surface soil. The procedure yielded community DNA in ~2.5mL of Tris-HCl, which was stored at −80°C until further analysis. The total microbial community DNA in each extraction was quantified using the Quant-iT PicoGreen (Invitrogen, Carlsbad, CA) assay according to the manufacturer's instructions. Fluorescence was measured on an *f*-Max Fluorimeter with SoftmaxPro software (Molecular Devices Corp., Sunnydale, CA), in which excitation energy was set at 485 nm and emission occurred at 538 nm.

The universal bacterial primer Eub338F (ACGGGCGGTGTGTACA) was paired with the group-specific primer Act1159R (TCCGAGTTRACCCCGGC) to amplify actinobacterial 16S rRNA for community analyses (Blackwood et al., 2005). These primers match 2510 out of 2563 *Actinobacteria* 16S rRNA sequences in the ARB2003 database (Blackwood et al., 2005). Individual polymerase chain reaction (PCR) volume totaled 25 μL: 0.286 μL (1.0 unit) High Fidelity Expand *Taq* polymerase (Roche Applied Science, Mannheim, Germany), 2.5 μL buffer (Roche Applied Science), 0.25 uL bovine serum albumin (Invitrogen), 0.5 μL (0.2 μmol $L^{-1}$) Eub338F primer and 0.5 μL (0.2 μmol $L^{-1}$) Act1195R primer, 2 μL of template DNA, and $H_2O$. Low-cycle PCR reactions were optimized using a Stratagene Robocycler gradient thermocycler (Agilent Technologies, La Jolla, CA). Optimization measures were taken to maximize amplification while minimizing the formation of primer dimmers, chimeric DNA, and cycle number. The optimized PCR protocol was: 95°C for 5 min for initial denaturing, then 25 rounds of amplification (94°C for 30 s, 57°C for 30 s, 72°C for 90 s) followed by 10 min at 72°C for elongation; reactions were held at 6°C before removal (*sensu* Blackwood et al., 2005). The PCR products were visualized though electrophoresis on a 1.5% (w/v) agarose gel and ethidium bromide staining. The PCR products were purified with a MoBio Ultra Clean PCR clean-up kit (MoBio Laboratories) according to manufacturer's instructions and then stored at −20°C for further analysis.

## Actinobacterial Abundance

To gain insight into actinobacterial abundance, we estimated the actinobacterial 16S rRNA gene copy number using quantitative fluorescence PCR with the primers Eub338F and Act1159R as described above. We conducted four separate replicate qPCR analyses on each sample to ensure consistent replicated results; any replicate value >2 standard deviations from the mean was eliminated. The procedure was conducted on a Stratagene MX3000P real-time PCR system interfaced with MaxPro version 3.00 software (Agilent Technologies, La Jolla, CA). Brilliant SYBR Green qPCR Master Mix, containing SYBR Green I reporting dye, was used in this procedure following the manufacturer's instructions. Fluorescence was measured at the end of each elongation step, and it was normalized to the fluorescence of 6-carboxyl-X-rhodamine (ROX), with excitation and emission wavelengths of 584 and 612 nm, respectively.

Separate standard curves for the forest floor and surface soil were created by compositing 25 μL of PCR-amplified actinobacterial 16S rDNA from each site and treatment. Actinobacterial DNA was amplified with the previously described primers, Eub338F and Act1159R. The composite DNA for each horizon was then quantified with Quant-iT Pico green (Invitrogen) and diluted to create DNA standards ranging from $10^{-4}$ to $10^{-10}$ mg DNA $L^{-1}$. In this way, DNA standards for each horizon were representative of the actinobacterial sequences found for each site and treatment.

Quantitative PCR reactions were performed in 200-μL, thin-walled, optical PCR tube strips (Agilent Technologies). The reaction volume totaled 25 μL; the reaction mixtures contained SYBR Green Master Mix, ROX reference dye, and 2 μL of previously extracted template DNA. The

PCR cycles consisted of 5 min of denaturation at 95°C, followed by 35 amplification cycles of 30 s at 94°C, 30 s at 57°C for annealing, then 90 s at 72°C, followed by a 10-min elongation step at 72°C. The reaction was then cooled to 52°C and then gradually heated to 75°C, while the fluorescence of annealing and denaturing products was measured.

Mass-based estimates of Actinobacterial 16s rRNA gene were converted to copy number per kilogram of soil (dry weight) by assuming a molecular weight of 660 g $mol^{-1}$ per base pair for the average length of 820 base pairs. *Actinobacteria* copy numbers were transformed ($log_2$) before statistical analysis to meet the assumption of homogeneity of variance. A two-way ANOVA of the transformed actinobacterial 16s rRNA (gene copy number $kg^{-1}$) was then used to determine if *Actinobacteria* were more abundant under experimental N deposition. This analysis included a site × treatment interaction, which enabled us to determine if the actinobacterial community composition responded in the same manner among the four replicate study sites. In this analysis, site was a fixed variable and treatment was random.

## Analysis of Actinobacterial Community Composition

The influence of experimental N deposition on actinobacterial community composition was analyzed within and across all four sites by constructing separate clone libraries for the forest floor and surface soil in each site. The libraries contained an equal number of clones from the ambient and experimental N deposition treatments. Rarefaction curves and Chao1 estimates of diversity were calculated for the ambient and experimental N deposition treatments of both soil horizons (Chao, 1984). The DNA from each PCR reaction was cloned using the Invitrogen TOPO TA cloning kit and TOP 10 chemically competent cells via PCR 2.1-TOPO vectors; the manufacturer's instructions were followed throughout the procedure. Ninety-six positive colonies from each cloning reaction were randomly selected using sterile toothpicks, placed in culture blocks containing a medium of Luria-Bertani broth (10% glycerol, 0.25 g $L^{-1}$ ampicillin, and 0.125 g $L^{-1}$ kanamycin), and grown overnight. Thirty-two clones from each culture block were then transferred to flat-bottom Cellstar sequencing plates, which were subsequently sequenced using the Sanger method at the Laboratory for Genomics and Bioinformatics at the University of Georgia. The average read length of the 16S rRNA gene was 820 base pairs.

Sequences were manually edited using FinchTV 1.4.0 (Geospiza, Seattle, WA), and contiguous sequences from forward and reverse sequencing were created in Geneious Pro 3.0.6 (Drummond et al., 2007). Eight clone libraries, one for each site in the forest floor and surface soil, were created by aligning the consensus sequences using ClustalW (Larkin et al., 2007). Each alignment contained sequences from both ambient and N deposition treatments. Alignments were edited in GeneDoc 2.6.002 (Nicholas et al., 1997). All clone sequences are available in GenBank (Accession no. FJ661102–FJ662388).

Distance matrices of the edited alignments were produced using the Kimura 2-parameter algorithm in PHYLIP 3.6.7 (Felsenstein, 2004). The computer program DOTUR was used to assign operational taxonomic units (OTUs) based on 97.5% sequence identity for each horizon (Schloss and Handelsman, 2005). Operational taxonomic unit consensus sequences were generated in Geneious Pro 3.6.0 by taking a consensus of the representative clone sequences for each OTU identified by DOTUR. The OTU consensus sequences were then aligned for each library using ClustalW; PHYLIP was then used to construct distance matrices from the OTU libraries for phylogenetic analysis.

One hundred and twenty actinobacterial 16S rRNA reference sequences were obtained from the Ribosomal Database Project's database (Cole et al., 2007) using RDPquery (Dyszynski and Sheldon, 2006) and used in the construction of phylogenetic trees: one for the forest floor and one for the surface soil. Approximately 40 reference sequences were selected to provide phylogenetic representation throughout the extent of the trees for visual and phylogenetic analysis; *Staphylococcus aureus,* a Gram positive bacterium, rooted the tree. The PAUP method (Swafford, 2003) was used to determine the best-fit model for the forest floor and surface soil OTU libraries by comparing all available models with the Akaike information criterion (Akaike, 1974). We chose the HKY 85 model with a $\gamma$ value of 0.625 to create a maximum likelihood tree with OTU consensus sequences and reference sequences for phylogenetic analysis with weighted UniFrac (Lozupone et al., 2006). The OTU consensus sequences were assigned to an actinobacterial family based on >92% sequence similarity with family type strains and >95% bootstrap support on family clades; 90 to 92% sequence similarity or 75 to 95% bootstrap support designated suborder clades.

Shifts in actinobacterial community composition were assessed by two approaches: LIBSHUFF and weighted UniFrac. The LIBSHUFF code uses a Monte Carlo procedure to determine if two populations are significantly different from one another by generating and comparing coverage curves (Schloss, 2008). These coverage curves are based on the difference in the evolutionary distance between sequences within and between the two libraries (Schloss, 2008). The LIBSHUFF program reports two *P* values, XY and YX, each representing the comparison of sequences in one library to the other. If one of these reported *P* values is <0.025, the populations are considered significantly different from one another. This test can detect significant differences with limited sequence data; therefore, it was used to determine the effect of experimental N deposition within each site as well as data pooled across all sites. The UniFrac code, which also utilizes Monte Carlo simulations, follows the assumption that closely related organisms have similar ecological traits. By analyzing a phylogenetic tree, UniFrac calculates the unique phylogenetic branch length leading to one community or the other but not both (Lozupone and Knight, 2005). The UniFrac code was used in addition to LIBSHUFF to gain added insight into the differences in phylogenetic diversity and relatedness of the actinobacterial communities due to experimental N deposition; UniFrac analyses were conducted on the forest floor and surface soil sequence data pooled across the four study sites.

# RESULTS

In the forest floor, experimental N deposition had no effect on the total actinobacterial rRNA gene copy number (Fig. 2; $P = 0.317$; ambient mean = $1.1 \times 10^{10} \pm 2.4 \times 10^{9}$ copies $kg^{-1}$; N deposition mean = $8.3 \times 10^{9} \pm 2.8 \times 10^{9}$ copies $kg^{-1}$) and there was no effect of experimental N deposition on total extractable DNA ($P = 0.401$; ambient mean = $2.4 \times 10^{7} \pm 3.3 \times 10^{6}$ μg DNA $kg^{-1}$; N deposition mean = $2.6 \times 10^{7} \pm 2.3 \times 10^{6}$ μg DNA $kg^{-1}$). In the surface soil, experimental N deposition significantly decreased the actinobacterial rRNA copy number by 47% (Fig. 2; $P = 0.025$; ambient mean = $5.4 \times 10^{8} \pm 1.28 \times 10^{8}$ copies $kg^{-1}$; N deposition mean = $2.8 \times 10^{8} \pm 8.5\ 10^{7}$ copies $kg^{-1}$) and total

extractable DNA by 31% ($P = 0.029$; ambient mean = $1.1 \times 10^7 \pm 7.8 \times 10^5$ μg DNA $kg^{-1}$, N deposition mean = $7.9 \times 10^6 \pm 1.2 \times 10^6$ μg DNA $kg^{-1}$). The two-way ANOVA of the qPCR results reported no site × treatment interaction, meaning that all sites responded similarly to the treatment. Amplification efficiencies, reported by MaxPro 3.0 software (Mx3000P, Agilent Technologies) were derived from the slope of the log-linear phase of amplification for each run. Efficiencies correspond to the proportion of template molecules doubled every cycle. Reported efficiencies did not differ by treatment and ranged from 79 to 86% in the surface soil and 80 to 82% in the forest floor.

Analysis of 605 actinobacterial sequences from the forest floor yielded 571 viable consensus sequences, from which 112 OTUs were determined; 45 of these OTUs were singletons. Analysis of 693 sequences from the surface soil clone library resulted in 688 consensus sequences and the formation of 121 OTUs, 41 of which were singletons. All sequences recovered from forest floor and surface soil were of actinobacterial origin. Within the class of *Actinobacteria*, we recovered sequences from two of the orders: the *Actinomycetales* and *Acidimirobiales*. Within the order *Actinomycetales*, sequences matched 11 of 43 families (Zhi et al., 2009). The relative occurrence and the phylogenetic distribution can be observed in Fig. 3 and 4. Examination of individual rarefaction curves for the forest floor and surface soil libraries suggested that the ambient treatment libraries contained a higher diversity of actinobacteria than those from the experimental N deposition treatment. The Chao1 estimates of diversity, however, revealed overlapping confidence intervals for ambient and experimental N deposition treatments; therefore, we have no evidence that experimental N deposition has altered actinobacterial richness (see Supplemental Material).

Weighted UniFrac, which measures the unique branch length and relative abundance of taxa along lineages for each community, revealed a significant difference between forest floor actinobacterial communities under ambient and experimental N deposition ($P = 0.01$). In a separate analysis, however, UniFrac did not detect a significant effect of experimental N deposition on the actinobacterial communities in the surface soil ($P = 0.10$), at least not at our accepted level of statistical significance.

The LIBSHUFF code, a qualitative β-diversity analysis based on the evolutionary distance between sequences within and between communities, revealed that experimental N deposition significantly altered actinobacterial community composition in both the forest floor ($P = 0.006$ and 0.001) and surface soil ($P = 0.001$ and 0.001). The effect of experimental N deposition was consistent when each individual site was analyzed separately, as well as when the libraries were combined across stands for separate analyses of the forest floor and surface soil communities (Table 2). Moreover, all four ambient surface soil *Actinobacteria* communities were significantly different from each other ($P = 0.006$–$0.069$ for all pairwise comparisons; data not shown). Regardless of the initial differences in actinobacterial communities among the four study sites, our analyses collectively indicate that experimental N deposition had significantly and consistently altered the actinobacterial community composition across a large geographic region.

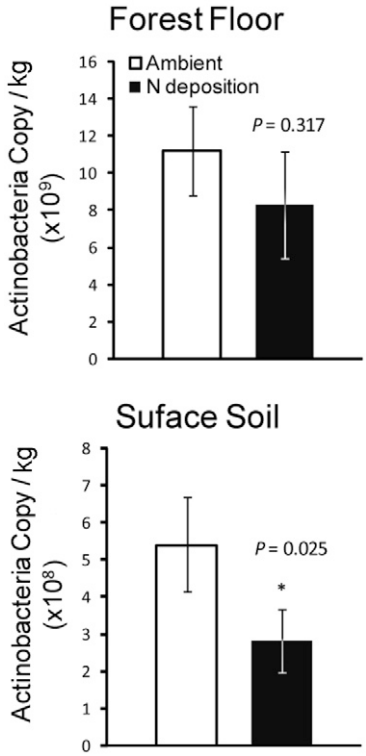


**Fig. 2. Gene copy number of actinobacterial rRNA under ambient and experimental N deposition. Values are treatment means for forest floor (Oi horizon) and surface soil (Oe, Oa, and A horizons) in four replicate northern hardwood stands in Michigan. Error bars represent standard error ($n = 4$).**

## DISCUSSION

The cycling of C in northern temperate forest ecosystems is subject to change as atmospheric N deposition continues to increase during the next century. Presently, we are just beginning to understand the mechanisms responsible for the slowing of plant litter decay and the accumulation of soil organic matter, as well as the greater leaching loss of phenolic DOC (Burton et al., 2004; Pregitzer et al., 2008; Zak et al., 2008). Collectively, these biochemical responses indicate the importance of microbial decomposers in regulating an ecosystem response to atmospheric N deposition (Zak et al., 2008). *Actinobacteria* are ecologically important mediators of lignin degradation and humus formation (Goodfellow and Williams, 1983; Wohl and McArthur, 1998; Eccleston et al., 2008), and changes in their abundance, composition, and physiology in response to increased atmospheric N deposition have the potential to reduce the rate as well as alter the end products of lignin degradation. Our results provide evidence that experimental N deposition can induce compositional shifts in actinobacterial communities and that these shifts occurred in parallel with the slowing of litter decay and the enhanced production of phenolic DOC (Pregitzer et al., 2004; Zak et al., 2008). Presently, our limited understanding of actinobacterial ecology and physiology does not allow us to discern whether changes in actinobacterial communities underlie biogeochemical responses to experimental N deposition. Nevertheless,

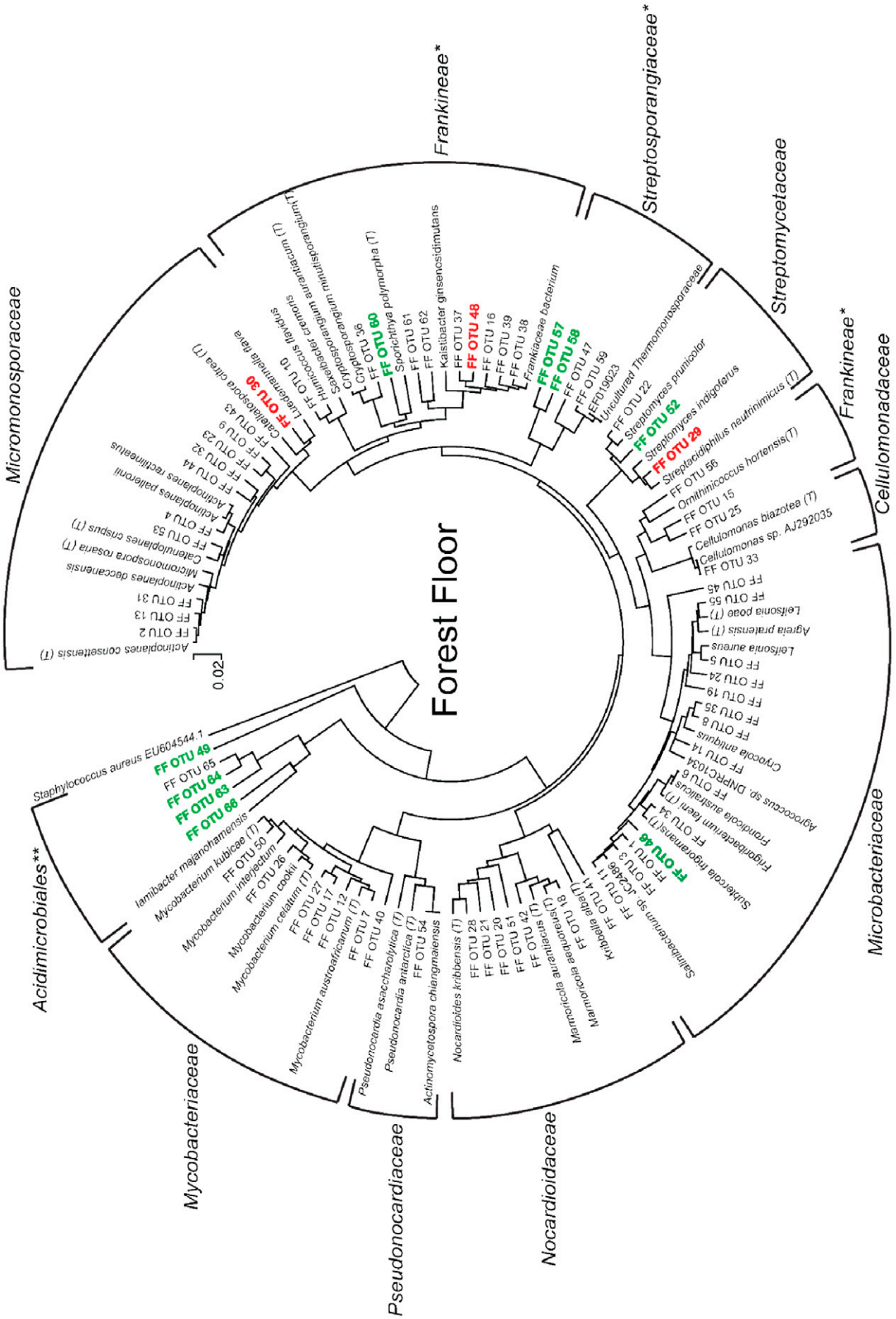


**Fig. 3. Neighbor-joining tree with Tamura–Nei substitution of forest floor actinobacteria operational taxonomic unit (OTU) consensus sequences. Families were based on >90% bootstrap support. Suborders are denoted by a single asterisk, and the order *Acidimicrobiales* is denoted by a double asterisk. The OTUs in red were unique to the ambient N deposition treatment, and those in green were unique to the experimental N deposition treatment.**

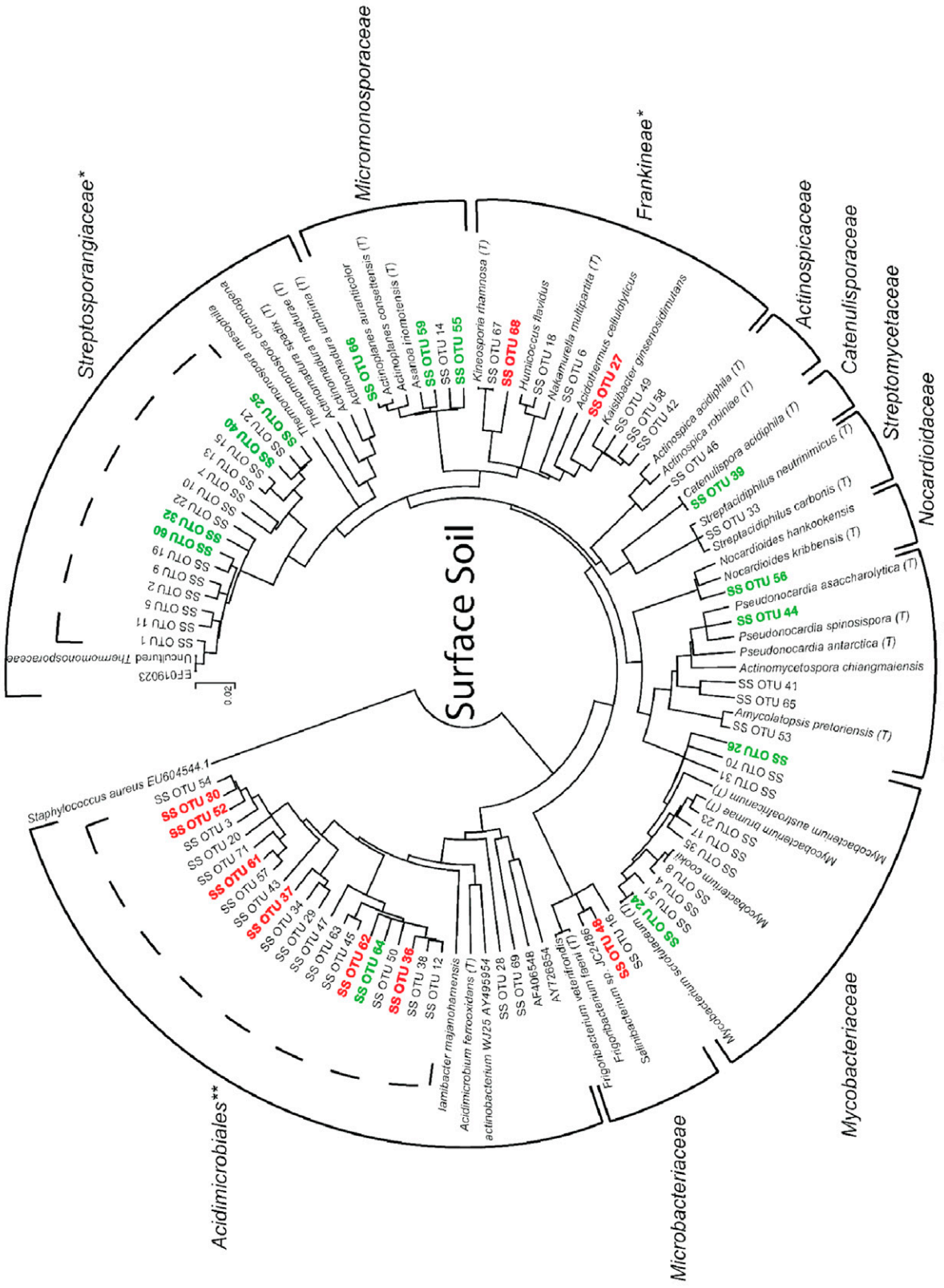


**Fig. 4. Neighbor-joining tree with Tamura–Nei substitution of surface soil actinobacteria operational taxonomic unit (OTU) consensus sequences. Families were based on >90% bootstrap support. Suborders are denoted by a single asterisk, and the order *Acidimicrobiales* is denoted by a double asterisk. The OTUs in red were unique to the ambient N deposition treatment, and those in green were unique to the experimental N deposition treatment.**

our observations are consistent with the idea that compositional shifts in soil microbial communities can elicit functional responses that potentially influence the rates of soil C cycling.

Changes in the soil microbial community under experimental N deposition did not result from a decrease in the abundance of *Actinobacteria*; rather, experimental N deposition altered the composition of these organisms in the forest floor and surface soil. For example, experimental N deposition did not have a significant effect on the abundance of *Actinobacteria* in the forest floor. Although we observed a 47% decrease in the surface soil *Actinobacteria* 16S rRNA copy number under N deposition, it should be noted that there was also a 31% decrease in total extractable DNA, suggesting that

**Table 2. Site-by-site comparison of actinobacterial communities under ambient and experimental N deposition, based on the phylogenetic test LIBSHUFF using designated operational taxonomic unit (OTU) consensus sequences including singletons.**

| Environment | Site | LIBSHUFF *P* value | |
|---|---|---|---|
| | | XY | YX |
| Forest floor | A† | 0.232 | 0.009 |
| | B | 0.058 | 0.164 |
| | C† | 0.006 | 0.048 |
| | D† | 0.522 | 0.012 |
| Surface soil | A† | 0.015 | 0.038 |
| | B† | 0.464 | 0.001 |
| | C† | 0.003 | 0.038 |
| | D† | 0.049 | 0.002 |

† Significant difference among sites.

actinobacterial relative abundance was unaffected by experimental N deposition. Therefore, we must reject the hypothesis that experimental N deposition favored *Actinobacteria* over other organisms mediating the process of litter decay, at least in terms of their overall abundance. Instead, it appears that experimental N deposition decreased the living microbial biomass (i.e., the total DNA) in the surface soil, an observation that is consistent with decreases in soil respiration (Burton et al., 2004), declines in extracellular enzyme activity, and other measures of microbial biomass (DeForest et al., 2004). The overall suppression of microbial activity in the surface soil could contribute to increased soil C storage by slowing decay; however, the suppression of lignolytic enzyme production, the increase in cellulolytic activity (DeForest et al., 2004; Sinsabaugh et al., 2005), and the threefold increase in DOC leaching under experimental N deposition (Pregitzer et al., 2004) are suggestive of a much broader effect on microbial community function.

In support of this assertion, we observed that experimental N deposition had significant and consistent effects on the composition of actinobacterial communities in the forest floor and surface soil, regardless of the initial differences among our study sites. Our results demonstrate that forest floor actinobacterial communities under experimental N deposition contained unique phylogenetic lineages (UniFrac $P = 0.01$) as well as membership (lowest LIBSHUFF $P = 0.00$), relative to those in the ambient treatment. If these unique actinobacterial lineages mediate different modes of lignin depolymerization, then changes in the actinobacterial community composition may, in part, be responsible for the biogeochemical changes induced by experimental N deposition; this remains a hypothesis to be tested. Furthermore, experimental N deposition also significantly altered the membership of the actinobacterial communities in the surface soil (lowest LIBSHUFF $P < 0.001$); however, the communities under ambient and experimental N deposition had similar phylogenetic lineages (UniFrac $P = 0.100$), implying that they also may mediate similar biogeochemical processes. The changes in phylogenetic lineages detected by UniFrac were not due to a change in diversity, nor was the actinobacterial community under experimental N deposition a subset of the ambient community (see Supplemental Material). Operational taxonomic units from both treatments were distributed throughout the phylogenetic tree, with some groups of *Actinobacteria* responding positively to experimental N deposition (i.e., *Acidimicrobiales* in the surface soil; Fig. 4) and others responding negatively (i.e., *Streptosporangiaceae*; Fig. 3 and 4). These shifts in the actinobacterial communities occurred in concert with the observed slowing of plant litter decay, and could in part be responsible for greater soil C storage and DOC export under experimental N deposition (Pregitzer et al., 2008; Zak et al., 2008). Our results are consistent with the idea that changes in the forest floor community composition may contribute to a greater production of phenolic DOC under experimental N deposition (Pregitzer et al., 2004; Smemo et al., 2006, 2007), whereas changes in the surface soil communities are more subtle and perhaps less important.

Due to limited knowledge of which *Actinobacteria* participate in leaf litter decomposition and the unknown extent of those that mediate lignin depolymerization, it is difficult to link changes in the community composition to the biogeochemical responses under experimental N deposition. That is, we are presently unable to draw a causal link between changes in the actinobacterial community composition and the biogeochemical response to experimental N deposition that we have documented. Examining the relative occurrence of actinobacterial families known to function in lignocellulose decomposition, however, can yield some insight into how these communities have been affected by experimental N deposition.

Forest floor actinobacterial communities were consistently dominated by the families *Micromonosporaceae* and *Microbacteriaceae* and the suborder *Frankineae.* Members of the *Frankineae* suborder, which do not function in leaf litter decomposition (Goodfellow and Williams, 1983), were widely dispersed through the phylogenetic tree and without much bootstrap support in their grouping (data not shown). The *Microbacteriaceae* occurred with high fidelity under both ambient and experimental N deposition, and they are known to inhabit both aquatic and terrestrial ecosystems (Goodfellow and Williams, 1983); unfortunately, we do not yet understand their role in forest floor decomposition. The *Micromonosporaceae*, on the other hand, are known to mediate lignin degradation (Mason et al.,1988; Godden et al.,1992; Eccleston et al., 2008) and composed approximately 20% of forest floor actinobacterial communities and 6% of the surface soil communities. In the surface soil, approximately 75% of the sequences associated with this family were retrieved from the ambient treatment, whereas, in the forest floor, *Micromonosporaceae* clones were recovered equally from both ambient and experimental N deposition treatments (data not shown). Even though this family is a small component of the surface soil actinobacterial community, it appears that experimental N deposition negatively impacted its occurrence and perhaps its function in lignin decomposition. *Micromonosporaceae* form aerial mycelia and are common in freshwater, benthic sediments, forest floor, and surface soil (Goodfellow and Williams, 1983; Eccleston et al., 2008); isolates from these environments have been observed to degrade both lignin and chitin (Eccleston et al., 2008). Further research into the activity of this *Actinobacteria* family and others that function in lignin depolymerization will

yield greater insight into which organisms are mediating the biogeochemical responses elicited by experimental N deposition.

*Acidimicrobiales* was a consistently dominant group in the surface soil; the 114 sequences recovered in this subclass were represented by 21 distinct OTUs (Fig. 4) and represented 32% of all OTUs in the surface soil. Approximately 70% of the *Acidimicrobiales* OTUs were comprised of sequences recovered from the soil receiving experimental N deposition. On the other hand, *Acidimicrobiales* composed only 8% of the forest floor community and 80% of these sequences were found only in the ambient treatment. The *Acidimicrobiales* have been identified as highly pervasive and active in soil communities (Felske et al., 1997); however, their function in soil C cycling is largely unknown. Our study suggests that experimental N deposition specifically altered this group of *Actinobacteria*, and furthermore it did so differently depending on the horizon in which they resided. To date, this order contains only one family with one genus; only three cultured isolates reside in the National Center for Biotechnology Information database. Our study revealed that the *Acidimicrobiales* are a deeply branched group of *Actinobacteria* with diversity previously unexplored, and highlighted the need for further understanding of this large and pervasive group of *Actinobacteria* in the forest floor and surface soil.

Surprisingly, the lignin-degrading family *Streptomycetaceae,* which is the most widely studied *Actinobacteria* family, accounted for only 4% of the forest floor community and <1% of those obtained from the surface soil. Even when considering PCR and cloning biases, they are not a dominant family in either the forest floor or the surface soil. Because there is little representation of this family in our libraries, we could not draw any conclusions regarding how experimental N deposition had altered their composition. Regardless, this family is known to degrade lignin and their genomes contain laccase genes, which encode the phenol oxidase enzyme (Endo et al., 2003; Hullo et al., 2001). Understanding the activity of this common *Actinobacteria* family in the forest floor and surface soil would provide further insight into how change in actinobacterial communities many be linked to the slowing of decomposition under experimental N deposition.

In summary, experimental N deposition significantly and consistently altered actinobacterial community composition in the forest floor and surface soil, regardless of initial differences among study sites, which lie along a 500-km climatic gradient. The influence of experimental N deposition on saprotrophic *Actinobacteria* may be one cause underlying the slowing of decomposition and the production and leaching of DOC (Pregitzer et al., 2004), albeit we are unable to draw a causal link at this point in time. Nonetheless, experimental N deposition changed the actinobacterial community composition in the forest floor and surface soil differently; the surface soil actinobacterial abundance (i.e.,16S rRNA copy number) and total microbial abundance (i.e., total DNA) were suppressed, whereas phylogenetic analysis revealed that forest floor communities contained evolutionary lineages that were unique relative to those in the ambient treatment. In conclusion, changes in actinobacterial communities occurred in parallel with the slowing of decomposition, the accumulation of organic matter in the surface soil, and increases in the production of phenolic DOC. Although further work is necessary to mechanistically link changes in the actinobacterial community composition to physiological function, our observations are consistent with the hypothesis that ecosystem-level responses to anthropogenic N deposition can be mediated by changes in microbial community composition.

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