

DOES ATMOSPHERIC NO_3^- DEPOSITION ALTER
ACTINOBACTERIAL ABUNDANCE AND COMMUNITY
COMPOSITION IN A NORTHERN HARDWOOD FOREST
ECOSYSTEM?

By

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A thesis submitted
in partial fulfillment of the requirements
for the degree of
Master of Science
(Natural Resources and Environment)
at the University of Michigan
August 2008

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ABSTRACT

Atmospheric nitrogen deposition can alter the cycling of carbon in forest ecosystems by slowing the microbially mediated decay of plant detritus, leading to the accumulation of organic matter in surface soil and the greater leaching of dissolved organic carbon (DOC) to ground and surface waters. However, we presently do not understand the microbial mechanisms affected by atmospheric nitrogen deposition that regulate these biogeochemical responses. Actinobacteria are one of the few groups of saprotrophic soil microorganisms which degrade lignin, uniquely producing soluble polyphenolics which can accumulate in the soil. The overall objective of this study was to examine the impact of atmospheric NO_3^- deposition on actinobacterial community composition and subsequent effects on soil carbon cycling. To address this objective, actinobacterial community structure was quantified in a large-scale field study in which atmospheric NO_3^- deposition has been experimentally increased for over a decade. Actinobacterial abundance was assessed using quantitative PCR of 16S rDNA and community composition was assessed through the compilation of clone libraries. Experimental atmospheric NO_3^- deposition had no effect on actinobacterial relative abundance in either forest floor or surface mineral soil. However, there were significant differences in community structure and the relative occurrence of key lignin degrading actinobacteria families. Specifically, *Streptomycetaceae* and *Micromonosporaceae*, decreased in occurrence under experimental NO_3^- deposition in the surface soil, whereas the occurrence of *Streptomycetaceae* in the forest floor increased under experimental NO_3^- deposition. Changes in the actinobacterial community composition appear to be one mechanism contributing to the ecosystem-level biogeochemical changes in response to increased nitrate deposition.

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ACKNOWLEDGEMENTS

My research was supported by grants from the National Science Foundation (DEB 0315138). I would like to thank my advisor, Donald Zak for complete guidance and patience, and Rima Upchurch for her invaluable assistance with phylogenetic analysis. Thank you to Jeremy Semrau for his contributions to this manuscript and confidence in my work. I would like to thank John Hassett for collaboration and support. In addition, this thesis would not have been possible without input and advice from Ivan Edwards, Chris Blackwood, Harld Kelner and Rebecca Mau.

INTRODUCTION

Most nitrogen (N) on Earth (70%) resides in an un-reactive pool in the atmosphere, i.e., as N₂. Lightning and biological fixation are the few natural processes which create reactive nitrogen (Nr) from N₂, which can then be used by living organisms. As a result, N availability is a limiting factor for the productivity of many plants in a wide array of terrestrial ecosystems as well as organisms living in the open ocean (Vitousek and Horwath, 1992). The increased use of N-containing fertilizers and N₂-fixing agricultural crops, biomass burning in the tropics, and fossil fuel combustion have increased the input of Nr to terrestrial ecosystems, the rate of which presently exceeds the rate of Nr removal from leaching and denitrification (Galloway et al., 2003). As a result, these anthropogenic inputs cause Nr to accumulate in many ecosystems, potentially producing a suite of complex ecological responses including eutrophication, aquatic hypoxia, as well as altered ecosystem C storage (Galloway et al., 2003).

Atmospheric N deposition -- the increased influx of Nr to ecosystems from the atmosphere -- is a global phenomenon with some regions of the Earth receiving higher deposition rates than others. For example, in the absence of human-derived inputs, atmospheric N deposition is estimated to be *ca.* 0.05 g N m⁻² yr⁻¹ (Dentener et al., 2006). Presently, there are large regions of the Earth, such as the Northeastern United States, Western Europe and parts of China, in which atmospheric N deposition reaches 2 g N m⁻² yr⁻¹ (Galloway et al., 2008). By 2050, atmospheric N deposition is predicted to double in most terrestrial ecosystems and certain regions of the Earth may receive atmospheric N deposition exceeding 5 g N m⁻² yr⁻¹ (Galloway et al., 2004). This influx of Nr is thought to interact with the mechanisms governing the cycling and storage of C in temperate

forest ecosystems (Fog, 1988; Nadelhoffer et al., 1999; Gruber and Galloway, 2008). Northern hardwood forests are a dominant ecosystem in the northeastern portion of North America, and are an important component of the global C budget. Carbon storage in this widespread ecosystem is subject to change under increasing atmospheric N deposition (Pregitzer et al., 2007; Zak et al., 2008; Galloway et al., 2008).

At a rate likely to occur in this century ($3 \text{ g NO}_3^- \text{-N m}^{-2} \text{ y}^{-1}$), a decade of simulated atmospheric NO_3^- deposition has dramatically altered the biogeochemical cycling of C and N in a Great Lake region northern hardwood ecosystem. Annual net primary production has increased by 10%, however, biomass exclusively accumulated in woody tissue (Burton et al., 2004; Pregitzer et al., 2007). Leaf litter N concentration increased by 15%, decomposition has slowed significantly, and the leaching of DOC has increase 300% (Pregitzer et al., 2007; Deforest et al., 2005, Smemo et al., 2006). These responses have lead to a 10% increase in surface soil C storage under experimental NO_3^- deposition (Zak et al., 2008). The combined effects of NO_3^- deposition in this forest ecosystem indicate that a decrease in microbially-mediated litter decay is a plausible mechanism for an accumulation of soil organic matter and greater DOC leaching (Zak et al., 2008). Extracellular enzymes deployed by microorganisms to decompose lignin have also decreased under elevated NO_3^- deposition, further indicating that a microbial mechanism(s) is responsible for the shift in C storage in this forest ecosystem (Carreiro et al., 2000; Deforest et al., 2004; Sinsabaugh et al., 2005).

Atmospheric N deposition has the potential to alter the complex interactions between microorganisms responsible for the decay of lignin in plant detritus, potentially leading to the observed change in soil C cycling described above. Lignin is the second

most common compound in plant litter, and it limits the overall rate of decay (Berg, 1999; Mellilo et al., 1989). A limited number of soil microorganisms can metabolize lignin, and the extent to which lignin is mineralized to CO₂ varies among microbial taxa. Saprotrophic species with lignolytic capabilities include white- and brown-rot basidiomycetes (D'Souza et al., 1996; Kirk and Ferrel 1987), soft-rot ascomycetes (Liers et al., 2006; Rodriguez et al., 1996), and a limited number of actinobacteria (McCarthy, 1986; Zimmerman, 1989). White-rot fungi (Basidiomycota and Ascomycota) enzymatically mineralize lignin to CO₂ (Kirk and Farrell 1987), whereas brown-rot basidiomycetes have a limited ability to metabolize lignin via the Fenton reaction, and thereby gain access to the lignin-protected cellulose (D'Souza et al., 1996). Ascomycete fungi have the ability to degrade polysaccharides and lignin; however, their lignolytic potential in forest ecosystems is not well understood (Liers et al., 2006). Actinobacteria incompletely depolymerize lignin resulting in water-soluble phenolic C compounds as the end products of lignin degradation (Paul and Clark, 1996). These end products can accumulate in SOM or be leached as DOC.

Actinobacteria degrade lignocellulose during primary metabolic activity, partially oxidizing lignin to utilize the carbohydrates protected therein (Godden, 1992; McCarthy 1986). The metabolism of these carbohydrates into biomass requires N, and *in vitro* studies have found that some species of actinobacteria increase lignolytic activity in N-rich environments (Vidal et al, 1989). In contrast, white-rot fungi metabolize lignocellulose during secondary metabolism, using lignin for energy and respiration (Kirk and Ferrell, 1987). Past research on pure cultures of lignin degrading fungi found inorganic N delayed lignolytic enzyme production and overall decomposition of lignin in

vitro (Kirk, 1987). Additionally, experimentally elevated N *in situ*, decreased active fungal biomass (27-61%) in Northern hardwood forest soils (Frey et al., 2004). Furthermore, in some white-rot fungi, the transcription of genes encoding for phenol oxidases (which oxidatively depolymerize lignin) is repressed under high N concentrations (Li et al., 1994), which is consistent with declines in lignolytic activity under elevated atmospheric N deposition.

The enzymatic process of lignin decay has been thoroughly studied in white-rot basidiomycetes, because they are the primary degraders of lignin in forest ecosystems (Kirk and Farrell, 1987) and lignin degradation mediates the overall rate of litter decay (Baldrian 2006; Berg, 1999). Lignin is degraded via coordinated attack by extracellular polyphenol oxidases that oxidize, depolymerize, and perform ring cleavage (Kirk and Farrell, 1987). Recent work has been conducted on actinobacteria to examine their role in lignin degradation, as several species occupy ecological niches similar to those preferred by white-rot basidiomycetes (Paul and Clark, 1996). Laccases and phenol oxidases, produced by white-rot fungi to mineralize lignin, are also found in the genomes of well-studied actinobacteria in the family *Streptomyetaceae* (Endo et al., 2003). Additionally, some actinobacteria can solubilize lignin in solid state fermentation situations (Vidal et al., 1988; Giroux et al., 1988; Godden et al., 1992). However, we understand relatively little about the roll actinobacterial have in lignin degradation in forest floor or surface soil. Berrocal et al., (1997) found different species of *Streptomyces* were capable of producing significantly different amounts of extracellular peroxidases and phenol oxidases, yielding different quantities of CO₂ and water-soluble, polyphenolic end products of lignin mineralization, indicating that actinobacteria decomposer

community structure, as well as size is important when considering the role of these cells in carbon cycling. Because actinobacteria in soil communities are capable of decomposing lignin with ample N, the effects of elevated N deposition on this community could alter soil carbon cycling and produce the observed changes in soil carbon storage and DOC production described above.

Under chronic N deposition, northern hardwood forests are experiencing an overall decline in forest floor decomposition, a reduction of lignolysis, an accumulation of soil organic matter, and greater DOC leaching; all these observations suggest a decrease in complete lignin mineralization (DeForest et al., 2004; Pregitzer et al., 2004; Careirro et al., 2000). This observation could result from a shift in the competitive interaction between white-rot basidiomycetes and actinobacteria during the decomposition of plant detritus. Anticipated positive effects on the actinobacterial community could be observable through an increase in abundance or a shift in the overall microbial community composition. Although actinobacteria are important to litter decomposition in northern hardwood forests, we understand relatively little about how actinobacteria respond to chronic atmospheric NO_3^- deposition and the extent to which these organisms contribute to biogeochemical changes elicited by chronic NO_3^- deposition.

Hypothesis

Experimental elevated NO_3^- deposition will increase the relative abundance of actinobacteria in the soil microbial community, making them more dominant members of the lignolytic community of microorganisms in forest floor and surface mineral soil.

Furthermore, I predict significant, observable changes in actinobacteria community composition will occur with elevated NO_3^- deposition. My analyses assume that closely related organisms perform similar biogeochemical functions, and account for phylogenetic divergence, diversity and clustering (Louzupone and Knight, 2008; Schloss, 2008; Martin, 2002). Finally, I hypothesize that changes in actinobacterial community composition with increased nitrogen deposition will be consistent with the broader phenomenological observations of slower leaf litter decomposition, increased soil organic matter and greater DOC leaching in situ.

MATERIALS AND METHODS

Study Sites

Four sugar maple sites (*Acer saccharum* Marsh.) dominated northern hardwood sites which lie along a climatic and atmospheric N deposition gradient in the Great Lakes region were used as study sites (MacDonald et al., 1995; Fig 1). This gradient spans Lower and Upper Michigan. Stands are of similar age, plant composition and soil development. Soils are well-drained sandy, typic haplorthod of the Kalkaska series. Sites range in temperature, growing season length, and ambient N deposition (Table 1; MacDonald et al., 1995).

In each of the four sites, there are six 30m x 30m plots; three receive ambient N deposition and three receive 3g NO₃⁻-N m⁻² yr⁻¹ above ambient; these treatments were initiated in 1994. The experimental NO₃⁻ deposition treatment was designed to simulate rates of atmospheric N deposition anticipated to occur in the northeastern U.S. and Europe by 2050 (Galloway et al., 2008). Plots receiving experimental NO₃⁻ deposition have been treated with solid NaNO₃ granules, which are broadcast over the forest floor in six increments (5 g N m⁻² yr⁻¹) over the growing season (April through September) Each plot has a 10-m treated buffer zone to reduce edge effect.

Forest floor and surface mineral soil samples were collected from the ambient and experimental NO₃⁻ deposition plots in each site. Sampling took place mid May, 2007 over the time span of three days. Forest floor (O_i) was collected from 10 randomly placed 10cm x10cm areas within each plot. While in the field, forest floor samples were composited within each plot and homogenized with sterilized hand held food processors. Surface soil (O_e, O_a, and A horizons) was collected from the center of each 10cm x 10cm

area using a 2.5-cm diameter soil core, which extended to a depth of 5 cm. Soil was composited by plot and homogenized by passing through a 2-mm sieve in the field. Samples were stored on ice in DNA extraction vials for transport to the University of Michigan, where they were held at -20°C prior to DNA extraction.

Community DNA Extraction

Within one week of field collection, microbial community DNA was extracted from all surface soil and forest floor samples with MoBio PowerMax Soil DNA isolation kits. Extractions followed manufacturer instruction, starting with approximately 1.5 g of field moist leaf litter (5.0g field moist mineral soil) for each extraction. The procedure yielded community DNA in ~2.5mL Tris-HCL, which was stored at -80 °C for further analysis. Extraction recovery was calculated for each sample in order to account for DNA loss in this multi-step procedure. Percent recovery was applied to quantitative data in order to predict the actual amount of DNA in the sample. Average extraction efficiency was 44% for forest floor and 55% for surface soil.

Soil Microbial Community DNA quantification

Total microbial community DNA in each extraction was quantified using the Quant-iT PicoGreen (Invitrogen, Inc.) assay according to manufacturer instructions. Florescence was measured on an *f*-Max Florameter (Molecular Devices Corp., Sunnydale, CA) with SoftmaxPro software in which excitation energy was set at 485 nm and emission occurred at 538 nm.

Actinobacteria rDNA Amplification

Actinobacterial 16S rDNA primers (2005; Eub338F- ACGGGCGGTGTGTACA and Act1159R – TCCGAGTTRACCCCGGC) were used to target all members of the *Actinobacteria* phylum, to gain insight into actinobacterial community composition in each plot (Blackwood et al. 2005). These primers match 2,510 out of 2,563 *Actinobacteria* 16S rDNA sequences in the ARB2003 database (Blackwood et al., 2005). Individual PCR reaction volume totaled 25 µL: 0.286µL (1.0 unit) High Fidelity Expand taq polymerase (Roche, Inc.), 2.5µL buffer (Roche, Inc.), 0.25uL BSA (Invitrogen, Inc.), 0.5µL (0.2uM) Eub338F primer and 0.5µL (0.2uM) Act1195R primer, 2µL of template DNA, and H₂O. Low cycle PCR reactions were optimized using a Stratgene Robocycler Gradient thermocycler (Stratgene, Inc). The PCR protocol was: 95 °C for 5 min for initial denaturing, then 25 rounds of amplification (94 °C for 30 sec, 57°C for 30 sec, 72°C for 90 sec) followed by 10 min at 72 °C for elongation, and finally held at 6 °C before removal (adapted from Blackwood et al., 2005). Optimization measures were taken to maximize amplification, while minimizing the formation of primer dimmers and chimeric DNA, genomic DNA amplification, and cycle number. PCR products were visualized though electrophoresis on a 1.5% (w/v) agarose gel, weighted with Promega blue orange dye. Gels were stained with ethidium bromide before viewing under UV light. PCR products were purified with Mobio Ultra Clean PCR Clean up Kit according to manufacture's instruction and then stored at -20 °C until further analysis.

Analysis of Actinobacterial Abundance

To gain insight into actinobacterial abundance, I estimated actinobacterial 16S rDNA copy number using quantitative fluorescence PCR. The procedure was conducted on a Stratagene MX3000P real-time PCR system interfaced with MxPro version 3.00 (Stratagene, La Jolla, CA). Forward primer (EUB338) and reverse primer (ACT1159) amplified the 16S actinobacterial rDNA region as described above. Brilliant SYBR Green qPCR Master Mix, containing SYBR Green I reporting dye, was used in this procedure following manufacture instruction. Fluorescence was measured at the end of each elongation step and was normalized to the fluorescence of ROX, a passive reference dye with excitation and emission wavelengths of 584nm and 612nm respectively.

A standard curve was created from a composite of 16S rDNA PCR products from forest floor and surface soil horizons. These PCR products were pooled by horizon then quantified with Pico Green, before being diluted to create DNA standards ranging from 1 ng DNA/ μL to 1×10^{-12} Ng DNA/ μL . Several optimization measures were implemented to ensure consistent standards, which spanned the concentration range of sampled DNA.

Quantitative PCR reactions were performed in 200 μL thin walled optical PCR tube strips (Stratagene, Inc.). Reaction volume totaled 25 μL ; reaction mixtures contained SYBR Green Master Mix, ROX reference dye, and 2 μL previously extracted template DNA. PCR cycles consisted of 5 min of denaturation at 95 °C, followed by 35 amplification cycles of 30 sec at 94 °C, 30 sec at 57 °C for annealing, then 90 sec at 72 °C, followed by a 10 min elongation step at 72 °C. After the polymerase chain reaction, the reaction was cooled to 52 °C then slowly heated to 75 °C, while measuring fluorescence of annealing and denaturing products. Using DNA extraction recovery and

total copy number of actinobacteria DNA sequences in each sample, I was able to estimate the total number of actinobacteria 16s rDNA copies in each gram of sample.

A two-way analysis of variance of actinobacterial 16s rDNA copy number was used to determine if actinobacteria are more abundant in the experimental NO_3^- deposition treatment compared to the ambient treatment. This analysis included a site by treatment interaction, which enabled me to determine if actinobacterial community composition responded in the same or different manner among the four study sites. Actinobacterial relative abundance was calculated as the proportion of actinobacterial DNA in the total DNA extracted from soil; actinobacterial relative abundance was analyzed using a two-way ANOVA, as described above.

Analysis of Actinobacterial Community Composition

The influence of atmospheric NO_3^- deposition on actinobacterial community composition was analyzed in the northernmost and southernmost sites, A and D (Fig. 1) by constructing clone libraries of forest floor and surface mineral soil. Shifts in actinobacterial community composition were assessed by three approaches, explained in detail in subsequent sections.

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; manufacturer instructions were followed throughout the procedure. Ninety-six positive colonies from each cloning reaction were randomly selected and grown overnight in culture blocks containing a medium of Luria-Bertani broth, 10% glycerol, ampicillin and kanamycin. Thirty-two clones from each culture block were transferred to flat bottom Cellstar

sequencing plates, which were analyzed at Genomics and Bioinformatics Laboratory at the University of Georgia. .

Sequence Analysis

Sequences from the eight sub-libraries were manually edited using FinchTV 1.4.0 (Geospiza, Inc). Contigs of forward and reverse sequences were created in Geneious Pro 3.0.6 (Drummond et al., 2007). Sequences were then aligned by soil horizon using ClustalW (Larking et al., 2007), which resulted in two overall libraries – one for forest floor and one for surface soil. Alignments were edited in GeneDoc 2.6.002 (Nicholas et al., 1997).

Distance matrices of edited alignments were produced using the Kimura 2-paramater algorithm in PHYLIP 3.6.7 (Felsenstein, J. 2004). DOTUR (Schloss et al., 2005) was employed to assign operational taxonomic units (OTUs) based on 97.5% sequence similarity. Consensus sequences for each OTU were created by aligning representative clone sequences in Geneious Pro. OTU consensus sequences were then aligned for each library using ClustalW.

Phylogenetic Tree Construction

Seventy actinobacterial 16S rDNA reference sequences were obtained from the RDP database (Cole et al., 2007) using RDPquery (Dyszynski and Sheldon, 2006). Sequence similarity was set at 97.5% for species, 95% for genus, 92% for family and 90% for order. Seventy type strain reference sequences from 15 Actinobacteria families supplemented the OTU and full sequence alignments; *Bacillus subtilis* and

Staphylococcus aureus rooted the tree. Phylogenetic Neighbor-Joining trees with Tamura-Nei substitution and bootstrap support were then created in *MEGA* version 4.0 (Tamura, et al., 2007) for visual and statistical analysis. OTU consensus sequences were assigned to an Actinobacterial family based on > 95% bootstrap support on family clades; 75-95% bootstrap support designated sub-order clades. If sequences did not group into a supported clade, they were compared against the NCBI database; an identity of 95% or higher with a type strain species was used to assign sub-order.

Redundancy Analysis

A canonical ordination redundancy analysis (RDA) using Conoco 4.5 (Biometris, Inc., The Netherlands) was conducted on the OTU libraries for each horizon; this analysis excluded singletons and was based on the relative abundance of sequences in the clone library. Relative abundance was determined by how many individual sequences were grouped (by 97.5% similarity) to represent an OTU sequence. This analysis also constrained axes to linear combinations of environmental variables (site and NO_3^- treatment). Using CanoDraw (Biometris, Inc., The Netherlands), a T-value-Biplot was created to visualize which OTUs were influenced by site and by treatment.

Evolutionary Distance Analysis

WebLIBSHUFF (<http://libshuff.mib.uga.edu>) was employed to ascertain qualitative beta diversity community differences based on evolutionary distances. PHYLIP-generated distance matrices of OTU sequence libraries were analyzed by webLIBSHUFF to examine all pair-wise comparisons of site, horizon, and treatment.

Phylogenetic Analysis

Previously constructed Neighbor-Joining trees of all sequences in forest floor and surface soil communities were analyzed by the P-test of significance (Martin, 2002) using UniFrac (Lozupone et al., 2006). Analyses were based on 100 random permutations of phylogenetic trees to assess if the distribution of sequences between communities exhibit covariation in their phylogeny (Martin, 2002). That is, this analysis allowed me to determine if sequences exhibited significantly different clustering between treatments based on phylogenetic tree topology. The UniFrac metric, which considers branch lengths unique to each community to assess differences in the evolutionary patterns, was not used in this study. The group specific primers and similarity of environments studied are not the conditions which necessitate UniFrac's metric of shared ancestry between communities.

RESULTS

Does nitrogen deposition increase the relative abundance of actinobacteria in forest floor or soil microbial communities?

In the forest floor, experimental NO_3^- deposition had no significant effect on actinobacterial rDNA copy number, total extractable DNA or the relative abundance of actinobacterial rDNA (Fig. 2a, c, and e). In contrast, experimental NO_3^- deposition significantly decreased ($P = 0.0025$) actinobacterial rDNA copy number and total extractable DNA in surface soil in each of the four sites consistently (Fig. 2b and d), but it had no effect on the relative abundance of actinobacterial rDNA (Fig. 2f).. Surface soil from the experimental NO_3^- deposition treatment had 31% ($P = 0.025$) fewer copy numbers of actinobacterial 16s DNA than the ambient treatment (Figure 2.b). There was also a significant 25% decrease ($P = 0.029$) in total extractable DNA (Figure 2.d).

Has nitrogen deposition altered actinobacteria community composition in forest floor and surface soil?

Analysis of 302 sequences from the forest floor of site A and D resulted in 74 OTUs, 39 of which were singletons. The most common OTU had 81 representatives spanning both sites and treatments. The closest NCBI match (99% Max Identity) to the consensus sequence was a *Frigiobacterium* spp. (AY571813.1) in the family *Microbacteriaceae*. Analysis of 361 sequences from the surface soil clone library resulted in the formation of 85 OTUs, 33 of which were singletons. The most common OTU had 65 representatives spanning both sites and treatments. The closest NCBI match

(99% Max Identity) was an uncluttered *Thermomonosporaceae* bacterium (EF019023.1) in the *Thermomonosporaceae* family.

Phylogenetic trees of forest floor and soil libraries placed OTU consensus sequences in 10 out of 39 actinobacteria families, in 9 out of 10 sub-orders, and in 2 out of 5 sub-classes (Figures 3 and 4). The forest floor actinobacteria community was dominated by the *Microbacteriaceae* family (49 % of all clones), whereas the soil community was dominated by *Thermomonosporaceae* family (46 % of all clones). Dominant families occurred with high fidelity in both treatments. Table 2 displays the representation of each family, sub-order, and order from in each of the libraries.

Redundancy ordination analysis of OTU community composition resulted in non-significant effects of treatment in forest floor ($P = 0.66$) and soil ($P = 0.33$). However, the T-value bi-plot identified three OTU consensus sequences in forest floor that were negatively influenced by experimental NO_3 deposition. These three OTUs had a total of 23 clone representatives, 5 in the *Mycobacteriaceae* family and 18 in the in the *Microbacteriaceae* family. According to phylogenetic family designations, these families did not respond to experimental NO_3^- deposition (Figure 5). However, the 18 identified *Microbacteriaceae* sequences matched with 95% similarity to type strains in the genus *Leifsonia*, implying community changes at a finer genetic resolution than at the family level. Similarly, three OTU consensus sequences in mineral soil were negatively influenced by experimental NO_3^- deposition. These three OTUs had a total of 12 clone representatives; 5 in the *Microbacteriaceae* and 3 in the sub-order *Frankineae*. Phylogenetic designation agrees with this analysis, indicating reduced clone sequence representation in these families under experimental NO_3^- deposition (Figure 6).

Surface soil OTUs negatively influenced by experimental NO_3^- deposition also had 4 clone representatives in the order *Acidimicrobiales*; however, according to phylogenetic tree analysis, *Acidimicrobiales* increased by 51% under experimental NO_3^- deposition. Presently, there are only three sequenced members of this order in the NCBI and RDP databases, all in the same family *Acidimicrobiaceae*. This analysis found 66 individual sequences, which comprise 23 distinct OTUs grouping into several clades with higher than 60% boot strap support. These results indicate drastically more diversity in *Acidimicrobiales* than is presently understood.

LIBSHUFF, which is based on OTU community membership, revealed that experimental NO_3^- deposition significantly altered actinobacterial community composition in forest floor ($P = 0.433$, $P = 0.001$). When the lower of the P-values is less than 0.025, the two communities are significantly different in composition with 95% confidence (Schloss, 2008). This result was consistent when the analysis was conducted for each stand as well as when clone libraries were combined across stands (Table 3). In surface soil, actinobacteria communities in the experimental NO_3^- deposition treatment were significantly different from those in the ambient treatment ($P = 0.002$, $P = 0.025$). Moreover, actinobacteria communities in the forest floor and surface soil of the ambient treatment differed significantly between the two sites (Forest Floor: $P = 0.017$, $P = 0.146$ Surface Soil: $P = 0.004$, $P = 0.024$). Although LIBSHUFF detected significant differences between communities in the ambient treatments of sites A and D, there were minor (4-7%) differences in the relative occurrence of actinobacterial families and sub-orders; this was true in forest floor and surface soil (Table 4). Regardless of this difference, these

analyses indicate that experimental NO_3^- deposition had a significant and consistent effect on actinobacterial community composition.

The P-test of significance, which is based on phylogenetic tree construction, revealed that forest floor actinobacteria communities were clustered significantly by NO_3^- deposition treatment ($P = 0.001$), further evidence that experimental NO_3^- deposition altered actinobacterial community composition. However, surface soil actinobacteria communities exhibited near significant clustering by treatment ($P = 0.070$). Phylogenetic clustering draws on the paradigm that closely related organisms provide closely related functions, suggesting that the actinobacteria communities in the experimental NO_3^- deposition treatment function differently than those in the ambient treatment.

DISCUSSION

The cycling of C and N in northern temperate forest ecosystems is subject to change as atmospheric N deposition continues to increase over the next century. Presently, we are just beginning to understand the mechanisms responsible for the slowing of decomposition which has resulted in greater forest floor turnover time, an accumulation of organic matter in surface soil, as well as the greater leaching loss of DOC (Burton et al., 2004; Pregitzer et al., 2007; Zak et al., 2008). These changes indicate that the importance of microbial decomposers in regulating ecosystem response to atmospheric N deposition is amplified with increasing nitrogen deposition. Actinobacteria are ecologically important in the metabolism of lignin and humus (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 can significantly reduce the rate as well as alter the product distribution of lignin degradation.. This study demonstrates that changes in actinobacterial community composition occurred in concert with widespread biogeochemical changes and therefore may be linked to ecosystem-level responses to anthropogenic sources of NO_3^- .

Changes in the soil microbial community, which effect ecosystem level C storage, are not due to a decrease in the relative abundance of actinobacteria, but more likely to a change in the active community composition. Experimental NO_3^- deposition had no effect on the abundance of actinobacteria in the forest floor. Although a 31% decrease in the surface soil actinobacteria 16S rDNA copy number under NO_3^- deposition was observed, it should be noted that there was also a 25% decrease in total extractable DNA,

with no observable change in actinobacterial relative abundance (Figure 2 e and f). Therefore, I must reject my hypothesis that experimental NO_3^- deposition increases actinobacterial relative abundance due to the proposed inhibition of fungal-mediated lignin decomposition. Instead, it appears experimental NO_3^- deposition inhibited overall microbial growth, an observation which is supported by other analyses of microbial biomass and composition (Deforest et al., 2004). This inhibition of overall microbial growth could contribute to increased C storage in the forest floor; however, the suppression of lignolytic enzyme production, the increase of cellulolytic activity (Deforest et al., 2004; Sinsebaugh et al., 2005) and the 3-fold increase of DOC leaching under NO_3^- deposition (Zak et al. 2008) indicate an impediment of litter decomposition is not the only effect elevated NO_3^- deposition has on microbial communities. Therefore, a shift in the existing decomposer community is most likely a mechanism responsible for the aforementioned ecosystem level biogeochemical changes.

In support of this rationale, it was observed that experimental NO_3^- deposition had significant and consistent effects on forest floor and surface soil actinobacterial community composition that could elicit the changes seen in ecosystem C storage. Significant differences occurred in the phylogenetic distribution and clustering of ambient and NO_3^- treated actinobacteria clones in both soil horizons (Table 3). A difference in the distribution and clustering of actinobacterial clones within a phylogenetic tree implies that functional differences may occur between the actinobacterial communities in the ambient and experimental NO_3^- deposition treatments. This is based on the assumption that evolutionarily similar species provide similar functions in the environment. Additionally LIBSHUFF, a qualitative analysis, identified

significant differences in the ambient and NO_3^- deposition treatments based on membership, not abundance (Table 3). The significant differences in actinobacteria community composition are consistent weather data was analyzed using presence-absence data (LIBSHUFF) or the abundance of clone representatives (P-test). Regardless of initial differences between ambient treatments in the two sites, experimental NO_3^- deposition exerted a consistent effect on the occurrence of particular actinobacteria families in forest floor and surface mineral soil. These results are consistent with the idea that a change in actinobacterial composition and physiology are responsible for the slowing of decomposition, increase in organic matter accumulation and the acceleration of DOC leaching under experimental NO_3^- deposition.

Examining the occurrence of OTUs representing families known to function in lignocellulose decomposition will yield insight to how these communities have changed under experimental NO_3^- deposition. In this study, functional traits of families were assumed for all OTUs phylogenetically placed within a family clade, backed up with greater than 90% bootstrap support. Due to the nature of these data, the effects of experimental NO_3^- deposition on particular actinobacteria families and sub-orders are not statistically quantifiable, instead, trends in the occurrence of clone representatives in these actinobacteria groups were considered. Soil actinobacteria communities were dominated (~46%) by members of the cellulase producing *Thermonosporaceae* family in the *Streptosporangineae* sub-order (Goodfellow and Williams, 1983; Robson et al, 1989). However, experimental NO_3^- had no effect on their abundance in the clone libraries. Over 90 clones were identified in this family, the majority having less than 92% sequence similarity with type strain *Thermomonosporaceae* representatives in the *Actinomadura*,

Actinocorallia or *Spirillospora* genera. These clones did not group with any other members of the Actinobacteria phyla, suggesting that there is more diversity in this dominant actinobacterial family than presently documented. It is possible that shifts occurred within this dominant and phylogenetically diverse family towards more pertinent cellulose decomposers; however this could not be detected, due to the lack of cultured *Thermomonosporaceae* isolates presently available,

Experimental NO_3^- decreased the occurrence of *Micromonosporaceae* and *Streptomycetaceae* families in surface soil, both of which are known to mediate lignin degradation (Mason et al., 1988; Godden et al., 1992; Eccleston et al., 2008).

Micromonosporaceae composed ~ 14% of forest floor actinobacteria communities and ~5% of surface soil communities. This family, which forms aerial mycelia are common in fresh water, benthic sediments, and forest floor and soil (Goodfellow and Wilson, 1983; Eccleston et al., 2008). Isolates from these environments have been observed to degrade both lignin and chitin (Eccleston et al., 2008). The data presented here suggest experimental NO_3^- deposition inhibited *Micromonosporaceae*, with a decrease from 7.5% to 1.5% of the surface soil actinobacterial community. This shift could contribute to, and is consistent with, the decreased lignolytic activity in soil.

The lignin degrading family *Streptomycetaceae* also was diminished by experimental NO_3^- deposition in the surface soil, further contributing to the suppression of recalcitrant leaf litter decomposition in this forest ecosystem. Even though *Streptomyces* are the most widely studied actinobacteria genera, this family composed only 1-2% of forest floor and surface soil actinobacterial communities. Even when taking into account PCR and cloning biases – they are not a dominant family in the forest

47 floor or surface soil. Regardless, this family is known to degrade lignin and their genomes contain phenol oxidase genes (Endo et al., 2003; Hullo et al., 2001). In this study, experimental NO_3^- deposition lead to an decrease in occurrence of OTUs representing this family in the surface soil, albeit by ~2%, which further supports the decrease of lignolytic enzyme production and increased forest floor mass under elevated NO_3^- deposition (Sinsabaugh et al., 2005; Zak et al., 2008).

Streptomycetaceae in forest floor actinobacterial communities increased from 1-3% of the total community under NO_3^- deposition, highlighting the difference in forest floor and surface soil actinobacterial communities in their response to experimental NO_3^- deposition. In this field experiment, T-RFLP analyses have revealed that actinobacterial communities in the forest floor are significantly different from surface soil actinobacterial communities (Zak and Upchurch, *unpublished data*). This study suggests that there are differences in the way these two communities respond to NO_3^- deposition as well as contribute to ecosystem scale biogeochemical changes. When actinobacteria degrade lignin, they release water soluble polyphenolic compounds which accumulate in the forest floor and leach through mineral soil as DOC. The increase of lignin degrading actinobacteria in the forest floor could therefore be in part responsible for the increase in DOC export in this forest ecosystem, whereas the decrease of these actinobacteria in the surface soil could lead to an increase of C storage in the soil. Further research into the activity of this actinobacteria family and others which function in lignin decomposition will yield greater insight to which organisms are effecting these ecosystem scale changes.

The greatest change in the soil actinobacteria community composition was the 14-25% increase in the occurrence of clone representatives in the Order *Acidimicrobales*.

The positive effect of experimental NO_3^- deposition on these microorganisms is evident; however, their role in soil C cycling in forest ecosystems is largely unknown. To date, this order only contains one family with one genus and three cultured isolates characterized in the NCBI database (<http://www.ncbi.nlm.nih.gov/>, 2008). This is a deeply branched group of actinobacteria which has been identified as highly pervasive and active in the soil communities (Felske et al., 1997). Without closely related cultured isolates, the function of these clone representatives cannot be confidently determined. There are several groups of actinobacteria in the surface soil which responded positively and negatively to experimental NO_3^- deposition, such as the increase of *Frankineae* and the decrease of *Mycobacteriaceae*, *Catenulisporaceae* and *Pseudonocardineae* (Fig 6). Yet little is known about their function in forest floor decomposition; therefore, I cannot speculate on the role these families play in the observed microbially mediated biogeochemical changes.

Experimental elevated NO_3^- deposition has led to a suppression of surface soil actinobacterial populations, significant changes in phylogenetic clustering and community composition, the inhibition of lignolytic actinobacteria families in the surface soil, and the increase in the occurrence of *Streptomyetaceae* in the forest floor. Moreover, experimental NO_3^- deposition exerted a consistent effect on actinobacterial community composition, regardless of initial differences in communities between the two study sites. These changes in the actinobacterial communities occurred in parallel with the slowing of decomposition, accumulation of organic matter in the surface soil, and increases in the production and leaching of DOC. In combination, these observations indicate that ecosystem-level responses to anthropogenic NO_3^- deposition are mediated by microbial communities in soil.

TABLES

Table 1. Climatic characteristics of four northern hardwood sites receiving experimental atmospheric NO₃⁻ deposition (Zak et al., 2008).

	Site			
	A	B	C	D
I. Location				
Latitude, N	46°52'	45°33'	44°23'	43°40'
Longitude, W	88°53'	84°52'	85°50'	86°09'
II. 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 m ⁻² y ⁻¹)	0.38	0.58	0.78	0.76
Wet + Dry Total N Deposition (g m ⁻² y ⁻¹)	0.68	0.91	1.17	1.18

Table 2. Actinobacteria community structure in the forest floor and surface soil. Family (>90% bootstrap support) *Sub-order (75-90% bootstrap support, or 94% Max Id with Type Strain) **Order (*Acidimicrobiales* determined by 60-99% bootstrap support) All other families belong to Order *Actinomycetales*

Family	Forest Floor				Surface Soil			
	All	OTUCount	Ambient	NO ₃ -	All	OTUCount	Ambient	NO ₃ -
<i>Acidimicrobiales</i> **	8	8	6	2	70	21	25	45
<i>Catenulisporaceae</i>	0	0	0	0	8	4	6	2
<i>Cellulomonadaceae</i>	3	1	1	2	1	1	1	0
<i>Frankineae</i> *	37	15	22	15	16	7	5	11
<i>Microbacteriaceae</i>	145	12	80	65	10	2	3	7
<i>Micromonosporaceae</i>	43	8	24	19	16	5	13	3
<i>Mycobacteriaceae</i>	26	5	15	11	47	11	28	19
<i>Propionibacterineae</i> *	22	12	12	10	1	1	1	0
<i>Pseudonocardineae</i> *	5	5	4	1	21	9	12	9
<i>Streptomycetaceae</i>	6	4	2	4	5	5	3	2
<i>Thermomonosporaceae</i>	4	3	2	2	166	19	80	86
<i>Unknown</i>	3	1	1	2	0	0	0	0
Total	302	74	169	133	361	85	177	184

Table 3. Results from Phylogenetic tests P-Test and LibShuff for Forest floor and surface soil actinobacterial clone libraries. P-values in parentheses are bonferoni corrected. LibShuff $p < 0.025$ is significant.

Environment Comparison		P-Test	LibShuff	
			<u>XY</u>	<u>YX</u>
<u>Forest Floor</u>	Ambient vs NO3-	0.001	0.433	0.001
	Alberta Ambient vs NO3-	0.01 (0.06)	0.575	0.002
	Oceana Ambient vs NO3-	0.01 (0.06)	0.225	0.011
<u>Surface Soil</u>	Ambient vs NO3-	0.07	0.002	0.025
	Alberta Ambient vs NO3-	0.05 (0.40)	0.011	0.012
	Oceana Ambient vs NO3-	0.48 (1.0)	0.007	0.003

Table 4. Actinobacteria community structure in the Ambient treatment of Sites A and D for forest floor and surface soil based on the number of clone representatives found. Family (> 90% bootstrap support) *Sub-order (75-90% bootstrap support, or 94% Max Id with Type Strain)**Order (Acidimicrobiales determined by 60-90% bootstrap support) All other families belong to order Actinomycetales

Family	Forest Floor		Surface Soil	
	Site A	Site D	Site A	Site D
<i>Acidimicrobiales**</i>	3	3	11	14
<i>Catenulisporaceae</i>	0	0	2	4
<i>Cellulomonadaceae</i>	1	0	0	1
<i>Frankineae*</i>	12	10	2	3
<i>Microbacteriaceae</i>	36	44	3	0
<i>Micromonosporaceae</i>	13	11	8	5
<i>Mycobacteriaceae</i>	4	11	16	12
<i>Propionibacterineae*</i>	7	5	1	0
<i>Pseudonocardiaceae*</i>	3	1	8	4
<i>Streptomycetaceae</i>	1	1	1	2
<i>Thermomonosporaceae</i>	0	1	42	38

FIGURES

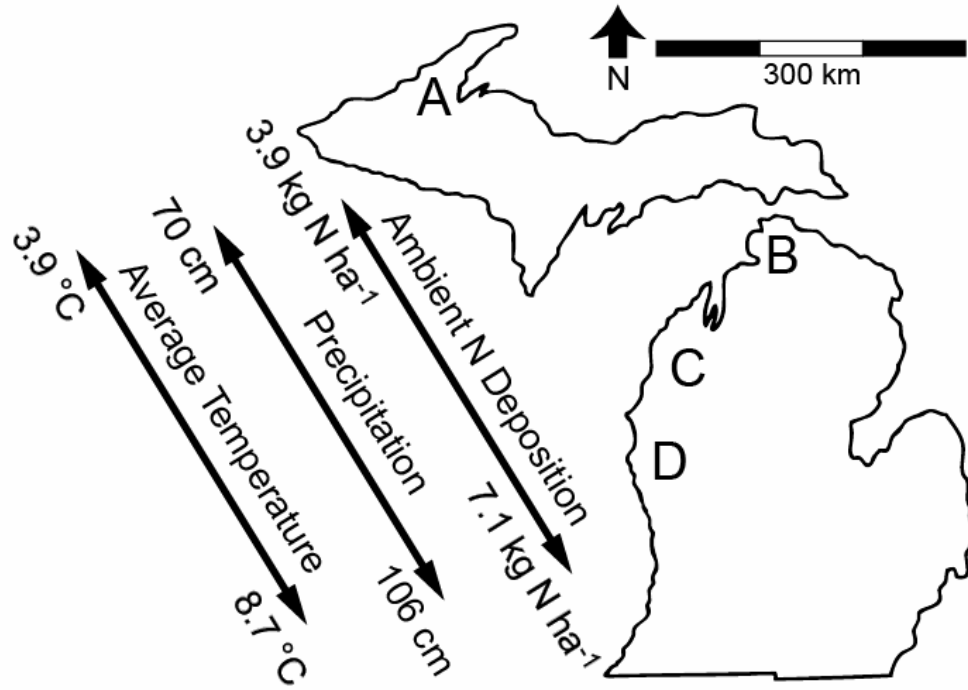


Figure 1. Approximate location and climactic information for Michigan gradient field sites, denoted sites A through D.

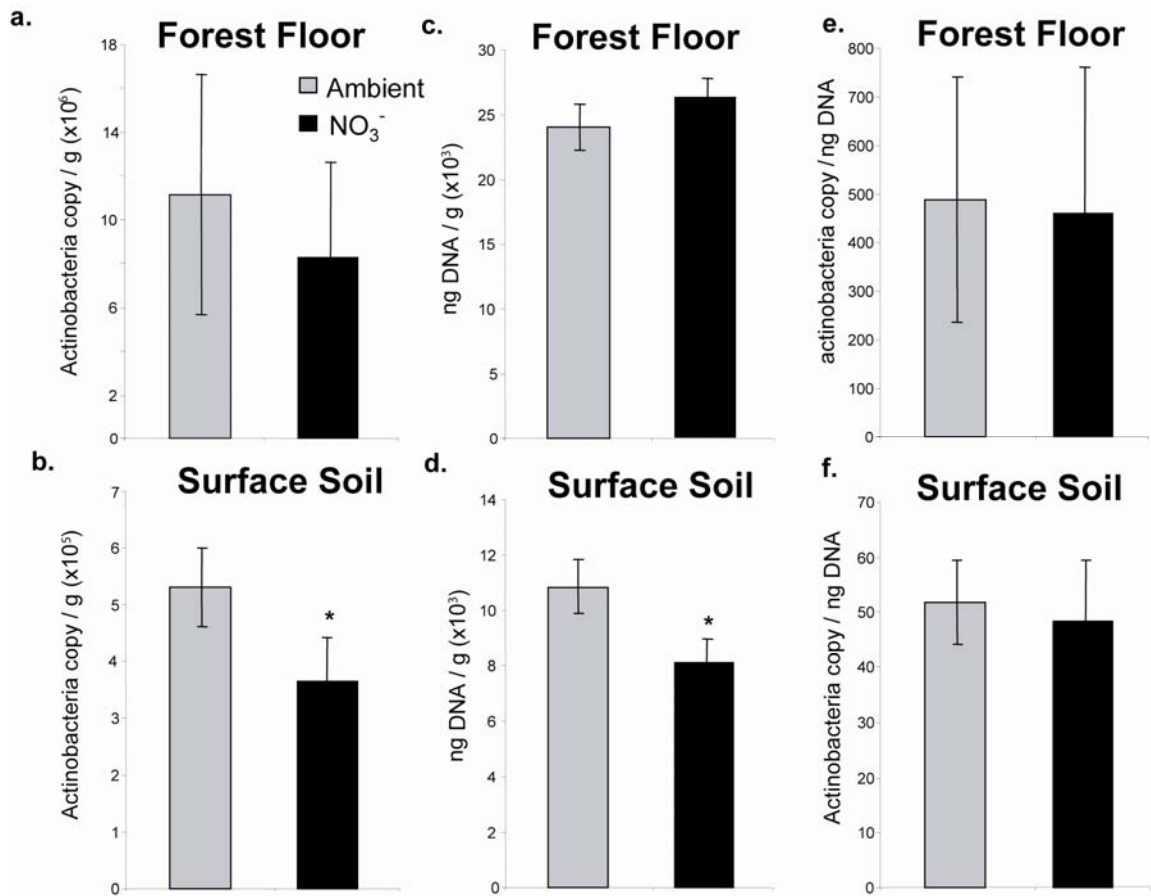


Figure 2. (a) Total actinobacteria copy number is 25.6% less in elevated NO₃⁻ treated forest floor than ambient. (b) Treatment had a significantly ($p=0.0025$) negative effect on surface soil actinobacteria copy number. (c) Total extractable DNA in forest floor was 5.6% greater under NO₃⁻ deposition. (d) Nitrogen deposition significantly ($p=0.029$) decreased total extractable DNA in the surface soil. (e) Slight decrease in relative actinobacterial abundance in forest floor due to Treatment. (f) Actinobacteria relative abundance is 5.7% lower under treatment in surface soil.

Forest Floor

(Without *Microbacteriaceae*)

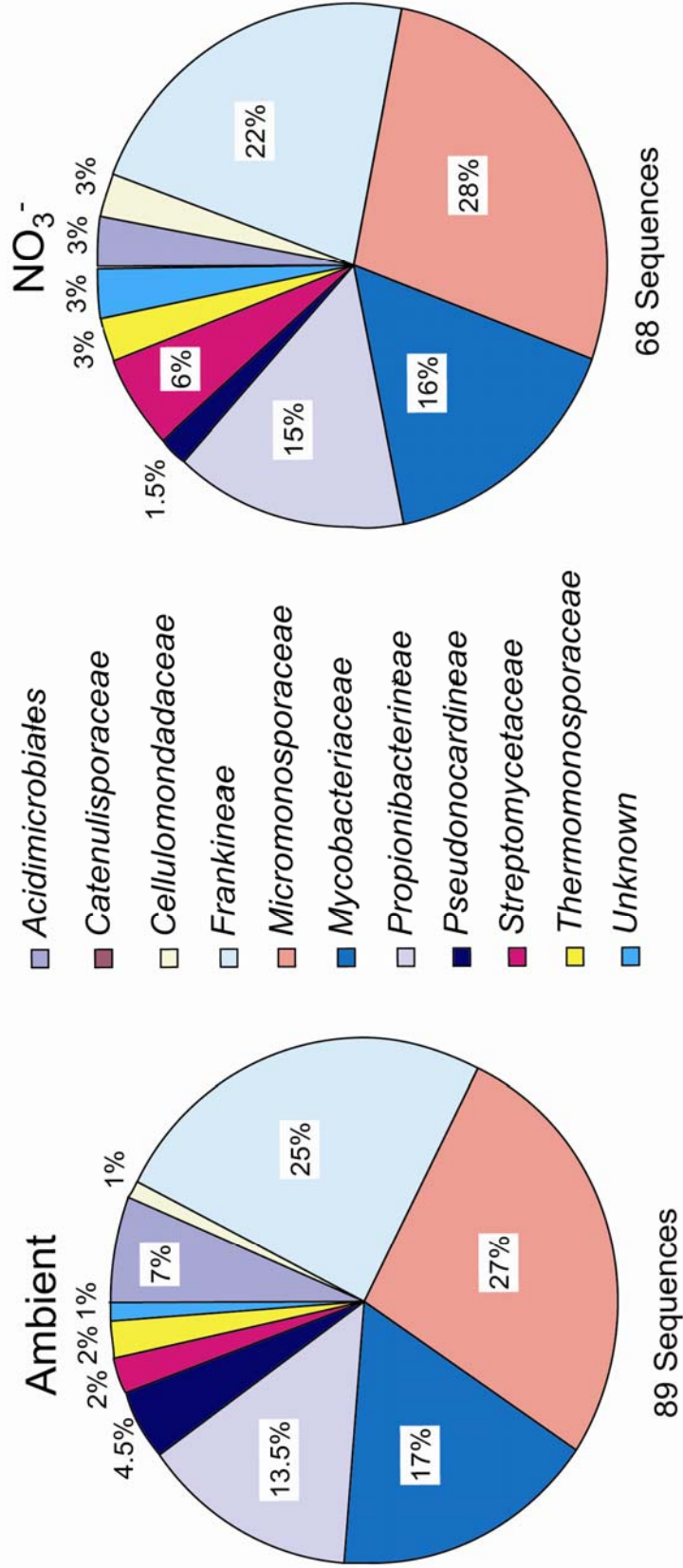


Figure 5. Forest floor actinobacterial community structure without dominant family *Microbacteriaceae* – which comprises 49% of the Ambient and NO₃⁻ treatment communities. Changes in community composition are subtle but significant based on phylogenetic inference.

Surface Soil

(Without *Thermomonosporaceae*)

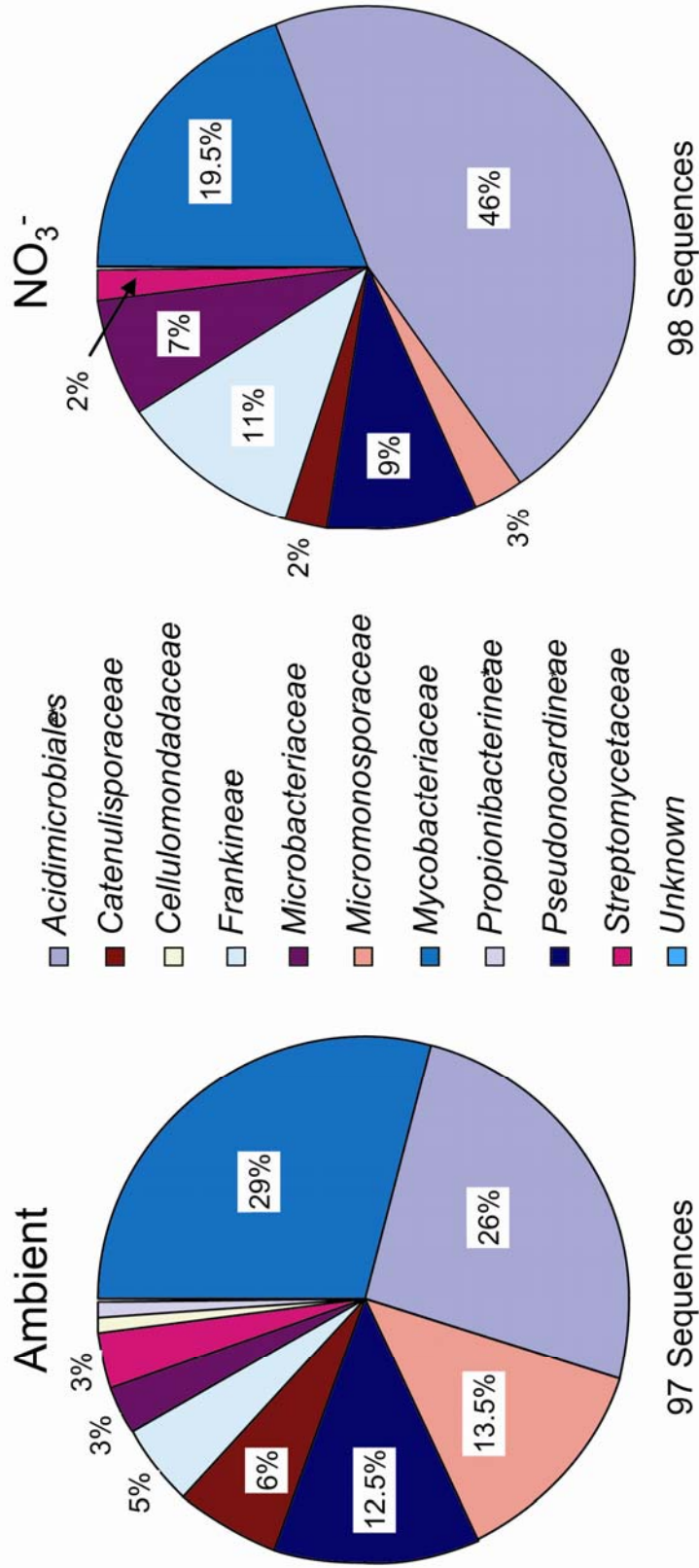


Figure 6. Surface soil actinobacterial community structure without dominant family *Thermomonosporaceae* – which comprises 46% of the Ambient and NO_3^- treatment communities. The nitrogen amended community has significantly different community membership and structure in comparison to the ambient community.

REFERENCES CITED

- Aber, J.D., C.L. Goodale, S.V. Ollinger, M.L. Smith, A. H. Magill, M.E. Martin, R.A. Hallett and J.L. Stoddard. 2003. Is nitrogen deposition altering the nitrogen status of northeastern forests? *BioScience*. **35**(4):375-389.
- Baldrian, P. 2006. Fungal laccases – occurrence and properties. *FEMS Microbial Review* **30**:215-242.
- Berrocal, M.M., J. Rodriguez, A.S. Ball, M.I. Perez-Leblic, and M.E. Arias. 1997. Solubilisation and mineralization of ¹⁴C lignocellulose from wheat straw by *Streptomyces cyaneus* CECT 3335 during growth in solid-state fermentation. *Applied Microbiological Biotechnology* **48**:379-384.
- Berg, B. 2000. Litter decomposition and organic matter turnover in northern forest soils. *Forest Ecology and Management*. **133**:13-22.
- Blackwood, C.B., A. Oaks, and J.S. Buyer. 2005. Phylum- and class-specific PCR primers for general microbial community analysis. *Applied and Environmental Microbiology*. **71**(10):6193-6198.
- Bouwman, A.F., D.P. Van Vuuren, R.G. Derwent, and M. Posch. 2002. A global analysis of acidification and eutrophication of terrestrial ecosystems. *Water, Air and Soil Pollution*. **141**:349-382.
- Braak and Smilauer, 2006. *Biometris – plant research international*, Wageningen, the Netherlands.
- Bredeiner M., K. Blanck, Y. J. Xu, A. Tietema, A.W. Boxman, B. Emmett, F. Moldan, P. Gundersen, P. Schleppi, and R.F. Wright. 1998. Input-output budgets and the NITREX sites. *Forest Ecology and Management*. **101**:57-64.

- Burton, A.J., J.S.Pregitzer, J.N. Crawford, G.P. Zogg, and D.R. Zak. 2004. Simulated chronic NO₃⁻ deposition reduces soil respiration in northern hardwood forests. *Global Change Biology*. **10**:1080-1091.
- Carreiro, M.M., R.L. Sinsabaugh, D.A. Repert, and D.F. Parkhurst. 2000. Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. *Ecology*. **81**(9):2359-2365.
- Cole, J. R., B. Chai, R. J. Farris, Q. Wang, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, A. M. Bandela, E. Cardenas, G. M. Garrity, and J. M. Tiedje. 2007. The ribosomal database project (RDP-II): introducing *myRDP* space and quality controlled public data. *Nucleic Acids Res.* 35 (Database issue): D169-D172; doi: 10.1093/nar/gkl889
- DeForest, J.L., D.R. Zak, K.S. Pregitzer, and A.J. Burton. 2004. Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forests. *Soil Biology & Biochemistry* **36**:965-971.
- DeForest, J.L., D.R. Zak, K.S. Pregitzer and A.J. Burton. 2005. Atmospheric Nitrate Deposition and Enhances Dissolved Organic Carbon Leaching: Test of A Potential Mechanism. *Soil Science Society of America*. **69**:1233-1237.
- Dentener, F., J. Drevet, J. Lamarque, I. Bey, B. Eickhout, A. Fiore, D. Hauglustaine, L. Horowitz, M. Krol, U. Kulshrestha, M. Lawrence, C. Galy-Lacaux, S. Rast, D. Shindell, D. Stevenson, T. Van Noije, C. Atherton, N. Bell, D. Bergman, T. Butler, J. Cofala, B. Collins, R. Doherty, K. Ellingsen, J. Galloway, M. Gauss, V. Montanaro, J. Muller, G. Pitari, J. Rodriguez, M. Sanderson, F. Solmon, S. Strahan, M. Schultz, K. Sudo, S. Szopa, and O. Wild. 2006. Nitrogen and sulfur

- deposition on regional and global scales: A multimodel evaluation. *Global Biogeochemical Cycles*. **20**: GB4003, doi:10.1029/2005GB002672.
- Drummond AJ, Ashton B, Cheung M, Heled J, Kearse M, Moir R, Stones-Havas S, Thierer T, Wilson A. 2007. Geneious v3.5, Available from <http://www.geneious.com/>. 2008.
- D'souza, T.M., K. Boominathan, and C.A. Reedy. 1996. Isolation of laccase gene-specific sequences from white rot and brown rot fungi by PCR. *Applied and Environmental Microbiology*. **62**:3739-3744.
- Dyszynski, G. and Sheldon, W.M. RDPquery: 2006. A Java program from the Sapelo program Microbial Observatory for automatic classification of bacterial 16S rRNA sequences based on Ribosomal Database Project taxonomy and Smith-Waterman alignment. (http://simo.marsci.uga.edu/public_db/rdp_query.htm, [2.7])
- Eccleston, G., P. Brooks, D. Kurtboke. 2008. The occurrence of bioactive micromonosporae in aquatic habitats of the sunshine coast in Australia. *Marine Drugs*. **6**:243-261
- Endo, K., Y. Hayashi, T. Hibi, K. Hosono, T. Beppu and K. Ueda. 2003. Enzymological characterization of EpoA, a laccase-like phenol oxidase produced by *Streptomyces griseus*. *Biochemistry*. **133**:671-677.
- Felsenstein, J. 2004. PHYLIP (Phylogeny Inference Package) Version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.
- Felske, A., H. Rheims, A. Wolterink, E. Stackerbrandt, A. Akkermans. 1997. Ribosome

- analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology*. **143**: 2983-2989.
- Frey, S.D., M. Knorr, J.L. Parrent, R.T. Simpson. 2004. Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. *Forest Ecology and Management*. **196**: 159-171.
- Galloway, J.N., J.D. Aber, J.W. Erisman, S.P. Seitzinger, R.W. Howarth, E.B. Cowling, and B.J. Cosby. 2004. The nitrogen cascade. *BioScience*. **53**(4):341-356.
- Galloway, J., A. Townsend, J. Erisman, M. Bekunda, Z. Cai, J. Freney, L. Martinelli, S. Seitzinger, M. Sutton. 2008. Transformation of the nitrogen cycle: recent trends, questions and potential solutions. *Science*. **320**: 889-892.
- Giroux, H., P. Vidal, J. Bouchard, and F. Lammy. 1998. Degradation of kraft indulin lignin by *Streptomyces viridosporus* and *Streptomyces badius*. *Applied Environmental Microbiology*. **54**:3064-3070.
- Godden, B., A.S. Ball, P. Helvenstein, A.J. McCarthy, and M.J. Penninckx. 1992. Towards elucidation of the lignin degradation pathway in actinobacteria. *Journal of General Microbiology*. **138**:2441-2448.
- Goodfellow, M. and S. Williams. 1983. Ecology of actinomycetes. *Annual Review of Microbiology*. **37**: 189-216
- Gubber, N. and N. Galloway. 2008. An Earth-system perspective of the global nitrogen cycle. *Nature*. **451**:293-296
- Henriksen, James R. 2004. webLIBSHUFF (<http://libshuff.mib.uga.edu>) 2008
- Hughes, J., J. Hellmann, T. Ricketts, and B. Bohannan. 2001. Counting the uncountable:

- statistical approaches to estimating microbial diversity. *Applied and Environmental Microbiology*. **67**(10):4399-4406.
- Hullo, M.F., I. Moszer, A. Danchin, and I.Martin-Verstraete. 2001. CotA of *Bacillus subtilis* is a copper-dependent laccase. *Journal of Bacteriology*. **138**:5426-5430.
- Kirk, T.K. and R.L. Farrell. 1987. Enzymatic "combustion": the microbial degradation of lignin. *Annual Review of Microbiology*. **41**:465-501.
- Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J. and Higgins D.G. 2007. ClustalW. (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>)
2008
- Li, D., M. Alic, and M.H. Gold. 1994. Nitrogen regulation of lignin peroxidase gene transcription. *Applied and Environmental Microbiology*. **60**:3447-3449.
- Liers, C., R. Ullrich, K.T. Steffen, A. Hatakka, and M. Hofrichter. 2006. Mineralization of ¹⁴C-labelled synthetic lignin and extracellular enzyme activities of the wood-colonizing ascomycetes *Xylaria hypoxylon* and *Xylaria polymorpha*. *Appl Microbial Biotechnology*. **69**:573-579.
- Lozupone, Hamady & Knight, "UniFrac - an online tool for comparing microbial community diversity in a phylogenetic context.", *BMC Bioinformatics* 2006, 7:371
- Lozupone, C. and R. Knight. 2008. Species divergence and the measurement of microbial diversity. *FEMS Microbiology Review*. (2008)1-22.
- Lozupone, C. and R. Knight. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*.

71(12):8228-8235.

- MacDonald, N.W., D.R. Zak and K.S. Pregitzer. 1995. Temperature effects on kinetics of microbial respiration and net nitrogen and sulfur mineralization. *Soil Science American Journal*. **59**:233-240.
- Magill, A.H. and J.D. Aber. 2000. Dissolved organic carbon and nitrogen relationships in forest litter as affected by nitrogen deposition. *Soil Biology & Biochemistry*. **32**:603-613.
- Martin, A.P. 2002. Phylogenetic approaches for describing and comparing the diversity of microbial communities. *Applied and Environmental Microbiology*. **68**(8):3673-3682.
- Mason, J., M. Richards, W. Zimmermann, P. Broda. 1988. Identification of extracellular proteins from actinomycetes responsible for the solubilisation of lignocellulose. *Applied Microbiology and Biotechnology* **28**:276-280.
- Melillo, J.M., J.D. Aber, A.E. Linkins, A. Rica, B. Fry, and K.J. Nadelhoffer. 1989. Carbon and nitrogen dynamics along the decay continuum: plant litter to soil organic matter. *Plant and Soil*. **115**:189-198
- McCarthy, A.J. 1987. Lignocellulose-degrading actinobacteria. *FEMS Microbiology Review* **46**:145-163.
- McDowell, W.H., A.H. Magill, J.A. Aitkenhead-Peterson, J.D. Aber, J.L. Merriam, and S.S. Kushal. 2004. Effects of chronic nitrogen amendment on dissolved organic matter and inorganic nitrogen in soil solution. *Forest Ecology and Management* **196**:29-41
- Nadelhoffer, K., B. Emmett, P. Gundersen, O. Kjonaas, C. Koopmans, P. Schlegli, A.

- Tietema, R. Wright. 1999. Nitrogen deposition makes a minor contribution to carbon sequestration in temperate forests. *Nature*. **398**:145-148
- Nicholas, K.B., Nicholas H.B. Jr., and Deerfield, D.W. II. 1997. GeneDoc: Analysis and visualization of genetic Vvariation, *EMBNEW.NEWS* 4:14.
<http://psc.edu/biomed/genedoc>
- Paul E.A. and F.E. Clark, 1996. *Soil microbiology and biochemistry*. Second Edition. Academic Press, Inc. San Diego, CA. Chapter 6. 130-152.
- Pregitzer, K.S., A.J. Burton, D.R. Zak, and A.F. Talhelm. 2007. Simulated cChronic nitrogen depositioniIncreases carbon storage in northern temperate forests. *Global Change Biology*. **14**:142-153.
- Pregitzer, K.S., D.R. Zak, A.J. Burton, J.A. Ashby, and N.W. MacDonald. 2004. Chronic nitrate additions dramtically increase the export of carbon and nitrogen from Northern Hardwood Ecosystems. *Biogeochemistry*. **68**:179-197.
- Robson, L. and G. Chambliss. 1989. Cellulases of bacterial origin. *Enzyme and Microbial Technology*. **11**(10): 626-644
- Rodriguez, A., V. Regaldo, F. Perestelo, A. Carnicero, G. De La Fuente, and M. A. Falcon. 1997. Lignin degradation and modification by the soil-inhabiting fungus *Fusarium proliferatum*. *Applied and Environmental Microbiology*. **63**(9):3716-3718.
- Schloss, P.D. 2008. Evaluating different approaches that test weather microbial communities have the same structure. *ISME Journal*. **2**:265-785.
- Schloss, P.D. and Handelsman, J. 2005. Introducing DOTUR, a computer program for

- defining operational taxonomic units and estimating species richness. *Applied and Environmental Microbiology*. **71**(3):1501-1506. Accessible at www.plantpath.wisc.edu/fac/joh/dotur.html
- Sherpherd, V.A., D.A. Orlobich and A.E. Ashford. 1993. A dynamic continuum of pleiomorphic tubules and vacuoles in growing hyphae of a fungus. *Journal of Cell Science*. **104**:495-507.
- Sinsabaugh, R.L., M.E. Gallo, C. Lauber, M.P. Waldrop and D.R. Zak. 2005. Extracellular enzyme activities and soil organic matter dynamics for northern Hardwood Forests Receiving simulated nitrogen deposition. *Biogeochemistry*. **75**:201-215.
- Smemo, K., D.R. Zak, and K. S. Pregitzer. 2006. Chronic experimental NO₃⁻ deposition reduces the retention of leaf litter DOC in a norther hardwood forest soil. *Soil Biology and Biogeochemistry*. **38**: 1340-1347.
- Tamura K., J.Dudly, M. Nei, and S. Kumar. 2007. *MEGA4*: Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*. **24**:1696-1599.
- Vidal, P.F., J. Bouchard, R.P.Overend, E. Chornet, H. Giroux, and F. Lamy. 1989. Bacterial degradation of kraft lignin: production and characterization of water soluble intermediates derived from *Streptomyces badius* and *Streptomyces vidiospourus*. *Plant cell polymers: biogenesis and biodegradation. ACS Symposium Series*. **399**:529-543.
- Vitousek, P.M., and R.W. Harwoth. 1992. Nitrogen limitation on land and in the sea: How can it occur? *Biogeochemistry*. **13**(2):87-115.

Wohl D. and J. McArthur. 1998. Actinomycete flora associated with submersed freshwater macrophytes. *FEMS Microbial Ecology*. **26**:135-140

Zak, D.R., W.E. Holmes, A.J. Burton, K.S. Pregitzer and A.F. Talhelm. 2008. Atmospheric NO_3^- deposition increases soil organic matter by slowing decomposition in a northern hardwood ecosystem. *Ecological Applications* *in press*.

Zimmerman, W. 1989. Degradation of lignin by bacteria - Minireview. *Journal of Biotechnology* **13**:119-130.