AEM Accepts, published online ahead of print on 7 December 2012
Appl. Environ. Microbiol. doi:10.1128/AEM.03156-12

Microbial mechanisms mediating increased soil C storage under elevated atmospheric N deposition

Sarah D. Eisenlord[1] # (seisen@umich.edu)
Zachary Freedman[1] (zacf@umich.edu)
Donald R. Zak[1,2] (drzak@umich.edu)
Kai Xue[3] (kx36@ou.edu)
Zhili He[3] (zhe@rccc.ou.edu)
Jizhong Zhou[3] (jzhou@ou.edu)

[1]School of Natural Resources and Environment, University of Michigan, Ann Arbor, MI 48109
[2]Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109
[3]Department of Botany and Microbiology, University of Oklahoma, Norman, OK 73019

Running Title:
Saprotrophic functional gene response to N deposition

Key Words:
N deposition, decomposition, C storage, *Actinobacteria*

Sarah D. Eisenlord
2560 Dana Bldg, 440 Church St
Ann Arbor, MI 48109
Phone/Fax (734) 647-5925
seisen@umich.edu

Abstract

Future rates of anthropogenic N deposition can slow the cycling and enhance the storage of C in forest ecosystems. In a northern hardwood forest ecosystem, experimental N deposition has decreased the extent of forest floor decay, leading to increased soil C storage. To better understand the microbial mechanisms mediating this response, we examined the functional genes derived from communities of Actinobacteria and fungi present in the forest floor using GeoChip 4.0, a high throughput functional gene microarray. The composition of functional genes derived from actinobacterial and fungal communities was significantly altered by experimental nitrogen deposition, with more heterogeneity detected in both groups. Experimental N deposition significantly decreased the richness and diversity of genes involved in the depolymerization of starch (~12%), hemicellulose (~16%), cellulose (~16%), chitin (~15%), and lignin (~16%). The decrease in richness occurred across all taxonomic groupings detected by the microarray. The composition of genes encoding oxidoreductases, which plausibly mediate lignin decay, were responsible for much of the observed dissimilarity between actinobacterial communities under ambient and experimental N deposition. This shift in composition and decrease in richness and diversity of genes encoding enzymes which mediate the decay process has occurred in parallel with a reduction in the extent of decay and accumulation of soil organic matter. Our observations indicate that compositional changes in actinobacterial and fungal communities, elicited by experimental N deposition, have functional implications for the cycling and storage of carbon in forest ecosystems.

Introduction

The extent to which terrestrial ecosystems will function as a future sink for anthropogenic CO_2 in the atmosphere will be modified by current and future rates of anthropogenic nitrogen (N) deposition from the atmosphere (1). In addition to increasing ecosystem carbon (C) storage by fostering more rapid rates of net primary production (NPP), anthropogenic N deposition may reduce the decomposition of plant detritus, thereby increasing the storage of C in long-lived soil organic matter. This response has been documented in a wide range of ecosystems (2), but such a response is not universal. We have studied the impact of elevated atmospheric N deposition on forest C cycling in a long-term replicated field experiment in Michigan, USA (Figure 1, Table S1). We have evidenced that experimental N deposition, at a rate expected by mid-century, has decreased litter decay and increased soil C storage (3). Although experimental N deposition has increased NPP (+10%), it has not altered the amount of leaf or fine root litter entering the forest floor (Table 1) (4). Meanwhile, leaf litter N concentration has increased by 25% and inorganic N concentrations in the forest floor have increased by 288% (3, 5). With the use of field observations and a biogeochemical simulation model, we further have determined that chronic N deposition has decreased the extent of microbial decay, thereby leading to an accumulation of soil organic matter (6). In combination, these observations indicate that a microbial mechanism underlies a decline in litter decay under experimental N deposition, which has enhanced soil C storage (+10%) (3, 7).

Saprotrophic basidiomycete and ascomycete fungi are the primary agents of litter decay, especially during the later stages dominated by degrading plant cell wall polymers (8).

These fungi facilitate the rate-limiting step of litter decay in which cell-wall lignin is depolymerized. Laboratory studies have found that increased concentrations of inorganic N can suppress the transcription of fungal genes required for the metabolism of lignin and lignocellulose (9). In our long-term field study, experimental N deposition has also decreased the transcription of fungal genes encoding ligninolytic enzymes, and induced a shift in the composition of basidiomycete fungi (10). Some Actinobacteria may also be able to solubilize and modify lignin and lignocellulose; however, not as completely as basidiomycete fungi which mineralize lignin to CO_2 (11-14). Rather, the end-products of actinobacterial lignin metabolism are dissolved organic compounds and soil organic matter. In our study, experimental N deposition has also altered the composition of Actinobacteria, which occurred in concert with reduced decay and increased production of phenolic dissolved organic matter (15).

Here, we focused on functional genes from the Dikarya fungi (basidiomycetes and ascomycetes) as well as Actinobacteria involved in plant and fungal cell-wall decay to help elucidate the potential microbial mechanisms mediating reduced decay under experimental N deposition. We quantified the abundance, diversity, and composition of genes involved in degrading four components of plant detritus: starch, hemicellulose, cellulose, and lignin; we also quantified genes encoding enzymes that metabolize chitin, a major component of fungal cell wall. Previously, we have explored the extent to which experimental N deposition has altered the cellulolytic and lignolytic capability of basidiomycete and ascomycete fungi (3, 10, 16); here, we examine a broader suite of Dikarya fungal and Actinobacterial genes mediating the decay of detritus to better understand how this agent of global change has reduced the extent of plant litter decay under experimental N deposition. We hypothesized experimental N

deposition has altered the functional capability, and therefore diminished the gene content, of forest floor microbial communities. To test our hypothesis, we used GeoChip 4.0 (17), a closed-format metagenomic approach to compare communities of Actinobacteria and fungi growing under ambient and experimental N deposition.

Methods

Study Sites

The impact of chronic atmospheric N deposition on northern hardwood forest ecosystems has been documented in a long-term experiment spanning the upper and lower peninsulas of Michigan (18). Four replicate sugar maple (*Acer saccharum*) forest stands similar in age, plant community composition, and edaphic properties, span a 500-km climactic and ambient N deposition gradient (Figure 1, Table S1). There are six 30-m X 30-m plots within each site; three plots receive ambient N deposition, and three have received an additional 3 g NO_3^--N m^{-2} y^{-1} of NO_3^- over the growing season. These treatments were initiated in 1994 and have been maintained to the present.

Sample Collection and DNA extraction

Forest floor samples were collected in early October 2009; samples from all four sites were gathered over a 4-day period. A 10-cm X 10-cm frame was randomly placed at 10 locations within each experimental plot. From the center of the frame, the freshly fallen intact Oi horizon was removed by hand, and the partially decomposed Oe/Oa horizon was collected down to the mineral soil. Material from all 10 random locations within each plot was composited and homogenized in the field with sterilized scissors; a 5-g sub-sample was

removed and frozen in liquid N_2. Samples were then stored in -80 °C prior to DNA extraction. DNA was extracted from approximately 2.5 g of forest floor using the MoBio Power Soil DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA, USA) following manufacturer instruction. Total microbial DNA was quantified with Quant-iT PicoGreen (Invitrogen) per manufacturer instruction.

Target preparation and GeoChip hybridization

GeoChip 4.0 was used to quantify a broad suite of key fungal and actinobacterial functional genes encoding enzymes that disassemble plant and fungal cell walls; hybridization of DNA and pre-processing of data is described by Lu *et al*., (17). Briefly, 1 µg of genomic DNA from each plot was purified by the Genomic DNA Clean & Concentrator kit (Zymo Research, Irvine, CA, USA) and labeled with fluorescent dye Cy-3 using random primers (19). The labeled gDNA was dried and rehydrated with 2.7 µl of a sample tracking control, followed by incubation at 50 °C for 5 min. This DNA solution was then mixed with 7.3 µl of hybridization buffer containing a universal standard DNA labeled with fluorescent dye Cy-5; it was denatured at 95 °C for 5 min, and maintained at 42 °C until DNA was loaded onto GeoChip 4.0 microarrays (NimbleGen, Madison, WI, USA). The hybridization was performed on a Hybridization Station (MAUI, Roche, CA, USA) at 42 °C for 16 h with agitation. After washings, the arrays were scanned using a MS 200 Microarray Scanner (NimbleGen) at laser power of 100% PMT (photomultiplier tube). Each biological replicate was run once on the micro-array.

GeoChip data pre-processing

The signal intensities of GeoChip hybridization spots were normalized by the Cy-5 labeled universal standard DNA across samples and by dividing the signal intensity of each spot by the average intensity of all positive spots within each sample. After normalization, unreliable spots were removed, if their original signal intensities were below the noise level (<2000), signal-to-noise ratio (SNR) was ≤ 2.0, or the coefficient of variation of the background was > 0.8 (17). All signal intensities were log-normal transformed. Details of further normalization steps can be found in the supplemental material of Lu *et al.,* (17).

Statistical Analysis

To examine the effect of experimental N deposition on functional gene composition, a subset of normalized GeoChip 4.0 signal intensity data was analyzed for diversity, functional richness, and composition. The set of genes considered encode enzymes involved in the depolymerization of the biochemical components of plant and fungal litter: starch, hemicellulose, cellulose, chitin and lignin. We chose specifically to analyze all variants of these genes belonging to the Actinobacteria and Dikarya fungi (herafter refered to as fungi). A full list of genes, the substrate category in which they belong as designated by He *et al.,* (20), and the number of their variants (hybridization spots on the microarray) included in these analyses can be found in the supplemental materials of this manuscript (Table S2). Actinobacterial and fungal data was analyzed separately.

The diversity of actinobacterial and fungal functional genes in each sample was calculated with the Shannon diversity index in PRIMER v6 (21) using the normalized signal

intensity of all relevant gene variants. The total functional richness of each sample was calculated as the sum of the presence-absence data of all gene variants across all genes considered *sensu* (22). We calculated the functional richness of the genes in each substrate category (e.g., lignin) by summing the presence of all gene variants for genes which code for enzymes acting upon that substrate. Differences in diversity and functional richness due to experimental N deposition and site were examined with two-way ANOVAs. If an interaction effect was present, it was further elucidated with the protected Fisher's Least Significant Difference (LSD) test. Univariate statistics were conducted in SPSS (IBM Statistics Version 20, IBM Corp., Armonk, NY, USA).

To assess the response of actinobacterial and fungal taxonomic groups (class, order, family) to experimental N deposition, we re-organized the data to sum the signal intensity for all genes within each taxonomic group under ambient and experimental N deposition. The response ratio of each taxonomic group was then calculated by taking the natural log of the ratio of the mean summed signal intensity of experimental N deposition samples to the mean summed signal intensity of ambient N deposition samples *sensu* (23, 24). We then calculated the variance and 90, 95, and 99% confidence intervals (CIs) for each response ratio to assess the significance of the response ratios (24).

Multivariate statistics were conducted in PRIMVER v6 (21). Similarity matrices for the Actinobacteria and Dikarya fungi were calculated with the Bray-Curtis similarity metric on presence-absence transformed data for all relevant gene variants. Principal coordinate analyses (PCOs) were created to visualize the differences in composition between ambient and elevated

N deposition functional gene assemblages. The correlation of each gene to the primary PCO axis was then calculated with the vector overlay function in PRIMER, averaging the Pearson correlation metric of all gene variants showing a correlation to the first PCO axis. We used the multivariate community similarity analyses ANOSIM (25), PERMANOVA (26), and PERMDISP (27) to analyze whether experimental N deposition induced compositional dissimilarities in functional gene variants present in the forest floor. To examine which genes contributed most to the difference in the composition of functional gene assemblages, we used the SIMPER tool in PRIMER v6 to calculate the contribution of each gene to the average Bray-Curtis dissimilarity between treatments. All genes were given an equal weight for this analysis, to account for the bias inherent in having a wide range of gene variants for each gene on the array.

Results

We examined 20 actinobacterial functional genes and 26 ascomycete and basidiomycete functional genes encoding for enzymes involved in degrading starch, hemicellulose, cellulose, chitin and lignin, all common biochemical constituents of plant and fungal detritus. Experimental N deposition consistently and significantly altered the composition of actinobacterial and fungal genes mediating plant and fungal cell wall depolymerization, as we explain below.

Diversity and Richness

The 20 actinobacterial genes of interest were represented by 635 gene variants (probes on the GeoChip) from 25 actinobacterial families on GeoChip 4.0 (Table S2). The mean Shannon diversity index of these actinobacterial genes declined under experimental N deposition (H =

6.01), relative to ambient N deposition (H = 6.51; *P* = 0.035). Additionally functional richness also declined under experimental N deposition (richness = 393), relative to ambient N deposition (richness = 440; *P* = 0.026). There were significant site differences for both actinobacterial diversity (*P* = 0.013) and functional richness (*P* = 0.003). The overall decrease in actinobacterial diversity and richness was driven by the 25-35% decrease in gene variants detected in the northern two sites A and B (Fisher's protected LSD *P* < 0.035), and no response in the southern sites C and D (Figure 2a,c). This lead to an interaction between site and treatment on diversity (*P* = 0.08) and richness (*P* = 0.043) as reported by a two-way ANOVA.

Actinobacterial functional genes encoding enzymes involved in degrading starch, hemicellulose, chitin and lignin decreased in richness under experimental N deposition in sites A and B, but not C and D (Figure 3a). Genes encoding enzymes for the breakdown of cellulose did not respond to the treatment. There was a disproportionately greater decrease in the richness of actinobacterial genes involved in chitin degradation, when compared to the other substrates (*P* = 0.037).

Twenty six fungal functional genes involved in the depolymerization of the biochemical components of plant and fungal cell wall were also measured on GeoChip. There were 1294 variants of these genes from 56 fungal families within the Dikarya represented on the microarray. The fungal functional genes responded in a nearly identical manner to experimental N deposition as did the Actinobacteria. Shannon's diversity of fungal functional genes declined significantly under experimental N deposition (H = 6 .71), relative to the ambient treatment (H = 6.52; *P* = 0.017); and the functional richness declined from 824 to 723 under ambient and

experimental N deposition, respectively (P = 0.018). There was a significant site effect for diversity and richness (P < 0.001). The overall decrease in diversity and richness was again driven by a ~30% decrease in the gene variants detected in the northern two sites (P < 0.001); there was no change diversity or richness in the southern sites C and D (Figure 2). The interaction effect was significant for both diversity (P = 0.032) and functional richness (P = 0.014). Different from the Actinobacteria, the decrease in richness of fungal functional genes equally occurred in all five C substrates (-14%). The decrease significantly occurred in sites A and B (P < 0.001), but not in C or D (Figure 3b).

Response Ratio of Taxonomic Groups

The response ratio (RR) of actinobacterial families to experimental N deposition was consistently negative (Figure 4a). A negative response ratio indicates a decrease in gene abundance under experimental N deposition relative to the set of genes exposed to ambient N deposition, whereas a positive response ratio would indicate the opposite. The *Corynebacteriaceae, Bifidobacteriaceae, Catenulisporaceae, Micrococcaceae, Jonesiaceae* and *Actinosymnemataceae* all significantly decreased under experimental N deposition (Figure 4a). The *Beutenbergaceae, Cellulomondaceae* and *Promicromonosporaceae* families have slightly positive response ratios (RR < 0.15); however with the standard error considered these values cannot be distinguished from a null response.

The response ratio of fungal ascomycete and basidiomycete groups to experimental N deposition was also mostly negative (Figure 4b), with the exception of members of the Ustilaginomycetes which responded positively (RR = 0.15), although not significantly. Members

of the Cantharellales, Auriculariales, Botryoosphaeriles and Microscales all had a significant negative response to experimental N deposition, where the 90%, 95% or 99% CIs did not cross zero.

Multivariate Analysis

Compositional shifts in actinobacterial functional gene assemblages were visualized using principal coordinate analyses (PCOs; Fig. 5a). The first PCO axes accounted for 55.1% of the total variation and the second PCO axis accounted for 15.3%. Gene assemblages under ambient and experimental N deposition in sites A and B separated on the first PCO axis. Experimental N deposition gene assemblages in sites C and D shifted in a slightly in the opposite direction along PCO axis one. The genes for phenol oxidase, glyoxal oxidase (*glx),* and endoglucanase had the strongest correlations to the first PCO axis with Pearson's r correlation values of -0.68, -0.56 and -0.65, respectively. All other genes had correlation values between -0.18 and -0.46 (Table S3). Functional genes exposed to experimental N deposition were more dispersed in multivariate space than those occurring under ambient N depositions (Figure 5a); the dispersion effect of experimental N was confirmed with a significant PERMDISP ($P = 0.004$).

ANOSIM (Analysis of Similarity) revealed there were significant differences between actinobacterial gene assemblages under ambient and experimental N deposition (R = 0.658; $P = 0.01$). Large R values (close to 1) indicate complete separation of the between sets of genes, whereas small R values (close to 0) imply completely random grouping. The assemblages of functional genes within each site were distinct; regardless of treatment, actinobacterial genes within a site were more similar to themselves than those in other sites (R = 0.613, $P = 0.01$).

The differences in the composition of functional genes were further elucidated with PERMANOVA, the multivariate adaptation of ANOVA. There were significant treatment (P = 0.022), site (P = 0.001) and interaction effects (P = 0.005) for actinobacterial functional genes. Pairwise comparisons with Monte Carlo simulations revealed significant treatment effects on functional gene composition in Sites B (P = 0.036) and C (P = 0.026). The composition of functional genes at site D did not significantly differ under experimental N deposition.

Within the bounds of the incomplete coverage of the complete microbial community due to the nature of the microarray; we observed an 18% difference in the composition of ambient and experimental N deposition actinobacterial functional gene variants based on the Bray-Curtis similarity metric with the SIMPER analysis. When all genes were given equal weight, regardless of gene variant number, the composition of phenol oxidase genes, which may be involved in lignin degradation, contributed the most (10%) to compositional differences. When summed, the composition of genes possibly involved in lignin degradation contributed to 27% of the difference in these functional gene assemblages. The overall influence these oxidoreductase-encoding genes was a negative response, however, actinobacterial peroxidase contributed to 6.3% of the difference; it increased under experimental N deposition. Differences in the composition of genes encoding enzymes involved in decomposing starch contributed to 19% of dissimilarity followed by, hemicellulose (17%), cellulose (16%), and chitin (13%; Table S4).

Differences in fungal functional gene composition between ambient and experimental N deposition were visualized with a PCO (Figure 5b). The first PCO axes accounted for 56.4% of

the total variation in fungal gene assemblages and the second PCO axis accounted for 13.2%. The gene encoding exochitinase had the strongest correlation (Pearson's r = -0.61) with the first axis; the remaining genes all had correlation values between -0.33 and -0.49 (Table S3). The separation of experimental N deposition and ambient gene assemblages in sites A and B primarily occurred on POC axis one as well. The set of fungal genes under experimental N deposition had more variation between samples than the ambient gene assemblages (PERMDISP; $P = 0.001$).

Similar to the actinobacterial set of functional genes, the ANOSIM found fungal functional gene assemblages under experimental N deposition to be significantly different from those under ambient N deposition (R = 0.72; $P = 0.01$). PERMANOVA results supported this; there were significant treatment ($P = 0.013$), site ($P = 0.001$) and interaction effects ($P = 0.001$) within the fungal functional gene assemblages. Pairwise comparisons with Monte Carlo simulations revealed significant treatment effects on functional genes in Sites B ($P = 0.047$) and C ($P = 0.027$).

The composition of fungal functional gene variants under elevated N deposition was 25% different from the ambient functional genes. According to the SIMPER analysis, differences in the composition of exochitinase, which encodes a chitin depolymerizing enzyme, contributed the greatest (8%) to the difference between ambient and experimental N deposition. All other genes contributed less than 5% when considered individually. When pooled by their substrate category, genes encoding enzymes for the depolymerization hemicellulose contributed to 25%

of the difference in ambient and N deposition fungal functional gene assemblages, followed by chitin (21%), cellulose (19%), lignin (16%), and starch (11%; Table S4).

Discussion

Experimental N deposition has elicited changes in the diversity, richness, and composition of both actinobacterial and fungal functional gene assemblages involved in the decomposition plant detritus in our long-term field experiment. Specifically, experimental N deposition decreased richness and diversity of genes in the northern two sites A and B, altered the composition of functional genes in sites B and C, and increased the heterogeneity of functional gene assemblages in all sites. These changes occurred in concert with previously documented decreased extent of forest floor decay, which has increased the accumulation of organic matter in forest floor and surface mineral soil (Table 1). The work we report here supports the idea that compositional shifts in the forest floor decomposer community have functional implications for the C cycling and C storage in forest ecosystems under future rates of anthropogenic N deposition from the atmosphere.

In the northern two sites, the decreases in functional richness occurred for both actinobacterial and fungal gene assemblages under experimental N deposition, indicating there were fewer genes detected that mediate the process of plant and fungal cell wall decay. The suppression of functional diversity by enhanced levels of N has been documented in boreal forest floor fungal communities (28) and arctic soil bacterial communities (29). Several studies have further linked decreases in diversity of microbial communities to a decline in function; for example, Salonius (30) correlated a decline in the metabolic capabilities of soil bacterial

328 communities to diminished diversity; this response was not result from decreased cell counts.
329 Decreased soil saprotrophic fungal richness has also been found to lower respiration in species
330 poor communities (31).

331 In our study, decreased functional richness and diversity of saprotrophic
332 microorganisms under experimentally elevated N deposition may be related to the decreased
333 extent of forest floor decay and increased C storage in soil organic matter. As explained by
334 Hanson *et al.,* (32), the decomposition of decay-resistant biopolymers, such as lignocellulose
335 requires a more diverse assemblage of fungi than is required for other components of plant
336 litter. This is due to the complexity of the lignocellulose molecule and the multiple enzymes
337 needed for depolymerization, many of which are produced by different species and act upon
338 different parts of the molecule (33-35). Therefore, the decrease in the alpha-diversity of
339 functional genes under experimental N deposition is one plausible mechanism underlying the
340 decreased extent of decay and organic matter accumulation in our experiment.

341 The decrease in fungal functional richness occurred across all gene categories by ~ 14%
342 (Figure 3b). The response of the Actinobacteria was similar, with a widespread decrease in
343 functional genes, except there was no decrease in the richness of genes encoding enzymes
344 responsible for the decomposition of cellulose. The lack of response from cellulolytic genes is
345 not surprising in that previous studies examining the impact of experimental N deposition on
346 the later stages of forest floor decay found no effect of N on either the activity of
347 cellobiohydrolase enzymes (36) or the transcription of fungal cellobiohydrolase genes (10). It
348 has been suggested that increased N availability is associated with higher cellulolytic activities

and initial rates of mass loss (37), however; high N availability neither promotes nor suppresses the breakdown of cellulose in the later stages of leaf decay in our experiment (10).

We also observed a disproportionately large (P = 0.037) decrease in actinobacterial genes encoding for chitin degrading enzymes, when compared to the decrease in other gene categories. Actinobacteria typically produce chitin degrading enzymes to requisition the energy (e.g., C) and N in fungal cell walls (38, 39). In N-enriched ecosystems, the decomposition of chitin for N may not be as important. Furthermore the decrease in genes encoding chitin degrading enzymes is intriguing when considering competitive interactions between Actinobacteria and fungi (40). High N concentrations decreased fungal biomass (36) and competitive ability (41); this altered environment may be selecting for Actinobacteria less dependent on decomposing fungal litter. However, previous studies have not detected any response of the enzyme activity of chitinase enzyme N-acetyl-glucosaminidase to experimental N deposition (36).

Experimental N deposition not only decreased the alpha-diversity of fungal and actinobacterial functional genes, it increased the beta-diversity between the ambient and experimental N deposition forest floor functional gene assemblages. This is evidenced by significant PERDISP (P < 0.004) results; functional gene assemblages under experimental N deposition were more dispersed in multivariate space than ambient genes (Figure 5). The incomplete depolymerization of lignin under experimental N deposition may feedback to increase heterogeneity in the decomposer community as well as suppress the diversity and

function of saprotrophic microorganisms, and could likely be contribute to the variation in site to site responses to experimental N deposition.

Lignins are polyphenolic compounds with low solubility; as they are depolymerized by saprotrophic organisms, soluble polyphenols are released into the environment (42). The accumulation of soluble polyphenols can either stimulate or inhibit fungal spore germination and hyphal growth (43). Basidiomycete fungi tend to depolymerize lignin completely to CO_2; however, ascomycetes and Actinobacteria are limited in their ability to mineralize lignin and therefore incompletely degrade lignin compounds, resulting in the variable production of such soluble polyphenolics (11, 44, 45). There are many catabolic pathways which require a diverse set of enzymes for the complete depolymerization of lignin and its components (33), so it stands to argue that saprotrophic communities which differ in species composition, as our study sites do (46), may change the rate and extent of lignin decomposition in response to experimental N deposition. This would result in a different composition of soluble polyphenolics in the forest floor, and therefore different potential feedbacks on the microbial communities. To understand more about this possible mechanism regulating soil C storage, further insight into the composition of polyphenolic compounds in partially decomposed litter under experimental N deposition is necessary.

Fungal and actinobacterial functional gene assemblages responded differently to experimental N deposition at each site, yet they all had the same functional response: decreased litter decay. This disconnect between the measured physiological response of decreased litter decay and the inconsistent shift in functional gene composition between sites

could result from the dispersion effect of experimental N deposition on the sapotrophic communities. While these forest floor communities likely contain functional redundancy (47), it seems both a suppression of richness and diversity (Sites A and B), and a shift in overall community composition (Sites B and C) leads to similar functional outcomes (i.e., reduced decay and organic matter accumulation). Seasonal differences between sites at the time of sampling could further contribute to the site specific responses. All sites were sampled within four days of each other, however due to the climactic gradient on which the sample sites lie; each forest site was at a different seasonal phase. It is well established that there is strong temporal variation in forest floor microbial communities(48); the site to site differences seen in this study could be attributed to the communities being in different stages of autumnal forest floor decomposition.

This study revealed that even small changes in community composition (25% difference in fungi; 18% in Actinobacteria) could lead to important biogeochemical implications. Given the high diversity of soil microbial communities and the limited coverage of this diversity by GeoChip, we are unable to draw conclusions regarding the generality of this response in nature. Nonetheless, a link between composition and function is supported by a review linking soil microbial diversity and composition to C cycling dynamics, which concluded that changes in microbial community composition resulting from global change are more likely to be relevant to ecosystem function than changes in species richness alone (47). The driving force leading to changes in actinobacterial functional gene assemblages under experimental N deposition was the response of genes encoding oxidoreductases possibly involved in lignin degradation. Our SIMPER results revealed the composition of genes possibly related to lignin degradation

account for 27% of the difference in actinobacterial gene assemblages. Furthermore, the composition genes encoding putative phenol oxidase and glyoxal oxidase (*glx*) were two of the genes most strongly correlated with the first axis of the PCO (Figure 5a), this axis accounted for 55.1% of the variation in assemblages.

The importance of genes possibly involved in ligninolysis in structuring the actinobacterial functional community is further indicated by the significant negative response of the *Micrococcaceae* family to experimental N deposition. The *Micrococcaceae* are represented on the microarray by genes encoding muconate lactonizing enzymes which are involved in the breakdown of lignin-derived aromatics in soil. Taylor *et al.,* (49) isolated two members of the *Micrococcaceae* able to grow on minimal media containing lignocellulose and they metabolized Kraft lignin into lower molecular weight products. Even though Actinobacteria are not the primary agent of the later stages of plant litter decay, the changes in actinobacterial functional gene composition observed here, occurring concomitantly with a decreased fungal presence could be a mechanism responsible for lower decay under experimental N deposition.

Most other taxonomic groups of Actinobacteria responded negatively to experimental N deposition (Figure 4a) the *Corynebacteriaceae, Bifidobacteriaceae, Catenulisporaceae, Jonesaceae* and the *Actinosynnemataceae* all had significantly negative response ratios. The lignolytic capabilities of members of these families are either non-existent or unknown; nonetheless, the *Catenulisporaceae* and *Actinosynnemataceae* are known to have vegetative and areal mycelia and have been isolated from fallen leaves and soil (50, 51). The *Bifidobacteriaceae* are able to break down complex carbohydrates, and their genomes contain

433 genes for complex sugar metabolism; however, these enzymes are not suspected to be
434 extracellular (52).

435 The actinobacterial families which responded positively are the *Beutenbergiaceae,*
436 *Cellulomonadaceae* and *Promicromonosporaceae,* all of which belong to the suborder
437 *Micrococcineae.* The *Beutenbergiaceae* have been found in cave ecosystems and are known to
438 use glucose and cellobiose as growth substrates; 12% of their genome is dedicated to
439 carbohydrate transport and metabolism (53). The members of the *Micrococcineae* suborder
440 exist in a wide variety of ecosystems, and have found to be positively correlated with the
441 decomposition of *Microcystis* blooms in aquatic ecosystems (54), although their autecology in
442 forest floor ecosystems is not well understood.

443 The composition of fungal functional gene assemblages under experimental N
444 deposition was found to be 25% different from ambient assemblages, and the gene encoding
445 for exochitinase contributed most to the difference between ambient and experimental N
446 deposition communities. The composition of this gene also had the strongest correlation of all
447 genes to the primary PCO axis along which the functional community split. The altered
448 composition of genes encoding for the degradation of chitin could be indicative of a different
449 saprotrophic competitive ability by fungi exposed to experimental N deposition (41).

450 The functional genes from the fungal orders Auriculariales, Botryosphaeriales,
451 Cantharellales, and Microscales all significantly declined under experimental N deposition. The
452 Cantharallales and Microscales orders were primarily represented by genes encoding for
453 lignolytic enzymes on the microarray. The Cantharellales are ectomycorrhizal fungi from which

distinctive laccases have been isolated from fruiting bodies (55) these fungi are active decomposers in our study sites (unpublished). The Microscale lignolytic genes represented on the microarray are from the family *Halosphaeriaceae* of the Sordariomycetes. These are primarily aquatic fungi (56), however the negative response of these genes encoding lignolytic enzymes gives evidence for experimental N deposition inhibiting the growth of lignolytic fungi. Even though changes in gene composition and richness of genes encoding for lignolytic enzymes has not been detected as a driving force of changes in fungal gene composition, the decrease in fungal taxa known to be active lignin decomposers supports our findings of decreased fungal lignolytic activity in forest floor ecosystems (10). Similar to our findings in Michigan, experimental N deposition decreases the lignolytic capability of organisms of microorganisms across nine biomes around the world (57).

In summary, 18 years of experimental atmospheric N deposition have reduced the functional capability of sapotrophic Actinobacteria, basidiomycetes and ascomycetes in the forest floor, consistent with a decreased extent of plant litter decay. Experimental N deposition has led to smaller and less diverse sapotrophic functional gene assemblages and increased the amount of variation between them. Changes in the actinobacterial functional gene composition were driven by changes in genes encoding enzymes possibly involved in lignin degradation. This supports the notion by Bugg *et al*., (58), that the bacterial metabolism of lignin may be more important to decomposition than previously thought. The observations we report here, in combination with a documented reduction in litter decay, provides a plausible mechanism by which chronic, experimental deposition has slowed decay, increase soil C storage as well as the enhanced production of phenolic dissolved organic C.

Acknowledgments

This research was made possible by support for the National Science Foundation's Long-Term Research in Environmental Biology program and the Department of Energy's Biological and Environmental Research Division. The authors would like to thank Rima A Upchurch and Lauren Cline from Don Zak's soil ecology lab for their valuable contribution to the analysis of the data presented here, and Tong Yuan from Jizhong Zhou's lab for her work in running the microarray.

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Figure 1. Approximate locations and climactic information of four replicate sugar maple dominated northern hardwood forest stands A-D across the upper and lower peninsulas of Michigan, USA.

Figure 2. Shannon Diversity index of (a) Actinobacterial and (b) fungal functional genes of soil microbial communities at each site A-D. Functional richness, determined by the average sum of the gene variants detected in (c) Actinobacteria (d) fungi. Diversity of functional genes decreased under experimental deposition in Actinobacteria (P= 0.035) and fungi (P=0.017). Richness declined under experimental N deposition in Actinobacteria (P=0.026) and fungi (P=0.018). Site and SitexTreatment interaction effects were present in all analyses. ** indicates significant Protected Fisher's LSD *P*<0.015; *indicates LSD *P*<0.035

Figure 3. The functional richness at each site A-D, as sum of all detected genes within each C substrate category for (a) Actinobacteria and (b) fungi. Error bars represent standard error. N deposition decreased total signal intensity for each substrate, except for Actinobacterial cellulose degrading genes, across all sites with a treatment effect ($P<0.04$), site effect ($P<0.005$) and interaction ($P=0.07$-0.006).** Indicates Protected Fisher's LSD $P < 0.025$, *$P < 0.05$

1 **Figure 4**. Response ratios of (a) actinobacterial and (b) Dikarya fungal taxa in soil microbial
2 communities under experimental N deposition relative to ambient N deposition. Lines
3 represent standard error. ***Indicates 99%, **95%,*90% CIs did not cross zero.
4

Figure 5. Principal coordinate analysis (PCO) displaying the effect of N deposition on the set of (a) Actinobacterial and (b) fungal functional genes involved in the decomposition of forest floor material. Dissimilarity matrices were constructed from the Bray-Curtis similarity metric based on the presence/absence of all relevant gene variants. For Actinobacterial and fungal functional genes, there was significant treatment (P=0.022,0.013), site (P=0.001, 0.001) and interaction (P=0.005,0.001) effects respectively. Pairwise comparisons with Monte Carlo simulations found significant treatment effects in sites B and C (P<0.05) for both Actinobacterial and fungi. Sites A and D did not have a significant response to treatment.

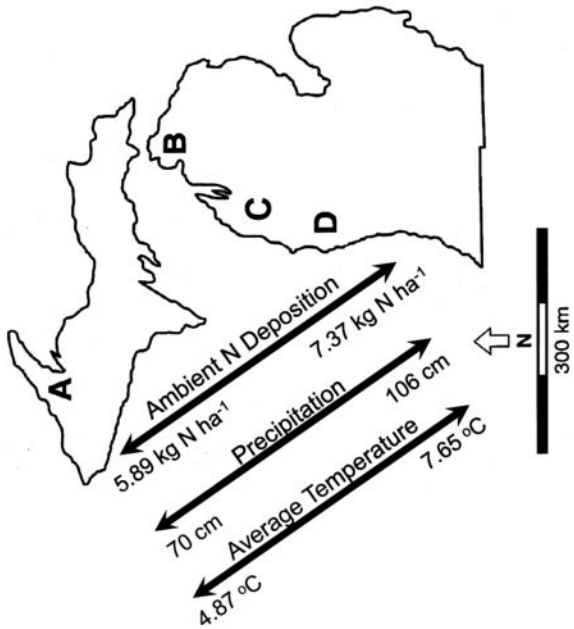

Figure 1.

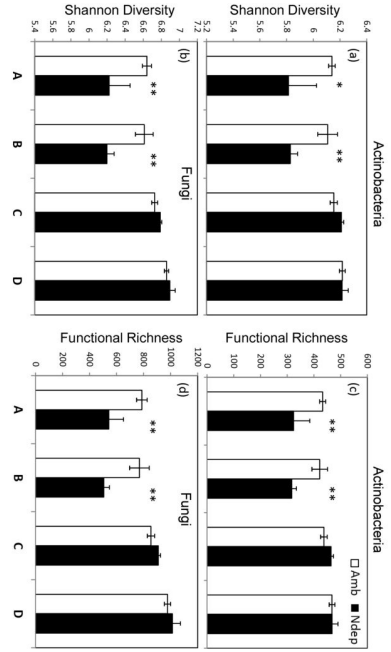

Figure 2.

Figure 3.

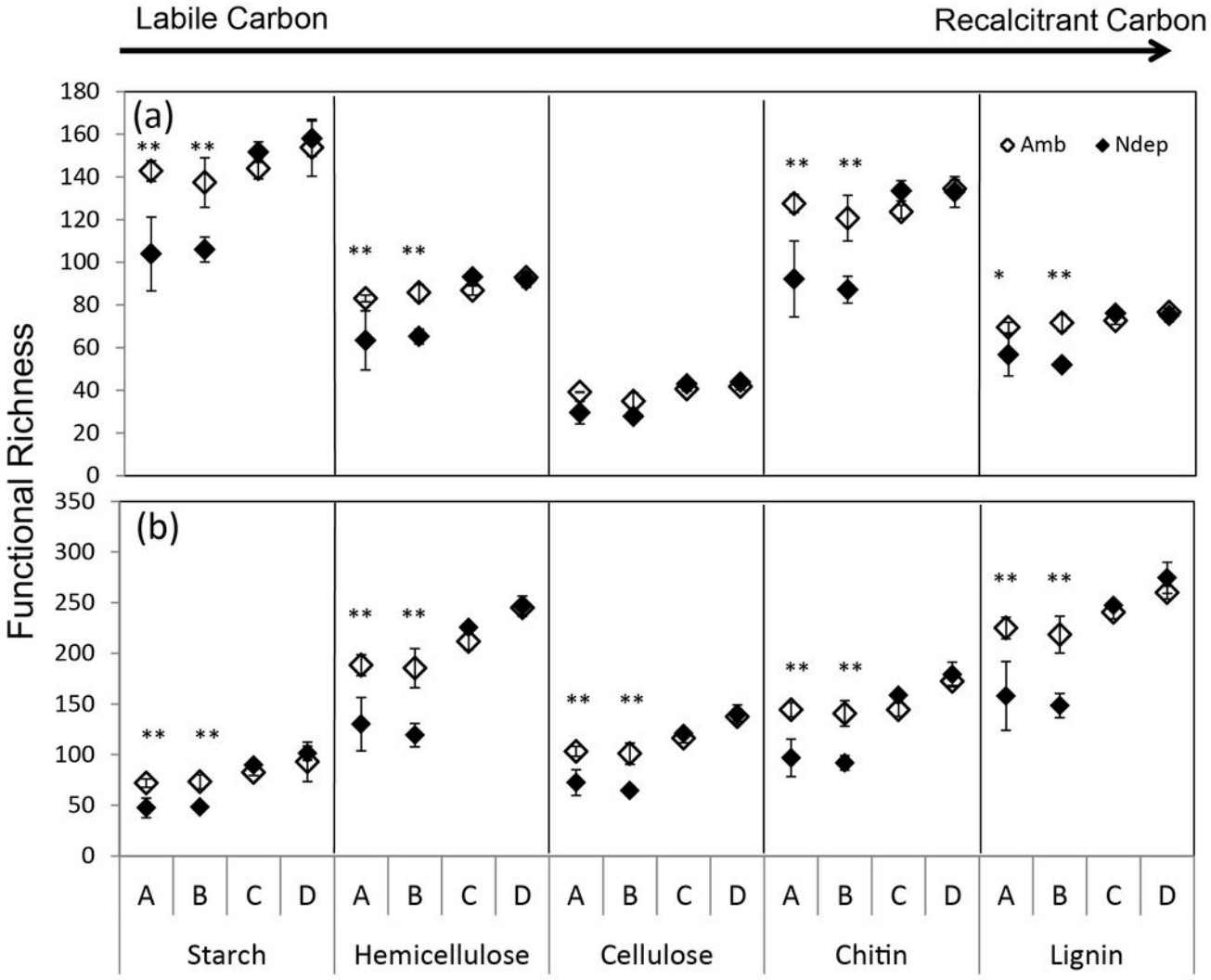

Figure 4.

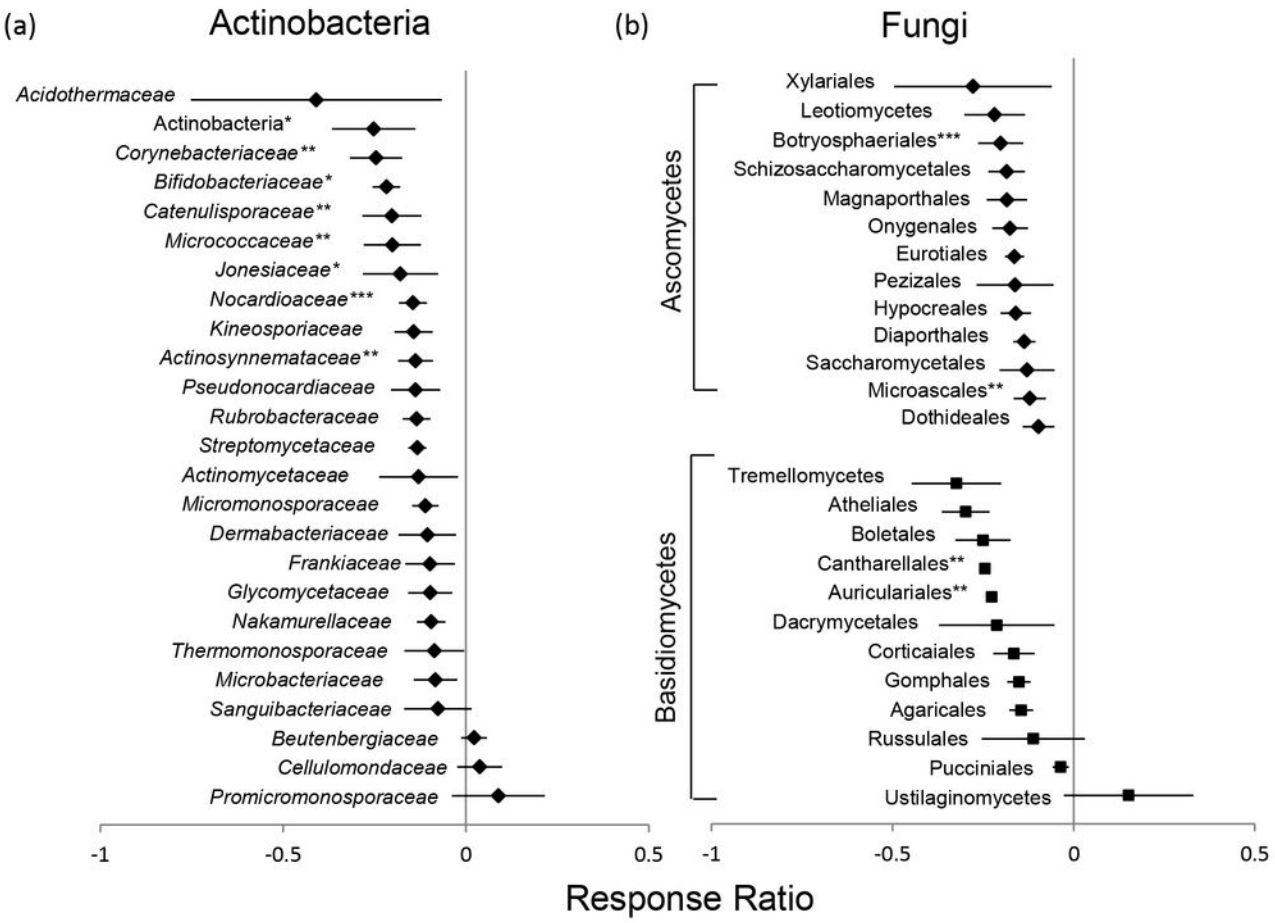

Figure 5.

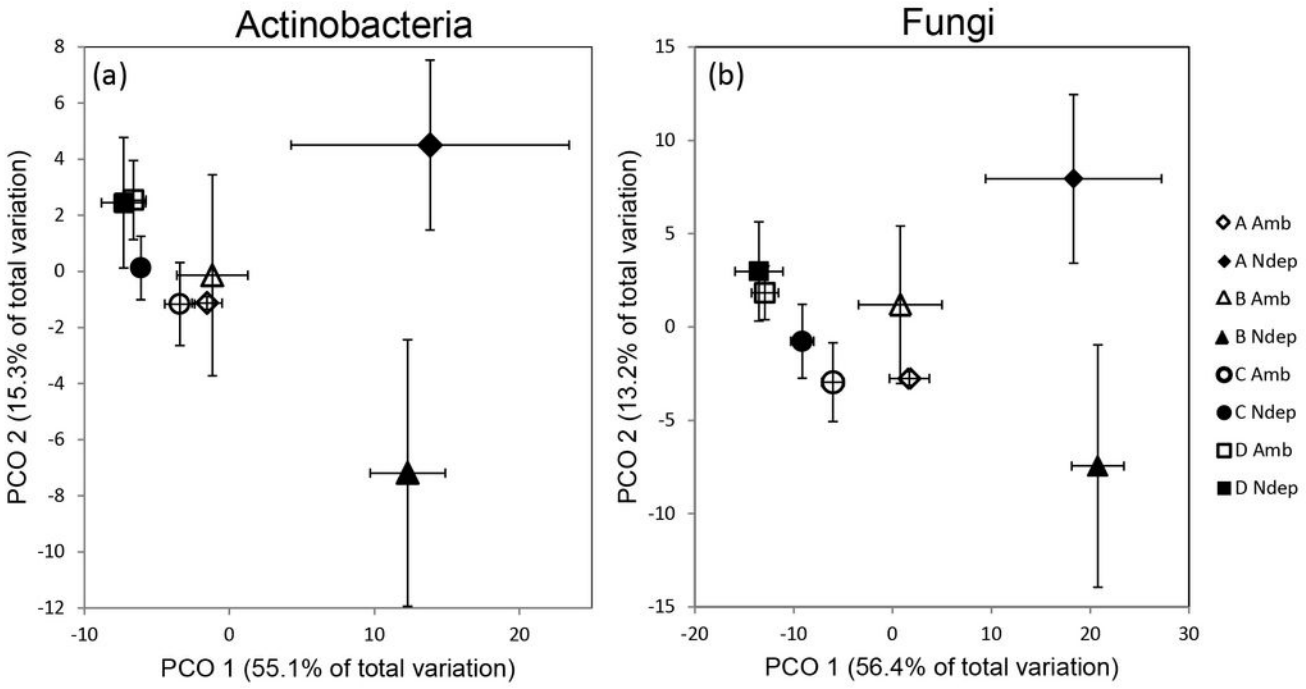

Table 1. Eighteen years of experimental N deposition have increased the storage and slowed the cycling of C in forest floor and surface mineral soil. Microbial responses are summarized for forest floor (Oe/Oa); mineral soil has exhibited similar directional trends. With the exception of laccase copy number, all of these responses are statistically significant.

Response to Chronic N Deposition	% Change	Citation
Plant		
Net Primary Production (NPP)	+10%	Pregitzer et al. 2008
Leaf Litter Production	0%	Pregitzer et al. 2008
Leaf Litter N Concentration	+25%	Zak et al. 2008
Fine Root Litter	0%	Burton et al. 2004
Fine Root N Concentration	0%	Burton et al. 2004
Biogeochemical		
Forest Floor Mass	+51%	Zak et al 2008
Forest Floor Turnover	-60%	Zak et al. 2008
Soil Organic Matter Content	+18%	Zak et al. 2008
Soil Solution NO_3^- Concentration	+288%	Zak et al. 2008
NO_3^- Leaching	+680%	Pregitzer et al 2004
DOC Leaching	+26%	Pregitzer et al. 2004
Microbial		
Soil Respiration	-15%	Burton et al. 2004
Active Microbial Biomass (PLFA)	-23%	DeForest et al 2004
Phenol Oxidase Activity	-33%	DeForest et al. 2004; 2005
Peroxidase Activity	-30%	DeForest et al. 2004; 2005
Basidiomycete Laccase Copy Number	-5 to -8%	Hassett et al. 2009

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Microbial Mechanisms Mediating Increased Soil C Storage under Elevated Atmospheric N Deposition

Sarah D. Eisenlord, Zachary Freedman, Donald R. Zak, Kai Xue, Zhili He, Jizhong Zhou

School of Natural Resources and Environment, University of Michigan, Ann Arbor, Michigan, USA; Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, Michigan, USA; Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma, USA

Volume 79, no. 4, p. 1191–1199, 2013. Page 1192: In Table 1, the % change value for "Active microbial biomass (PLFA^b)" should be "−23" and not "+23."

doi:10.1128/AEM.00468-13