Published September 20, 2013

Long-Term Experimental Nitrogen Deposition Alters the Composition of the Active Fungal Community in the Forest Floor

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Global increases in the rate of atmospheric N deposition have the potential to alter the composition and function of soil microbial communities. Here, we tested the hypothesis that experimental N deposition has altered the composition of active communities of Dikarya fungi. Such a change may underlie previously observed reductions in decomposition and increases in soil organic matter in a long-term field experiment. The actively metabolizing forest floor fungal community was characterized from cDNA clone libraries constructed from 28S fungal rRNA extracted from the forest floor of two northern hardwood stands in the lower peninsula of Michigan. We demonstrate that long-term experimental N deposition altered the composition of the active communities of Dikarya fungi in the forest floor in each of these forest stands. Because forest floor fungi are important decomposers, the alteration of forest floor fungal communities by increasing N deposition may have implications for the cycling and storage of C in forest ecosystems.

Abreviations: GTR: general time reversible; MAFFT, multiple sequence alignment based on fast Fourier transform; NCBI BLAST, National Center for Biotechnology Information Basic Local Alignment Search Tool; OTU, operational taxonomic unit; PCR, polymerase chain reaction.

Emissions of reactive N have increased 300 to 500% over the last century, a biogeochemical change that directly results from anthropogenic activities (Denman et al., 2007). Moreover, atmospheric N deposition in terrestrial ecosystems has been projected to further increase by 250% over the next century (Lamarque et al., 2005). Temperate forests are a globally important C sink, and future rates of atmospheric N deposition have the potential to influence their function. However, the majority of attention has focused on how atmospheric N deposition may enhance net primary productivity in these N-limited ecosystems (Nadelhoffer et al., 1999; Currie et al., 2004; LeBauer and Treseder, 2008), albeit there remains considerable debate regarding this response (Magnani et al., 2007). Nevertheless, ecosystem C storage is determined not only by rates of net primary production, but also by rates of decomposition and the formation of soil organic matter. Soils globally contain ~75% of the C stored in terrestrial ecosystems (Prentice et al., 2001); mounting evidence indicates that soil organic matter accumulation may be a widespread response to increasing N deposition in forests (Janssens et al., 2010). Thus, understanding the mechanisms through which C storage in forest soils may be affected by increasing rates of N deposition is of importance for understanding ecosystem function under global change.

One mechanism through which increasing rates of N deposition could reduce the rate or extent of decomposition is by inducing a change in the composition

Soil Sci. Soc. Am. J. 77:1648–1658
doi:10.2136/sssaj2013.05.0179
Received14 May 2013.
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or diversity of the microbial community. Decomposition is a microbially mediated process which can be altered by changes in the composition and diversity of the microbial decomposer community (Strickland et al., 2009a, 2009b; Fukami et al., 2010; McGuire et al., 2010; Wallenstein et al., 2010). Fungi are especially important decomposers of plant litter in terrestrial ecosystems. The majority of forest floor fungal saprotrophs belong to the fungal phyla Basidiomycota and Ascomycota, which together make up the subkingdom Dikarya. These organisms exhibit considerable variation in the number and type of genes they possess which encode for enzymes involved in the degradation of plant litter (Baldrian, 2006; Hoegger et al., 2006; Morgenstern et al., 2008; Hofrichter et al., 2010; Kellner et al., 2010; Floudas et al., 2012). Additionally, fungal taxa have been observed to vary in the efficiency in which they decompose plant litter and its constituents (Osono and Takeda, 2001, 2002, 2006; Osono et al., 2003, 2009; Osono, 2007; Valášková et al., 2007; Šnajdr et al., 2010). Thus, a change in the diversity or composition of the fungal decomposer community could have important functional consequences for decomposition.

Particular attention has been given to the idea that fungi which decompose lignin could become less competitive under elevated N deposition, leading to a change in fungal community composition (Fog, 1988; DeForest et al., 2004b; Blackwood et al., 2007; Hassett et al., 2009). Lignin is a decay-resistant component of plant cell walls that protects the more energy-rich cellulose and hemicellulose constituents from microbial attack. Lignin decay limits the overall rate of forest litter decomposition

Table 1. Climatic, floristic, and edaphic properties of two northern hardwood forests receiving experimental N deposition.

Characteristic	Site B	Site D
Location		
Latitude (N)	45°33′	43°40′
Longitude (W)	84°52′	86°09′
Climate		
Mean annual precipitation (mm)†	874	824
Mean annual temperature (°C)‡	6.2	7.7
Wet + dry total N deposition (g N m^{-2} yr^{-1})§	0.91	1.18
Vegetation		
Overstory age (2008)	95	100
Soil Chemistry¶		
Exchangeable calcium (cmol(+) kg^{-1})	3.43	2.36
Exchangeable magnesium (cmol(+) kg^{-1})	0.49	0.44
Exchangeable aluminum	0.19	0.63
Base Saturation (%)	69	82
pH (10-cm mineral soil)	4.92	4.60

† Mean annual precipitation, for the years 1994 to 2008, was recorded using weighing rain gages (Model 5-780, Belfort Instrument Co., Baltimore, MD) located in open areas within 5 km of each site.

‡ Mean annual temperature, for the years 1994 to 2008, was recorded on site at 2 m using thermistors which were read every 30 min throughout the year, with averages recorded every 3 h using data loggers (EasyLogger Models 824 and 925, Data Loggers, Logan, UT).

§ MacDonald et al. (1992)

¶ D.R. Zak, unpublished data, 2009.

(Osono and Takeda, 2005), and accumulating evidence indicates that atmospheric N deposition may negatively influence lignin decay in forest ecosystems (Berg and Matzner, 1997; Waldrop and Zak, 2006). Additionally, evidence from laboratory and field studies suggests that higher N conditions can reduce the expression and activity of fungal enzymes involved in lignin decomposition, as well as the rate of lignin decomposition by some fungal species (Weinstein et al., 1980; Fenn and Kirk, 1981; Boominathan et al., 1990; Vanderwoude et al., 1993; Carreiro et al., 2000; Soden and Dobson, 2001, 2003; DeForest et al., 2004a, 2004b; Waldrop et al., 2004; Edwards et al., 2011).

If atmospheric N deposition does change fungal community composition, there are several different ways in which such a change could proceed. A reduction in species richness or a decline in taxonomic diversity (α-diversity) under higher rates of N deposition could occur. Alternatively, richness and α-diversity could be unaffected by increasing N deposition, but increasing rates of N deposition could alter the taxonomic composition (β-diversity) of the community (Whittaker, 1972; Lozupone and Knight, 2008). Lastly, the composition of taxa present could be unaffected by increasing N deposition, but a change in community composition could occur if the relative abundances of taxa change.

To test the hypothesis that long-term increases in N deposition alter the composition of an important group of decomposers, we have examined the composition of the active community of Dikarya fungi in a long-term field study in which northern hardwood stands have received N additions at a rate predicted to occur in the near future (3 g NO_3^--N m^{-2} yr^{-1}). In this long-term field experiment, plant litter decay has slowed and the accumulation of soil organic matter has increased under experimental N deposition (Zak et al., 2008; Pregitzer et al., 2008). Furthermore, the production of phenolic dissolved organic carbon has increased under experimental N deposition, suggesting that elevated N deposition might be leading to incomplete microbial lignin decay (Pregitzer et al., 2004; Smemo et al., 2006). Additionally, the activity of lignolytic enzymes (DeForest et al., 2004b) and abundance of mRNA transcripts for lignolytic enzymes (Edwards et al., 2011) has been reduced under experimental N deposition in our study. We have reasoned that changes in the community composition of Dikarya fungi, which are active in the forest floor, are occurring alongside these previously observed declines in extracellular enzyme activity and gene expression. Our objective was to characterize and compare the active forest floor fungal community. Here, we constructed and sequenced cDNA clone libraries of the conserved fungal 28S rRNA extracted from the forest floor to examine the richness, diversity, composition, and structure of the active Dikarya fungi.

MATERIALS AND METHODS

Site Description

Our study sites consisted of two northern hardwood forest stands that are dominated by sugar maple (*Acer saccharum* Marsh.) in Michigan (Table 1). These two sites are two of the

four sites in the Michigan Gradient Experiment, a long-term elevated atmospheric N deposition experiment spanning a climatic and ambient N deposition gradient in Michigan. The southern Site D experiences higher mean annual temperatures, longer growing seasons, and higher annual inputs of ambient atmospheric N deposition than the northern Site B (Table 1). Soils are well-drained sandy typic Haplothords of the Kalkaska series. The sites have similar overstory ages and floristic compositions. Sites do not significantly differ in soil pH (Table 1). The O_i horizon at each site is dominated by sugar maple leaf litter.

Each site contains six 30-m by 30-m plots; three plots receive ambient N deposition, whereas the other three receive ambient N deposition plus 3 g NO_3^--N m^{-2} yr^{-1}. This amount has been added since 1994 and is consistent with levels expected to be reached in northeastern North America and portions of Europe by 2050 (Galloway et al., 2004). Treatments are applied as $NaNO_3$ pellets in six equal additions of 0.5 g N m^{-2} during the growing season (April–September). Each treatment plot is surrounded by a 10-m treated buffer zone to reduce edge effect, which also receives the aforementioned N deposition treatments.

Sample Collection

Forest floor from the O_e and O_a horizons was collected from three plots receiving ambient N deposition and three plots receiving experimental N deposition at both sites. Samples were composited at the plot level. Within each plot, the O_e and O_a horizons were manually collected from 10 random 100-cm^2 subplots, after the O_i horizon was removed. The O_e and O_a material from these 10 random subplots was combined and homogenized with sterilized scissors. For each plot, duplicate 50-mL sterile polypropylene tubes were filled with as much of this homogenized material as they could contain and immediately flash frozen in the field in liquid N_2. Samples were kept frozen while transported to the laboratory, where they were stored at −80°C.

RNA Extraction and Purification

For each composite sample from each plot, total RNA was extracted from 3 g of forest floor using an initial Tris-phenol extraction to separate nucleic acids from contaminants, followed by subsequent extraction of the aqueous phase using a Qiagen RNA/DNA Midi kit following a previously published method (Luis et al., 2005). Extracted RNA solutions were treated with DNase I to remove any DNA that may be present in the RNA solution and were then stored at −80°C. Before reverse transcription, samples were purified using the Plant RNAeasy Mini column kit (Qiagen, Venlo, Netherlands). Purification was performed according to a modified manufacturer's protocol for isolation of RNA directly from tissue with 2.5 mg of activated charcoal added to 350 μL of the manufacturer supplied "RLC" buffer. Purified RNA was quantified using a Quant-iT Ribogreen kit (Invitrogen, Carlsbad, CA) and Molecular Devices Fmax fluorescent microplate reader (Sunnyvale, CA), according to Ribogreen manufacturer instructions.

Reverse Transcription and Amplification of cDNA

The primers LR3 (5′CCG TGT TTC AAGAC GGG 3′) and LR0R (5′ ACCC GCT GAA CTT AAGC 3′) were selected to target the 28S rRNA region of interest (http://biology.duke.edu/fungi/mycolab/primers.htm). First strand cDNA was synthesized from 28S rRNA via a reverse transcription reaction using the reverse primer LR3, 65 ng of purified extracted total RNA, and SuperScript II reverse transcriptase, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Following first strand synthesis, cDNA of fungal 28S rRNA was amplified via polymerase chain reaction (PCR) on a Robocycler 96 thermocycler (Stratagene, La Jolla, CA) with initial denaturation at 95°C for 3 min, followed by 10 cycles of denaturing at 94°C for 30 sec, annealing at 50°C for 45 sec, and elongation at 72°C for 90 sec, and a final elongation at 72°C for 10 min. The PCR mixtures each contained 1 μL of first strand cDNA, 0.625 μL forward primer of 10 μM LR0R, 0.625 μL 10 μM reverse primer LR3, 2.5 μL dNTPs (2 μM), 2.5 μL 10X PCR buffer (1.5 mM $MgCl_2$), and 16.5 μL molecular grade water. Duplicate 25-μL reactions were performed and combined for a total of 50 μL PCR product per plot. Polymerase chain reaction products were purified using a MoBio Ultraclean PCR Clean-up kit (MoBio, Carlsbad, CA) and stored at −20°C.

Clone Library Construction and Sequencing

Amplified 28S cDNA segments were cloned into vector PCR 2.1 TOPO using a TOPO TA Cloning kit, with manufacturer's protocol modified to reduce all reagents to one half of recommended volume (Invitrogen, Carlsbad, CA). Vectors containing inserts were transformed into TOPO TA Cloning TOP10 chemically competent cells. Ninety-six positive colonies were selected for each sample and grown overnight at 37°C in Luria-Bertani broth containing 10% glycerol, 0.025 g L^{-1} ampicilin, 0.0125 g L^{-1} kanamycin. Libraries were screened and frozen at −80°C until sequencing could occur. For each sample, 96 sequences were submitted for bidirectional sequencing. Sequencing was performed at the DNA Sequencing Lab at the University of Georgia (Athens, GA) and Seqwright (Houston, TX).

Sequence Editing and Alignment

Sequences were edited and contiguous sequences were constructed in Geneious version 5.5.7 (Biomatters, www.geneious.com/). All high-quality sequences were aligned in MAFFT v.6.814b plug-in for Geneious (Katoh et al., 2002) Neighbor-joining trees were constructed using the Geneious Tree-Builder. Sequences that were nonfungal, fungal but neither ascomycetes nor basidiomycetes, or incomplete sequences were identified using the neighbor-joining trees to identify sequences that did not group with fungal sequences and by performing searches in the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) database; these sequences were removed from subsequent analyses.

Assessment of Sampling Effort and Subsampling

The remaining sequences were realigned in MAFFT with 181 Dikarya reference sequences that had been previously identified from NCBI databases (see Supplemental Materials and Methods). Within multiple sequence alignment based on fast Fourier transform (MAFFT), the FFT-NS-2 algorithm was selected because the size of the data set exceeded 200 sequences. This alignment was imported into MOTHUR v.1.27.0 and converted into a distance matrix (Schloss et al., 2009). Sampling effort was assessed by creating rarefaction curves and calculating Good's coverage estimator (Good, 1953) in MOTHUR. Sequences were clustered into 99% sequence similarity operational taxonomic units (OTUs) in MOTHUR and a representative sequence from each OTU was randomly selected using the get.oturep command. These are available in GenBank under accession numbers KC701765 to KC701965 (Supplemental Table S1).

An unequal number of Dikarya sequences were recovered among sites and N deposition treatments. Therefore, it was necessary to subsample sequences before estimating richness, calculating diversity indices, and performing β-diversity analyses. We subsampled each treatment to contain the same number of Dikarya sequences as that in the treatment with the lowest recovery. For each N deposition treatment within Sites B and D, a set of 121 sequences were selected using the sub.sample command in MOTHUR. A separate subsampling procedure was conducted before examining the relative abundance of different taxonomic groups because this required comparisons of the relative abundance of taxa among plots within each treatment. The number of sequences recovered was uneven across plots, so subsampling of 36 sequences per plot for Site B and 49 sequences per plot for Site D was conducted on the original set of 626 recovered sequences in MOTHUR. These values represent the number of recovered sequences in the smallest sample at each site. Subsampling for Sites B and D was conducted separately, because the number of sequences per plot between the sites was significantly different (Student's t test, 2-tailed, $P = 0.001$). Clustering of OTUs and random selection of representative sequences on subsampled sets of sequences was also performed in MOTHUR.

Phylogenetic Tree Construction and Taxonomic Assignment

Phylogenetic trees were created by aligning randomly selected representative sequences from each 99% sequence similarity OTU in the manner described above but with the additional inclusion of a Glomeromycota reference sequence as an outgroup (Supplemental Materials and Methods). Maximum likelihood trees were created using the PhyML plug-in (Guindon and Gascuel, 2003) for Geneious with a general time reversible (GTR) substitution model (Tavaré, 1986). The trees were manually rooted in MEGA version v.5.05 (Tamura et al., 2011). A phylogenetic tree was created before subsampling for assigning taxonomy to sequences. A second phylogenetic tree was created after subsampling as described above for use in β-diversity analysis (Supplemental Fig. S1).

Richness Estimation and Diversity Indices

Chao1 richness (Chao, 1984), Shannon diversity (Shannon, 1948), and Simpson diversity (Simpson, 1949) were calculated in MOTHUR for 99, 95, and 90% sequence similarity OTUs. Chao1 richness uses the low-frequency count OTUs to estimate the number of missing OTUs (Chao, 1984; Chao and Shen, 2012). The Shannon index provides us with a measure of the uncertainty in predicting the OTU to which the next randomly-selected sequence belongs (Shannon, 1948). Simpson diversity was calculated to examine the probability that any two sampled sequences would belong to the same OTU (Simpson, 1949). Simpson diversity is calculated in the original form in MOTHUR. Because of this, the Simpson values (λ) calculated in MOTHUR were inversed to obtain the more commonly used inverse form of the Simpson index ($1/\lambda$). Examining three OTU levels was a proxy for examining these measures over a range of taxonomic levels.

β-Diversity Analyses

Analyses of β-diversity were performed using the online Unifrac portal at http://bmf2.colorado.edu/unifrac/ (Lozupone and Knight, 2005; Lozupone et al., 2006, 2007). These analyses include Unifrac significance, weighted Unifrac significance, and Martin's P-test (Martin, 2002; Lozupone and Knight, 2005, 2008; Lozupone et al., 2006, 2007). The "each pair of environments" option was selected to determine if experimental N deposition libraries were significantly different than ambient N deposition libraries; this option removes sequences which are not found in either of the two libraries being compared. Analyses were run for 100 permutations.

The Unifrac metric measures the fraction of unique phylogenetic branch length leading to sequences found in one environment but not in others. Significantly different communities are those in which less than 5% of trees in which sequences were randomly assigned to environments had a higher Unifrac metric (i.e., fraction of unique branch length) than the real tree (Lozupone and Knight, 2005). Unifrac is a useful tool for comparing microbial communities because it incorporates phylogenetic relatedness by using branch length. The degree of relatedness is an important consideration because more closely related sequences share more evolutionary history, and perhaps ecological characteristics. Unifrac significance determines if communities contain more unique lineages than would be expected by chance based on presence or absence of those lineages (Lozupone et al., 2007). Weighted Unifrac significance, which incorporates abundance information, examines whether individuals in a community are more phylogenetically similar to each other than to those in another community (Lozupone et al., 2007).

Unlike Unifrac, Martin's P-test does not examine branch length or unique lineages (Martin, 2002; Lozupone et al., 2006). Instead, Martin's P-test examines the covariation of sequence distribution and phylogeny. The relationships between sequences are randomized to estimate the number of switches between communities that would occur under the null hypothesis that

phylogeny and sequence distribution between communities do not covary. The results are significant if the number of switches between communities needed to explain the observed distribution of sequences is less than the number of changes estimated to occur when sequence distribution is randomized. Martin's P-test can inform us if taxa present under ambient and experimental N deposition are distinctly separated in a phylogenetic tree.

Abundance of Taxonomic Groups

Taxonomy was assigned to each recovered sequence based on construction of a phylogenetic tree with reference sequences, BLAST searches, and through consulting recent literature. We were able to assign most ascomycete sequences to class and most basidiomycete sequences to order. We used a subsampled set of sequences to examine the relative abundances of different taxonomic groups under experimental N deposition. Student's 1-tailed *t* test was used to test the hypothesis that the abundance of particular taxonomic groups would be suppressed under experimental N deposition. However, it is also possible that other taxonomic groups might increase in abundance, either in response to the decline of another group or because they are favored under high nitrogen conditions. Therefore, we used the Student's 2-tailed *t* test to address the hypothesis that the abundances of taxonomic groups would differ in libraries from ambient and experimental N deposition plots. The *t* tests were performed in Microsoft Excel 2007. We compared the relative abundance of Ascomycota and Basidiomycota sequences under experimental N deposition at Sites B and D. We also tested the abundance of taxonomic groups within the Ascomycota and Basidiomycota; these taxa included several ascomycete classes from the subdivision Pezizomycotina (Dothideomycetes, Eurotiomycetes, Leotiomycetes, Orbiliomycetes, Pezizomycetes, Sordariomycetes), several orders from the basidiomycete subdivision Agaricomycotina and the class Agaricomycetes (Agaricales, Amylocorticiales, Auriculariales, Cantharellales, Gomphales, Polyporales, Trechisporales), two basidiomycete classes from subdivision Agaricomycotina and class Tremellomycetes (Cystofilobasidiales, Tremellales), and the basidiomycete order Microbotryomycetes in the subdivision Pucciniomycotina. We also examined the abundance of a basal clade of ascomycetes with unknown taxonomy mostly known from uncultured, environmental clones. Relative abundance testing of these taxonomic groups (Student's *t* tests) was performed separately for sequences from Site B and from Site D.

RESULTS

Dikarya Sequence Recovery

Of the 1152 clones submitted for sequencing, 1086 clones contained inserts that produced readable sequences containing both primer regions. Of these, 626 sequences represented Dikarya. The other 460 sequences were removed from analysis because they were nonfungal (385), represented fungi but not Dikarya (69), or were of insufficient length to cover the entire amplified region (6). Rarefaction curves for ambient and experimental N deposition treatments are presented in Supplemental Fig. S2 to S5. Good's coverage varied across both the sites and N deposition treatments before subsampling, with Site D showing higher coverage than Site B (Supplemental Table S2).

Richness Estimation and Diversity Indices

Chao1 richness and inverse Simpson diversity were not significantly affected by experimental N deposition in either Site B or Site D (Tables 2 and 3). Shannon diversity was not significantly different under ambient and experimental N deposition at Site B (Table 4). At Site D, Shannon diversity was significantly lower under experimental N deposition for 90% sequence similarity OTUs ($P < 0.05$), but there was no significant difference in Shannon diversity for either 99 or 95% sequence similarity OTUs under experimental N (Table 4).

Table 2. Chao1 richness for Dikarya 28S rRNA cDNA libraries from two hardwood forest stands (B and D) under ambient and experimental N deposition.†

Site	OTU similarity level	N deposition treatment	
		Ambient	Experimental
B	99%	243 (132–526)	151 (105–251)
	95%	183 (98–422)	84 (59–156)
	90%	87 (47–221)	36 (29–64)
D	99%	213 (119–445)	136 (87–257)
	95%	111 (74–203)	86 (57–167)
	90%	50 (38–86)	69 (37–181)

† Libraries were subsampled to equalize sample sizes across stands and N deposition treatments before clustering. Clustering of operational taxonomic units (OTUs) at three sequence similarity levels (99, 95, and 90%) and calculation of Chao1 richness were performed in MOTHUR v. 1.27.0. Values for the lower-upper confidence intervals are presented in parentheses. There were no significant differences ($P < 0.05$) for comparisons between N deposition treatments within a site and OTU level.

Table 3. Inverse Simpson diversity for Dikarya 28S rRNA cDNA libraries from two hardwood forest stands (B and D) under ambient and experimental N deposition.†

Site	OTU similarity level	N deposition treatment	
		Ambient	Experimental
B	99%	48 (36–72)	55 (39–98)
	95%	30 (23–44)	30 (24–41)
	90%	5 (4–8)	9 (7–13)
D	99%	7 (5–12)	15 (11–28)
	95%	7 (5–12)	13 (9–20)
	90%	6 (4–9)	3 (3–5)

† Libraries were subsampled to equalize sample sizes across stands and N deposition treatments before clustering. Clustering of operational taxonomic units (OTUs) at three sequence similarity levels (99, 95, and 90%) and calculation of Simpson diversity were performed in MOTHUR v. 1.27.0. Values for the lower-upper confidence intervals are presented in parentheses. There were no significant differences ($P < 0.05$) for comparisons between N deposition treatments within a site and OTU level.

Table 4. Shannon diversity for Dikarya 28S rRNA cDNA libraries from two hardwood forest stands (B and D) under ambient and experimental N deposition.†

Site	OTU similarity level	N deposition treatment	
		Ambient	Experimental
B	99%	3.8 (3.7–4.0)	4.0 (3.8–4.1)
	95%	3.5 (3.4–3.7)	3.5 (3.3–3.6)
	90%	2.4 (2.2–2.7)	2.7 (2.4–2.9)
D	99%	3.0 (2.7–3.4)	3.4 (3.1–3.6)
	95%	3.0 (2.7–3.3)	3.0 (2.8–3.2)
	90%	2.5 (2.3–2.8)	1.9* (1.6–2.2)

* Comparisons between N deposition treatments within a site and operational taxonomic unit (OTU) level for which confidence intervals do not overlap ($P < 0.05$).

† Libraries were subsampled to equalize sample sizes across stands and N deposition treatments before clustering. Clustering of OTUs at three sequence similarity levels (99, 95, and 90%) and calculation of Shannon diversity were performed in MOTHUR v. 1.27.0. Values for the lower-upper confidence intervals are presented in parentheses.

β Diversity Analyses

Pair-wise UniFrac significance tests indicated that there was less shared branch length between fungal communities from ambient and experimental N deposition treatments in Site D than would be expected by chance (Unifrac, $P = 0.02$, Table 5). There was no significant difference in the amount of shared branch length between ambient and experimental N deposition environments at Site B (Unifrac, $P = 0.15$, Table 5). When branches were weighted with abundances, ambient N deposition and experimental N deposition libraries differed significantly in the amounts of shared branch length at Site B (weighted Unifrac, $P = 0.02$, Table 5), but not at Site D (weighted Unifrac, $P = 0.76$, Table 5). Phylogeny and sequence distribution covaried for ambient and experimental N deposition environments at Site D (Martin's P-test, $P < 0.0001$, Table 5), but this was not true at Site B (Martin's P-test, $P = 0.15$, Table 5).

Relative Abundance of Taxonomic Groups

The proportion of basidiomycete sequences declined significantly under experimental N deposition at site B (Student's *t* test, 1-tail, $P = 0.03$); however, this response did not occur in Site D. At site B, Agaricales declined significantly under experimental N deposition (Student's *t* test, 1-tail, $P = 0.03$, Fig. 1a). No other taxonomic groups responded significantly to N deposition at Site B (Fig. 1a). At Site D, the Cantharellales (Student's *t* test 1-tail, $P = 0.02$) and Tremellales (Student's *t* test, 1-tail, $P = 0.03$) declined significantly under experimental N deposition (Fig. 1b); Cantharellales were also lower in relative abundance under experimental N deposition at Site D when a two-tailed Student's *t* test was applied ($P = 0.04$, Fig. 1b). The Agaricales (Student's *t* test, 2-tail, $P = 0.02$) and Gomphales (Student's *t* test, 2-tail, $P = 0.02$) were relatively more abundant in the active community under experimental N deposition at Site D (Fig. 1b).

Table 5. Significance values for pair-wise Unifrac, weighted Unifrac, and Martin's P-test for cDNA clone libraries of 28S rRNA for Dikarya fungi from two northern hardwood forests (Sites B and D) receiving ambient and experimental N deposition.

Pair-wise comparisons				
Site, N deposition treatment	Site, N deposition treatment	Unifrac significance	Weighted Unifrac significance	Martin's P-test significance
B, ambient	B, experimental	0.15	0.02*	0.15
D, ambient	D, experimental	0.02*	0.76	<0.0001***
B, ambient	D, ambient	0.03*	0.68	<0.0001***
B, experimental	D, experimental	0.46	0.09	0.98
B, ambient	D, experimental	0.21	0.99	0.21
B, experimental	D, ambient	0.02*	0.49	<0.0001***

* Significant at the 0.05 probability level.

*** Significant at the 0.001 probability level.

DISCUSSION

Atmospheric N deposition will continue to increase over the next century (Galloway et al., 2004). It is important to understand the response of litter-decaying fungi to long-term increases in atmospheric N deposition because these organisms transform plant detritus into soil organic matter (Osono, 2007), thereby mediating the process of soil C storage in forests. Here, we document different compositional changes in the active communities of Dikarya fungi in two forest stands in response to long-term experimental N deposition. In our long-term field study, experimental N deposition has reduced plant litter decay and increased soil organic matter accumulation (Zak et al., 2008; Pregitzer et al., 2008), but these consistent biogeochemical responses appear to have arisen from disparate compositional shifts between our study sites.

By targeting rRNA instead of rDNA, we have examined the members of the fungal community which were metabolically active. Baldrian et al. (2012) observed that the composition of the active fungal community (RNA) differed from that of the total community (DNA), with some OTUs exclusively recovered from the active community. Furthermore, when functions were assigned to these OTUs, Baldrian et al. (2012) found that saprotrophic and parasitic fungi were more abundant in the active community than the total community. Kellner et al. (2009) similarly observed that the composition of expressed cellulose-degrading genes formed a distinct population from the cellulose-degrading genes present based on DNA. These observations (Kellner et al., 2009; Baldrian et al., 2012) further suggest that organisms with a low abundance can be active and important in soil processes. Targeting the active community, rather than the total community, is important because the active community is mediating the functions (i.e., decomposition) which we seek to understand.

Richness and Diversity Indices

Experimental N deposition did not impact Chao1 richness estimates in the active community. Furthermore, experimental N deposition only had a significant effect on Shannon diversity for 90% sequence similarity OTUs at Site D. Shannon and inverse Simpson diversity were unaffected by experimental N deposition for all other comparisons (Tables 3 and 4). Thus, experimental N deposition appears to have

only a limited effect on the α-diversity of the active Dikarya community. However, Site B exhibited higher Shannon and inverse Simpson diversity than Site D in several of the comparisons (Tables 3 and 4). The differences in ambient atmospheric N deposition between the sites are small relative to our experimental N deposition treatment (Table 1) and are unlikely the cause of site-to-site variation. However, the higher diversity of the active Dikarya in Site B compared to Site D could be related to the climatic differences between these two sites (Table 1). That Site B and D exhibited differences in α-diversity indices is interesting because these two sites responded in a site-specific manner to experimental N deposition in the other measures which we examined, for example, β-diversity and OTU abundances.

β Diversity

Experimental N deposition significantly altered the β-diversity of active fungal communities in both sites, albeit in different ways. The changes in the community composition of active Dikarya fungi that we predicted could be characterized by the presence-absence of unique lineages under experimental N deposition (Unifrac), the covariation of lineages with N deposition treatment (Martin's P-test), or from individuals being more closely related to those within their N deposition treatment than to those from the other N deposition treatment (weighted Unifrac). A significant response to even one of these β-diversity metrics would be consistent with our predictions; which metric responded simply informs us about the nature of the observed compositional changes taking place in response to experimental N deposition.

When the ambient and experimental N deposition treatments were compared at Site B, neither a significant Unifrac nor a significant Martin's P-test result were obtained; however, weighted Unifrac was significant. Weighted Unifrac can be significant even when Unifrac, which is based solely on presence-absence of unique lineages is not, because it incorporates abundance information to weight the branches (Lozupone et al., 2007). The fungi in each N deposition treatment at Site B were phylogenetically more similar to one another than to those in the other N deposition treatment. A higher degree of phylogenetic relatedness with an N deposition treatment suggests that these individuals share traits that may be favored under different N regimes.

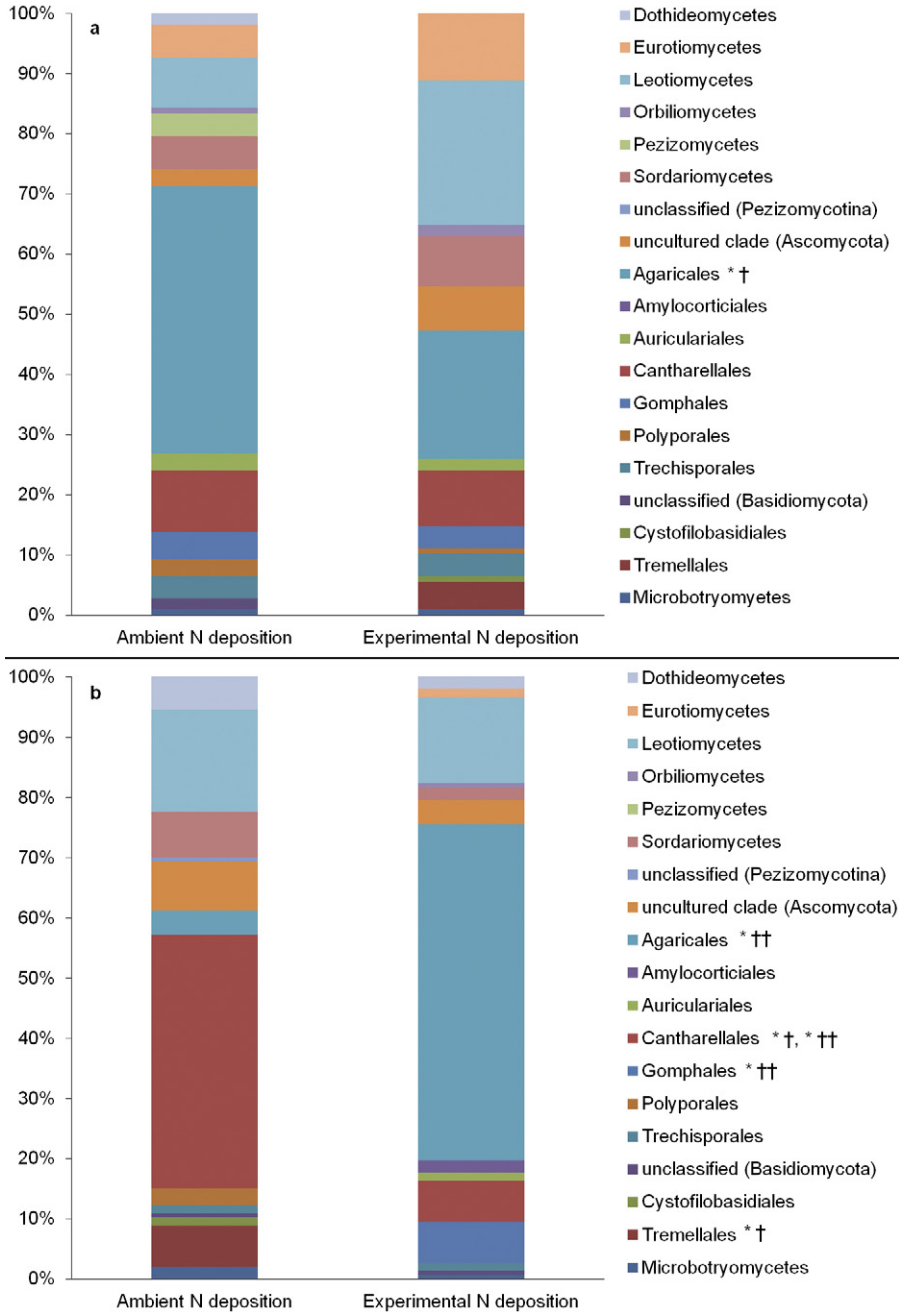

Fig. 1. Relative abundance (%) of taxa in Dikarya communities under ambient N deposition and experimental N deposition (+3 g NO_3^-–N m^{-2} yr^{-1}) in two northern hardwood forests, Site B (a) and Site D (b). More information on these sites is provided in Table 1. Taxa for which a significant change ($P < 0.05$) in abundance occurred are marked with an asterisk (*). A significant decline in relative abundance under experimental N deposition according to a one-tailed Student's *t* test is denoted with one dagger (†). A significant difference in abundance between ambient and experimental N deposition treatments according to a two-tailed Student's *t* test is denoted with two daggers (††). Dothideomycetes through uncultured group (Ascomycota) represent taxonomic groups from the Ascomycota, while Agaricales through Microbotryomycetes represent taxonomic groups from the Basidiomycota.

In contrast, the active fungal communities under ambient N deposition and experimental N deposition at Site D were significantly different according to both Martin's P-test and Unifrac significance tests, but not according to weighted Unifrac (Table 5). Martin's P-test indicated that the sequences from the

ambient N deposition treatment were clustered distinctly from those of the experimental N deposition treatment. Further, Unifrac indicated that there was more unique branch length than would be expected by chance leading to sequences from one N deposition treatment but not the other. The presence of a significant Unifrac and Martin's P-test result together indicates that the monophyletic lineages not shared between the N deposition treatments contribute substantial branch length or, in other words, that the unique lineages are rooted deeper within the tree and not near the tips (Lozupone et al., 2006). This suggests that the compositional differences between the ambient and N deposition treatments at Site D are more than superficial and that different evolutionary lineages with potentially different traits are present in each. Weighted Unifrac was not significant for comparisons between the N deposition treatments at Site D. This suggests that perhaps the significant result for Unifrac (unweighted) was driven by the presence-absence of rare taxa and not by the dominant taxa, because significance disappeared when abundance weights were incorporated. We observed that no sequences for the Gomphales (Basidiomycota) or Eurotiomycetes (Ascomycota) were recovered in the ambient treatment at Site D, but these taxa were present in the experimental N deposition treatment (Fig. 1b). The absence of these groups from the ambient N deposition treatments may have influenced differences in unique branch length and clustering measured with Unifrac and Martin's P-test.

It is interesting that the composition of active fungal communities at Sites B and D was altered by long-term experimental N deposition, but that different metrics of β-diversity were significant at each site. It is important to note, however, that the ambient communities at Sites B and D were also different from each other in terms of diversity indices (Tables 2–4) and β-diversity measures (Table 5). Thus, it is not surprising that we did not obtain consistent changes in the active community in response to long-term experimental N deposition because the ambient communities present at these sites are different from each other.

Taxa which Responded to Experimental N Deposition

Dikarya taxonomic groups at Sites B and D did not respond to experimental N deposition in a similar manner. The Agaricales was the only taxonomic group to respond significantly at both sites, but experimental N deposition had a negative effect on its relative abundance at Site B and a positive effect at Site D. Furthermore, several groups responded to experimental N deposition at Site D which did not respond significantly at Site B, limiting our interpretation of the universality of the responses of these groups to increasing rates of atmospheric N deposition. Furthermore, there was a decline in the basidiomycete/ascomycete ratio under experimental N deposition at Site B, but no significant change in the proportions of basidiomycete and ascomycete sequences at Site D. It should be noted that the communities under ambient N deposition at each of these sites contained different proportions of Dikarya taxa (Fig. 1).

Ecological Role of Fungi Responding to N Deposition

We had hypothesized that not only would the composition of the Dikarya community shift but that the composition of the community would change in such a way that lignolytic fungi would decline and less lignolytic fungi would increase in relative representation. The most effective litter decomposers with lignolytic capacities are found in the basidiomycete order Agaricales, particularly in the *Marasmius*, *Gymnopus*, *Mycena*, *Clitocybe* and *Collybia* (Osono and Takeda, 2006; Osono, 2007; Valášková et al., 2007; Šnajdr et al., 2010). The basidiomycete class Agaricales declined in relative abundance in the active community under experimental N deposition at Site B, but counter to our expectations, Agaricales increased in relative abundance in Site D. Because these organisms responded in an opposing manner to N deposition at each of the sites, we cannot conclude whether increasing rates of N deposition have a positive or negative effect on a group that are important mediators of lignin decay.

Other groups responding to experimental N deposition at Site D have multiple nutritional modes, with little being known about their role in decomposition. For example, the Cantharellales declined significantly under experimental N deposition at Site D and were primarily composed of sequences associated with *Sistotrema* and *Ceratobasidium*. These genera include saprotrophs, mycorrhizal associates, and pathogens (Olive, 1957; Nilsson et al., 2006; Yurchenko, 2006; Di Marino et al., 2008; Mosquera-Espinosa et al., 2013). Saprotrophic *Ceratobasidium* and *Sistotrema* species have not been included in the majority of studies quantifying the decomposition abilities of other common saprotrophs (i.e., Osono and Takeda, 2002; Osono, 2007; Steffen et al., 2007), so relatively little is known about their capacity to degrade lignin. A related fungus, a *Sistotrema-Clavulina* strain, efficiently decomposed cellulose but did not decompose lignin in *Pinus sylvestris* needles (Boberg et al., 2011). Thus, the ecological role of one of major taxonomic groups recovered in this study which was significantly affected by experimental N deposition is unclear. Similarly, the Gomphales recovered here were all associated with the genus *Ramaria*. *Ramaria* species can be ectomycorrhizal or saprotrophs on wood or litter (Agerer et al., 2012). Thus, what the increase in Gomphales under experimental N deposition at Site D means in terms of ecological function is undeterminable, because the nutritional mode of the recovered *Ramaria*-associated sequences is not known.

Finally, basidiomycete yeasts in the order Tremellales declined under experimental N deposition at Site D, but these organisms do not play a role in lignin decomposition. Instead, soil yeasts assimilate carbon from both root-exudates and from compounds produced during plant litter decay through the action of enzymes of other fungi and bacteria (Botha, 2006, 2011). More recently, however, it has been suggested that Tremellales yeasts are cellulolytic (Štursová et al., 2012).

Thus, only Site B showed a decline in representation of fungi implicated in lignin decomposition (Agaricales). Interestingly, it is Site B which has responded most strongly in terms of increased

soil C under experimental N deposition (Pregitzer et al., 2008). At Site D, we observed an increase in fungi important in lignin decomposition (Agaricales) as well as responses by taxonomic group whose role in lignin decomposition is either unknown or negligible. Therefore, experimental N deposition did not consistently suppress lignolytic fungi in our study system.

Summary and Conclusions

Atmospheric N deposition is an important agent of global change which has the potential to affect C cycling and storage in terrestrial ecosystems by slowing decomposition. Experimental N deposition had negatively affected plant litter decay and enhanced soil organic matter accumulation in this long-term experiment (Pregitzer et al., 2008; Zak et al., 2008), a response that may be widespread in forests (Janssens et al., 2010). A shift in decomposer community composition has been put forward as one possible mechanism which could explain this response (Fog, 1988; Waldrop et al., 2004; Janssens et al., 2010). Previous research examining microbial response to experimental N deposition has found either alterations (Allison et al., 2007; Eisenlord and Zak, 2010; Edwards et al., 2011) or no changes (DeForest et al., 2004b; Hassett et al., 2009) in the composition of decomposer communities. In our study, long-term experimental N deposition has not altered richness and has had only minor effects on fungal diversity. We have also demonstrated that long-term experimental N deposition has altered the β-diversity in each of these two forests, albeit in different ways. While each site contained Dikarya taxonomic groups that responded to long-term experimental N deposition, the responses of particular taxonomic groups were not consistent between the two sites. These results suggest that local fungal community composition plays an important role in how these communities shift in response to increasing rates of N deposition. Although experimental N deposition has cumulatively reduced forest floor decay and increased organic matter accumulation in a long-term experiment (Pregitzer et al., 2008; Zak et al., 2008), these biogeochemical responses have been mediated locally by different groups of active saprotrophic fungi.

ACKNOWLEDGMENTS

This research was supported by grants from the National Science Foundation and the Department of Energy's Division of Environmental Biology. We are grateful to Sarah Eisenlord and Rima Upchurch who provided us with valuable assistance in the field and laboratory. Tim James provided valuable insights throughout this project, especially with phylogenetic methods. We also wish to thank other members of the Zak lab for their thoughtful feedback on this project.

SUPPLEMENTAL MATERIALS

The supplement includes additional materials related to the sequences obtained in this study, assessments of sequencing effort, sequence alignment, and phylogenetic tree construction.

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