Anthropogenic Nitrogen Deposition and Decomposer Fungi: Altered Composition and Function Fosters Greater Soil Carbon Storage

by

Elizabeth Mae Entwistle

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Doctoral committee:

Professor Donald R. Zak, Chair
Associate Professor Gregory Dick
Associate Professor Inés Ibáñez
Associate Professor Timothy Y. James

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ABSTRACT

Anthropogenic N Deposition and Decomposer Fungi: Altered Composition and Function Fosters Greater Soil C Storage

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Elizabeth Mae Entwistle

Chair: Donald R. Zak

The anthropogenic deposition of reactive nitrogen (N) onto terrestrial ecosystems has accelerated dramatically in the last 160 years as the result of human activity, with future rates of deposition projected to increase further. In some temperate forests, experimental increases in N deposition have reduced decomposition and concomitantly increased soil carbon (C) storage. One mechanism proposed to explain this response is that experimental N deposition negatively affects fungal decomposers of lignin, a recalcitrant constituent of plant cells which limits the overall rate of plant litter decay. More specifically, anthropogenic N deposition is hypothesized to reduce fungal lignolysis, and, as a result, reduce the representation of lignolytic fungi while favoring fungal taxa which are less efficient lignin decomposers. I tested this mechanism by examining the composition and diversity of fungi and the lignolytic genes that they express in a long-term field experiment in a series of northern hardwood forests, which have received experimental N deposition (3 g N m^{-2} y^{-1}) for nearly 20 years. First, I found that experimental N deposition altered the composition of the active fungal community in the forest floor, whereas it had

miminal effect on fungal richness and diversity. In my second experiment, experimental N deposition reduced the abundance of lignolytic fungi occurring on high-lignin and wood substrates; this appeared to be part of an overall change in fungal community composition in response to experimental N deposition, wherein lignolytic taxa declined and cellulolytic fungi increased in relative abundance. In Chapter 3, I found that experimental N deposition altered the composition, but not the richness or diversity, of expressed class II fungal peroxidases in the forest floor. Together, my results revealed that experimental N deposition reduces decomposition of plant litter and increases soil C storage by altering the composition and activity of fungal decomposers. This research improves our understanding of the biological mechanism through which an agent of global change alters biogeochemical cycling in temperate forest ecosystems.

CHAPTER 1
Introduction

Nitrogen deposition as an agent of global change

Nitrogen (N) makes up ~80% of the Earth's atmosphere as inert N_2 gas. However, N_2 is not a biologically available form of this nutrient, which essential to all life. For most of Earth's history, the transfer of N from the atmospheric pool to the biosphere was limited by the rates of microbial nitrogen fixation, and, to a lesser extent, fixation by lightening. Nitrogen fixation is an energetically expensive process restricted to specialized groups of bacteria. Moreover, N availability within ecosystems depended upon transfers between biological pools until N was returned to the atmosphere via denitrification (Schlesinger 1997, Ward 2011).

However, human activity has dramatically altered fluxes of N between atmospheric, terrestrial, aquatic, and marine pools. Anthropogenic inputs of reactive N (N_r) now exceed those provided by natural processes (Vitousek et al. 1997a, Vitousek et al. 1997b). During the last century, N_r emissions have increased between three-fold and five-fold as a result of human activities (Denman and Brasseur 2007). Anthropogenic emissions of ammonia (NH_3), nitric oxide (NO), and nitrogen dioxide (NO_2) gases are returned to the surface of the Earth via wet and dry N deposition. Volatilization of ammonia fertilizer to NH_3 has led to increased NH_4^+ deposition. Internal combustion of fossil fuels has been a major source of increased NO_x emissions in the industrialized world; biomass burning and volatization of NO_x gases from agricultural systems also contribute increased NO_x fluxes to the atmosphere (Galloway et al.

2004, Chapin et al. 2011). The majority of atmospheric N deposition occurs in terrestrial and coastal ecosystems (Galloway et al. 2004). In terrestrial ecosystems, atmospheric N deposition is predicted to increase by a factor of 2.5 over the next century (Lamarque et al. 2005). This represents a major biogeochemical change for terrestrial ecosystems.

Nitrogen deposition and N retention in temperate forests

Ecosystem response to increased levels of N deposition depends upon whether the amount and duration of deposition has exceeded the ecosystem's capacity to retain N (Aber et al. 1989, Fenn et al. 1998). Nitrogen is retained in terrestrial ecosystems through plant uptake, microbial immobilization, and abiotic immobilization (Zak et al. 1990, Johnson 1992, Nadelhoffer et al. 1995, Fenn et al. 1998). Most retention of nitrogen is biotic (Davidson et al. 1992, Johnson 1992); however, the presence of high quantities of soil organic matter (SOM) can contribute to abiotic retention of N (Mortland and Wolcott 1965, Nõmmik 1965).

Factors such as stand age, overstory composition, and soil properties affect biotic retention and, therefore, total ecosystem N retention (Vitousek and Reiners 1975, Vanmiegroet and Cole 1984, Aber et al. 1995, Högberg 2012). Land-use and disturbance history can influence these factors and indirectly affect the ability of a system to retain N (Magill et al. 1996, Magill et al. 1997). Historic land-use that removed large amounts of N deposition can be a better predictor of ecosystem N status than current N deposition levels (Aber et al. 1998). While disturbed ecosystems lack the biomass to retain additional N, early successional ecosystems are accumulating N as biomass increases following disturbance and would be expected to internally retain additional N from atmospheric deposition. However, late-successional systems with large internal N pools in which there is efficient N cycling may have limited ability to retain any

additional N within the system (Vitousek and Reiners 1975, Aber et al. 1989, Fenn et al. 1998). Moreover, ecosystems in which N is in steady-state through cycling, rather than accumulating N, will be those most likely to reach N saturation via additional anthropogenic inputs of N (Aber et al., 1989). However, increasing NPP in the Earth's forests suggests that many forests are not yet N-saturated (Magnani et al. 2007, Chapin et al. 2011). The effects of N deposition on ecosystems may change over time as an ecosystem moves from N limited to N saturated (Aber et al., 1989).

Effects of increased N deposition on temperate forest ecosystems

Increases in the atmospheric deposition of N can have far-reaching consequences on forest ecosystems. One well-known consequence of elevated N deposition is its potential to affect soil cation nutrient concentrations, soil pH and, thus, plant health. Excess N from atmospheric deposition is often leached from soil as nitrate (NO_3^-) (MacDonald et al. 2002). The negatively charged NO_3^- can co-leach positively charged ions (Ca^{2+}, Mg^{2+}, K^+) which are important plant nutrients (Currie et al. 1999). Additionally, wet deposition of N as HNO_3 is part of acid rain (Likens et al. 1996). Increased soil acidity from acid deposition can further exacerbate losses of these nutrients in poorly-buffered soils with a limited capacity for cation retention (Likens et al. 1996). This can have a deleterious effect on plants requiring high amounts of these nutrients, growing on soils in which availability of these nutrients was already limited (Zaccherio and Finzi 2007). Furthermore, reductions in soil pH can release Al^{3+} in soil, which can be toxic for plants (Johnson et al. 1991, Matzner and Murach 1995). However, the effects of co-leaching and acidity are not as pronounced on well-buffered soils with large cation exchange capacities and high nutrient concentrations, conditions which are present in the forest stands studied in this dissertation (Patterson et al. 2012).

However, increased rates of N deposition also impact temperate forest ecosystems by increasing N availability. Plant growth in terrestrial ecosystems is often N limited (LeBauer and Treseder 2008). Because of this, elevated N deposition leads to increases in net primary productivity (NPP) (Nadelhoffer et al. 1999, Magnani et al. 2007). Together, increased N availability and increased productivity can lead to changes in carbon (C) allocation within plant tissues. Increased root turnover, lower root biomass, increased foliar biomass, and increased woody biomass were originally proposed as possible plant physiological responses to rising N deposition in forests (Peterson and Melillo 1985, Aber et al. 1989). Furthermore, plant allocation to mycorrhizal fungi and rhizosphere microbes are likely to decline as anthropogenic N deposition increases (Aber et al. 1989). Such potential changes in plant allocation to tissues and to microbes have the potential to further impact ecosystem C storage and nutrient cycling.

The increase in plant available N that results from elevated N deposition also causes an increase in N concentrations in foliage (Koerselman and Meuleman 1996, Verhoeven et al. 1996). This, in turn, leads to leaf litter which is enriched in N because not all N is translocated from the foliage upon leaf senescence (Flanagan and Vancleve 1983). Foliar increases in N can, therefore, impact not only productivity, but also the process of microbial N mineralization and decomposition. Decomposer microbes carry out N mineralization, the process of converting organic N in plant litter into NH_4^+, which makes N available for plant assimilation; microbial N-mineralization is thus coupled with the C-cyling process of decomposition. However, microorganisms can also directly assimilate inorganic N and compete with plants for this resource (Zak et al. 1990, Zogg et al. 2000). Therefore, N deposition has the ability to alter microbial N and C cycling by changing the availability of inorganic and organic N in litter and soil pools.

Initially, it was predicted that increases in N deposition would stimulate decomposition by either 1) reducing the C:N ratio in plant litter and, thereby, increasing its lability to microbes or 2) increasing the amount of N available in the soil and, thus, allowing for increased microbial decomposition of litter with high C:N ratios. (Aber et al. 1989, Taylor et al. 1989, Prescott 1995). For example, under conditions of N limitation, N-rich leaf litter decomposes more rapidly than litter with low N content (Gosz 1981, Taylor et al. 1989). However, others suggested that increases in inorganic N availability, resulting from elevated rates of N deposition, would instead inhibit microbial decomposition by reducing N-mineralization rates (Fog 1988). Indeed, increased N availability tends to stimulate early stages of decomposition, but retard the later stages of litter decomposition (Berg and Staaf 1980, Berg and Staaf 1981, Berg et al. 1987, Fog 1988, Nilsson 1995). Similarly, increasing rates of N deposition can stimulate microbial decomposition of labile litter, but slow the decomposition of recalcitrant litter (Waldrop et al. 2004); thus, the effects of increased N deposition on plant litter decomposition can vary across forest ecosystems (Waldrop et al. 2004). Nevertheless, after decades of study, metaanalyses suggest that N deposition often has an inhibitory effect on this ecosystem process, rather than a stimulating one (Janssens et al. 2010, Liu and Greaver 2010).

Efforts to understand the effects of N deposition on decomposition have often focused specifically on lignin decay because lignin is the constituent of plant litter which limits the rate of decomposition (Fogel and Cromack 1977, Berg and Staaf 1980, Melillo et al. 1982). Lignin is a recalcitrant plant compound composed of phenolpropane units linked by carbon-carbon and ether linkages. Lignin makes up 15-30% of the mass of lignocellulose, a complex formed from cellulose, lignin, and hemicelluloses. Together, cellulose and lignin constitute the two most abundant biopolymers on Earth (Lee 1997, Kogel-Knabner 2002, Yadav and Malanson 2007).

Fungi mediate critical processes during lignin decay in terrestrial ecosystems (de Boer et al. 2005, Bugg et al. 2011, Floudas et al. 2012). Saprotrophic fungal decomposers break down lignin in order to access the energy-rich cellulose and hemicellulose which are protected by lignin; lignin alone is not a substrate for fungal growth. However, fungi also attack lignin to obtain N which would otherwise be unavailable because it is protected by lignin or complexed with partially-decomposed lignin derivatives in soil organic matter (Cairney and Burke 1994, Bending and Read 1997, Martin and Selosse 2008, Courty et al. 2009). For example, some ectomycorrhizal fungi possess lignolytic genes (Bodeker et al. 2009, Courty et al. 2009) and it has been suggested that ectomycorrhizae might express these genes in order to obtain N (Burke and Cairney 2002).

Experimental evidence suggests that increasing N availability reduces fungal lignin decomposition. High levels of inorganic N levels can suppress lignin degradation by the wood-rotting basidiomycete *Phanerochaete chrysoporium* (Weinstein et al. 1980, Fenn and Kirk 1981), although this response is not always consistent across fungal taxa (Leatham and Kirk 1983, Boyle et al. 1992). Furthermore, N concentrations may regulate fungal production of lignin degrading enzymes and their activity in some situations (Boominathan et al. 1990, Vanderwoude et al. 1993, Soden and Dobson 2001, 2003). Declines in the enzyme activity and gene expression of laccase, an enzyme involved in lignin depolymerization, have been reported under field conditions (Carreiro et al. 2000, DeForest et al. 2004b, a, Waldrop et al. 2004, Edwards et al. 2011). These changes in fungal activity may underlie observed reductions in decomposition under higher rates of N deposition.

If atmospheric N deposition suppresses the production and activity of fungal lignolytic enzymes, then fungi that decompose lignin might have less access to energy-souces (*i.e.,*

cellulose, hemicelluloses) that are protected by lignin. These fungi could be placed at a competitive disadvantage, relative to other fungi that target accessible cellulose and hemicellulose without first breaking-down the protective lignin. If lignolytic fungal taxa are at a disadvantage under higher rates of N deposition, this could result in a decline of their representation within the saprotrophic fungal community and a change in fungal community composition. Determining the underlying mechanism of this response was the primary focus of my dissertation research.

Summary of previous findings of a long-term atmospheric N deposition experiment in northern hardwood forests of Michigan

A long-term field study in Michigan has been examining the effects of experimental N deposition on northern hardwood forests, and I used this experiment to test my ideas regarding the effect of anthropogenic N depositon on fungal decomposers. Since 1994, a series of northern hardwood stands have received N additions at a rate predicted to occur in the near future (3 g NO_3^--N m^{-2} y^{-1}). These forest stands span a gradient of temperature, precipitation, and ambient atmospheric N deposition (Figure 1.1, Table 1.1).

This long-term experiment has produced some results consistent with hypotheses for N deposition on forests (Aber et al. 1989, Aber et al. 1998) and some findings that run counter to those expectations. For example, as predicted, both NPP and foliar N have increased in response to experimental N deposition (Zak et al. 2004, Pregitzer et al. 2008). The biomass of wood, but not foliage, has increased under experimental N deposition (Pregitzer et al. 2008). Additionally, the biomass, turnover, or respiration of fine roots does not appear to have changed under experimental N deposition (Burton et al. 2004).

This experiment has provided many insights into the effect of N deposition on the process of decomposition. Chronic experimental N deposition has slowed plant litter decay and increased soil organic matter (Pregitzer et al. 2008, Zak et al. 2008). Forest floor biomass has increased under experimental N deposition, a result which is due to decreased decomposition rather than increased leaf production (Pregitzer et al. 2008, Zak 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). In addition to biogeochemical changes, N deposition has caused observable changes in the activity of fungal decomposers. For example, experimental N deposition has decreased both the activity of lignolytic enzymes (DeForest et al. 2004b) and the abundance of mRNA transcripts for lignolytic enzymes (Edwards et al. 2011).

This long-term N deposition experiment offered me a unique opportunity to investigate whether changes in decomposer community composition are co-occurring with decreases in decomposer activity. I have conducted several experiments to examine the response of the fungal community to long-term experimental N deposition. While this long-term experiment consists of four replicate sites, for logistical reasons, the experiments outlined in Chapter 2 were conducted in only two of the four sites (Sites D & B, Figure 1.1, Table 1.1), and the experiments described in Chapter 3 were conducted for the southernmost site only (Site D, Figure 1.1, Table 1.1). The experiments in Chapter 4, however, include all four of the experimental sites (Figure 1.1, Table 1.1).

Chapter 2 asks "Does atmospheric N deposition alter the diversity, composition, or structure of the active fungal community in the forest floor?" To help me understand whether changes in the fungal community under N deposition are related to lignin decomposition,

Chapter 3 asks "Does experimental N deposition reduce the abundance of lignolytic fungi?" The experiments in this chapter examined the fungal communities on decomposing substrates that vary in their initial lignin, cellulose, and hemicellulose content over the course of decomposition. Chapter 4 focuses on potential effects of experimental N deposition on the function of fungal communities by asking "Does experimental N deposition alter the composition, richness, or diversity of expressed lignolytic genes?" Specifically, this chapter examines fungal class II peroxidases, an important fungal enzyme for lignin decay. If experimental N deposition alters the composition or diversity of expressed fungal lignolytic peroxidases, this could reflect reduced enzymatic capabilities of the fungal community to attack lignin.

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TABLES

Table 1.1. Climatic, floristic, and edaphic properties of two northern hardwood forests receiving experimental N deposition. Both of these sites were included in Chapter 1. Field experiments for Chapters 2 and 3 were conducted in Site D only. Two other sites, A and C, are also part of this long-term N depositional field experiment. However, they are not included in this table because they were not included in the studies proposed here.

Characteristic	**Site**	
	B	**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 minutes throughout the year, with averages recorded every 3 h using data loggers (EasyLogger Models 824 and 925, Data Loggers, Inc., Logan UT).

§MacDonald et al. 1992

¶D.R. Zak, *unpublished data*

FIGURES

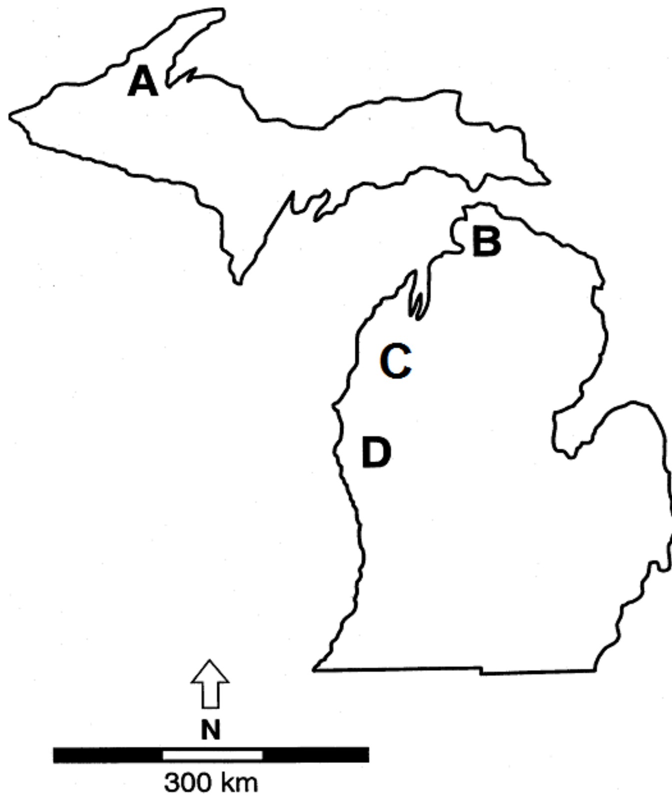

Figure 1.1. Map of four replicate northern hardwood forests part of a long-term N deposition experiment. These four forests lie along a gradient of climate and ambient N deposition. Temperature and ambient N deposition decrease across this gradient from south to north. Precipitation increases along the gradient from south to north. Chapter 1 experiments were conducted in Sites B and D; experiments in Chapters 2 and 3 were conducted in Site D only.

CHAPTER 2

Long-term experimental nitrogen deposition alters the composition of the active fungal community in the forest floor

ABSTRACT

Global increases in the rate of atmospheric nitrogen 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, USA. 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.

INTRODUCTION

Emissions of reactive nitrogen (N) have increased 300-500% 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 carbon (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, 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 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 that 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 (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} y^{-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 in order to examine the richness, diversity, composition, and structure of the active Dikarya fungi.

METHODS

Site Description

Our study sites consisted of two northern hardwood forest stands that are dominated by sugar maple (*Acer saccharum* Marsh.) in Michigan, USA (Table 2.1). 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, USA. 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 2.1). 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 2.1). The O_i horizon at each site is dominated by sugar maple leaf litter.

Each site contains six 30-m x 30-m plots; three plots receive ambient N deposition, whereas the other 3 receive ambient N deposition plus 3 g NO_3^- -N m^{-2} y^{-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 6 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 3 plots receiving ambient N deposition and 3 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 ten 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. Prior to 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 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 minutes. 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. PCR 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, http://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 non-fungal, 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 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 (Appendix A). Within 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 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 - KC701965 (Appendix B).

An unequal number of Dikarya sequences were recovered among sites and N deposition treatments. Therefore, it was necessary to subsample sequences prior to 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 prior to 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 (Appendix A). Maximum-likelihood trees were created using the PhyML plug-in (Guindon and Gascuel, 2003) for Geneious with a 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 prior to 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. S2.1).

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/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. *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. S2.2. 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 S2.1).

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 2.2 & 2.3). Shannon diversity was not significantly different under ambient and experimental N deposition at Site B (Table 2.4). 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 2.4).

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 2.5). 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 2.5). 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 2.5), but not at Site D (weighted Unifrac, $P = 0.76$, Table 2.5). Phylogeny and sequence distribution covaried for ambient and experimental N deposition environments at Site D (Martin's P-test, $P < 0.0001$, Table 2.2), but this was not true at Site B (Martin's P-test, $P = 0.15$, Table 2.5).

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. 2.1a). No other taxonomic groups responded significantly to N deposition at Site B (Fig. 2.1a). 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. 2.1b); 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. 2.1b). 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. 2.1b).

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. In a spruce (*Picea abies*) forest, 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. In a beech-oak forest, 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 in our study because the active community is mediating the function (*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 (Table 2.3 &2. 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 (Table 2.3 & 2.4). The differences in ambient atmospheric N deposition between the sites are small relative to our experimental N deposition treatment (Table 2.1) 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 2.1). 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, e.g., 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. We had hypothesized experimental N deposition could alter the composition of active Dikarya fungi through through the loss or gain of lineages under experimental N deposition (Unifrac), the covariation of lineages with N deposition treatment (Martin's P-test), or the individuals of a single N deposition treatment being more closely related to each other than they are to members of the other N deposition treatment (weighted Unifrac). A significant response to even one of these β-diversity metrics would be consistent with our prediction that experimental N deposition alters active fungal community composition; 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.

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 2.5). 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 2.2-2.4) and β-diversity measures (Table 2.5). 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. 2.1).

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.

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TABLES

Table 2.1. Climatic, floristic, and edaphic properties of two northern hardwood forests receiving experimental N deposition.

Characteristic	Site	
	B	**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 minutes throughout the year, with averages recorded every 3 h using data loggers (EasyLogger Models 824 and 925, Data Loggers, Inc., Logan UT).

§MacDonald et al. 1992

¶D.R. Zak, *unpublished data*

Table 2.2. Chao1 richness for Dikarya 28S rRNA cDNA libraries from two hardwood forest stands (B and D) under ambient and experimental N deposition. Libraries were subsampled to equalize sample sizes across stands and N deposition treatments prior to clustering. Clustering of operational taxonomic units (OTUs) at three sequence similarity levels (99%, 95%, 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.

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)

Table 2.3. Inverse Simpson diversity for Dikarya 28S rRNA cDNA libraries from two hardwood forest stands (B and D) under ambient and experimental N deposition. Libraries were subsampled to equalize sample sizes across stands and N deposition treatments prior to clustering. Clustering of operational taxonomic units (OTUs) at three sequence similarity levels (99%, 95%, 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.

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

Table 2.4. Shannon diversity for Dikarya 28S rRNA cDNA libraries from two hardwood forest stands (B and D) under ambient and experimental N deposition. Libraries were subsampled to equalize sample sizes across stands and N deposition treatments prior to clustering. Clustering of operational taxonomic units (OTUs) at three sequence similarity levels (99%, 95%, 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. Comparisons between N deposition treatments within a site and OTU level for which confidence intervals do not overlap ($P < 0.05$) are marked with an asterisk (*).

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)

Table 2.5. 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 signficance**
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***

FIGURES

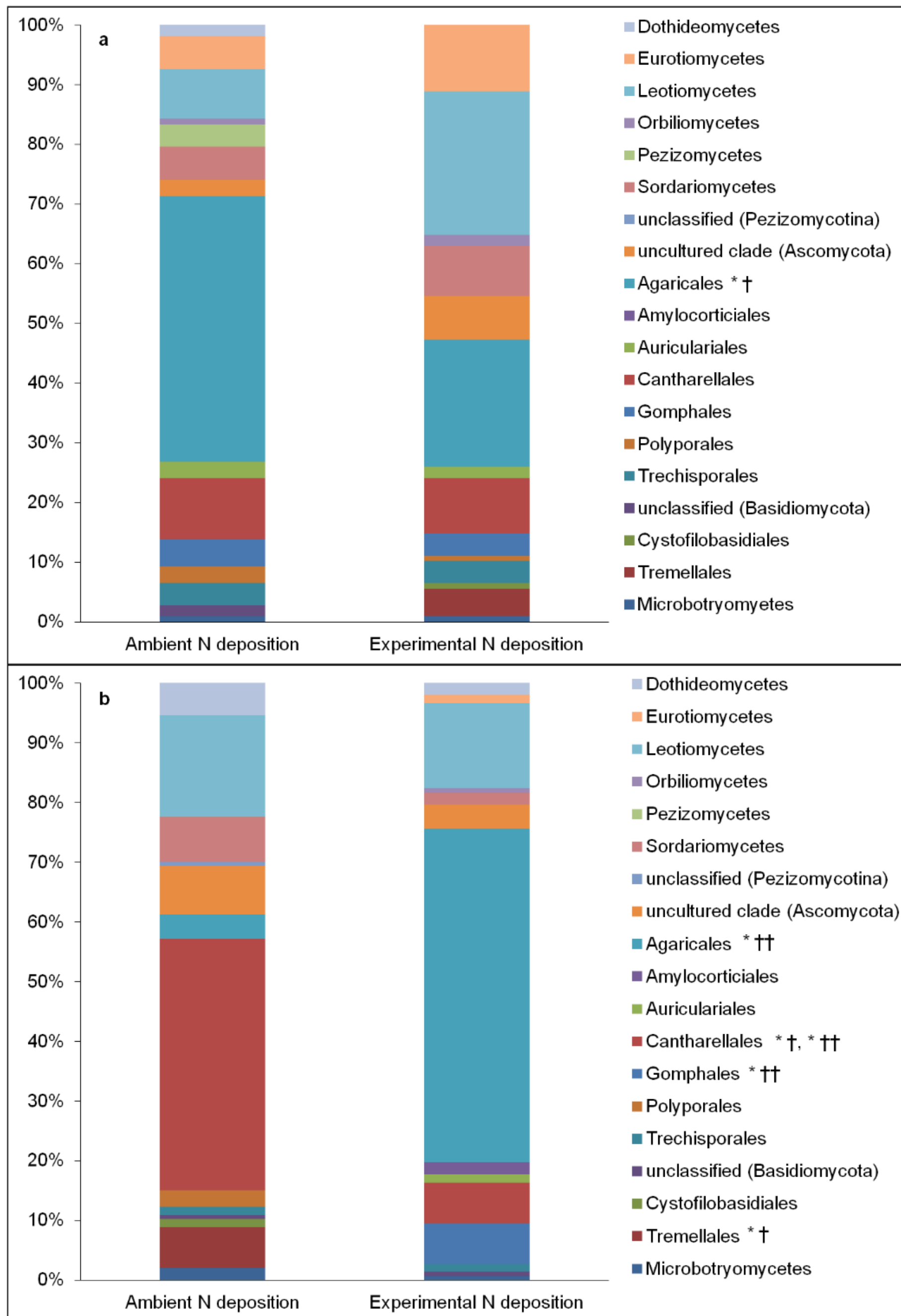
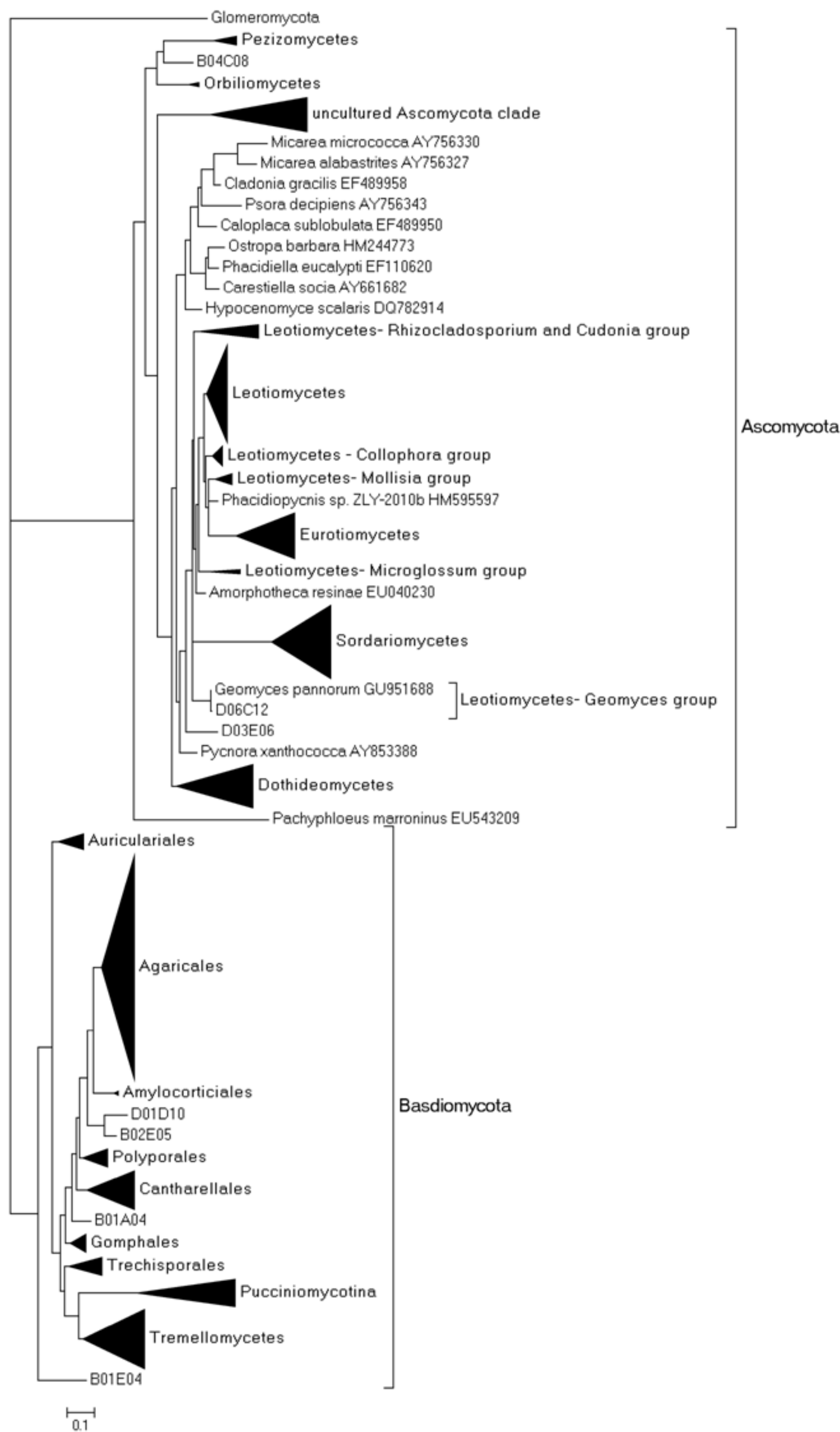


Figure 2.1. Relative abundances (%) of taxa in Dikarya communities under ambient N deposition and experimental N deposition (+ 3 g NO_3^- -N m^{-2} y^{-1}) in two northern hardwood forests, a) Site B and b) Site D. More information on these sites is provided in Table 2.1. Taxa for which a significant 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 an †. A significant difference in abundance between ambient and experimental N deposition treatments according to a two-tailed Student's *t*-test is denoted with a ††. Dothideomycetes through uncultured group (Ascomycota) represent taxonomic groups from the Ascomycota, while Agaricales through Microbotryomycetes represent taxonomic groups from the Basidiomycota.

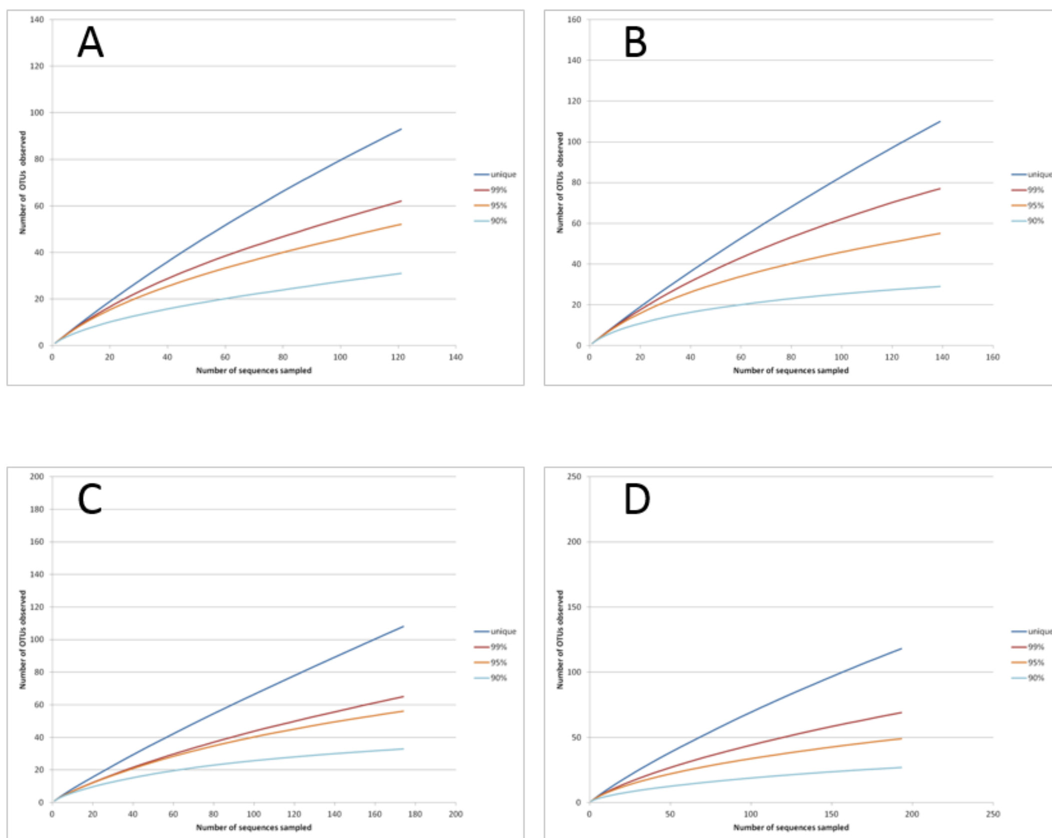
Supplemental Table S2.1. Estimation of Good's coverage for Dikarya sequences recovered in fungal 28S rRNA cDNA clone libraries from two northern hardwood forest stands under ambient and experimental N deposition.

Site, N deposition treatment	OTU similarity level			
	100%	**99%**	**95%**	**90%**
B, ambient	0.38	0.64	0.72	0.83
B, experimental	0.34	0.65	0.78	0.92
D, ambient	0.46	0.74	0.82	0.93
D, experimental	0.52	0.77	0.87	0.93



Supplemental Figure S2.1. Maximum likelihood tree of Dikarya fungi from two northern hardwood forests receiving ambient and experimental N deposition. Tree includes randomly selected representatives of subsampled 99% sequence similarity OTUs, 183 Dikarya reference sequences from GenBank, and a Glomeromycota outgroup sequence. Branches have been

collapsed and labeled so that the tree can be displayed on a single page. Most Leotiomycetes formed a single clade in our tree, however, *Rhizocladosporium* & *Cudonia*, *Collophora*, *Mollisia*, and *Microglossum* clades were separate from the main Leotiomycetes clade. We labeled these clades as Leotiomycetes here because they have been considered Leotiomycetes in the literature. In Crous et al., 2007, *Rhizocladosporium* formed a clade separate, but related to Helotiales; *Cudonia* has been placed in the Rhystimataceae (Wang et al., 2002; Wang et al., 2006b) which is considered part of the Leotiomycetes (Wang et al., 2006b; Lantz et al., 2011). *Mollisia* has been placed in the Helotiales (Crous et al., 2003; Wang et al., 2006a). *Collophora* has been placed in the Leotiomycetes (Damm et al., 2010). *Microglossum* has also been included in the Leotiomycetes (Spatafora et al., 2006; Wang et al., 2006a).



Supplemental Figure S2.2. Rarefaction curve of observed OTU richness in northern hardwood forest stands at Site B (panels A-B) and Site D (panels C-D) receiving ambient (panels A & C) and experimental (panels B & D) rates of N deposition. Rarefaction curves were generated by a re-sampling without replacement approach in MOTHUR. Points on the curve represent average richness obtained for 1000 iterations. Lines represent rarefaction curves for unique sequences, 99% similarity OTUs, 97% similarity OTUs, 95% similarity OTUs, 90% similarity OTUs, respectively.

CHAPTER 3

Anthropogenic N deposition increases soil C storage by reducing the relative abundance of lignolytic fungi

ABSTRACT

The rate of atmospheric nitrogen (N) deposition has increased dramatically since preindustrial times and continues to increase across many regions of the Earth. In temperate forests, this agent of global change has increased soil carbon (C) storage, but the mechanisms underlying this response is not understood. One long-standing hypothesis proposed to explain the accumulation of soil C proposes that higher inorganic N availability may suppress both the activity and abundance of fungi which decay lignin and other polyphenols in soil. In field studies, elevated rates of N deposition reduce the activity of enzymes mediating lignin decay, but a decline in the abundance of lignolytic fungi has not been definitively documented to date. Here, we tested the hypothesis that elevated rates of anthropogenic N deposition reduce the abundance of lignolytic fungi. We conducted a field experiment in which we compared fungal communities colonizing low-lignin, high-lignin, and wood substrates in a northern hardwood forest that is part of a long-term N deposition experiment. We reasoned that, if lignolytic fungi decline under experimental N deposition, this effect should be most evident among fungi colonizing high-lignin and wood substrates. Using molecular approaches, we provide evidence that anthropogenic N deposition reduces the relative abundance of lignolytic fungi on both wood and a high-lignin substrate. Furthermore, experimental N deposition increased total fungal abundance on a low-lignin substrate, reduced fungal abundance on wood, and had no significant effect on a high-lignin

substrate. We simultaneously examined these responses in the surrounding soil and forest floor, in which we did not observe significant reductions in the relative abundance of lignolytic fungi or in the size of the fungal community; however, we did detect a change in community composition in the forest floor that appears to be driven by a shift away from lignolytic fungi and towards cellulolytic fungi. Our results provide direct evidence that reductions in the abundance of lignolytic fungi are part of the mechanism by which elevated rates of anthropogenic N deposition increase soil C storage.

INTRODUCTION

Human activity has dramatically increased the production of reactive N (N_r) compounds, with global N_r emissions projected to further double by mid-century (Galloway et al. 2004). As a result, the annual amount of atmospheric N_r deposition in terrestrial and coastal ecosystems has increased dramatically over historical levels and continues to rise in some parts of the Earth (Galloway et al. 2004). Rising rates of N_r deposition reduce soil respiration (Janssens et al. 2010) and increase soil C content (Frey et al. 2014) in temperate forest soils. Therefore, atmospheric N deposition has the potential to influence the storage of C in soils, which are a large and globally important C pool (Prentice et al. 2001).

Field studies have suggested that elevated rates of N deposition may increase soil C content by depressing the rate of lignin decay (Berg and Matzner 1997, Waldrop and Zak 2006). Lignin is a recalcitrant, protective compound found in plant secondary cell walls which limits the rate of plant litter decay (Melillo et al. 1989). Fungi are the primary mediators of lignin decay in terrestrial ecosystems (de Boer et al. 2005, Bugg et al. 2011, Floudas et al. 2012) and changes in soil fungal communities, mediated by greater rates of N deposition, may be one plausible mechanism by which soil C storage has increased. For example, high inorganic N concentrations

can suppress fungal lignin decay in culture (Fenn and Kirk 1981, Leatham and Kirk 1983) as well as on inoculated natural substrates (Osono et al. 2006); however this effect is not universal among fungal species (Kaal et al. 1995). Similarly, increasing rates of N deposition reduce lignolytic enzyme activity in forest leaf litter, suggesting that high inorganic N availability also suppresses fungal lignolytic enzyme activity under field conditions (Carreiro et al. 2000, DeForest et al. 2004a). Furthermore, it has been hypothesized that, by reducing the activity of lignolytic fungi, anthropogenic N deposition will weaken the competitive advantage of these organisms over other decomposers and result in a decrease in their abundance within the microbial community (DeForest et al. 2004b). The biological mechanism proposed to explain increased soil C storage under elevated N deposition posits that higher N availability reduces both the activity and abundance of fungi involved in lignin decay (DeForest et al. 2004b, a).

However, it has remained uncertain whether lignolytic fungi actually decline under elevated rates of N deposition. For example, elevated N deposition has been observed both to significantly alter (Eisenlord et al. 2013, Entwistle et al. 2013, Hesse et al. 2015) and to have no effect (Edwards et al. 2011, Freedman et al. 2015) on fungal community composition. Furthermore, even when elevated N deposition has been observed to alter fungal community composition, it is not clear whether these changes involve a decline in lignolytic fungi. However, these previous studies of fungal response to N deposition examined fungal taxonomy only at only the order, class, or phylum level; because these higher taxonomic groupings contain fungi with multiple nutritional modes and diverse abilities to decay lignin, this has thus far limited the degree of insight into the physiology and ecology of fungal taxa affected by anthropogenic N deposition that has been obtained. For some of these studies (Edwards et al. 2011, Entwistle et al. 2013), small library sizes necessitated grouping sequences at higher

taxonomic levels, a limitation which we have overcome in this study by employing next-generation sequencing. Additionally, we have taken advantage of improved tools for classifying fungal sequences (Liu et al. 2012, Schloss et al. 2016) and recent advances in our understanding of the phylogenomic distribution of fungal genes involved in lignin decay (Floudas et al. 2012, Nagy et al. 2015) in order to achieve a better understanding of how experimental N deposition has affected lignolytic fungi.

One way to directly observe the role of lignin and its decomposers in response to N deposition is to compare fungal communities colonizing low-lignin and high-lignin substrates under ambient and elevated rates of N deposition. We hypothesized that anthropogenic N deposition slows decomposition by reducing the abundance of fungi that mediate the process of lignin decay, specifically the abundance of fungi with known lignolytic physiologies. If increasing rates of N deposition alter fungal community composition in a manner consistent with our proposed mechanism, we reasoned that these effects should be more evident in fungal communities colonizing lignified substrates than on those colonizing low-lignin substrates. Furthermore, we hypothesized that these effects would become more apparent with time because, over the course of decomposition, cellulose and hemicellulose are decomposed more rapidly than lignin, rendering partially decayed materials relatively enriched in lignin. Additionally, if lignolytic fungi are suppressed by higher rates of N deposition, we would anticipate that a greater percentage of plant carbohydrates would remain protected by unmetabolized lignin and, therefore, would be unavailable as energy resources to most fungi; for this reason, we hypothesize that the overall size of the fungal community will be smaller under higher rates of N deposition.

To test our hypotheses, we allowed low-lignin, high-lignin, and wood substrates to be colonized by fungi in in a northern hardwood forest that has received experimental N deposition since 1994. We investigated whether experimental N deposition altered fungal community composition on decomposing substrates that vary in their biochemical composition, and subsequently examined which types of fungal physiologies primarily account for dissimilarities in fungal community composition under experimental N deposition. Furthermore, we directly tested whether fungal groups involved in lignin decay, specifically Agaricomycetes, as well as highly lignolytic taxa within the Agaricomycetes, decline in response to experimental rates of N deposition. Lastly, we assessed fungal abundance on each of our substrates over time using quantitative PCR (qPCR). We also assessed these responses for fungal communities in the mineral soil and the forest floor.

METHODS

Site Description. Our study site consists of a sugar maple (*Acer saccharum* Marsh.) dominated northern hardwood forest in Lower Michigan, located at 43°40' N, 86°09' W. This site represents one of the four sites in the Michigan Gradient Experiment, a long-term elevated atmospheric N deposition experiment in Michigan, USA. Soils are well-drained sandy typic Haplothords of the Kalkaska series; the forest floor (O_i) is dominated by sugar maple leaf litter.

The site contains six 30-m x 30-m plots; three plots receive ambient N deposition, whereas the other 3 receive ambient N deposition plus 3 g NO_3^- -N m^{-2} y^{-1}. This amount has been added since 1994 and is consistent with future levels expected portions of northeastern North America and Europe by 2050 (Galloway et al. 2004). Treatments are applied as $NaNO_3$ pellets in 6 equal additions of 0.5 g N m^{-2} from April to September. Each treatment plot is surrounded by a 10-m treated buffer to reduce edge effect, which also receives the

aforementioned N deposition treatments. Experimental N application has not altered soil pH, base saturation, matric potential, or forest floor conductivity (Patterson et al. 2012).

Substrate selection and deployment. Different products of the forest industry provided us with materials which vary in their biochemistry, enabling us to test our hypotheses. For example, newsprint is produced by mechanical pulping, with the resulting paper having a high lignin content (Ragnar et al. 2000). In contrast, kraft paper is subjected to chemical pulping processes which removes 50% of its lignin (Kleppe 1970), as well as breaking bonds between lignins, cellulose, and hemicelluloses (Ragnar et al. 2000). Wood, by contrast, has not been subject to any form of physical or chemical alteration; it has intact cell walls and intact lignocellulose. In our study, we use birch wood (*Betula* spp.; Woodsies, Loew Cornell, Cincinnati, OH), a high-lignin paper substrate (newsprint, white 3401, Pacon Corp., Appleton, WI), and a low-lignin paper substrate (kraft paper; #60, Caremail, ShurTech Brands, LLC Avon, OH) in a field decomposition experiment to make comparisons between fungal communities targeting weakly and highly lignified substrates. To measure the initial biochemistry of these materials, we submitted dried, ground kraft paper, newsprint, and wood samples to the Soil & Forage Lab at the University of Wisconsin-Madison for analysis of lignin, cellulose, hemicelluloses, and nitrogen content (Table 3.1).

Bags of nylon screen with a mesh size of 1.13 mm x 1.30 mm were filled with ~6 g of either kraft paper or newsprint, an amount represented by four pieces kraft paper or eight pieces of newsprint (10.8 cm x 13.97 cm), in order to give the low and high lignin paper substrates similar initial mass and surface area. The wood substrate consisted of a stick 15 cm x 2 cm x 0.15 cm; we drilled a hole near one of the stick to attach a tether. We recorded the initial mass of

each substrate sample. One substrate of each type was tethered to a single plastic stake with < 0.5 m of monofilament.

Our decomposition experiment was initiated in November 2011. Within each ambient and experimental N deposition plot, substrates were placed at 5 points: 3 m from each corner of the rectangular plot and within 1 m of the plot center. At each of these five locations, we placed 2 stakes each with the 3 tethered substrates, enabling us to collect decomposing substrates over two dates. We removed the O horizon and placed the substrates on top of the mineral soil, and then covered the substrates by returning the O horizon.

Field collection. We collected substrates from the field after 7 and 18 months of decay. During our first collection date, we also collected the forest floor (O_i,O_e,O_a horizons) from a 100 cm^2 area and a 5 cm-deep mineral soil core from within 1 m of each substrate location to assess the initial composition and abundance of soil fungi. We transported collected substrates and environmental samples to the lab on ice. Samples were stored -80 °C prior to DNA extraction.

Sample preparation, homogenization, moisture content, and mass loss. Post-collection, samples were weighed to determine mass loss. Soil cores were manually homogenized inside a sterile plastic bag. We cut leaf litter and high and low-lignin substrates with sterilized scissors into ~25-mm^2 pieces and manually homogenized the material. Wood was first cut into ~8 mm^2 pieces with sterilized pruning shears, which were then placed inside a sterile plastic bag, mixed, and pulverized with a hammer. A subsample was dried for 24 h at 105 ° C to determine moisture content. Another subsample of homogenized samples was taken for DNA extraction.

DNA extraction. We extracted DNA from each sample in triplicate using a MoBio PowerLyzer® PowerSoil® DNA isolation kit. We added ~0.125 g forest floor, ~0.18 g wood, or ~0.25 g soil, ~0.25 g high-lignin substrate, or ~0.25 g low-lignin substrate to a MoBio

Powerlyzer® Glass Bead Tube with 0.1 mm glass beads. We bead-beat the material for extraction on a MoBio PowerLyzer® 24 bench top bead-based homogenizer for 45s at 2500 rpm for soil, 1 min at 3500 rpm for forest floor, 1 min at 4500 rpm for wood, or 45s at 4500 rpm for the low-lignin and high-lignin substrates. We followed manufacturer's instructions with the several modifications. Specifically, we increased the time for incubations at 4 °C from 5 to 10 minutes, added two centrifugations in new microcentrifuge tubes to remove residual wash buffer and ethanol, and introduced a 10 minute ethanol evaporation step prior to DNA elution. Furthermore, DNA elution buffer was allowed to sit on the membrane for one minute before centrifugation; the supernatant containing eluted DNA was placed on the membrane for an additional minute and spun through a second time to maximize the quantity of DNA obtained. We measured the quality of the extracted DNA on a ThermoScientific NanoDrop™ 8000 UV-Vis Spectrophotometer. Triplicate DNA extractions were pooled prior to amplification.

Fungal 28S rDNA amplification and sequencing. We amplified the 28S rRNA gene using 10 barcoded pairs of fungal primers LR0R (5' ACCCGCTGAACTTAAGC 3') and LR3 (5' CCGTGTTTCAAGACGGG 3') (http://sites.biology.duke.edu/fungi/mycolab/primers.htm

). Barcode pairs 1, 42, 3, 40, 7, 33, 11, 17, 20, and 24 were selected from among 96 paired barcodes available for multiplexing on the Pacific BioSciences® RS II sequencer (https://github.com/PacificBiosciences/Bioinformatics-

Training/blob/master/barcoding/pacbio_barcodes_paired_nopadding.fasta). Reactions contained 2.5 µL dNTPs (2 mM each), 2.5 µL Roche Expand™ High Fidelity (10x concentrated) Buffer with 15 mM $MgCl_2$, 0.5 µL bovine serum albumin (20 mg mL^{-1}), 0.5 µL of barcoded LR0R (20 µM), 0.5 µL barcoded LR3 (20 µM), 16 µL molecular biology-grade water, 0.5 µL Roche Expand™ High Fidelity enzyme mix, and 2 µL of 10x diluted target DNA. For samples that

amplified weakly, PCR was repeated with template DNA with a lower dilution factor or no dilution until products of satisfactory concentration were obtained. Thermocycling conditions were: initial denaturation (5 min, 95 ˚C), followed by 25 cycles consisting of denaturation (30 s, 95 ˚C), primer annealing (30 s, 54 ˚C), extension (75 s, 72 ˚C), with cycling followed by a final extension (7 min, 72 ˚C). Each sample was amplified in triplicate and these triplicate products were pooled prior to purification.

We purified the PCR products using a Qiagen MinElute PCR Purification Kit. The quality of PCR DNA was measured with ThermoScientific NanoDrop™ 8000 UV-Vis Spectrophotometer, while the concentration of PCR products was measured fluorometrically using a Quant-IT™ PicoGreen® dsDNA kit on a BioTek® Synergy HT microplate reader. We combined 10 barcoded samples in equimolar concentrations for multiplexing on a PacBio® SMRT® chip. These were submitted to the University of Michigan sequencing core for analysis on a PacBio® RS II sequencer with P6-C4 chemistry.

28S rDNA sequence data processing. We processed our 28S rDNA sequences in mothur v.1.31.2 (Schloss et al. 2009). Briefly, we removed low quality sequences (qwindowsize=50, qwindowaverage=25), sequences with > 1 mismatch to the primer or barcode, sequences with > 1 ambiguous nucleotide, sequences with homopolymers of > 8 nucleotides, and sequences longer or shorter than our expected amplicon size (550-750 bp). We aligned sequences with the Ribosomal Database Project (RDP) version 7 fungal 28S rRNA (LSU) training set (Liu et al. 2012, Cole et al. 2014), which had been previously obtained from http://mothur.org/wiki/RDP_reference_files and aligned (Freedman et al. 2015) in Clustal Omega (Sievers et al. 2011) according to default parameters. Chimeras were identified with UCHIME (Edgar et al. 2011) and were removed. Sequences were classified using the mothur-

compatible taxonomy file that accompanied the RDP v.7 LSU fungal training set (available at: http://mothur.org/wiki/RDP_reference_files) using a bootstrap cutoff of 51. Non-fungal sequences and sequences that were not able to be classified beyond kingdom were removed. We clustered operational taxonomic units (OTUs) using the cluster.split command with sequences split for clustering at the class level. In order to help us identify the taxonomy of our OTUs, we selected the most abundant sequence in each OTU as a representative sequence which we could use to help us identify the OTU by conducting searches with National Center for Biotechnology Information (NCBI) BLAST®.

Fungal community composition and dissimilarity analyses. To test the hypothesis that experimental N deposition alters fungal community composition, we compared fungal communities from our ambient and experimental N deposition treatments with permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001). We followed PERMANOVA with similarity percentages (SIMPER) analyses (Clarke 1993) to test whether changes in fungal community composition in response to experimental N deposition observed with PERMANOVA were consistent with a shift in community composition from strong lignin decomposers towards weaker decomposers of lignin. We examined the composition of fungal communities under ambient and experimental N deposition on our substrates as well as in soil and forest floor using OTUs clustered at 97% sequence similarity. We removed singleton OTUs from the data set before calculating the proportional abundance of each OTU. We performed multivariate statistical analyses in the PRIMER-E, version 6, with the PERMANOVA+ add-on (Clarke and Gorley 2006). To prepare the data for analysis, we restricted analysis to include OTUs that comprised $\geq 0.5\%$ of at least one sample, applied the square root transformation, and calculated a Bray-Curtis similarity index.

We ran two-factor PERMANOVA with 9999 permutations and the default options for sum of squares and permutation of residuals. We were unable to perform individual comparisons for samples for which we have data for only one time-point (*i.e.,* the low-lignin substrate, soil, and forest floor) because the statistical power of our experiment (n = 3) is too low for PERMANOVA to be able to detect differences between communities even where they are present. Therefore, we analyzed all samples collected after 7 months of decay together with sample type and N deposition treatment as factors. For the high-lignin and wood substrates, we had data for samples collected at 7 and 18 months, allowing us to analyze communities on these substrates individually with N deposition treatment and time as factors.

We performed one-factor SIMPER analyses and considered each sample type and collection date separately. For each comparison, we reported the results for the ten OTUs accounting for the highest amount of dissimilarity between fungal communities under ambient and experimental N deposition. For each of these OTUs, we identified the closest known species for each OTU with a megablast search through the NCBI BLAST® portal. Based on our knowledge of the biology of the species identified by BLAST®, we assigned each OTU as 1) white-rot and lignolytic litter decay fungi, 2) soft-rot & cellulolytic/hemicelluloytic litter decay fungi, 3) brown-rot fungi, 4) weakly lignolytic fungi, or 5) mycorrhizal & biotrophic; we summarize which taxa were assigned to each of these categories in Appendix C. In order to determine whether fungi from each of these physiologies were responding positively or negatively to elevated rates of N deposition, we calculated the mean abundance of each of these physiologies under ambient and experimental N deposition, the 95% confidence intervals around those means, and the change in relative abundance for these physiologies under experimental N deposition.

Relative abundance of Agaricomycetes and highly lignolytic taxa. We hypothesized that experimental N deposition would reduce the relative abundance of lignolytic fungi, especially on high-lignin and wood substrates. To test this hypothesis, we examined the relative abundance of Agaricomyetes, because this class contains the most important lignin decomposers, and the relative abundance and of a subset of Agaricomycete taxa with highly lignolytic physiologies. We identified this suite of highly lignolytic Agaricomycete fungi *a priori* (Table 3.2 & Appendix D) based on the following criteria: 1) taxa had consistently demonstrated high lignin loss and/or selectivity for lignin in published studies and 2) were represented in our data by > 20 sequences. Highly lignolytic taxa are described in Table 3.2 and Appendix D, while a list of taxa excluded and our justifications for their exclusion are provided in Appendix E. Highly lignolytic taxa were summed together, rather than considered individually.

We examined the relative abundance of Agaricomycetes and highly lignolytic taxa under ambient and experimental N deposition on the three substrates, as well as in soil and forest floor. For each sample, we calculated the relative abundance of Agaricomycetes as a proportion of total fungal sequences and the relative abundance of highly lignolytic taxa as a proportion of Agaricomycete sequences. Statistical analysis of this data is described below.

Fungal ITS1 qPCR. Because experimental N deposition suppresses lignin decay, we reasoned that a greater proportion of cellulose and hemicellulose would remain protected by undecayed lignin and would thus remain inaccessible to the fungal community as an energy source. We hypothesized, then, that the overall size of the fungal community would be smaller under experimental N deposition, particularly on substrates with intact lignocellulose (*i.e.*, the wood and high-lignin substrate). Because leaf and root litter also contain lignocellulose, and because experimental N deposition reduces energy flow from detrital and microbial pools (Gan et al.

2013), we anticipated that experimental N deposition would reduce fungal abundance in soil and forest floor. We tested these hypotheses by conducting qPCR on the fungal ITS1 region to measure fungal abundance.

We selected the fungal ITS1 region for fungal qPCR because it is short (~300 bp) and it does not vary significantly in length among fungal phyla (Bellemain et al. 2010, Toju et al. 2012). Fungal primers ITS1f (5' CTTGGTCATTTAGAGGAAGTAA 3') and 5.8s (5' CGCTGCGTTCTTCATCG 3') used to amplify this region (Gardes and Bruns 1993, Vilgalys undated). We calculated ITS1 copy number for each sample as both number of copies per μg of extracted DNA and number of copies per g of soil, which enabled us to assess both the abundance of fungi as a proportion of the total microbial community DNA and total fungal abundance in soil, forest floor, and decomposing substrate biomass, respectively.

A qPCR standard was prepared by cloning the ITS1 region of *Agaricus bisporus* with the TOPO® TA Cloning ® Kit for Sequencing and the insert was subsequently amplified with M13 primer to create a linear target appropriate for qPCR (Hou et al. 2010). Standard DNA concentration was quantified fluorometrically as previously described and copy numbers were calculated according to manufacturer instructions (AppliedBiosystems 2013). Quantitative PCR was calibrated with a standard curve ranging from ranging from 1.71×10^3 to 1.03×10^6 copies/μL.

Prior to qPCR, we quantified initial DNA concentrations of all samples fluorometrically as previously described and diluted samples as necessary to bring target DNA concentration into the range of 0.8 – 11 ng μL^{-1}. Each 25 μL qPCR consisted of 1 μL of template DNA from samples or standards, 0.625 μL of each primer (20 μM), 0.375 μL ROX reference dye (500x dilution), 0.5 μL bovine serum albumin (20 mg mL^{-1}), 9.375 μL molecular biology-grade water,

and 12.5 µL Brilliant III Ultra-Fast SYBR® Green qPCR master mix (Agilent Technologies, cat. #600882). We amplified qPCRs on an Agilent Technologies Stratagene Mx3000P qPCR System using the following thermocycling program: initial denaturation (10 min, 95 ˚C), followed by 40 cycles of denaturation (45s, 95 ˚C), annealing (30s, 53˚C), and extension (30s, 72 ˚C), followed by a final dissociation curve set at manufacturer default settings. We ran all standards and no template controls in triplicate and all unknown samples in duplicate, allowing us to run to run all samples from the same set (*i.e.,* same sample type & collection date) in a single 96-well run. Each run was performed three times for quality control. All qPCRs used for data generation had efficiencies of 80 to 94% and R^2 values of 0.991 to 0.999.

Statistical analyses. We performed statistical analyses for substrate mass loss, the relative abundances of Agaricomycetes and highly lignolytic taxa, and qPCR data with IBM SPPS Statistics v. 23 software. Prior to analysis, values for individual samples were averaged across each plot. No data transformations were applied to either mass loss or relative abundance data, but qPCR data were $\log_{10}$ transformed. We conducted independent sample t-tests for comparisons with only one factor (*i.e*., N deposition treatment) and two-way ANOVA for comparisons with two factors (*i.e*., N deposition treatment and either collection date or sample type). For comparisons in which there was a significant interaction between N deposition and collection date, we also considered collection dates separately with independent-sample t-tests.

RESULTS

Field collection and mass loss. After 7 months of decay, low-lignin substrates exhibited high mass loss and showed evidence of faunal grazing (*e.g.,* distinct holes in substrate). The high-lignin substrate, by contrast, was entirely intact after 7 months of decay, with visible evidence of fungal colonization, but no apparent damage by soil fauna. Wood samples at 7 months were

intact with only some showing visible evidence of fungal colonization. We were able to collect high-lignin and wood substrates after 18 months of decay, but relatively rapid decay of the low-lignin substrate prevented collection at this second time-point. At 18 months, the high-lignin substrate was still intact, but visible evidence of fungal colonization was more extensive. After 18 months of decay, many wood samples were structurally weak, suggesting more extensive decay had occurred.

After 7 months of decay, the low-lignin (61%), high-lignin (26%), and wood substrates (10%) displayed a range of mass loss and these differences among substrates types were significant (two-way ANOVA, $F = 218.947$, $P < 0.001$). This result validated that our selection of substrates of varying biochemistry did, in fact, represent a range of decomposability in the field, with the wood and high-lignin substrates decomposing at much slower pace than the low-lignin substrate. After 18 months of decay, the high-lignin substrate had lost 30% of its mass, an additional loss of only 4% compared to that at 7 months, demonstrating that the high-lignin substrate was highly resistant to decay.

Because decomposition is reduced under experimental N deposition in our long-term experiment (Zak et al. 2008), we anticipated that experimental N deposition would also significantly slow the rate of decomposition of our substrates. Mass losses from the low-lignin (-8.3%), high-lignin (-3.6%), and wood (+0.4%) substrates were not significantly lower under experimental N deposition after 7 months of decay in the field (Figure 3.1). After 18 months, mass loss of the high lignin substrate was lower (-4.5%), but again not significantly so under experimental N deposition. We were unable to accurately assess mass loss of wood after 18 months of decay because a number of the wood substrate samples were broken prior to or during collection of samples for this time-point.

Fungal 28S sequencing. Sequencing produced a total of 575,339 reads with 5x circular consensus coverage. Forty-seven percent of sequences were removed during quality-control steps. Ninety-five percent of the resulting sequences were fungal, yielding a total of 289,920 high quality fungal sequences; of these, 126,371 sequences were unique. These fungal sequences clustered to 9115 operational taxonomic units (OTUs) at the 97% sequence similarity level; of these, 2589 OTUs were represented by more than a single sequence, while 861 and 247 OTUs were represented by >10 and >100 sequences, respectively. Mean Good's coverage for 97% sequence similarity OTUs was 94% for substrates and 88% for soil and forest floor samples.

Fungal community composition. Experimental N deposition significantly altered fungal community composition on both wood and the high-lignin substrate (Table 3.3). Fungal community composition changed over time on the high-lignin substrate (Table 3.3), but time did not have a statistically significant effect on fungal community composition of wood (P = 0.0553, Table 3.3). However, the interaction of N deposition treatment and time was not a significant factor affecting fungal community composition for either wood nor the high-lignin substrate (Table 3.3), suggesting that experimental N deposition altered fungal community composition across time.

When we compared community composition across all substrates collected after 7 months of decay, as well as in the soil and forest floor co-collected on the same date, experimental N deposition significantly altered fungal community composition across sample types (Table 3.3). Unsurprisingly, fungal community composition was significantly different among substrates, soil, and forest floor (Table 3.3); however, there were no significant interactions between N deposition treatment and sample type, suggesting that a change in

community composition occurred in all substrates as well as in the soil and forest floor under experimental N deposition. We anticipated that change in community composition would be greatest on high-lignin and wood substrates; instead, it appears experimental N deposition caused changes in in fungal community composition broadly across our samples, irrespective of the degree to which they are protected by lignin.

Fungal community dissimilarity. We followed PERMANOVA with SIMPER analyses to determine whether the changes in fungal community composition that occurred in response to experimental N deposition were driven by a decline in lignolytic fungi (Appendix F). For communities on the high-lignin substrate, wood, and forest floor, many of the OTUs were white-rot and lignolytic litter decay taxa (Table 3.4) and, overall, these white-rot and lignolytic litter decay fungi were less abundant under experimental N deposition (Figure 3.2); this response was significant on wood after 7 months of decay, while a negative but nonsignificant trend was observed for wood after 18 months of decay, the high-lignin substrate, and the forest floor (Figure 3.2). In contrast, no white-rot or lignolytic litter decay fungi were among the top ten SIMPER results for the low-lignin substrate (Table 3.4, Figure 3.2). The only positive response of white-rot and lignolytic litter decay taxa occurred in mineral soil in which a single OTU (Table 3.4) slightly, but significantly, increased in response to experimental N deposition (Figure 3.2).

At the same time, the OTUs that were associated with fungi that decompose cellulose and hemicellulose, but that decay little to no lignin, increased in abundance under experimental N deposition. For example, the relative abundances of OTUs associated with soft-rot and cellulolytic and hemicelluloytic litter decay fungi significantly increased on wood and in forest floor under experimental N deposition. There were also several comparisons for which the mean

abundance of OTUs associated with fungi specializing in cellulose and hemicellulose decay, but not lignin, were higher under experimental N deposition than under ambient N deposition, although not siginificantly so. For example, on the low-lignin substrate there were more brown-rot fungi as well as a small and nonsignficant increase in soft rot & cellulolytic and hemicellulolytic litter decay fungi. On wood, there was a nonsignificant increase in weakly lignolytic fungi. Thus, experimental N deposition appears to stimulate a variety of cellulose-decomposing fungi on substrates and in the forest floor.

In soil, most of the OTUs identified by SIMPER as highly dissimilar under experimental N deposition were associated with biotrophic or mycorrhizal species (Table 3.4, Figure 3.2); overall, their abundance in soil declined, but not significantly (Figure 3.2). While there is evidence that ectomycorrhizal (ECM) fungi may decay organic matter (Shah et al. 2015), there is presently little is known regarding the extent to which ECM species might contribute to this function. Because of this, we were unable to ecologically interpret SIMPER results for biotrophic and mycorrhizal fungi in terms of their implications for lignin decay.

These SIMPER results suggest that the changes in fungal community composition under experimental N deposition which we had previously detected with PERMANOVA are consistent with the mechanism proposed to explain reduced lignin decay under elevated N deposition: a decline in the relative abundance of lignolytic fungi and a shift toward fungal groups which are largely not capable of decaying lignin. While the examination of the top-ten OTUs identified by SIMPER suggests that experimental N deposition negatively affected lignolytic fungi, it was necessarily to perform relative-abundance analyses to determine whether declines in lignolytic fungi were broadly occurring in the fungal community.

Relative abundance of Agaricomycetes. Consistent with our hypothesis, experimental N deposition reduced the relative abundance of Agaricomycetes on wood across both collection dates (-46%, two-way ANOVA, $F = 18.368$, $P = 0.003$, Figure 3.3a). On the high-lignin substrate, however, experimental N deposition had no effect on the relative abundance of Agaricomycetes (Figure 3.3c), a finding inconsistent with our hypothesis and our results for wood. On the low-lignin substrate, the relative abundance of Agaricomycetes was higher under experimental N deposition (Figure 3.3c), although this increase was not statistically significant (independent-samples t-test, $P = 0.057$). Because the low-lignin substrate had low amounts of intact lignocellulose, this result is more likely to reflect an increase in cellulolytic Agaricomycetes than an increase in lignolytic taxa and is, therefore, not necessarily inconsistent with our hypothesis. Finally, mean relative abundance of Agaricomycetes was also lower under experimental N deposition in both soil (-15%) and forest floor (-19%), but these differences also were not significant (Figure 3.4a,c).

We had further hypothesized that reductions in Agaricomycete abundance under experimental rates of N deposition would be enhanced with time. Instead, we found the opposite was true in wood for which Agaricomycete relative abundance was significantly higher (37%) at 7 months of decay than at 18 months of decay (two-way ANOVA, $F = 10.757$, , $P = 0.011$) on wood. On the high-lignin substrate, we found no change in Agaricomycete abundance over time; there also was no interaction between N deposition and time with a two-way ANOVA. Thus, the negative effects of experimental N deposition on Agaricomycete abundance does not appear to be enhanced with time.

Relative abundance of highly lignolytic taxa. We hypothesized that experimental N deposition would reduce the relative abundance of fungi with highly lignolytic physiologies, with this effect

anticipated to be most evident on lignified substrates. Experimental N deposition significantly reduced the proportion of highly lignolytic fungi present on wood across both collection dates (Figure 3.3b, two-way ANOVA, $F = 8.254$, $P = 0.021$), with no significant N deposition by time interaction. Experimental N deposition did not have a significant main effect on the abundance of highly lignolytic taxa on the high-lignin substrate (Figure 3.3d), but there was a significant interaction between N deposition treatment and collection date (two-way ANOVA, $F = 8.641$, $P = 0.019$). When we analyzed collection dates separately, the relative abundance of highly lignolytic taxa on the high-lignin substrate was significantly lower (-67%, independent samples t-test, $P = 0.015$) under experimental N deposition at 7 months of decay, but this was no longer true after 18 months of decomposition (Figure 3.5). By contrast, highly lignolytic taxa were not abundant (~9% of Agaricomycetes) on our low-lignin substrate and their relative abundance was not significantly affected by experimental N deposition (Figure 3.3f). Consistent with our hypothesis, experimental N deposition reduced the abundance of high lignolytic taxa on both lignin-rich substrates, wood (for both time points) and the high-lignin substrate (for one time point), while having no effect on their abundance on the low-lignin substrates. Moreover, we found that the relative abundance of highly lignolyic taxa in the forest floor was lower (-20%) under experimental N deposition, but that this decline was not statistically significant (Figure 3.4b). Taxa which we had identified as highly lignolytic were uncommon in mineral soil (~3% of fungi, ~6% of Agaricomycetes) and their relative abundance was not significantly altered by experimental N deposition (Figure 3.4d).

We had further hypothesized that experimental N deposition would have the strongest negative effects on the abundance of highly lignolytic taxa at later points in decay, when substrates become relatively enriched in lignin compared to their initial state. Our results,

however, did not support this hypothesis. In contrast to our expectations, experimental N deposition only reduced the abundance of highly lignolytic taxa on the high-lignin substrate after 7 months (Figure 3.5). By 18 months, experimental N deposition no longer had a negative effect on the relative abundance of highly lignolytic taxa inhabiting the high-lignin substrate (Figure 3.5). On wood, neither time nor the interaction between time and N deposition had significant effects on the abundance of highly lignolytic taxa. While experimental N deposition significantly suppressed the relative abundance of highly lignolytic taxa on wood, this effect was not any stronger after 18 months of decay than after 7 months of decay. Thus, the suppression of highly lignolytic taxa under experimental N deposition does not appear become greater over the course of decay.

Fungal abundance. We hypothesized that experimental N deposition would reduce fungal biomass on substrates with intact lignocellulose (*i.e.*, the wood and high-lignin substrate) and in soil and forest floor as a result of reduced availability of energy to the fungal community under experimental N deposition. Consistent with this hypothesis, experimental N deposition reduced fungal ITS1 copy number per µg of extracted DNA on wood (Figure 3.6a, two-way ANOVA, $F = 8.309$, $P = 0.020$); when we calculated fungal ITS1 copy number per g of substrate, this trend was still the same for wood, although this difference was not significant (Figure 3.6b). However, experimental N deposition did not alter fungal ITS1 copy number on the high-lignin substrate (Figure 3.6c,d). For the low-lignin substrate, experimental N deposition significantly increased fungal ITS1 copy number per g of substrate (Figure 3.6f, independent samples t-test, $P = 0.003$), while having no significant effect on fungal ITS1 copy number per µg of extracted DNA (Figure 3.6e); this is not inconsistent with our hypothesis because the low-lignin substrate is largely unprotected cellulose (Table 3.1).

In contrast to our expectations, experimental N deposition did not significantly reduce fungal ITS1 copy number in either forest floor or soil (Figure 3.7). However, there were non-significant trends toward reduced fungal ITS1copy numbers under experimental N deposition in soil both per μg of extracted DNA (Figure 3.7c) and per g of sample (Figure 3.7d), as well as for forest floor when copy number was expressed per g of sample (Figure 3.7b).

Fungal ITS1 copy number (per μg of extracted DNA) on wood was higher after 7 months than after 18 months of decomposition in the field (two-way ANOVA, $F = 16.263$, $P = 0.004$), but time did not significantly affect fungal ITS1 copy number on the high-lignin substrate. The interactions of time and N deposition treatment did not significantly affect fungal ITS1 copy numbers for either wood or the high-lignin substrate.

DISCUSSION

Here, we demonstrate that the experimental deposition of N, at rates predicted to occur by midcentury (Galloway et al. 2004), reduced the relative abundance of fungi with lignolytic physiologies on both wood and a high-lignin substrate. Furthermore, a decline in lignolytic fungi under experimental N deposition was accompanied by a shift toward cellulolytic fungi on wood, as well as in the forest floor. Experimental N deposition changed fungal community composition overall across substrates, soil, and forest floor and across time on both the high-lignin and wood substrates. These findings are consistent with a longstanding mechanism which posits that high rates of N deposition can reduce lignolytic fungal activity, leading to a decline in the abundance of lignolytic fungi and a shift toward fungal species with limited capacities for lignin decay, thereby increasing soil C storage (Fog 1988, DeForest et al. 2004b, DeForest et al. 2005). Finally, we found that experimental rates of N deposition decreased fungal abundance on wood, but increased the abundance of fungi on a low-lignin, cellulose-rich substrate. These

observations are consistent with previous studies wherein elevated rates of N deposition suppressed lignin decay, but stimulated cellulose decay (Berg and Matzner 1997, Carreiro et al. 2000, Talbot and Treseder 2012). The evidence we have accumulated is consistent with the hypothesis that greater soil C storage under chronic N deposition arises from a decline in the abundance of soil fungi with lignolytic physiologies.

In our long-term experiment, experimental N deposition reduced decay and increased soil organic matter (Pregitzer et al. 2008, Zak et al. 2008). Several lines of evidence demonstrate that these responses are the result of reduced microbial decomposition under elevated rates of N deposition. First, changes in plant litter production under experimental N deposition can be excluded from consideration, because experimental N deposition has not substantially altered the production or recalcitrance of leaf and root litter (Burton et al. 2004, Xia et al. 2015). Further evidence that microbial activity has been suppressed under experimental N deposition comes from the observations that this treatment has reduced soil respiration without altering root respiration (Burton et al. 2004, Burton et al. 2012). Finally, stable isotope tracing with ^{13}C-labelled plant litter has further revealed that experimental N deposition reduced the amount of C transferred from plant litter into the soil microbial community (Gan et al. 2013). Thus, reduced decomposition under experimental N deposition results from reduced microbial decay, not from increases in either the amount or recalcitrance of plant litter produced.

Collectively, the evidence presented and summarized above points toward a reduction in the decay of lignin by microorganisms as the explanation for increased soil C storage under experimental N deposition. Lignin is a recalcitrant, aromatic compound that limits the rate of decay of plant litter; because of this, it stands to reason a reduction in its decay could affect the overall rate of decomposition as well as the accumulation of SOM. Furthermore, elevated rates

of N deposition have been observed to retard lignin decay in plant litter in other field experiments (Berg and Matzner 1997). In our long-term field experiment, elevated rates of N deposition have increased the phenolic and aromatic content of dissolved organic C (DOC), with partially modified lignin thought to be the source of these additional compounds (Smemo et al. 2007). Moreover, accumulation of lignin in soil in response to increased N deposition has been observed in another northern hardwood forest ecosystem (Frey et al. 2014) and may, in fact, be a widespread response to experimental N deposition (Liu et al. 2016). Finally, elevated rates of N deposition reduced lignolytic enzyme activity both in our long-term experiment (DeForest et al. 2004a, Freedman and Zak 2014) as well as in other systems (Carreiro et al. 2000). When we consider these results in combination with the observations we present here, it appears that reduced microbial decay of lignin is a general mechanism through which elevated N deposition slows plant litter decomposition and leads to greater accumulation of SOM.

While enzyme assays assess both bacterial and fungal enzymatic activity, molecular examination has disentangled the effects of N deposition on fungi and bacteria and has revealed that N deposition positively affects saprotrophic bacteria (Freedman and Zak 2014, Freedman et al. 2016). For example, experimental N deposition has increased the abundance of genes associated with pathways for saprotrophic bacterial metabolism in soil metagenomes (Freedman et al. 2016), suggesting an overall positive effect of experimental N deposition on the abundance of bacterial metabolisms for plant cell-wall decay. Furthermore, the abundance of bacteria with laccase-like multicopper oxidase (LMCO) genes increased in response to experimental N deposition, indicating that the potential for bacteria to decay lignin increases with rising rates of anthropogenic N deposition (Freedman and Zak 2014). However, such an expanded role of bacteria in lignin decay under experimental N deposition would likely not offset reductions in

fungal lignin decay because, while both bacteria and fungi share laccase or laccase-like enzymes that act upon polyphenolics (Baldrian 2006, Ausec et al. 2011, Lu et al. 2014), fungi additionally possess other, more powerful lignolytic enzymes (*e.g.,* fungal class II peroxidases) which bacteria do not, giving fungi greater oxidative capacity in lignin decay than bacteria (Kirk and Farrell 1987, Floudas et al. 2012).

According to our working mechanism (DeForest et al. 2004a, DeForest et al. 2005), the abundance of microorganisms which are less efficient at lignin decay may increase under experimental N deposition as a result of reduced competition with lignolytic fungi, which have declined due to N-inhibition of their lignolytic enzymes. However, it is also possible that an alternative mechanism in which N stimulates fungi which primarily target labile- C but which also are capable of lignin decay, albeit with lower efficiency, and that that these fast-growing microorganisms outcompete slower-growing specialists on lignified substrates (Fontaine et al. 2003). Experimental N deposition reduced the relative abundance of lignolytic fungi after 7 months of decay when 74% of the high-lignin and 90% wood substrate still remained; in light of this, it seems more plausible than an increase in bacteria with LMCOs is more likely an effect, rather than a cause, of the reduced abundance of lignolytic fungi under experimental N deposition. Regardless of the mechanism at work, this shift appears to be co-occurring alongside the decline in lignolytic fungi observed in this study and previously observed increases in SOM and phenolic DOC production. However, increased bacterial potential for plant cell-wall decay cannot alone account for the decreases in lignin decay under experimental N deposition in our study system.

Instead, several pieces of evidence from our field study suggest that experimental N deposition is suppressing fungal lignin decay. For example, experimental rates of N deposition

reduced the diversity of fungal lignocellulolytic genes, indicating that experimental N deposition has reduced fungal functional potential for lignin decay (Eisenlord et al. 2013). Furthermore, long-term increases in experimental N deposition have reduced the number of transcripts for fungal laccase, an enzyme involved in the depolymerization of lignin, especially those originating from the Basidiomycota, which includes many lignin-decaying fungi and all of the most powerful lignin decomposers (Edwards et al. 2011, Hesse et al. 2015). In this study, experimental N deposition had a negative effect on the relative abundance of lignolytic fungi and appeared to shift fungal community composition towards fungi which are poor lignin-decomposers.

Substrate-specific responses

We observed the most consistent and significant negative responses to experimental N deposition on wood. Many of the most powerful lignolytic fungi are wood-decomposers, making wood an excellent substrate on which to observe the effects of N deposition on lignin decomposers. Because experimental N deposition simultaneously reduced the relative abundance of lignolytic fungi (Figure 3.3) and the size of the fungal community (Figure 3.6) on wood, the *absolute* abundance of lignolytic fungi on wood was lower under elevated rates of N deposition. Our results are consistent with recent observations that experimental N deposition has slowed the decay of coarse woody debris in our long-term experiment (B. Lyons, unpublished data). Furthermore, experimental N deposition has also increased woody biomass production (Pregitzer et al. 2008, Ibáñez et al. 2016). If higher rates of N deposition both increase the production and slow the decay of wood in temperate forests, this could dramatically increase ecosystem C storage.

Even though the high-lignin substrate contains twice as much lignin as wood (Table 3.1), experimental N deposition reduced the relative abundance of highly lignolytic taxa only early in its decay (7 months) and not at later stages of decay (18 months, Figure 3.5). This may partially reflect that, at later stages of decay (*i.e.*, 18 months), experimental N deposition had negative effects on lignolytic fungi which were not included among our list of highly lignolytic taxa. In community dissimilarity analyses, several OTUs associated with *Sphaerobolus* and *Trechispora* had high contributions to community dissimilarity at 18 months of decay on the high-lignin substrate (Appendix F) and these genera are likely important for lignin decay (Nobles 1958, Harkin et al. 1974, Robinson et al. 1993, Worrall et al. 1997, Nagy et al. 2015, Kreetachat et al. 2016). However, few studies exist which measure their ability to decay lignin and, as a result, these taxa were not included in our relative abundance analyses of highly lignolytic taxa which we restricted to include only taxa for which high amounts of lignin decay have been well-documented.

Experimental N deposition had few significant effects on fungal communities in the forest floor, an unexpected result given the responses of fungal communities on wood and the high-lignin substrate. We did observe a significant increase in soft-rot and celluolytic and hemicellulolytic litter decay fungi among fungi which contributed highly to community dissimilarity under experimental N deposition (Figure 3.2), but while there appeared to be a concurrent decrease in the mean abundance of white-rot and lignolytic litter fungi under experimental N deposition, this was not significant (Figure 3.2). Experimental N deposition did not significantly decrease the relative abundance of Agaricomycetes (Figure 3.4), the relative abundance of highly lignolytic taxa (Figure 3.4), or total fungal abundance in the forest floor (Figure 3.7). We have recently learned that, in our study system, fine roots provide the majority

of plant-litter lignin (Xia et al. 2015) and are the greatest source of lignin-derived SOM (Thomas et al. 2012). In other temperate forest systems, experimentally elevated N deposition has slowed root decomposition (Sun et al. 2015). If experimental N deposition increases soil C storage by suppressing lignolytic fungi on naturally occurring plant litter, this change is more likely occurring on root-litter than on leaf litter and this may explain why the forest floor did not respond in the manner which we had anticipated.

On the low-lignin substrate, experimental N deposition had little to no effect on lignin-decaying fungi (Figures 3.2 & 3.3). This is not surprising given the low-lignin substrate is cellulose-rich resource which is largely unprotected by lignin and on which highly lignolytic taxa were rare (Figure 3.3). Experimental N deposition increased overall fungal abundance (Figure 3.6) and may have had slight positive effects on cellulolytic & hemicellulolytic fungi and brown-rot fungi (Figure 3.2) on the low-lignin substrate. These results are consistent with previous observations that elevated N deposition stimulates cellulose decay by removing microbial N limitation (Talbot and Treseder 2012).

Fungal communities in mineral soil have few saprotrophs and are dominated by mycorrhizal species (Lindahl et al. 2007). Unsurprisingly, then, there was little effect of N deposition on saprotrophic fungi which decay either lignin or cellulose in mineral soil (Figures 3.2 & 3.4). In community dissimilarity analysis, mycorrhizal and biotrophic fungi in soil were less abundant, but not significantly so, under experimental N deposition (Figure 3.2, Table 3.2). Long-term increases in N deposition have previously been observed to alter the composition of ECM fungi (Avis et al. 2003, Frey et al. 2004). Interestingly, ectomycorrhizal fungi (ECM) may use lignolytic enzymes to obtain N complexed with soil organic matter (Talbot et al. 2013, Shah et al. 2015). Therefore, ECM species may be potentially sensitive to rising rates of N deposition

and may also play an important role in SOM decomposition in mineral soil. However, little is known yet about what roles individual ectomycorrhizal species may play in lignin decomposition in nature.

Effects of experimental N deposition over time

Contrary to our expectations, we found no evidence to support the hypothesis that there would be greater disparities in the relative abundance of Agaricomycetes or highly lignolytic taxa between ambient and experimental N deposition treatments at later stages of decay, when decomposing materials should be relatively more enriched in lignin compared to their initial state. Instead, we observed either no change with time or, surprisingly, larger disparities in abundance of lignolytic fungi early in decay. This is surprising in light of the fact that elevated N deposition has been observed to retard lignin decay at later stages of decomposition, while stimulating cellulose decay early on (Berg and Matzner 1997, Talbot and Treseder 2012). Interestingly, community dissimilarity analysis indicated experimental N deposition had a positive effect on the abundance of cellulose-decomposing fungi in our experiment (Figure 3.2). If cellulose is removed at higher rates early in decay under experimental N deposition, this may remove the energy-rich C sources needed to "prime" lignin decay at later stages (Fontaine et al. 2003), which could ultimately lead to lower amounts of lignin being decayed under elevated rates of N deposition.

Fungal responses to experimental N deposition

In order to explain why fungi decompose lower amounts of lignin in response to higher rates of N deposition, it has been proposed that higher N availability may lead to reduced fungal foraging for organic N compounds that are only obtainable through the activity of lignolytic enzymes, *i.e.,* organic N which is either protected by lignin or complexed with SOM (Craine et

al. 2007). However, this mechanism is unlikely to explain the reductions in lignolytic fungi that we have observed on wood and on the high-lignin substrate in this study. Wood and high-lignin substrates were both N-poor, but energy-rich resources for fungi (Table 3.1), yet experimental N deposition still led to lower abundance of lignolytic fungi on these low-N substrates. Therefore, reduced fungal foraging for litter-N does not seem to explain this response because it is unlikely that that lignolytic taxa were principally mining these substrates for N. This is consistent with the findings of another plant litter decomposition experiment conducted in a boreal forest in which elevated N deposition reduced lignin decay, but had no effect on N loss from plant litter (Talbot and Treseder 2012).

Furthermore, we found no evidence to suggest that this mechanism explains declines in lignolytic saprotrophs under experimental N deposition. Foraging for organic N in SOM is primarily an activity associated with ectomycorrhizal fungi in soil, not with the saprotrophic decomposer fungi commonly found in the litter layer (Lindahl et al. 2007, Talbot et al. 2013). The lignolytic fungi on our substrates which were negatively affected by experimental N deposition were all saprotrophs and our substrates were primarily colonized by saprotrophic fungi, with few ECM fungi present. ECM fungi, by contrast, were only abundant in mineral soil samples. While it is possible that experimental N deposition could reduce foraging for recalcitrant forms of organic-N by ECM fungi in soil, we were unable to further examine whether experimental N deposition has had effects on ECM fungi which would be consistent with this hypothesis because the role of ECM fungi in the decomposition of organic matter is still poorly understood. Regardless, reduced fungal mining for SOM-N does not appear to explain the declines in the relative abundance of lignolytic saprotrophs that we observed in this study.

It is not clear then why saprotrophic lignolytic fungi would reduce lignin decay in response to experimental N deposition, particularly when doing so would seem to limit their ability to obtain energy. However, for at least some species of fungi, suppression of lignin decay appears to be a physiological responses to high inorganic N concentrations (Leatham and Kirk 1983, Commanday and Macy 1985), with white-rot wood decay fungus *Phanerochaete chrysosoporium* being the most extensively studied species in this regard. In culture, lignolytic activity can be induced by N starvation in the absence of lignin (Keyser et al. 1978) and suppressed by high N conditions even when lignin is present (Jeffries et al. 1981). Furthermore, the addition of lignin alone, without addition of a carbohydrate, does not induce lignolytic activity in *P. chrysosporium* (Kirk et al. 1976). Low N conditions in culture may stimulate lignin decay because those conditions are similar to those of its natural environment, *i.e.* wood (Keyser et al. 1978). It is possible inorganic N concentration may serve as an indicator of environmental conditions appropriate for lignin decay. However, leaf litter is a more N-rich substrate than wood, yet some litter decay fungi also reduce their rate lignin decay when N is added during decomposition (Kuyper and Bokeloh 1994, Osono et al. 2006). Thus, there is evidence that N availability has physiological effects on fungal lignolytic activity, although the physiological and ecological reasons as to why this might be so remain unclear.

It is possible that fungal communities experiencing high rates of N deposition could regain lignolytic function through ecological or evolutionary processes. For some species of fungi, lignolytic activity is either unaffected or increases under high N conditions (Leatham and Kirk 1983, Kaal et al. 1995). Such nitrotolerant or nitrophilic lignolytic species could become more abundant through competitive release. It is also possible that lignolytic fungal species that are sensitive to N may evolve higher nitrogen tolerance under pressure from chronically higher

rates of N deposition. However, after nearly 20 years of chronic N deposition, we were able to observe significant decreases in lignolytic fungi colonizing our wood and high-lignin substrates, indicating that these responses have not yet occurred.

Conclusions

In temperate forests, experimental additions of N often increase soil C content (Nave et al. 2009, Janssens et al. 2010, Frey et al. 2014). If elevated N deposition has widespread negative effects on lignin-decomposing fungi in temperate forests, like those observed here, then shifts in fungal community composition may underlie the accumulation of soil C. Since rates of N deposition are projected to increase further in some parts of the world, this response could influence future rates of soil C storage in temperate forests. Furthermore, as large increases in atmospheric N deposition have also occurred globally over the past century and a half, it is possible that anthropogenic N deposition may have already had widespread effects on fungal biodiversity as well as elicited increases in soil C in temperate forests.

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TABLES

Table 3.1. Summary of results of biochemical analysis of low-lignin, high-lignin, and wood substrates. Measurements were performed by the Soil & Forage Lab, UW-Madison. Results are reported as a percentage of dry weight.

Substrate	Material	Lignin (%)	Cellulose (%)	Hemicellulose (%)	Nitrogen (%)
Low-lignin	Kraft paper	14.1[†]	64.1	14.1	0.14
High-lignin	Newsprint	27.6	46.7	23.1	0.09
Wood	Birch wood	11.8	52.4	28.9	0.13

[†]Because chemical pulping breaks bonds between lignins, cellulose, and hemicelluloses (Ragnar et al. 2000), the low-lignin substrate is more labile than its lignin content alone suggests.

Table 3.2. Agaricomycete taxa selected as highly lignolytic. All taxa are represented by >20 sequences in our data set. More details on taxa selected below as well as those we excluded are provided in the Appendices D & E.

Order	Family or genera included from this order	Citations documenting lignolytic activity
Agaricales		
	Marasmius[1]	(Osono and Takeda 2002, Steffen et al. 2007)
	Gymnopus[2]	(Osono et al. 2003, Osono and Takeda 2006, Valaskova et al. 2007, Šnajdr et al. 2010, Cline and Zak 2015)
	Mycena[3]	(Worrall et al. 1997, Osono and Takeda 2002, Steffen et al. 2007, Liers et al. 2011, Cline and Zak 2015)
	Clitocybe[4]	(Osono et al. 2003, Osono et al. 2011)
	Crepidotaceae	(Gutiérrez et al. 1999, Del Rıo et al. 2001, Martínez Ferrer et al. 2005)
Polyporales[5]		
	Ganodermataceae	(Blanchette 1984)
	Antrodiella	(Patel and Rao 1993)
	Phanerochaete[6]	(Kirk and Farrell 1987, Hatakka 1994, Del Rıo et al. 2002)
	Scopuloides[6]	(Kuuskeri et al. 2015)
Russulales[7,8]		
	Lachnocladiaceae	(Cline and Zak 2015)
	Auriscalpiaceae	(Miller and Stewart 1971, Miller and Methven 2000)

1. Also included sequences that were assigned to the closely related genus *Amyloflagellula* (Douanla-Meli and Langer 2008).
2. Some *Gymnopus* species were formerly *Collybia*.
3. Also included sequences assigned to closely related genus *Poromycena* (Moncalvo et al. 2002).
4. Also included sequences classified to closely related genus *Lepista* (Matheny et al. 2006).
5. White-rot Polyporales are broadly highly lignolytic (Campbell 1932, Ander and Eriksson 1977, Ruiz-Duenas et al. 2013). The selected Polyporales taxa above are simply the white-rot Polyporales present in our data set (Binder et al. 2013).
6. Sequences in our data set which were assigned to the "Aphyllophorales" by the RDP v7 actually represent these genera (see Appendix D for more details). "Aphyllophorales" is no longer a recognized taxonomic group (Hibbett and Donoghue 1995, Binder and Hibbett 2002).
7. These families now recognized as saptroptrophic Russulales (Hibbett and Donoghue 1995, Larsson and Larsson 2003, Miller et al. 2006), but are placed in Polyporales by RDP v7 fungal LSU classifier.
8. Saprotrophic Russulales are white-rot and have broadly been observed to be highly lignolytic (Blanchette 1984, Otjen and Blanchette 1984, Del Rıo et al. 2002, Speranza et al. 2009, Floudas et al. 2012, Nagy et al. 2015). The selected Russulales taxa above are the saprotrophic Russulales present in our data set.

Table 3.3. PERMANOVA results for comparisons of fungal communities under ambient and experimental rates of N deposition. Comparisons for wood and the high-lignin substrate are across both time-points. Comparisons for all substrates plus soil and forest floor are across sample types for substrates collected after 7 months of decomposition and soil and forest floor samples co-collected on the same date. Columns represent PERMANOVA values for the degrees of freedom (df), sum of squares (SS), mean square (MS), pseudo-F statistic and permutational *P* value (P(perm)).

Samples included	Factors	df	SS	MS	Pseudo-F	*P*(perm)
Wood	**N deposition treatment**	1	2686.7	2686.7	1.8946	**0.0476**
	Time	1	2443.4	2443.4	1.723	0.0553
	N deposition treatment x Time	1	994.72	994.72	0.70145	0.7487
High-lignin substrate	**N deposition treatment**	1	2600.9	2600.9	1.7597	**0.0266**
	Time	1	4405.4	4405.4	2.9806	**0.0023**
	N deposition treatment x Time	1	1362	1362	0.92149	0.6118
Low-lignin, high-lignin, & wood substrates; soil, forest floor	**N deposition treatment**	1	2856.8	2856.8	2.5307	**0.0001**
	Sample type	4	39395	9848.7	8.7244	**0.0001**
	N deposition treatment x Sample type	4	4807.8	1202	1.0647	0.2909

Table 3.4. SIMPER results for the ten OTUs with the highest average dissimilarity between fungal communities under ambient and experimental N deposition, including their cumulative contribution to total dissimilarity and the fungal physiologies they represent. Detailed results for individual OTUs can be found in Appendix F.

	Months of decomposition	Sample type	Cumulative contribution to dissimilarity (%)	Number of OTUs assigned to each fungal physiology				
				White-rot & lignolytic litter decay	Soft-rot & cellulolytic/ hemicellulolytic litter decay	Brown-rot	Weakly lignolytic	Mycorrhizal/ biotrophic
Substrates	7	low-lignin	24.39	0	6	1	1	2
		high-lignin	30.25	4	3	2	0	1
		wood	44.74	5	2	0	2	1
	18	high-lignin	33.18	7	1	1	1	0
		wood	47.19	3	3	0	4	0
Environmental samples	NA	forest floor	33.11	7	3	0	0	0
		soil	18.92	1	1	0	3	5

NA, not applicable

FIGURES

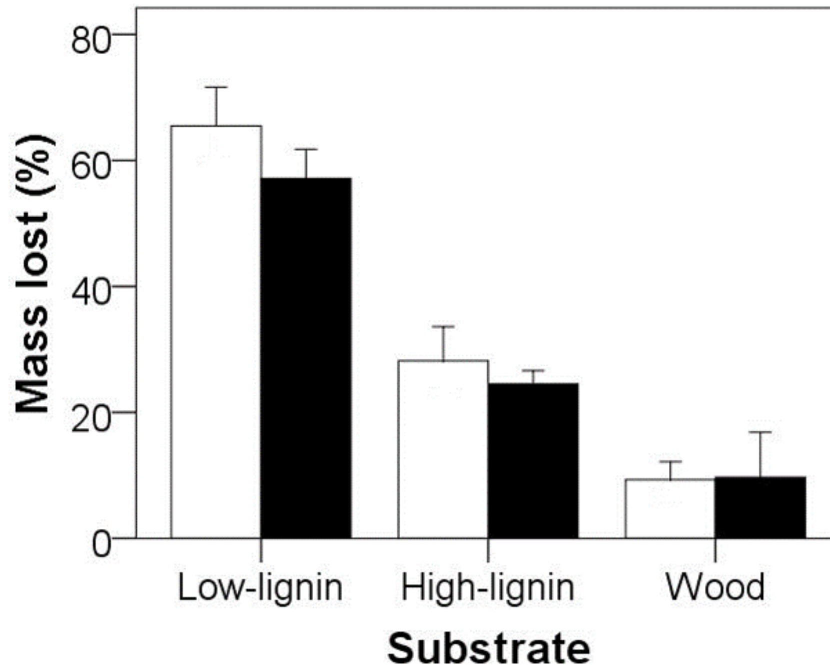

Figure 3.1. Mass loss of wood, high-lignin, and low-lignin substrates under ambient (open bars) and experimental N deposition (filled bars) after 7 months of decomposition in the field. Error bars represent 2 SE. Experimental N deposition did not significantly alter mass loss any substrate (independent sample t-tests).

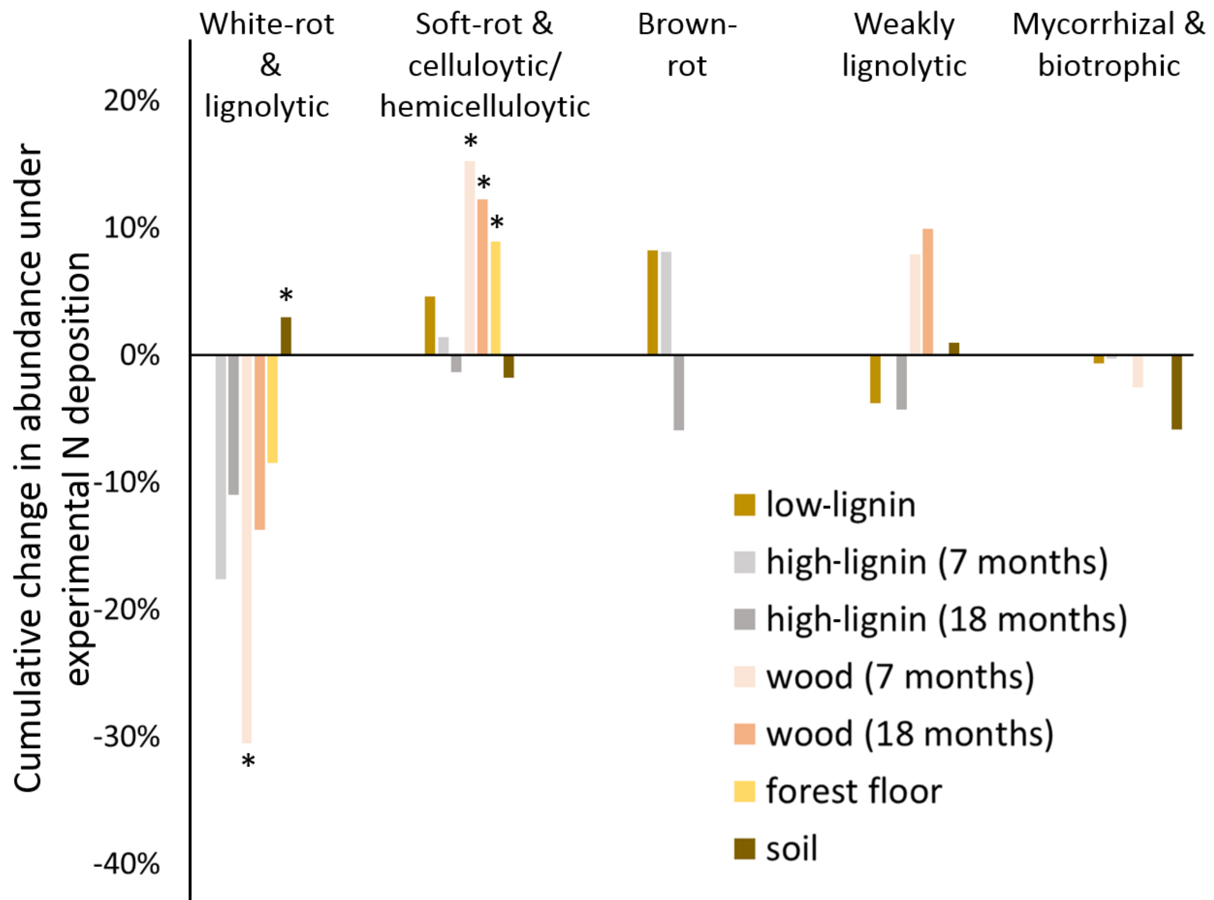

Figure 3.2. The response (% change in relative abundance) of fungi of different physiologies to experimental N deposition among the ten OTUs with the largest dissimilarity scores according to SIMPER analysis. Significant comparisons (marked with an asterisk) are those for which the 95% confidence intervals for relative abundance under ambient and experimental N deposition did not overlap.

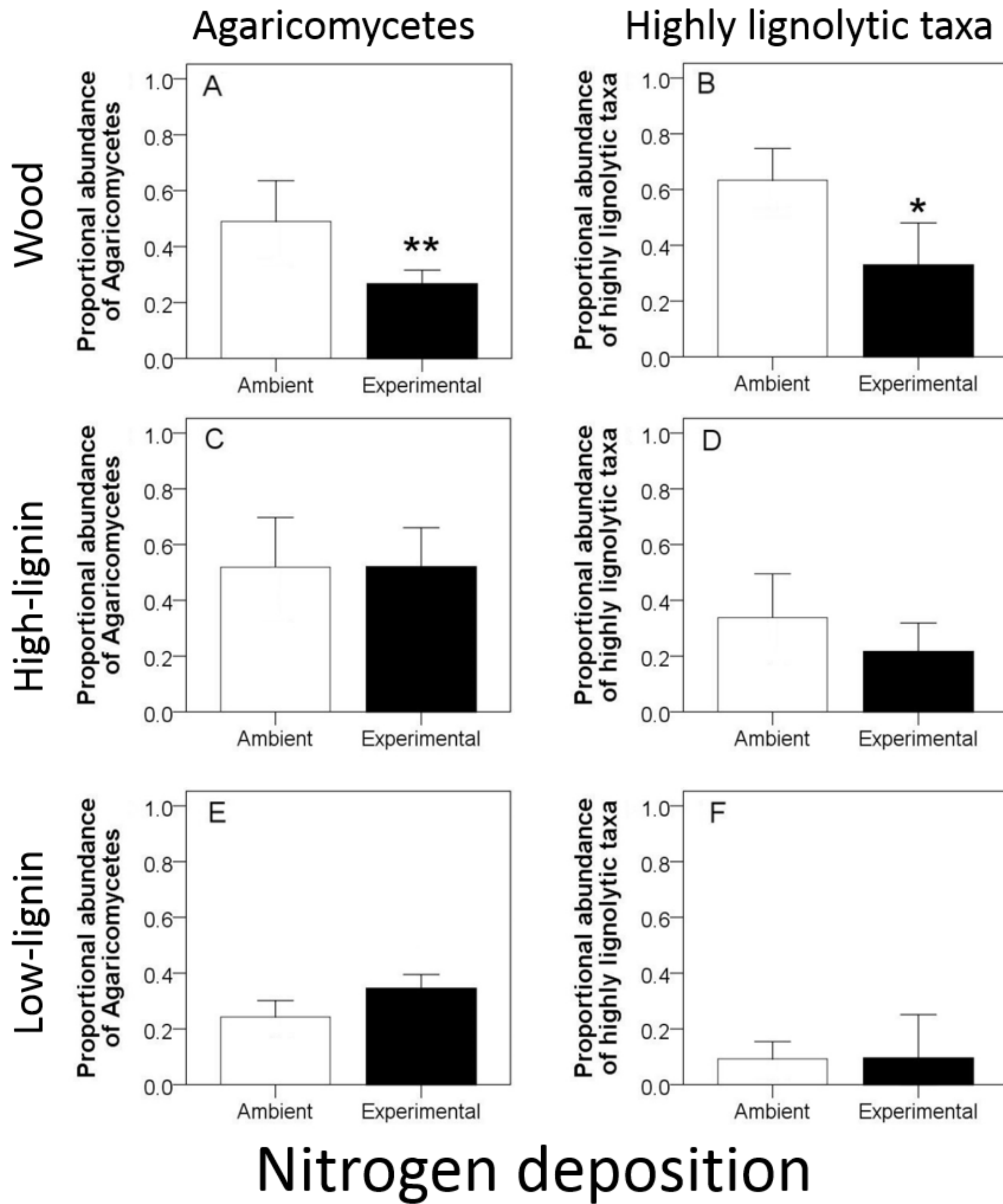

Figure 3.3. Relative abundance of Agaricomycetes (left) and highly lignolytic taxa (right) in fungal communities on a wood (top), high-lignin (middle), and low-lignin (bottom) substrates which after 7 and 18 months of decomposition in the field. Relative abundance of Agariomycetes was calculated as a proportion of fungi, while relative abundance of highly

lignolytic taxa was calculated as a proportion of Agaricomycetes. Error bars indicate 2 SE. Fungal communities on the low-lignin substrate paper, for which we have only one time point, were analyzed with independent sample t-tests. High-lignin and wood were analyzed across collection dates with a two-way ANOVA. Significance is indicated by * $P < 0.05$, ** $P < 0.01$.

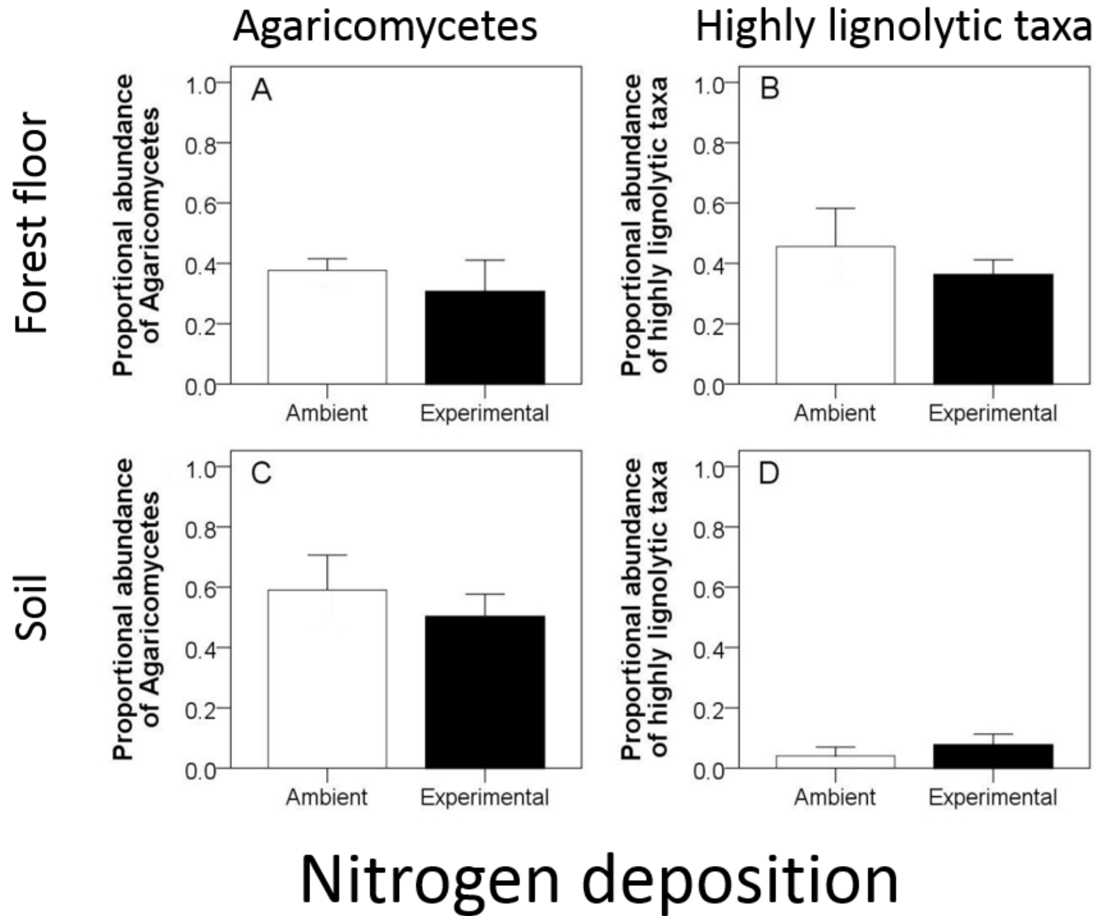

Figure 3.4. Relative abundance of Agaricomycetes (left) and highly lignolytic taxa (right) of fungal communities in forest floor (top) and soil (bottom). Relative abundance of Agariomycetes was calculated as a proportion of fungi, while relative abundance of highly lignolytic taxa was calculated as a proportion of Agaricomycetes. Error bars indicate 2 SE.

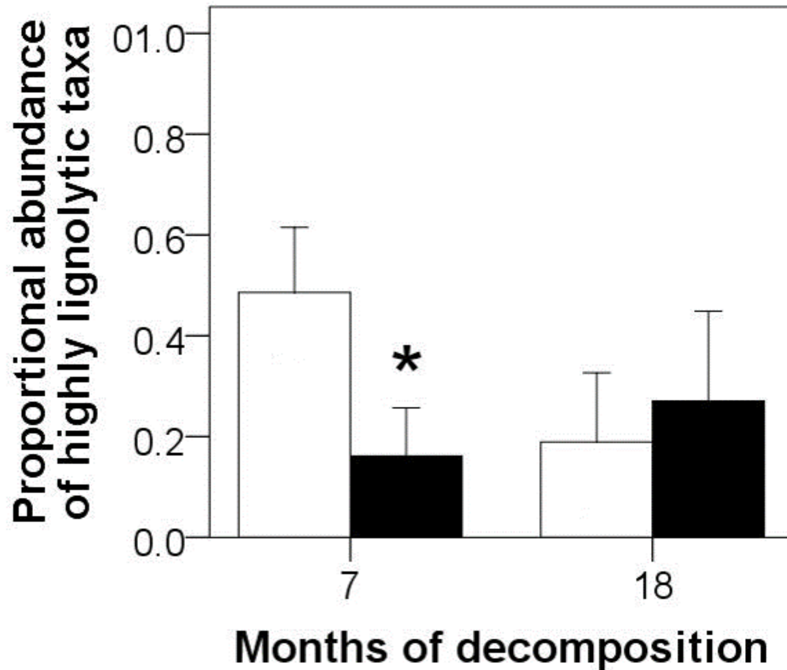

Figure 3.5. Relative abundance of highly lignolytic taxa on the high-lignin substrate collected after 7 and 18 months of decomposition under ambient (open bars) and experimental (solid bars) rates of N deposition. Relative abundance of highly lignolytic taxa was calculated as a proportion of Agaricomycetes.Error bars indicate 2 SE. Each collection date was analyzed with independent samples t-tests, for which significance is indicated with * $P < 0.05$.

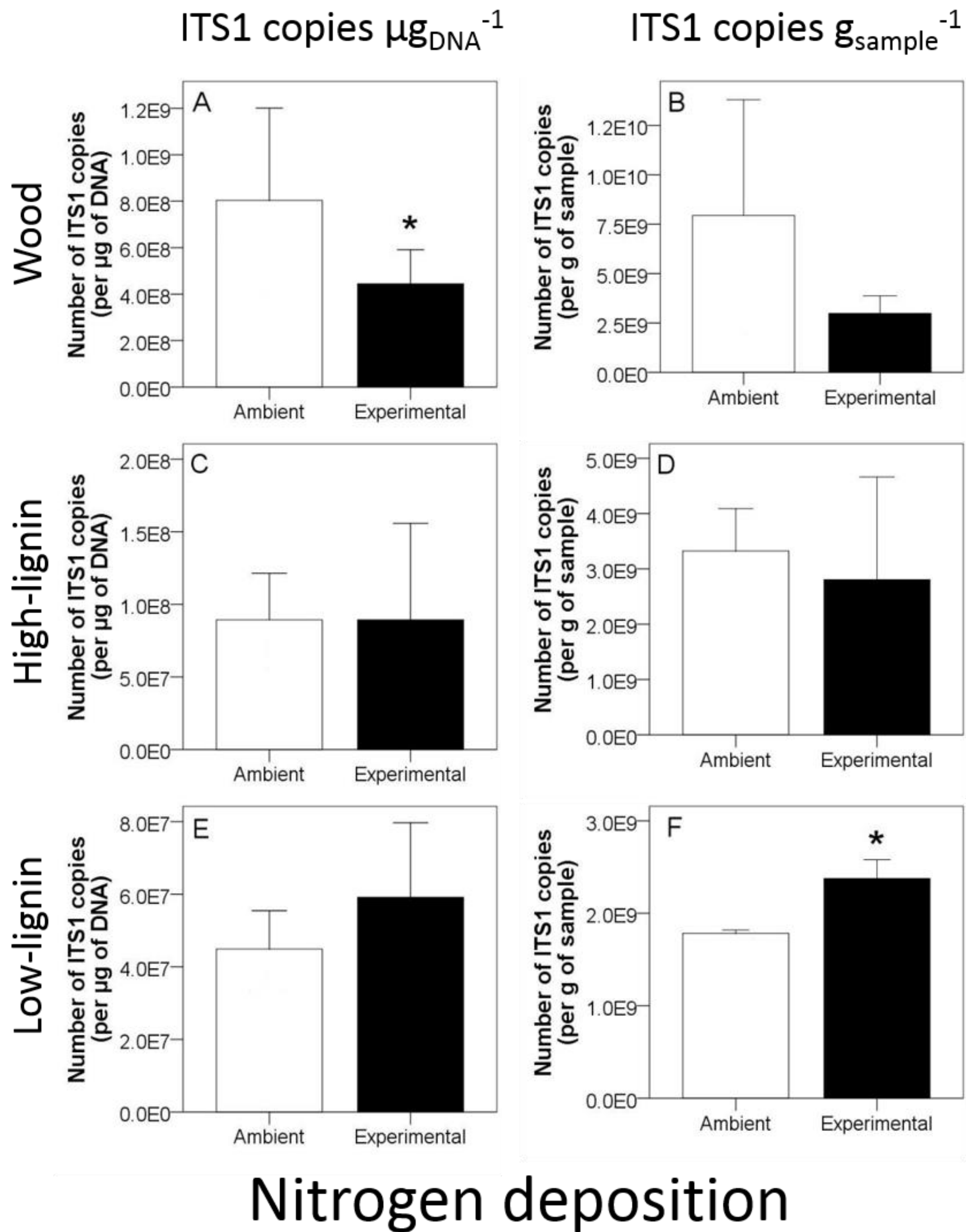

Figure 3.6. Fungal abundance as measured by quantitative PCR and presented as ITS1 copies μg^{-1} DNA (left) and ITS1 copies g^{-1} sample mass (right) on decomposing substrates of varying recalcitrance (wood, high-lignin, low-lignin) collected after 7 and 18 months of decomposition in

the field. We compared ITS1 copy number on the wood (top) and high-lignin (middle) between time points and N deposition treatments with two-way ANOVAs; we present data across time-points because there was no significant time-by-N deposition interaction. Differences in ITS1 copy number under experimental N deposition for the low-lignin substrate (bottom), for which we have only one time-point, were tested with an independent samples t-test. Error bars represent 2 SE. Significant comparisons are indicated with * $P < 0.05$.

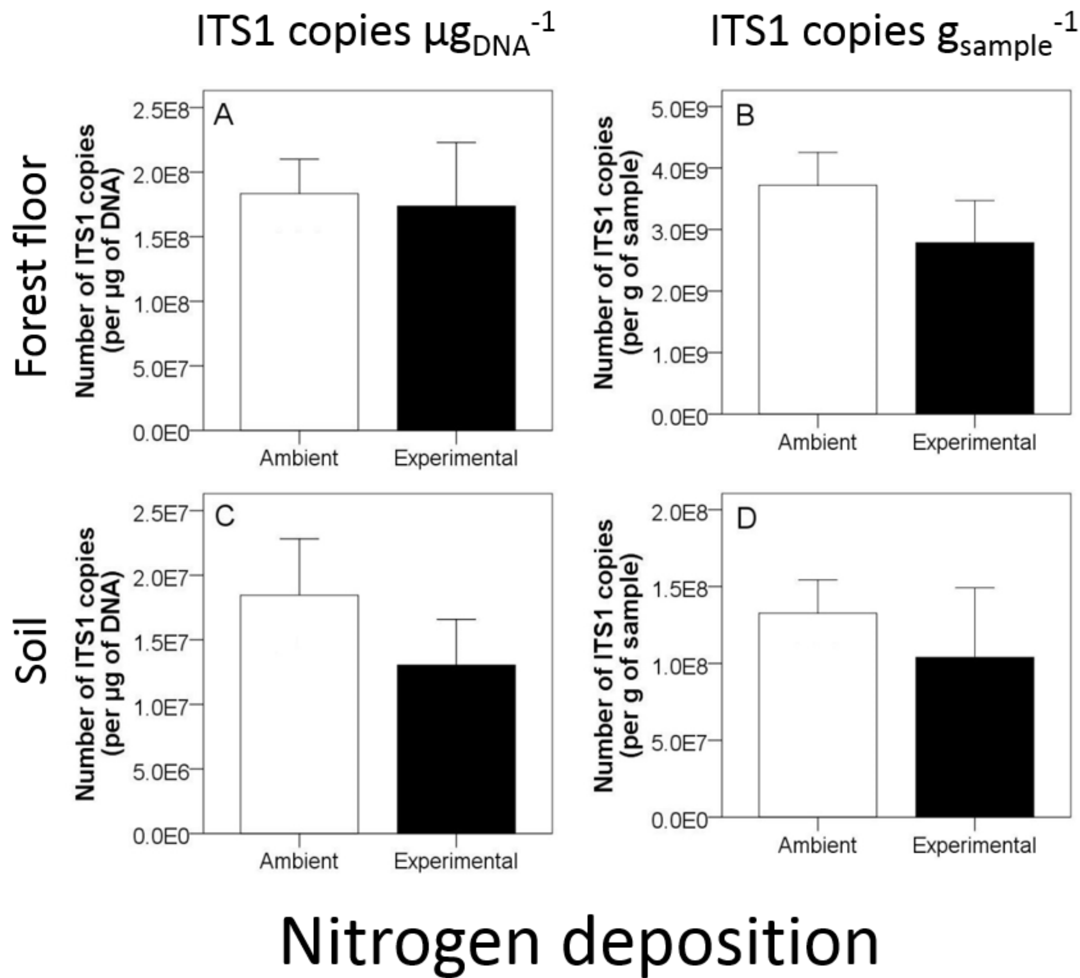

Figure 3.7. Fungal abundance as measured by quantitative PCR and presented as ITS1 copies μg^{-1} DNA (left) and ITS1 copies g^{-1} sample mass (right) in forest floor (top) and soil (bottom). Error bars indicate 2 SE. ITS1 copy numbers under ambient and experimental N deposition were compared with with independent samples t-tests, but no comparisons were significant.

CHAPTER 4

Anthropogenic N deposition alters the composition, but not the diversity of expressed class II fungal peroxidases

ABSTRACT

Here, we present evidence that *ca.* 20 yrs of experimental N deposition did not halt the expression of class II fungal peroxidases, despite the fact that it has slowed decay in a long-term field experiment in northern hardwood forest stands. Importantly, experimental N deposition altered the composition of expressed class II fungal peroxidases. Such a change in composition could potentially reflect differences in the substrate specificities of the enzymes being expressed under experimental N deposition or a change in the taxonomy or ecology of the fungi responsible for expressing these enzymes. However, the potential effects this may have on function are not obvious as transcripts for enzymes capable of oxidizing the phenolic bonds of lignin (MnPs) appear to be abundant under both ambient and experimental N deposition, while transcripts for enzymes capable of oxidizing the nonphenolic bonds of lignin (LiPs) or simple phenols (GPs) were uncommon but present in both N deposition treatments.

INTRODUCTION

Class II fungal peroxidases are important enzymes for the ecosystem process of decomposition because they play a key role in mineralizing lignin to carbon dioxide (Kirk and Farrell 1987, Hofrichter 2002, Floudas et al. 2012). Peroxidase activity has long been studied in relation to soil C cycling (Sinsabaugh et al. 1992, Waldrop and Firestone 2004); however, the diversity of fungal peroxidases expressed in nature has only recently received attention (Barbi et al. 2014, Bödeker et al. 2014, Kellner et al. 2014). Because of their pivotal role in the decomposition of lignin, a change in the richness, diversity, or composition of expressed fungal peroxidases could change soil C cycling and storage.

High rates of reactive nitrogen (N) deposition reduce peroxidase enzyme activity in soil (Sinsabaugh 2010) and negatively affect the relative abundance of fungi which express these oxidative enzymes (Chapter 2); furthermore, experimental N deposition retards the decomposition of lignin in plant litter and increase soil C storage (Frey et al. 2014). Rates of atmospheric N deposition have increased dramatically over historical levels and continue to increase in some parts of the Earth as the result of human activity (Galloway and Cowling 2002). This important agent of global change has the potential to increase soil C storage by negatively affecting the enzymes and organisms responsible for lignin decay. For example, if elevated rates of N deposition induce a change in either the diversity or composition of the expressed fungal peroxidases, this change could elicit changes in plant litter decomposition.

Fungal class II peroxidases occur in the Agaricomycetes and the type and number of these genes vary widely among taxa in this class (Morgenstern et al. 2008, Nagy et al. 2015). The major types of class II peroxidases are manganese (MnP), lignin (LiP), versatile (VP), and generic (GP) peroxidases. With the exception of GPs, all class II peroxidases decay lignin and

other polyphenols in soil organic matter (Ruiz-Duenas et al. 2013); however, some evidence suggests that GPs may play a role in oxidizing simple phenols (Ruiz-Duenas et al. 2009). MnPs, LiPs, and VPs vary in the types of reactions which they catalyze. MnPs oxidize manganese, which diffuses away to oxidize phenolics (Hofrichter 2002). LiPs, by contrast, directly oxidize the nonphenolic bonds of lignin, whereas VPs have the enzymatic capabilities of both MnPs and LiPs (Hofrichter et al. 2010). MnPs are ancestral to LiPs and VPs, and they are the most widespread among lignolytic taxa and the most diverse (Morgenstern et al. 2008, Floudas et al. 2012). Furthermore, a recent study of peroxidases in the environment have documented novel clades of class II peroxidases, demonstrating that the diversity of this enzyme in nature is greater than was previously known (Kellner et al. 2014). Because these enzymes may target different compounds and types of bonds, a change in the diversity or composition of expressed fungal class II peroxidases could have important functional consequences for the decay of plant litter,as well as polyphenols in soil organic matter.

In this study, we examined the composition and diversity of fungal peroxidase transcripts in the forest floor collected from an ongoing long-term field experiment in a series of northern hardwood stands in which atmospheric N deposition has been experimentally increased to simulate a rates of atmospheric N deposition predicted for midcentury (Galloway et al. 2004). In these study sites, transcripts of fungal peroxidases have been detected under both ambient and experimental rates of N deposition (Kellner et al. 2010, Kellner et al. 2014); however, this study is the first widespread survey of expressed peroxidases under experimental N deposition. We have hypothesized that experimental N deposition will reduce the richness and diversity of peroxidases. We have also hypothesized that experimental N deposition will change the composition of fungal peroxidases expressed. Here, we used a targeted functional gene approach

to gain unique insight into how an agent of global change affects the expressed diversity and composition of an enzyme that plays a critical role in a biologically-mediated ecosystem process.

METHODS

Site Description. Our study sites consists of four northern hardwood forests stands stretching across the lower and upper peninsulas of Michigan, USA. This long-term field experiment was designed to study the effects of chronic elevated levels of N deposition in a widespread type of temperate forest ecosystem. These four sites span a gradient of temperature, precipitation, and ambient atmospheric deposition of N (Figure 4.1). The overstory is dominated by sugar maple (*Acer saccharum* Marsh.). Soils are well-drained sandy typic Haplothords of the Kalkaska series. Plant community composition and soil chemical characteristics are similar across sites (Burton et al. 1991, Macdonald et al. 1991).

At each site, six 30-m x 30-m plots were established in 1994. Three plots receive ambient deposition of N from the atmosphere. The other three plots receive ambient atmospheric N deposition plus an additional application of 3 g NO_3^- -N m^{-2} y^{-1}, which is applied as $NaNO_3^-$ pellets in 6 equal additions of 0.5 g N m^{-2} during the growing season (April-September). This experimental N deposition treatment is consistent with levels of atmospheric N deposition predicted for northeastern North America and portions of Europe by 2050 (Galloway et al. 2004). Each treatment plot is surrounded by a 10-m treated buffer zone to reduce edge effect, which also receives the aforementioned N deposition treatments. Treated plots in this experiment have been receiving this rate of additional N annually since 1994. The long-term experimental application of N has not changed soil pH, base saturation, matric potential, or forest floor conductivity (Patterson et al. 2012).

Sample collection and processing. The O_e/O_a horizons were collected from ten random 10 cm^2 quadrats in each plot during September 2014. Collected forest floor samples were homogenized with sterile scissors and immediately immersed in liquid N_2 in the field. Samples were stored at -80˚C until nucleic extraction.

Nucleic acid extraction. For each experimental plot, we performed ten replicate nucleic acid extractions. For each extraction, we added ~0.25 g of forest floor material into a MoBio Powerlyzer® Glass Bead Tube with 0.1 mm glass beads. We extracted nucleic acids according to a previously published method (Freedman et al. 2015) using an initial phenol-chloroform extraction followed by extraction with a MoBio PowerLyzer® PowerSoil® DNA Isolation kit and an overnight ethanol precipitation. Extracted nucleic acids were purified with a MoBio PowerClean® DNA Clean-Up Kit. Purified nucleic acids were subsequently treated with DNase I enzyme in order to remove DNA from the total nucleic acids and obtain a solution containing RNA only.

Synthesis of random-hexamer cDNA. For each sample, we performed ten replicate reverse transcription (RT) reactions. For each RT reaction, we added 5 µL of extracted RNA to generate cDNA with random hexamer (RH) primers according to the manufacturer protocol for the SuperScript Vilo Master Mix (cat. #11755-010). The resulting first strand cDNA from RT with RH primers is subsequently referred to as random-hexamer cDNA (RH-cDNA).

PCR and cloning of fungal peroxidases from forest floor RH-cDNA. Prior to next generation sequencing, we initially amplified and cloned RH-cDNA in order to verify that PCR products represented fungal peroxidases. We recently modified an existing pair of class II peroxidase primers (Bodeker et al. 2009) to create primers *fpx*F (5' GGWGGWGCYGAYGGITC 3') and *fpx*R (5' GGRGTYGARTCGAAIGG 3'), which are more appropriate for use in environmental

samples. We first used these primers to amplify and clone expressed fungal peroxidases from a subset of our samples.

Each PCR contained 0.5 µL dNTPs (20 mM each), 2.5 µL SigmaAldrich® JumpStart™ 10x buffer, 1.5 µL $MgCl_2$ (25 mM), 1.5 µL bovine serum albumin (25 mg mL^{-1}), 1.5 µL *fpx*F (20 µM), 1.5 µL *fpx*R (20 µM), 13.5 µL molecular biology-grade water, 0.5 µL JumpStart™ *Taq* DNA polymerase, and 2 µL RH-cDNA template. In order to verify that all genomic DNA was removed at the DNase I treatment step, we also included PCRs in which the template was nucleic acid extractions that had been treated with DNase I, but which were not subsequently reverse transcribed. Reactions were assembled on a cold block, immediately transferred to a thermocycler block that had been preheated to 95 ˚C, and run with the following thermocycler protocol: initial denaturation (5 min, 95 ˚C), followed by 35 cycles of amplification which included denaturation (30 s, 95 ˚C), primer annealing (30 s, 51 ˚C), extension (75 s, 72 ˚C), and a final extension (7 min, 72 ˚C) to complete the protocol.

We excised a ~400 bp band from each product, removed agarose and contaminants with a Qiagen QIAquick® Gel Extraction Kit, and then cloned the amplicons with an Invitrogen TOPO® TA Cloning® kit. We selected colonies and prepared the plasmids with a Promega Wizard® Plus SV Miniprep kit which were submitted for Sanger sequencing at the University of Michigan sequencing core. After obtaining results of sequencing, we conducted discontinuous megablasts using the NCBI BLAST portal to determine whether inserts represented fungal peroxidases. We selected 10 of these plasmids, each of which contained a unique insert which had produced BLAST matches to one or more fungal peroxidases. We pooled these plasmids in equal proporotions (10^{11} copies each) to make a multigene mixture that would serve as a "mock

community" (Highlander 2014, Lluch et al. 2015), in order to help us assess sequencing error rate.

PCR and sequencing of peroxidases from forest floor RH-cDNA. We performed PCR on all samples, as well as on our mock community DNA, using barcoded primers which allowed us to multiplex multiple samples on a single PacBio SMRT chip for sequencing on a PacBio RSII sequencer. Barcodes selected were pairs 1, 2, 3, 8, and 9 from among 96 pairs of barcodes designed by the manufacturer for multiplexing on the PacBio RSII instrument (https://github.com/PacificBiosciences/Bioinformatics-Training/blob/master/barcoding/pacbio_barcodes_paired_nopadding.fasta). For each sample, we performed hot-start PCR in triplicate according to the conditions described above, ran the products on a 2% agarose gel, and excised the appropriate band from each lane with a clean, sterilized scalpel.

We removed agarose and other contaminants from the excised gel bands using a Qiagen QIAquick® Gel Extraction Kit with products from triplicate PCRs pooled prior to the elution step. We repeated the PCR and gel extraction process again for each sample and pooled products from both rounds prior to purifying the PCR products using a Qiagen MinElute PCR Purification Kit, and concentrated the samples by eluting with a volume of 20 µL of water. For most samples, it was necessary to repeat the entire PCR, gel extraction, and purification procedures twice in order to obtain enough product to sequence. PCR products from the mock community did not require gel excision and were purified with the Qiagen MinElute PCR Purification Kit. We measured quality of our final products on a ThermoScientific NanoDrop™ 8000 UV-Vis Spectrophotometer and quantity with an Invitrogen Quant-IT™ PicoGreen® dsDNA kit on a BioTek® Synergy HT microplate reader.

For multiplex sequencing, we pooled products to be sequenced on the same PacBio® SMRT® chip. For pooled products that had less than < 350 ng total DNA, we spiked the pooled products with PCR product from the mock community to bring it up to 350 ng and ensure sufficient DNA quantity for sequencing. Pooled PCR products were submitted to the University of Michigan sequencing core where they received two rounds of sequencing with magnetic-bead loading on the PacBio® RS II sequencer using P6-C4 chemistry. The resulting data files from each of these rounds of sequencing were combined during sequence processing in mothur. We obtained sequence files with 10X circular consensus sequence (CCS) coverage.

cDNA sequence processing. We processed our cDNA sequences in mothur (Schloss et al. 2009). First, we removed sequences with low quality scores (qwindowsize=50, qwindowaverage=25), sequences with mismatches to the barcode or primer regions, sequences with ambiguous nucleotides, sequences with homopolymers of > 9 nucleotides, and sequences below 350 bp and exceeding 450 bp in length. We trimmed primers and barcodes from sequences prior to downstream processing. The cDNA sequences were aligned in mothur using a reference alignment that consisted of 445 fungal class II peroxidase sequences which we had previously obtained from GenBank, Fungene (Fish et al. 2013), and MycoCosm (Grigoriev et al. 2014) and had aligned with MAFFT (Katoh et al. 2002). Following alignment of the cDNA sequences, we removed sequences which began or ended at inappropriate positions within the alignment. Finally, we removed chimeras which had been identified with UCHIME (Edgar et al. 2011). Unique sequences were preclustered in order to denoise the data set. Following the precluster step, we either exported unique sequences for translation or, for nucleotide-based OTU analyses, proceeded to distance matrix calculation and clustering. For the mock community, we calculated the error rate using the seq.error function.

Translation of cDNA sequences. In order to translate cDNA nucleotide sequences into amino acid sequences, we uploaded unique, preclustered, and degapped nucleotide sequences in a fasta-formatted file to the FunGene FrameBot (Fish et al. 2013) online interface (http://fungene.cme.msu.edu/FunGenePipeline/framebot/form.spr). To guide the translation, we provided a fasta file of 323 reference fungal class II peroxidases protein sequences; we had obtained these reference sequences from FunGene (Fish et al. 2013) and Mycocosm (Grigoriev et al. 2014) and had trimmed off the conserved amino acids of the primer regions (Bodeker et al. 2009) to create the reference file.

We downloaded our translated cDNA sequences from Fungene Framebot, imported them into Geneious 6.1.8, and aligned them with these same 323 fungal peroxidase protein reference sequences using MAFFT (Katoh et al. 2002). We inspected sequences to determine if they contained the conserved catalytic residues associated with Mn^{2+} oxidation (Asp-175) or LiP-like activity (Trp-171) as described by others (Morgenstern et al. 2008, Kellner et al. 2014). We trimmed the alignment to the last nucleotide position for which no sequences in our data set had a gap and manually removed the reference protein sequences from the alignment. We then exported aligned amino acid sequences for downstream use in either distance matrix calculation or Unifrac analysis.

OTU clustering and analyses. For cDNA nucleotide sequences, we were able to calculate distance matrices in mothur. However, mothur will not calculate distances for amino acid sequences, so we calculated distances for amino acid sequences by using the seqinr package for R and subsequently imported a column-formatted matrix into mothur to cluster sequences into operational taxonomic units (OTUs). We initially clustered our mock community nucleotide sequences at levels up to 0.10 and examined the number of OTUs obtained at the unique, 96%,

and 90% sequence similarity levels. Distances for amino acid sequences were very large and mothur was not able to cluster sequences beyond the unique level. However, we were able to cluster nucleotide sequences beyond this level and selected 96% and 90% OTUs for analysis. Prior to downstream analyses, singleton OTUs were removed.

We initially determined how many OTUs were present at an abundance of ≥ 1% of all sequences under both ambient and experimental N deposition across the entire data set using the get.coremicrobiome command in mothur. Following this, we removed all plots with < 100 sequences, normalized the number of sequences across the remaining plots by subsampling, and subsequently repeated core microbiome analysis. Using the subsampled data set, we calculated observed richness, Shannon diversity, and inverse Simpson diversity and created shared files for analysis of OTU composition.

We manually calculated evenness (E_H) from Shannon diversity (H) as E_H = H/ln(observed richness). We assessed the effect of N deposition treatment and site on observed richness, Shannon diversity, and inverse Simpson diversity were assessed with two-way ANOVAs in SPSS statistical package v. 23. Because the Venn diagrams created in mothur are not scaled, we created scaled diagrams using the VennDiagram package for R.

To statistically assess whether experimental N deposition altered the composition of expressed peroxidases, we performed one-factor permutational multivariate ANOVA (PERMANOVA) and PERMDISP in the PRIMER-E statistics program. A one-factor rather than a two-factor analysis was performed because we had an insufficient number of replicates to successfully examine the main effect of site with permutational analyses. We analyzed both untransformed data and data to which a presence-absence transformation had been applied; by not transforming data, the most abundant OTUs are heavily weighted and will most strongly

influence results, whereas with a presence-absence transformation all OTUs have the same weight regardless of their abundance (Anderson 2001). We calculated a Bray-Curtis distance matrix based on OTU abundances and performed PERMANOVA and PERMDISP with 9999 iterations with PERMDISP distances calculated from centroids. We report permutational *P* values for these analyses.

Phylogenetic composition of expressed peroxidases. OTU-based analyses treat closely and distantly related to OTUs the same, making it desirable to also examine peroxidase composition and structure with analyses that take the phylogenetic relationship between the sequences present into account. To that end, we performed both unweighted and weighted Unifrac analyses to test the hypotheses that experimental rates of N deposition altered the phylogenetic composition and structure of the expressed peroxidases.

Prior to translating our cDNA sequences, we excluded plots that had yielded fewer than 81 sequences. Prior to analyzing data, we subsampled all plots to normalize the number of sequences across plots and performed two analyses: one for all sites and a separate analysis for Sites B & C only; because we did not have a balanced design (*i.e.*, equal *n* for ambient and experimental N deposition) across all sites as a result of several low-yielding plots at Sites A & D which we had excluded, we performed an additional analysis restricted to Sites B & C in order to have a test with a balanced design with which to additionally consider the effects of Site and N-deposition-by-Site for on Unifrac metrics. For our analysis across all sites, an additional subsampling step was necessary in order to obtain an equal number of sequences in each N deposition treatment, which we accomplished by first merging plots by N deposition treatment and then further subsampled to normalize the number of sequences in each treatment. For analysis of Sites B & C only, no additional subsampling was necessary because we had already

normalized sequences at the plot level and the number of plots in each N deposition treatment was equal.

We exported unique nucleotide sequences, translated with Framebot, aligned the resulting amino acid sequences with MAFFT, and trimmed the alignment in the same manner described above. To perform Unifrac analysis, we reimported the alignment of our translated peroxidase sequences into mothur. In mothur, we created a relaxed neighbor-joining phylogenetic tree with clearcut (Evans et al. 2006) and ran the unweighted and weighted Unifrac commands.

In order to further examine lineages of peroxidases present under ambient and experimental N deposition, we performed a neighbor-joining analysis to identify peroxidases in our data set which were closely related to each other and to our selected reference sequences. To reduce the number of sequences to a feasible number for this analysis, we clustered peroxidase cDNA nucleotide sequences to 0.10, selected the most abundant sequence in each OTU as a representative sequence, manually removed singletons, and translated the representative sequences with Framebot as previously described. We aligned the resulting amino acid sequences as described previously with the additional inclusion of an outgroup sequence, *Magnaporthe oryzae* ligninase C (GenBank accession number ELQ39692.1), which was selected based on previously published phylogenies of class II fungal peroxidases (Morgenstern et al. 2008). While both neighbor-joining and maximum likelihood techniques were able to resolve the relationships of closely related sequences with high bootstrap support, the relationships between distantly related sequences remained unresolved with both techniques (data not shown); therefore, we did not attempt to resolve deep nodes of the tree, but instead constructed a neighbor-joining tree in Geneious Tree Builder with 100 bootstraps and imposed a bootstrap minimum of 25 in order to identify clades of closely related sequences only without attempting

to determine the relationships between distantly related clades. We number OTUs from most to least abundant. OTUs were considered to be as LiPs if they possessed Trp-171, but not Asp -175 and GPs if they possessed neither Trp-171 or Asp-175. All OTUs which were not LiPs or GPs possessed the conserved Asp-175 for oxidation of Mn^{2+}. We identified OTUs and lineages which occurred under ambient N deposition, under experimental N deposition, or both.

RESULTS

Error rate. The error rate of the raw sequences was 0.14%, meaning that ~21% of sequences contained 1 or more errors. Denoising with a precluster step reduced the error rate to 0.08%, meaning ~11% of sequences contained 1 or more errors. Because of this, the OTU count for our mock community was highly inflated compared to the ~10 OTUs we anticipated observing based on the initial ten sequences which we used to assemble it. However, OTUs resulting from sequencing error were also of low abundance. Therefore, by removing singletons and subsequently subsampling the mock community at a similar number of sequences (*i.e.*, 100) as used for our analyses of richness, diversity, and composition, we obtained 17 OTUs at the unique level, 12 OTUs at the 96% sequence similarity level, and 9 OTUs at the 90% sequence similarity level in the mock community.

Sequencing yield. Following our sequencing pipeline, we obtained 24,472 total fungal peroxidase cDNA sequences across our data set. Sequence yield varied highly across plots, with five plots yielded fewer than 100 sequences. However, 19 out of 24 plots in our experiment yielded >100 sequences and 6 plots yielded >1000 sequences. These 24,472 nucleotide sequences represented 3810 unique sequences and clustered to 380 and 185 OTUs at the 96% and 90% sequence similarity levels, respectively. Translation of the unique cDNA nucleotide sequences, subsequently yielded 3809 unique amino acid OTUs.

Singletons in our data set made up a small portion of our total sequences, but a large portion of our OTUs. For amino acid sequences clustered to the unique level, 13% of total sequences in our data set were singletons, but these represented 81% of all OTUs. For nucleotide sequences clustered to the 96% sequence similarity level, singletons accounted for only 0.7% of all sequences, but comprised 47% of all OTUs. For nucleotide sequences clustered at 90% similarity, singletons represented 0.2% of all sequences, but still represented 31% of OTUs. When singletons were removed, the total number of OTUs in the data set dropped to 718 for the unique amino acid OTUs, and 193 and 127 for the 96% and 90% nucleotide sequence similarity OTUs, respectively.

Inspection of the unique translated cDNA sequences revealed nearly all (99%) of these unique sequences possessed the conserved residue (Asp-175) for Mn^{2+} oxidation (Kellner et al. 2014). Very few of these sequences (0.3%) contained the conserved residue (Trp-171) for LiP-like activity associated with lignin and versatile peroxidases (Kellner et al. 2014). Thus, it appears that nearly all transcripts obtained in our study represent enzymes which can oxidize Mn^{2+}, whereas only a few may possess LiP-like activity. Therefore, transcripts obtained in our study largely appear to represent MnPs.

Richness and diversity indices. To address our hypothesis that experimental N deposition would reduce richness and diversity of expressed peroxidases, we calculated richness, the Shannon diversity index, the Shannon evenness index, and the inverse Simpson diversity index for each plot receiving ambient and experimental N deposition. Following removal of singletons and subsampling, we obtained a total richness of 267 (unique), 125 (96% sequence similarity) and 89 (90% sequence similarity) OTUs, respectively. At the plot level, the observed richness of peroxidases ranged from 1 to 30 OTUs (Table 4.1).

The ranges for Simpson diversity (1 – 11.6) and Shannon evenness (0.08 – 0.93) indicate that there was considerable variation among samples in the extent to which they were dominated by expression of a single peroxidase or reflected the expression of a variety of peroxidases at approximately equal rates (Table 4.1). When a sample is dominated by one highly expressed OTU, inverse Simpson diversity ≈ 1 and Shannon evenness ≈ 0. However, Simpson diversity increases and Shannon evenness ≈ 1 for samples in which multiple OTUs are present in equal amounts. Shannon diversity, which incorporates both abundance and evenness, exhibited a narrower range (0 – 2.62) among samples than did Simpson diversity (Table 4.1), which may reflect the fact that total observed richness was relatively low.

However, experimental N deposition had no significant effect on richness, Simpson diversity, or Shannon diversity of the expressed peroxidases at any of the OTU levels examined (Figure 4.2, Table 4.2). Furthermore, site and the interaction of N deposition treatment with site were also not significant factors for these comparisons (Table 4.2). However, experimental N deposition did significantly increase Shannon evenness of 90% nucleotide sequence similarity OTUs (Figure 4.2, Table 4.2); nonetheless, experimental N deposition did not significantly increase evenness for the 96% sequence similarity or unique OTUs, although these values were close to our significance cut-offs (Table 4.2). Shannon evenness also varied significantly among Sites for the 90% nucleotide sequence similarity OTUs, but this was not true for the other OTU levels (Table 4.2). There was no significant interaction between N deposition treatment and Site for comparisons of Shannon evenness at any of the OTU levels examined (Table 4.2).

OTU-composition. The peroxidases expressed under experimental N deposition were neither entirely separate nor completely a subset of those expressed under ambient N deposition (Figure 4.3). Instead, some peroxidases were expressed in both N deposition treatments, whereas a

larger number of OTUs are distinct to one N deposition treatment or the other. The proportion of peroxidases expressed under both ambient and experimental N deposition increased with decreasing percentage similarity of cDNA sequences (Figure 4.3).

However, when comparing OTUs which are abundant (*i.e.*, comprise at least 1% of all expressed sequences under both ambient and experimental N deposition), very few OTUs (3 – 5) were abundant under both ambient and experimental N deposition (Table 4.3). Therefore, most peroxidases expressed under both ambient and experimental N deposition treatments are those that are of low abundance, with only a few of the shared peroxidases were abundant in both N deposition treatments.

For unique OTUs clustered from translated cDNA sequences, experimental N deposition significantly altered composition of expressed peroxidases according to PERMANOVA (Table 4.4) and PERMDISP (Table 4.5) when presence-absence was examined; however, experimental N deposition had no significant effect using untransformed data (Tables 4.4, 4.5). This suggests that experimental N deposition had a significant effect on the composition of the rare, but not the dominant OTUs. By contrast, experimental N deposition had no significant effect on the composition 96% or 90% OTUs clustered from nucleotide sequences, either when untransformed data were examined or when the presence-absence transformation was applied, according to PERMANOVA (Table 4.4) or PERMDISP (Table 4.5). However, PERMANOVA results for 96% and 90% OTUs were close to our significance cut-off when data were analyzed for presence-absence (Table 4.4).

Phylogenetic composition. Experimental N deposition significantly altered the fraction of branch length in the phylogenetic tree which led to peroxidases from one N deposition treatment but not the other, both with and without weighting by sequence abundance (Table 4.6). When

we limited analysis to Sites B & C only in order to obtain a balanced design necessary to consider the effects of Site and N-deposition-by-Site (see Methods for more details), experimental N deposition had a significant effect on weighted and unweighted Unifrac, both within and across sites; furthermore, each site contained unique lineages of expressed peroxidases (Table 4.6). Thus, our results for unweighted and weighted Unifrac are consistent with our expectations that experimental N deposition would alter the phylogenetic composition of the peroxidases expressed by the fungal community.

To determine which lineages contribute to this effect, we performed a neighbor-joining analysis with translated representative sequences from 90% sequence similarity OTUs. Neighbor-joining revealed that most peroxidases were either present under both ambient and experimental N deposition as shared OTUs or were part of clades which included closely related peroxidases from both ambient and experimental N deposition treatments (Figure 4.4). However, some lineages were unique to a particular N deposition treatment.

We identified 14 lineages containing 17 OTUs that were present only under ambient N deposition and 8 lineages representing 11 OTUs which were only observed under experimental N deposition (Figure 4.4). All of the unshared lineages contained OTUs which were identified as either MnPs or GPs based on the presence and absence of conserved catalytic residues for Mn^{2+} oxidation and LiP activity (Morgenstern et al. 2008, Kellner et al. 2014). The LiP OTUs which we obtained were either part of OTUs which were expressed under both ambient and experimental N deposition or were closely related to these shared OTUs (Figure 4.4).

While most of these unshared lineages were not closely related to reference sequences from known species, several of the lineages unique to the ambient N deposition treatment included reference sequences from one or more known species; these were associated with MnPs

from the Agaricales (*Agaricus bisporus*, *Armillaria gallica*, and *Volvariella volvacea*), the Hymenochaetales (*Phylloporia ribis*), the Polyporales (*Phanerochaete flavidoalba* and *Grifola frondosa*), and the Corticiales (*Cytidia salicina*). While no MnPs that were distinct to the experimental N deposition treatment were closely related reference sequences, OTU077 (Figure 4.4) was placed within a larger clade containing MnPs from a variety of Polyporales.

DISCUSSION

Our prior work has revealed that *ca.* 20 years of experimental N deposition has altered the composition of the active fungal community in the forest floor (Chapter 1) and decreased the abundance of lignolytic fungi (Chapter 2). Furthermore, experimental N deposition decreased the richness of fungal lignocellulolytic genes in two out of four experimental sites in our study system (Eisenlord et al. 2013) and reduced the abundance of transcripts for fungal lignocellulolytic genes in a metatranscriptomic survey (Hesse et al. 2015). Based on these previous observations, we would anticipate that experimental N deposition would also reduce the richness and diversity as well as alter the composition of expressed class II fungal peroxidases. Such changes in the richness, diversity, or composition of transcripts for this important class of lignolytic enzymes could potentially explain reductions in lignin-decay observed in our study (Pregitzer et al. 2008, Zak et al. 2008). Yet, experimental N deposition did not reduce the richness or diversity of expressed fungal class II peroxidases in this study, but did alter the composition of the peroxidases which were expressed.

Rather than decreasing the diversity of expressed peroxidases as we hypothesized, experimental N deposition increased the diversity of peroxidases being expressed in the forest floor, although this increase was not statistically significant (Figure 4.2). Furthermore, experimental N deposition increased the Shannon evenness of peroxidases being expressed in the

forest floor (Figure 4.2). Taken together, this suggests that a greater variety of fungal genes are contributing substantially to the process of lignin decay under experimental rates of N deposition, relative to the ambient condition. One plausible explanation for this observation is that experimental N deposition suppressed the expression of genes which typically dominate this process, thereby opening up niche space for other fungi and allowing them to play a greater role in lignin decay. Previous studies have found a greater potential for bacterial lignin decay under experimental N deposition (Freedman and Zak 2014), possibly as a result of reduced competition from fungal lignin decomposers, which are more efficient in this regard (de Boer et al. 2005). It is, therefore, possible that a similar mechanism occurs among lignin-decaying fungi wherein less efficient lignin-decomposers play a greater role under experimental N deposition because of suppression of more efficient fungal decomposers. However, more information regarding the origins and functions of the specific enzymes declining and increasing in representation would be needed to determine whether this is the case.

Peroxidases expressed under experimental N deposition were not simply a subset of those expressed under ambient N deposition nor were the peroxidases expressed under experimental N deposition an entirely separate set of enzymes from those expressed under ambient N deposition (Figure 4.3). Instead, some peroxidases were distinct to each N deposition treatment, whereas others co-occurred in both treatments (Figure 4.3). Experimental N deposition altered the composition of fungal peroxidases expressed when all OTUs, regardless of abundance, were weighted equally, but not when the most abundant OTUs were highly weighted (Tables 4.4 & 4.5). However, most OTUs in the data set represented only relatively small percentages (2% or less each) of total sequences, whereas only a few OTUs represented large portions of sequences (5% or more, data not shown). The strongest effects on community composition, however, were

determined by Unifrac analyses in which the fraction of unique branch length (*i.e.*, unshared lineages) varied significantly between the N deposition treatments. Such unique lineages could potentially have different substrate specificities or arise from taxa with different physiological and ecological roles in lignin and polyphenol decomposition. Taken together, changes in both the composition of peroxidase OTUs and in the phylogenetic composition of expressed peroxidases suggest a potential change in the function of these expressed enzymes has occurred under experimental N deposition, consistent with our hypotheses and a decline in matter decay and increases soil C storage that we have previously documented (Pregitzer et al. 2008, Zak et al. 2008).

However, when we examined the peroxidases observed in our study, transcripts for MnPs, which oxidize the phenolic bonds in lignin, were abundant and diverse under both ambient and experimental N deposition (Figure 4.4). Furthermore, most MnP lineages occurred across both treatments. These observations suggest that that experimental N deposition did not have a negative effect on the expression of MnPs. Furthermore, some unique MnPs occurred in both treatments, suggesting that differences in composition were not solely driven by losses of enzymes expressed under ambient N deposition, but also by gains of enzymes not expressed under ambient N deposition; however, the number of lineages absent from the experimental N deposition did exceed those absent from the ambient N deposition treatment (Figure 4.4).

While many of the MnP lineages occurring only under ambient N deposition were unrelated to any known MnP, a few were related to well-studied MnPs from known species, allowing us to make inferences regarding the ecology and function of these lineages. For example, OTU071 occurred in the same clade as two MnP sequences from *Volvariella volvacea* (AFR44748, AFR44749), a litter decay fungus which resembles white-rot wood decay fungi in

its enzymatic capacity for decay of lignin and crystalline cellulose (Floudas et al. 2015). Other OTUs found only under ambient N deposition were in clades with MnPs from known wood-decay fungi including *Armillaria gallica*, *Cytidia salicina*, *Phanerochaete flavidoalba*, *Grifola frondosa*, and *Phylloporia ribis*. Finally, one OTU occurring only under ambient N deposition was in a clade litter decomposer *Agaricus bisporus,* which is adapted for late-stage decay and humic-rich environments (Morin et al. 2012). Thus, we can infer that these lineages found only under ambient, but not experimental, N deposition may represent MnPs from a variety of powerfully lignolytic wood and litter decay fungi and from late-stage decomposers specializing in the decay of highly humic litter. Most of the peroxidases occurring only under experimental N deposition were also not closely related to any known reference sequences, but one OTU grouped within a larger clade containing MnPs from a variety of wood-decay fungi of the Polyporales, suggesting this lineage also represents MnPs from highly lignolytic white-rot fungi.

Although a few LiP sequences were recovered in our study, expressed LiPs were uncommon under both ambient and experimental N deposition. Thus, there is also no evidence that their associated function (decay of nonphenolic bonds of lignin) has been lost under experimental N deposition. The low recovery of LiPs in our study is probably not the result of amplification bias against these enzymes, as the primer sequences in this study were present in LiP reference sequences (data not shown). Instead, it seems likely that LiP is rarely expressed in the forest floor, at least in our field sites and at the time of our sampling. Finally, we recovered some GPs, which may decay simple phenols, from both ambient and experimental N deposition, as well, suggesting that this function is also unaffected by experimental N deposition.

In this study, we were able to amplify fungal class II peroxidases transcripts from forest floor in stands receiving both ambient and experimentally elevated rates of N deposition. As a

result of this, we know that experimental N deposition does not completely inhibit peroxidase transcription, because we were able to extract and amplify sequences for these enzymes. However, it is plausible that experimental N deposition could reduce the rate at which these enzymes are transcribed and produced. While we did not quantify the abundance of fungal class II peroxidases transcripts in the present study, measuring the abundance of expressed peroxidases under ambient and experimental rates of N deposition is an important priority for future research.

Overall, we observed changes in the composition and evenness of transcripts a group of enzymes which play a critical role in lignin decay in a long-term experiment in which experimental N deposition has reduced lignin decay. Given the changes observed in C-cycling in response to experimental rates of N deposition, we might have anticipated more dramatic losses of peroxidase diversity and function than we have observed here. For example, while we did see changes in the evenness of expressed genes, we did not observe any loss in diversity or richness. Furthermore, while the changes in composition of expressed peroxidases, especially the loss of several lineages associated with known peroxidases, under experimental N deposition suggests that changes in function may have also occurred, we still obtained a diverse array of peroxidases apparently capable of oxidizing phenolic and, to a lesser extent, nonphenolic bonds of lignin across both N deposition treatments. Thus, in constrast to our expectations, we were unable to detect any obvious reduction in function in response to experimental N deposition.

The potential functional implications of the changes in composition and evenness of expressed peroxidases observed here are not entirely clear, because we have a limited understanding of the biology and ecology of enzymes recovered from the environment as well as the identity of the organisms which may be expressing them. Most of the sequences we obtained

were only distantly related to reference sequences from known and well-studied fungal peroxidases, a result which has also been observed in a previous environmental survey of these enzymes (Barbi et al. 2014). Others, including several of the larger OTUs in our study, were closely related only to peroxidases from fungal species for which genome sequencing has only recently revealed the potentially important roles in lignin decay by these organisms. For example, both OTU001 and OTU004 were closely related to a peroxidase sequence from the genome of *Sphaerobolus stellatus*, whose incredible diversity of peroxidases was unknown until last year (Nagy et al. 2015). Furthermore, we were unable to resolve the underlying phylogentic relationships between disparate clades of peroxidases, which may have also provided context for the evolution and function of different groups of MnPs. Thus, we presently lack the appropriate context to interpret the changes in composition observed here in more detail.

If experimental N deposition is dramatically altering the composition and diversity of fungal lignolytic genes, it is possible that we would have to observe a broader array of fungal lignocellulolytic genes in order to fully observe the extent of these effects. For example, peroxidases are among a suite of fungal enzymes which include laccases (Theuerl and Buscot 2010), dye-decolorizing peroxidases (DyPs) (Liers et al. 2010, Liers et al. 2013), unspecific or aromatic peroxygenases (UPOs/APOs) (Pecyna et al. 2009, Liers et al. 2011), and chloroperoxidases (CPOs) (Ortiz-Bermúdez et al. 2003, Kellner et al. 2010) for which roles in lignin-decay are either known or suspected. Furthermore, the extent of a fungal species to decay lignin is strongly linked to its mode of cellulose utilization; the fungi with the most lignolytic potential possess not only the most peroxidase genes, but also the most genes for utilization of crystalline cellulose (Riley et al. 2014, Floudas et al. 2015). It is, therefore, possible that experimental N deposition effects expression of entire suites of fungal lignocellulolytic genes

and that examination of more than one set of C-cycling genes may be necessary to more fully observe changes in function.

We recently documented that experimental N deposition reduced the abundance of lignolytic fungi on wood and high-lignin substrates more dramatically after 7 months of decay in the field than after 18 months (Chapter 3), which suggests that the strongest negative effects of experimental N deposition on lignolytic fungi occur earlier, rather than later, in the decay process. In this study, we examined forest floor (O_e/O_a) horizons which represent the latter stages of decay. Therefore, it is possible that larger differences in terms of peroxidase composition may have been observed if we had also included the more recently deposited (O_i) litter layers. Moreover, it is possible that experimental N deposition may have only subtle effects on the expression of lignolytic enzymes in leaf litter because leaf litter decay is not driving SOM accumulation under experimental rates of N deposition. While experimental N deposition has increased soil C content (Pregitzer et al. 2008, Zak et al. 2008), we have recently learned that root litter, not leaf litter, is the largest source of these inputs (Thomas et al. 2012, Xia et al. 2015), despite there being no increase in either the production or recalcitrance of root litter under experimental N deposition (Burton et al. 2012, Xia et al. 2015). In light of this, the response of fungal lignocelluloytic gene expression under experimental N deposition may ultimately be more important to observe on belowground litter than on aboveground litter.

Our findings here add to a growing body of knowledge regarding the effects of long-term increases in the rate of N deposition on soil and forest floor microbial communities. Thus far, we know that experimental N deposition has reduced the activity of peroxidase (DeForest et al. 2004, Freedman et al. 2015, Freedman et al. 2016), laccase (DeForest et al. 2004, Freedman and Zak 2014), and cellbiohydrolase enzymes (Freedman et al. 2015, Freedman et al. 2016) in our

study system, strongly suggesting that a decline in the activity of highly lignolytic fungi accounts for observed increases in soil organic matter under experimental N deposition. Furthermore, experimental N deposition has negatively affected the abundance of fungal transcripts for lignocellulose decay (Hesse et al. 2015), suggesting that declines in not only the activity, but also the expression of fungal lignocellulolytic genes, are occurring under experimental N deposition. At the same time, experimental N deposition has altered the composition of fungi active in the forest floor (Entwistle et al. 2013, Freedman et al. 2015), indicating that different fungal taxa are active in decomposition under experimental N deposition. Finally, experimental N deposition negatively affected the abundance of lignolytic fungi and positive affected on the abundance of fungi which specialize in the decay of cellulose and hemicelluloses without substantial amounts of concurrent lignolysis (Chapter 3). Because of this, experimental N deposition may have also altered the potential for expression of fungal lignolytic genes, although previous studies regarding this potential have provided conflicting results (Eisenlord et al. 2013, Freedman et al. 2016). Here, we have observed changes in composition of expressed fungal peroxidases, a highly important class of enzymes for lignin decay, but no accompanying loss of diversity. This change in composition of an expressed functional gene implies that co-occurring changes in function are occurring; indeed, we did observe the absence of some lineages of known importance under experimental N deposition, but the expression of genes capable of manganese-oxidation and, thus, the oxidation of the phenolic bonds of lignin remained robust across N deposition treatments. Further work to elucidate the origin of the peroxidase genes observed in our experiment, and to determine the rate at which these genes are transcribed under both ambient and experimental N deposition, may provide additional insight into how an important agent of global change alters the ecosystem process of decomposition at the molecular level.

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TABLES

Table 4.1. Observed ranges for richness, Shannon diversity, Shannon evenness, and Simpson diversity of expressed fungal peroxidases from unique OTUs which were clustered from amino acid sequences and 96% and 90% sequence similarity OTUs which clustered from cDNA nucleotide sequences.

Index	Similarity level of OTUs	Sequence type from which OTUs were created	maximum	minimum
Observed richness	Unique	Amino acid	12	30
	96%	Nucleotide	3	19
	90%	Nucleotide	1	16
Shannon diversity	Unique	Amino acid	0.72	2.62
	96%	Nucleotide	0.09	2.36
	90%	Nucleotide	0	2.22
Shannon evenness	Unique	Amino acid	0.29	0.85
	96%	Nucleotide	0.08	0.83
	90%	Nucleotide	0.15*	0.93
Simpson diversity	Unique	Amino acid	1.3	11.6
	96%	Nucleotide	1	8.2
	90%	Nucleotide	1	10.7

* Shannon diversity could not be calculated for plot for which observed richness was 1 and Shannon diversity was 0.

Table 4.2. Observed richness, Shannon diversity, Shannon evenness and inverse Simpson diversity of expressed fungal peroxidases analyzed with two-way ANOVA. Nucleotide cDNA sequences were clustered to the 96% and 90% sequence similarity level. Translated cDNA amino acid sequences were clustered to the unique level. Singletons were removed and all plots with fewer than 100 sequences were excluded prior to subsampling to normalize sequence counts across plots. Significant comparisons ($P < 0.05$) are in bold.

Unique amino acid OTUs					
	Factor	Degrees of freedom	Mean Square	F	*P*
Unique peroxidase amino acid sequences observed	N deposition	1	26.080	0.844	0.382
	Site	3	24.468	0.792	0.529
	N deposition x Site	3	25.559	0.827	0.512
Shannon diversity	N deposition	1	0.903	3.765	0.084
	Site	3	0.414	1.726	0.231
	N deposition x Site	3	0.545	2.274	0.149
Shannon evenness	N deposition	1	0.079	4.765	0.057
	Site	3	0.034	2.056	0.177
	N deposition x Site	3	0.050	3.009	0.087
Inverse Simpson diversity	N deposition	1	15.419	1.571	0.242
	Site	3	5.810	0.592	0.636
	N deposition x Site	3	4.046	0.412	0.748
96% sequence similarity nucleotide OTUs					
Richness or diversity index	Factor	Degrees of freedom	Mean Square	F	*P*
Unique peroxidase amino acid sequences observed	N deposition	1	0.840	0.059	0.814
	Site	3	52.102	3.631	0.053
	N deposition x Site	3	15.767	1.099	0.394
Shannon diversity	N deposition	1	0.687	1.973	0.190
	Site	3	0.937	2.689	0.103
	N deposition x Site	3	0.621	1.783	0.214
Shannon evenness	N deposition	1	0.151	3.980	0.074
	Site	3	0.071	1.870	0.199
	N deposition x Site	3	0.087	2.292	0.140
Inverse Simpson diversity	N deposition	1	5.268	1.352	0.272
	Site	3	6.532	1.677	0.234
	N deposition x Site	3	2.368	0.608	0.625
90% sequence similarity nucleotide OTUs					
Richness or diversity index	Factor	Degrees of freedom	Mean Square	F	*P*
Unique peroxidase amino acid sequences observed	N deposition	1	6.094	0.434	0.524
	Site	3	32.392	2.306	0.133
	N deposition x Site	3	20.652	1.470	0.276
Shannon diversity	N deposition	1	1.072	3.400	0.092
	Site	3	0.899	2.851	0.086
	N deposition x Site	3	0.696	2.209	0.144

Shannon evenness	N deposition	1	0.310	20.684	**0.001**
	Site	3	0.120	8.029	**0.005**
	N deposition x Site	3	0.036	2.412	0.127
Inverse Simpson diversity	N deposition	1	7.682	1.877	0.198
	Site	3	6.547	1.600	0.246
	N deposition x Site	3	2.610	0.638	0.606

Table 4.3. Fungal peroxidases which were abundant (≥1% of all sequences) under both ambient and experimental N deposition, in terms of the number and percentage of all OTUs (excluding singletons) shared across N deposition treatments. These numbers were determined for all sequences and again when plots containing < 100 sequences were removed and all remaining plots were subsampled to normalize sequence counts.

OTU level (sequence similarity)	Sequence type clustered	Sequences included in analysis	Number of OTUs shared	Percentage of all OTUs shared (%)
Unique	Amino acid	All	3	0.42
		Subsampled	5	2.10
96%	Nucleotide	All	4	1.97
		Subsampled	5	4.00
90%	Nucleotide	All	4	3.15
		Subsampled	5	5.68

Table 4.4. Effect of experimental N deposition on composition of expressed fungal peroxidases nucleotide as measured with PERMANOVA. This effect was examined for OTUs clustered to the unique level from translated cDNA sequences and OTUs clustered from cDNA nucleotide sequences to the 96% and 90% sequence similarity level for OTUs clustered. Significant comparisons ($P < 0.05$) are indicated in bold. Degrees of freedom for all comparisons is 1.

OTU level (sequence similarity)	Sequences clustered	Tranform applied	Sum of squares	Mean square	Pseudo-F	Permutational *P*
Unique	amino acid	none	5987.9	5987.9	1.4133	0.1101
		presence-absence	5869.9	5869.9	1.3733	**0.0466**
96%	nucleotide	none	6044.2	6044.2	1.4855	0.1295
		presence-absence	5869	5869	1.5401	0.081
90%	nucleotide	none	5284.7	5284.7	1.2719	0.2034
		presence-absence	5752.9	5752.9	1.5758	0.075

Table 4.5. Effect of experimental N deposition on composition of expressed fungal peroxidases as measured with PERMDISP. This effect was examined for OTUs clustered to the unique level from translated cDNA sequences and OTUs clustered from cDNA nucleotide sequences to the 96% and 90% sequence similarity level. Significant comparisons ($P < 0.05$) are indicated in bold.

OTU level (sequence similarity)	Sequences clustered	Degrees of freedom	Transform applied	F	Permutational *P*
Unique	amino acid	1, 15	none	0.8998	0.399
			presence-absence	5.8919	**0.0268**
96%	nucleotide	1, 16	none	0.92272	0.3666
			presence-absence	3.7192	0.1006
90%	nucleotide	1, 17	none	1.0405	0.3556
			presence-absence	2.5786	0.1372

Table 4.6. Effects of N deposition treatment and Site on Unifrac metric (unweighted and weighted) for expressed fungal peroxidase amino acid sequences. Analysis was conducted with all sites (unbalanced plot design) and repeated with data from Sites B and C only (balanced plot design) in order to examine the effect of Site. All comparisons were statistically significant ($P < 0.05$).

Factor		Unweighted Unifrac		Weighted Unifrac	
		Score	Significance	Score	Significance
All Sites					
N deposition		0.740583	$P < 0.001$	0.429579	$P < 0.001$
Sites B & C only					
N deposition treatment (across Sites)		0.717528	$P < 0.001$	0.376550	$P < 0.001$
Site		0.757523	$P < 0.001$	0.511148	$P < 0.001$
N deposition treatment (within Sites)	Site B	0.737063	$P < 0.001$	0.445275	$P < 0.001$
	Site C	0.719585	$P < 0.001$	0.390356	$P < 0.001$

FIGURES

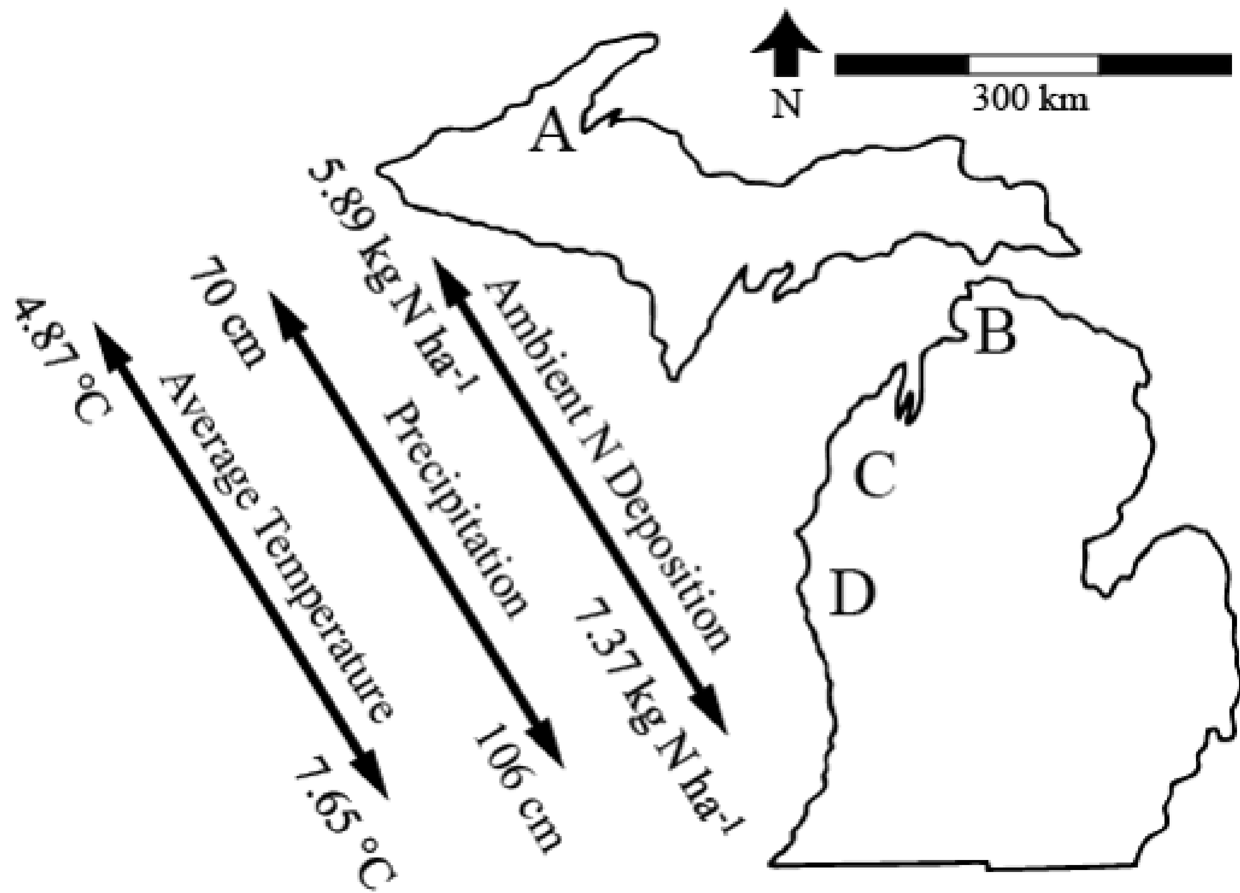


Figure 4.1. The location of four northern hardwood forest stands in our long-term N deposition experiment in Michigan, USA.

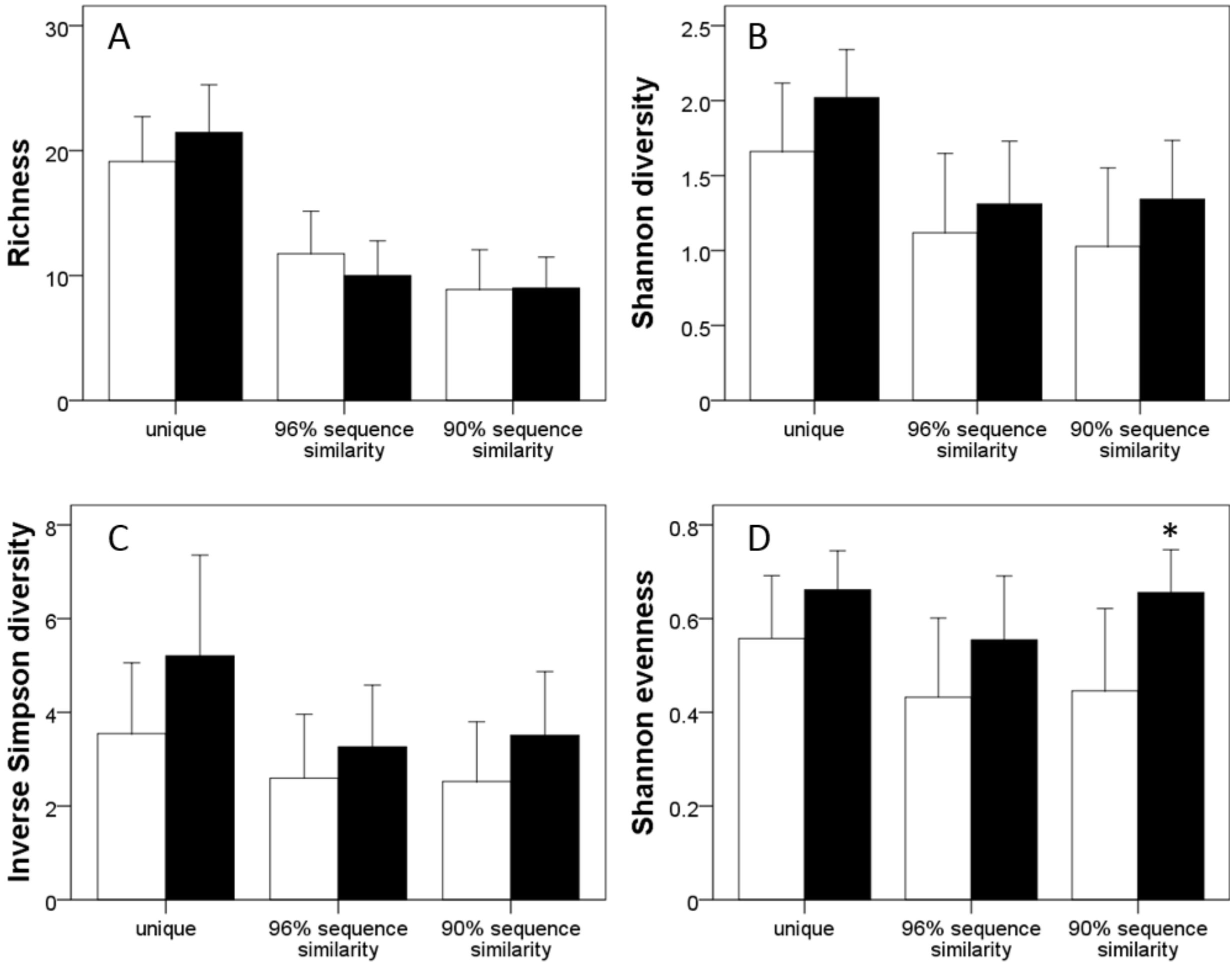


Figure 4.2. Richness, diversity, and evenness of fungal peroxidases expressed under ambient (open bars) and experimental (shaded bars) rates of N deposition for OTUs clustered at the unique, 96% sequence similarity, and 90% sequence similarity levels. Panels are A) observed richness, B) Shannon diversity, C) Inverse Simpson diversity, and D) Shannon evenness. The 96% and 90% sequence similarity OTUs were clustered from cDNA nucleotide sequences, while the unique OTUs were clustered from translated cDNA sequences. Error bars represent a standard error of 2. Significant comparisons ($P < 0.05$) are indicated with an asterisk.

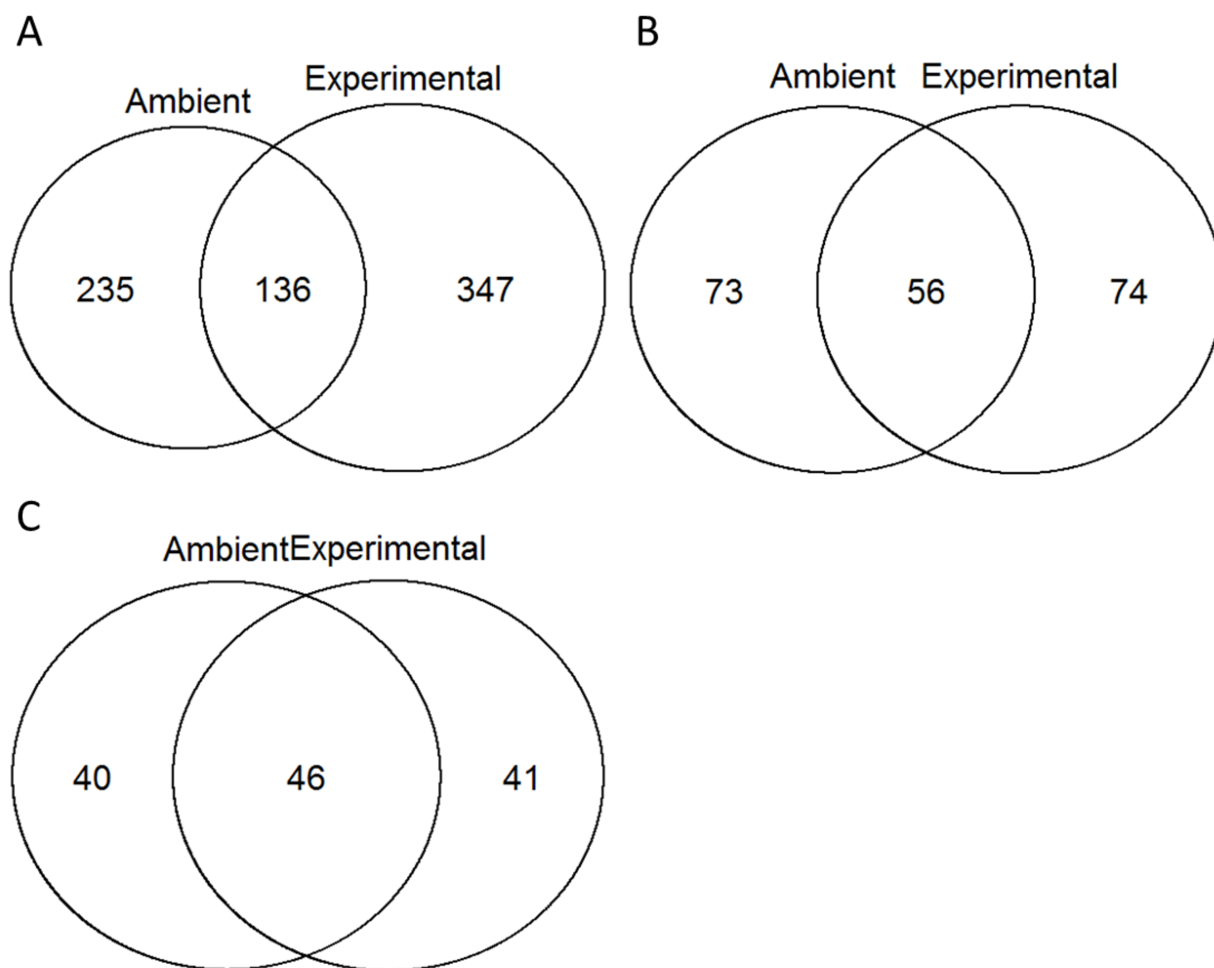
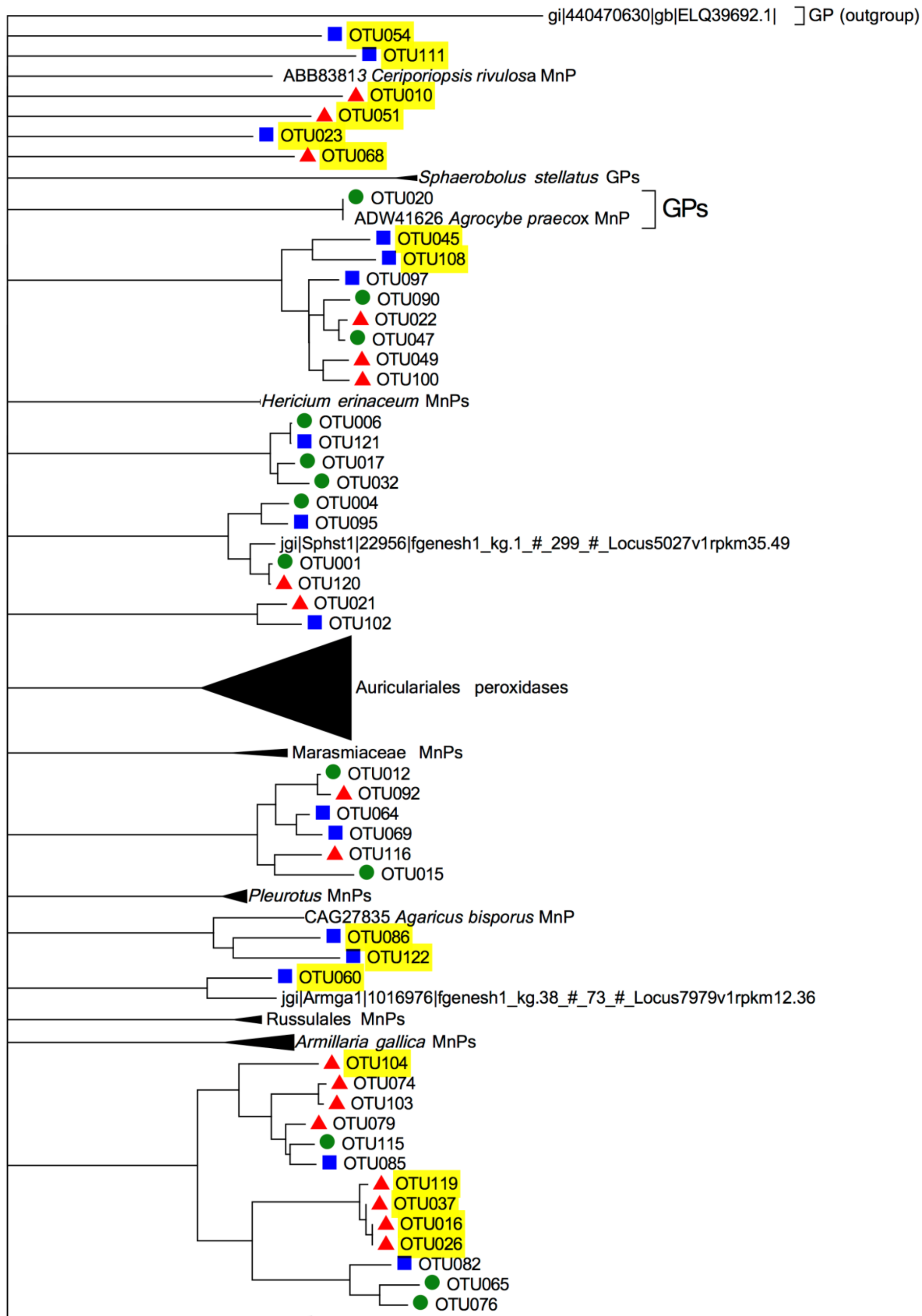
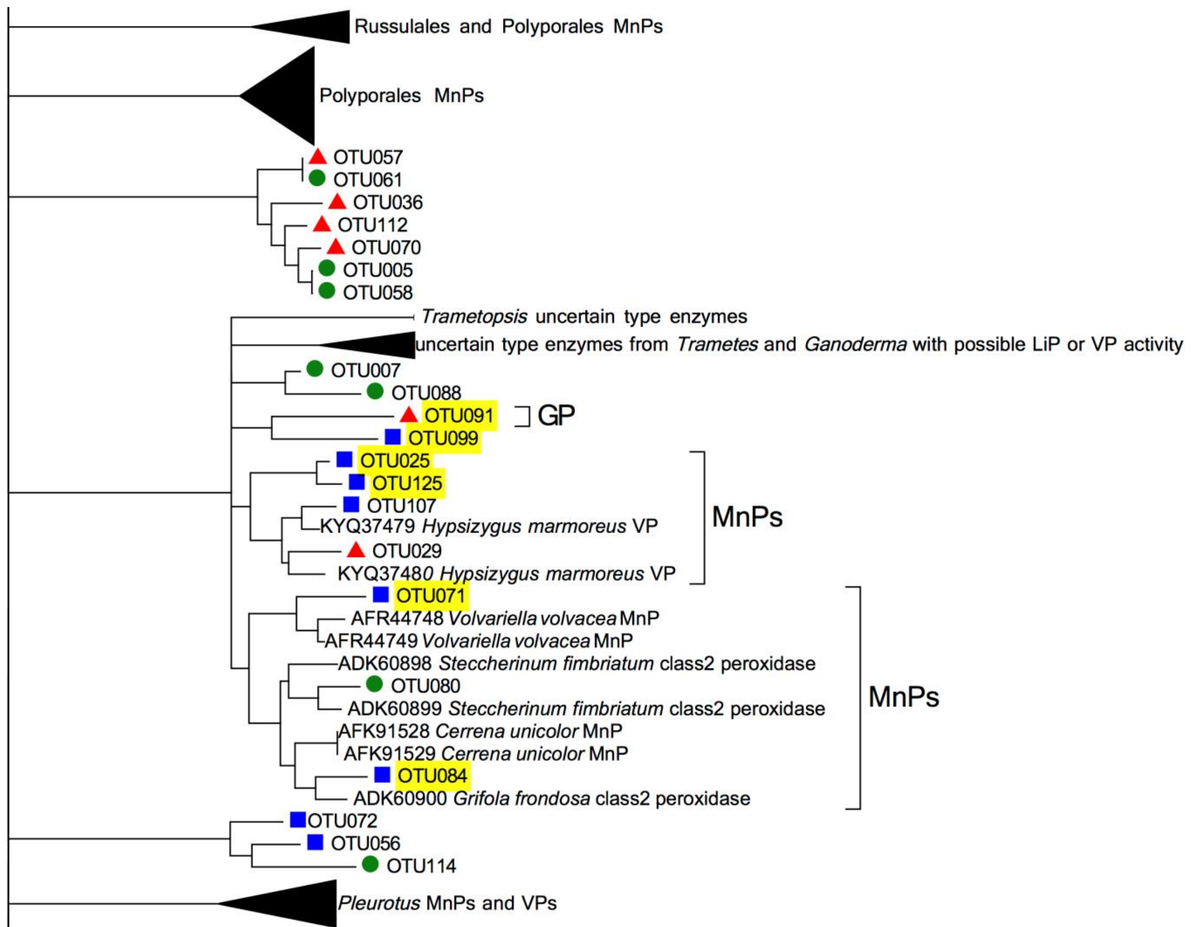
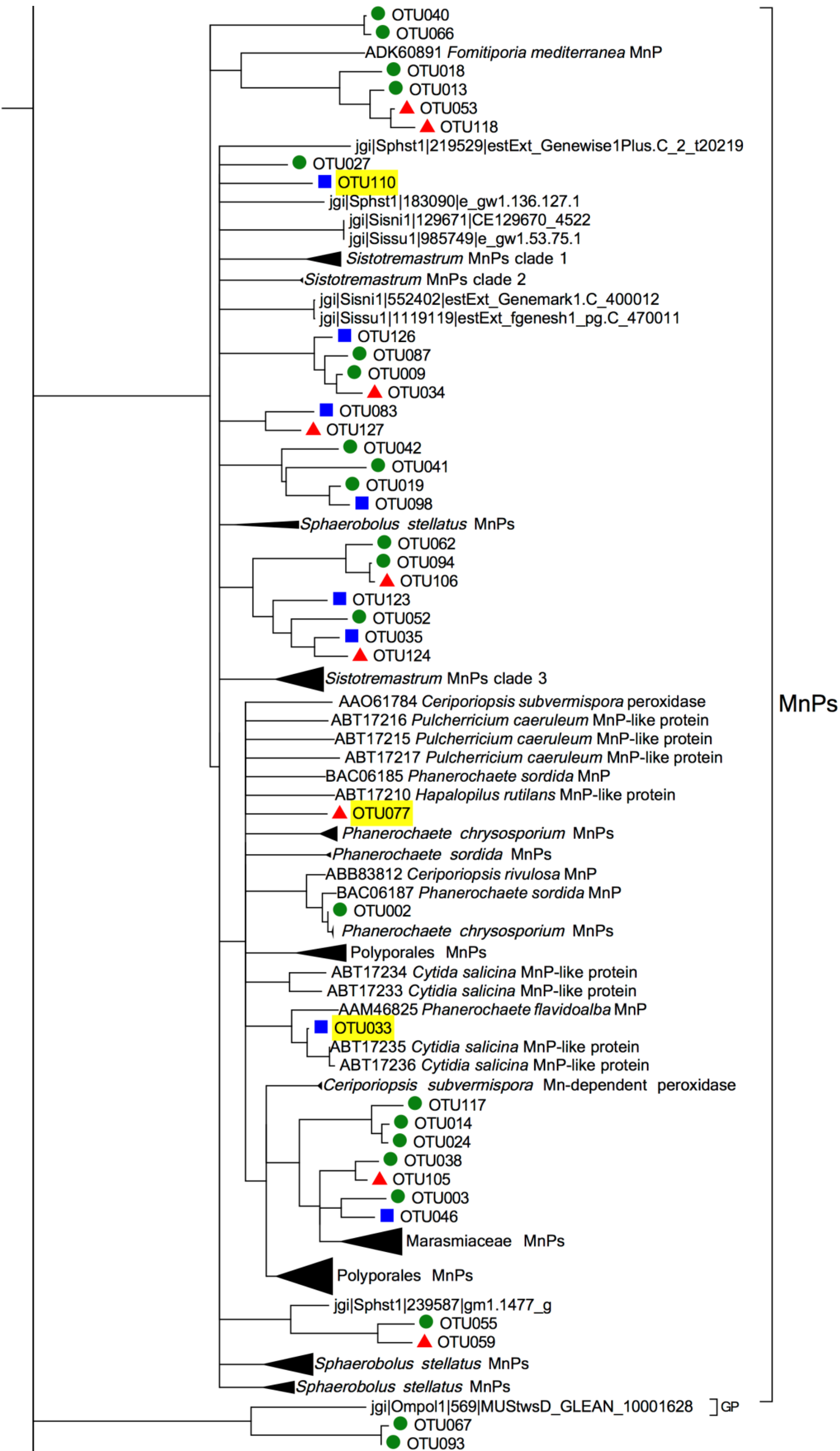


Figure 4.3. Scaled Venn diagrams for expressed peroxidase OTUs occurring under ambient and experimental rates of N deposition for a) unique OTUs clustered from translated cDNA sequences, b) 96% sequence similarity OTUs clustered from cDNA nucleotide sequences, and c) 90% sequence similarity OTUs clustered from cDNA nucleotide sequences. Singletons were removed from data set prior to creation of Venn diagrams. All plots were included with no subsampling.



gi|440470630|gb|ELQ39692.1|
GP (outgroup)
OTU054
OTU111
ABB83813 Ceriporiopsis rivulosa MnP
OTU010
OTU051
OTU023
OTU068
Sphaerobolus stellatus GPs
OTU020
ADW41626 Agrocybe praecox MnP
GPs
OTU045
OTU108
OTU097
OTU090
OTU022
OTU047
OTU049
OTU100
Hericium erinaceum MnPs
OTU006
OTU121
OTU017
OTU032
OTU004
OTU095
jgi|Sphst1|22956|fgenesh1_kg.1_#_299_#_Locus5027v1rpkm35.49
OTU001
OTU120
OTU021
OTU102
Auriculariales peroxidases
Marasmiaceae MnPs
OTU012
OTU092
OTU064
OTU069
OTU116
OTU015
Pleurotus MnPs
CAG27835 Agaricus bisporus MnP
OTU086
OTU122
OTU060
jgi|Armga1|1016976|fgenesh1_kg.38_#_73_#_Locus7979v1rpkm12.36
Russulales MnPs
Armillaria gallica MnPs
OTU104
OTU074
OTU103
OTU079
OTU115
OTU085
OTU119
OTU037
OTU016
OTU026
OTU082
OTU065
OTU076


Russulales and Polyporales MnPs
Polyporales MnPs
OTU057
OTU061
OTU036
OTU112
OTU070
OTU005
OTU058
Trametopsis uncertain type enzymes
uncertain type enzymes from Trametes and Ganoderma with possible LiP or VP activity
OTU007
OTU088
OTU091
OTU099
GP
OTU025
OTU125
OTU107
KYQ37479 Hypsizygus marmoreus VP
OTU029
KYQ37480 Hypsizygus marmoreus VP
MnPs
OTU071
AFR44748 Volvariella volvacea MnP
AFR44749 Volvariella volvacea MnP
ADK60898 Steccherinum fimbriatum class2 peroxidase
OTU080
ADK60899 Steccherinum fimbriatum class2 peroxidase
AFK91528 Cerrena unicolor MnP
AFK91529 Cerrena unicolor MnP
OTU084
ADK60900 Grifola frondosa class2 peroxidase
MnPs
OTU072
OTU056
OTU114
Pleurotus MnPs and VPs


OTU040
OTU066
ADK60891 Fomitiporia mediterranea MnP
OTU018
OTU013
OTU053
OTU118
jgi|Sphst1|219529|estExt_Genewise1Plus.C_2_t20219
OTU027
OTU110
jgi|Sphst1|183090|e_gw1.136.127.1
jgi|Sisni1|129671|CE129670_4522
jgi|Sissu1|985749|e_gw1.53.75.1
Sistotremastrum MnPs clade 1
Sistotremastrum MnPs clade 2
jgi|Sisni1|552402|estExt_Genemark1.C_400012
jgi|Sissu1|1119119|estExt_fgenesh1_pg.C_470011
OTU126
OTU087
OTU009
OTU034
OTU083
OTU127
OTU042
OTU041
OTU019
OTU098
Sphaerobolus stellatus MnPs
OTU062
OTU094
OTU106
OTU123
OTU052
OTU035
OTU124
Sistotremastrum MnPs clade 3
AAO61784 Ceriporiopsis subvermispora peroxidase
ABT17216 Pulcherricium caeruleum MnP-like protein
ABT17215 Pulcherricium caeruleum MnP-like protein
ABT17217 Pulcherricium caeruleum MnP-like protein
BAC06185 Phanerochaete sordida MnP
ABT17210 Hapalopilus rutilans MnP-like protein
OTU077
Phanerochaete chrysosporium MnPs
Phanerochaete sordida MnPs
ABB83812 Ceriporiopsis rivulosa MnP
BAC06187 Phanerochaete sordida MnP
OTU002
Phanerochaete chrysosporium MnPs
Polyporales MnPs
ABT17234 Cytida salicina MnP-like protein
ABT17233 Cytidia salicina MnP-like protein
AAM46825 Phanerochaete flavidoalba MnP
OTU033
ABT17235 Cytidia salicina MnP-like protein
ABT17236 Cytidia salicina MnP-like protein
Ceriporiopsis subvermispora Mn-dependent peroxidase
OTU117
OTU014
OTU024
OTU038
OTU105
OTU003
OTU046
Marasmiaceae MnPs
Polyporales MnPs
jgi|Sphst1|239587|gm1.1477_g
OTU055
OTU059
Sphaerobolus stellatus MnPs
Sphaerobolus stellatus MnPs
jgi|Ompol1|569|MUStwsD_GLEAN_10001628
GP
OTU067
OTU093
MnPs

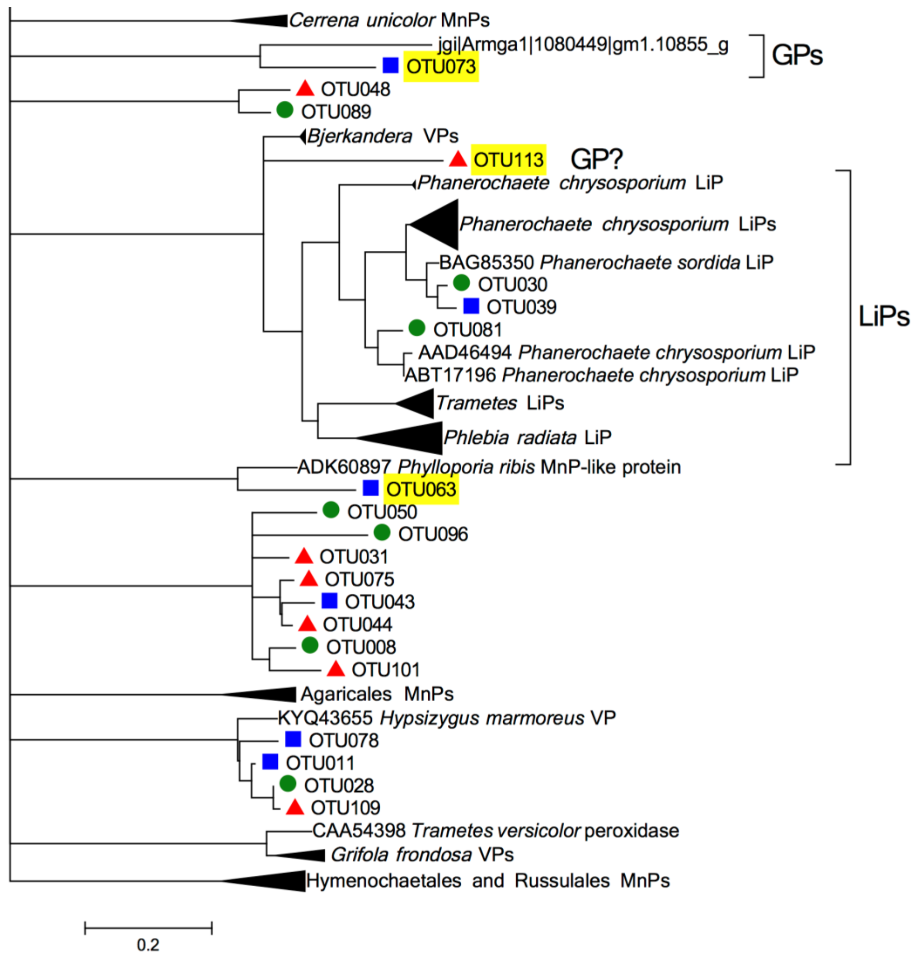


Figure 4.4. Neighbor-joining diagram of 127 OTU sequences occurring under ambient N deposition (■), experimental N deposition (▲), or both (●). Highlighted OTUs are in lineages belonging to only one N deposition treatment. OTUs were clustered at the 90% nucleotide sequence similarity level, translated to amino acid sequences, and aligned with 324 fungal peroxidase reference sequences prior to neighbor-joining for 100 repetitions with bootstrap cutoffs of 25. OTUs are numbered from most to least abundant. Collapsed nodes contain only reference sequences and no OTUs. Sequences were identified as LiPs if they possessed conserved catalytic residue Trp-171 and putative GPs if they lacked both Trp-171 for direct oxidation of nonphenolic bonds and the Asp-175 residue for manganese oxidation. All other OTU sequences not labelled as LiPs or GPs possessed an Asp-175 residue, but no Trp-171, and

are putative MnPs. Reference sequences obtained from FunGene are labelled with Genbank ID number, name of organism and gene type. Reference sequences obtained from MycoCosm are labelled with Joint Genome Institute ID numbers for sequences from the genomes of *Sphaerobolus stellatus* (Sphst), *Armillaria gallica* (Armga), *Sistotremastrum niveocremeum* (Sisni), *Sistotremastrum suecicum* (Sissu), and *Omphalotus olearius* (Ompol).

CHAPTER 5

Conclusions

The overall goal of my dissertation was to improve our understanding of the biological mechanism by which elevated rates of N deposition increase soil C storage. To gain this understanding, I used a long-term experiment which was designed to examine the effects of this agent of anthropogenic global change on northern hardwood forests, a widespread temperate forest ecosystem in North America. I examined the effects of experimental N deposition on the composition and diversity of fungi actively decomposing the forest floor, on the abundance of lignolytic fungi colonizing substrates of varying lignin content, and on the composition and diversity of fungal genes which are of critical importance in the decay of lignin. My working hypothesis was that experimental N deposition would reduce the richness and diversity and alter the composition of fungi and their expressed lignolytic genes. Overall, I found that experimental N deposition reduced decomposition and increases soil organic matter content by inducing changes in the composition of the fungal community which have important functional consequences for the decay of lignin and other polyphenols in soil organic matter.

In Chapter 2, I asked whether experimental N deposition reduces the diversity or changes the composition or structure of the active fungal community in the forest floor. I investigated this response in two of the four sites in our long-term N deposition experiment. Experimental N deposition had no effect on richness and limited effects on the diversity of active fungi in forest floor. In one forest stand, experimental N deposition altered the composition of active fungi; in the other stand, experimental N deposition changed the structure of active fungi. In addition, at

each site, the abundance of at least one order of fungi significantly changed in response to experimental N deposition; however, these responses were not consistent across sites. The relatively low sequencing depth of the clone library approach limited my ability to more deeply investigate how fungal taxa below the order level had responded to experimental N deposition.

Because of these limitations, I conducted a more thorough survey in Chapter 3 designed to specifically examine whether experimental N deposition decreases the abundance of lignolytic fungi. Such a response would be consistent with a previously proposed mechanism that posits that experimental N deposition reduces the decay of lignin via a shift in fungal community composition wherein lignolytic fungi decline and weak decomposers of lignin increase. I examined fungal communities that colonized substrates of varying lignin-content over the course of decay, as well as examined the fungal communities present in the surrounding mineral and organic soil horizons. I found that fungal taxa responsible for lignin decay decreased significantly on wood and a high-lignin substrate, but that these taxa were rare on a low-lignin substrate where no change in their abundance was observed. Furthermore, I found that experimental N deposition significantly altered fungal community composition and that the nature of this change was consistent with a shift within the fungal community; experimental N deposition suppresses the abundance of lignolytic fungi and increases the abundance of fungi with limited capacity for lignin decay. Finally, experimental N deposition reduced the size of the fungal community on wood, but increased it on a low-lignin substrate. These results are consistent with the mechanism which I was testing as well as with previous research that shows that elevated rates of N deposition inhibit fungal lignin decay, but stimulate fungal decay of cellulose. Contrary to research suggesting that experimental N deposition initially stimulates decay of cellulose and subsequently inhibits lignin decay in the late stage of decomposition

(Berg and Matzner 1997, Talbot and Treseder 2012), I found that the negative effects of experimental N deposition on lignolytic fungi were not enhanced at later stages of decay relative to earlier stages of decay.

In Chapter 4, I investigated whether experimental N deposition impacts the composition or diversity of gene transcripts encoding an important class of lignolytic enzymes, fungal class II peroxidases. I asked if experimental N deposition reduces the richness or diversity or alters the composition of lignin-decaying peroxidases expressed by fungi in the forest floor. Furthermore, I examined whether any changes in function could be inferred from changes observed in the composition of expressed peroxidases. While experimental N deposition did not reduce either the richness or diversity of the peroxidases expressed, it did increase the evenness of those peroxidases which were expressed. This observation suggests that experimental N deposition has negative effects on the fungi which typically dominate this process. Furthermore, experimental N deposition altered the composition of peroxidases which were expressed. There were no apparent differences among peroxidases expressed in terms of capacity for indirect oxidation of phenolics via Mn^{2+} oxidation or direct oxidation of nonphenolic bonds, a surprising result given the reductions in decomposition and increases in SOM which have previously been observed in this experiment. While most clades of fungal peroxidases appeared to be expressed across N deposition treatments, several lineages associated with peroxidases of known white-rot wood decay fungi or important litter decomposers were notably absent under experimental N deposition. Interestingly, several lineages of unknown origin were only present under experimental N deposition. Thus, while no broad functional changes were apparent, the presence and absence of some lineages under experimental N deposition suggests that there are potentially functional differences in the peroxidases being expressed; for example, enzymes of different

lineages may have different substrate specificities or originate from fungi with different ecological roles in decomposition.

Collectively, my results indicate that experimental N deposition altered the composition and function of the fungal community in a manner consistent with previously observed reductions in plant litter decomposition and increased soil C accumulation. Given that northern hardwood forests are a widespread ecosystem in the Northern Hemisphere and that elevated rates of N deposition are a widespread aspect of anthropogenic global change impacting terrestrial and coastal ecosystems, my results reveal a mechanism by which experimental N deposition has increased soil C storage. While I have examined this response in northern hardwood forests in Michigan, increased SOM accumulation in response to long-term increases in N deposition has been observed in northern hardwood forests elsewhere (Frey et al. 2014), in other types of temperate forests (Janssens et al. 2010) and may, in fact, be a widespread response to elevated rates of N deposition (Nave et al. 2009), albeit not a universal one (Waldrop et al. 2004a, Waldrop et al. 2004b, Keeler et al. 2009). Because rates of anthropogenic N deposition have already increased dramatically since the preindustrial era (Galloway and Cowling 2002), it is possible that present levels of SOM in temperate forests in North America may already reflect this response. The future implications of anthropogenic N deposition on forest soils will thus depend upon whether future rates are similar to those that have been predicted (Galloway et al. 2004). For instance, rates of N deposition in North America may have already peaked (Lloret and Valiela 2016), but rates of N deposition have continued to rise in parts of the recently developed world (Liu et al. 2013) and may continue to do so over the next several decades.

From this body of research, several lines of inquiry emerge as potentially important avenues for further investigation. In Chapter 3, I observed simultaneous decreases in the relative

abundance of lignolytic fungi within the fungal community and in the total size of the fungal community on wood. Other research has indicated that experimental N deposition increases woody biomass (Ibáñez et al. 2016) and may slow the decay of naturally occurring woody debris (Lyons 2012) in our study system. While elevated rates of N deposition have been observed to increase C storage in aboveground plant biomass (Magnani et al. 2007), the extent of this effect is contentious (Nadelhoffer et al. 1999, de Vries et al. 2008). Importantly, the potential effect of anthropogenic N deposition on the decay of dead wood under field conditions has not received much attention. Instead, the vast majority of research on decomposition to date has focused on the decay of leaf litter. My results suggest that further study on the effects experimental N deposition on wood decay is warranted because reduced decomposition of wood debris is another mechanism through which experimental N deposition could increase C storage in northern temperate forests.

My dissertation has examined how elevated rates of N deposition effect fungal decomposers and, thereby, increase soil C storage in temperate forests. Predicting how rising rates of N deposition will effect soil C storage globally and what impact this may have on climate requires additional consideration of not only the responses of temperate forests, but also other types of ecosystems (Bragazza et al. 2006) as well as consideration of the effects of elevated N deposition on the biogeochemical cycles of other greenhouse gases (Liu and Greaver 2009). However, determining the mechanisms through which ecosystems respond to elevated rates of N deposition is important for deepening our understanding of ecosystem responses to this agent of global change. My dissertation contributes to this our scientific understanding of this process by providing new insight into the biological mechanism by which anthropogenic N

deposition has increased soil C storage in a long-term field experiment in a widespread type of temperate forest ecosystem.

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APPENDIX A

Dikarya reference sequences

Dikarya sequences obtained in this study were aligned with reference sequences. Alignments for initial OTU clustering were performed with 181 Dikarya reference sequences to help to guide alignment construction. Alignments which were used for phylogenetic tree construction were also aligned with these 181 Dikarya reference sequences, but additionally included a Glomeromycota sequence as an outgroup in order to root the phylogenetic tree. All references sequences were obtained from the NCBI GenBank database. Sequences are identified according to the species or sequence name provided in Genbank with the Genbank accession number in parentheses. The outgroup (Glomeromycota) sequence is indicated in bold. The reference sequences used are listed below:

Acanthostigma filiforme (GQ850495), ***Acaulospora laevis* (FM876785)**, *Amanita jacksonii* (AF097376), *Amorphotheca resinae* (EU040230), *Anthostomella brabeji* (EU552098), *Antrodia infirma* (JQ700294), *Anungitopsis speciosa* (EU035401), *Articulospora tetracladia* (EU998929), *Athelia decipiens* (AY586632), *Aureobasidium pullulans* (AJ876762), *Aureobasidium pullulans* (EF595769), *Auricularia auricula-judae* (DQ520099), *Auritella foveata* (GU062739), *Boletellus projectellus* (NG027638), *Botryobasidium subcoronatum* (EU909344), *Bovista dermoxantha* (DQ112579), *Burgoa turficola* (DQ915467), *Burgoa verzuoliana* (DQ915475), *Caloplaca sublobulata* (EF489950), *Calosphaeria barbirostris* (EF577059), *Capronia semiimmersa* (EU514693), *Carestiella socia* (AY661682), *Catenulifera brevicollaris* (GU727561), *Ceratobasidium sp*. AGH (AF354089), *Ceratobasidium* sp. CAG1 (AF354086), *Ceratobasidium* sp. CAG3 (AF354080), *Ceriporiopsis gilvescens* (AF347110), *Chaetomidium leptoderma* (FJ666353), *Chamaeleomyces viridis* (HM635079), *Cistella acuum* (GU727552), *Cistella spicicola* (GU727553), *Cladonia gracilis* (EF489958), *Cladophialophora chaetospira* (EU035403), *Cladophialophora hachijoensis* (AF050263), *Clavaria fragilis* (EF535278), *Clavariadelphus ligula* (AF347099), *Clavicorona taxophila* (AF115333), *Clavulina cristata* (AM259212), *Clavulinopsis helvola* (AY586647), *Clitocybe lateritia* (U66431), *Clitocybe*

odora (AF261390), *Clitocybe hesleri* (HQ179664), *Coleophoma empetri* (FJ588250), *Coleosporium asterum (DQ354559), Colletotrichum boninense (DQ286161), Collophora* sp. TP-Snow-Y69 (JN400810), *Collybia dryophila* (AF042595), *Coniochaeta* sp. M136 (HM595603), *Coprinellus xanthothrix* (FJ755223), *Coprinopsis atramentaria* (DQ457661), *Corticium salmonicolor* (AF506709), *Cortinariaceous ectomycorrhiza* (AF430290), *Cortinarious icterinus* (AF539720), *Cortinarius picoides* (GU233424), *Corynascus sepedonium* (FJ666364), *Crepidotus calolepis* (FJ904178), *Crepidotus mollis* (AM882996)*, Cristinia rhenana* (GU187663), *Cryptococcus laurentii* (AJ555467), *Cryptococcus oeirensis* (AM160646), *Cryptococcus* sp. HA 2257 (FM991886), *Cryptococcus* sp. YSAR16 (AM922287), *Cryptococcus taibaiensis* (AY557601), *Cryptosporiopsis actinidiae* (HM595594), *Cudonia lutea* (AF433140), *Cudoniella* sp. ZW-Geo49-Clark (AY789338), *Cylindrosympodium lauri* (EU035414), *Cystofilobasidium infirmominiatum* (DQ645523), *Dactylellina parvicollis* (EF445986), *Discocistella grevillei* (GU727554), *Entoloma pallidocarpum* (JQ410331), Ericoid mycorrhizal sp. PPO-2 (AY599240), *Erysiphe hypophylla* (AB292712), *Exidiopsis plumbescens* (AF395309), *Exophiala bergeri* (AB479516), *Exophiala* sp. DAOM 216391 (AF050267), *Filobasidium uniguttulatum* (AF075468), *Flammula alnicola* (AF195588), *Fomitopsis pinicola* (AF347106), *Fusarium solani* (AY097316), *Geomyces pannorum* (GU951688), *Gloeostereum incarnatum* (AF141637), *Gloiocephala aquatica* (NG027642), *Graddonia coracina* (JN012011), *Gymnopus bicolor* (AY639411), Gyromitra infula (AJ698473), Haplographium catenatum (FJ839657), *Harknessia rhabdosphaera* (DQ923532), *Hebeloma affine* (FJ436324), *Hemimycena gracilis* (DQ457671), *Hemipholiota destruens* (AF261647), *Hyalodendriella betulae* (EU040232), *Hydnangium carneum* (HQ832455), *Hygrocybe citrinopallida* (HCU66435), *Hygrophorus chrysodon* (AY586661), *Hyphodontia barba-jovis* (AY293188), *Hyphodontia subalutacea* (DQ873631), *Hypocenomyce scalaris* (DQ782914), *Hypocrea lutea* (AB027384), *Hypsizygus ulmarius* (AF042584), *Inocybe fulvilubrica* (JQ085929), *Kabatiella microsticta* (EU167608), *Karstenella vernalis* (FJ499391), *Lasallia pustulata* (AY300839), *Lepista nebularis* (AY586685), *Leucosporidium* sp. AY30 (GQ336996), *Marasmius oreades* (DQ156126), *Marasmius rotula* (DQ457686),*Micarea alabastrites* (AY756327), *Micarea micrococca* (AY756330), *Microglossum viride* (AY789337), *Mollisia incrustata* (GU727556), *Monilinia fructicola* (AY544683), *Moniliophthora roreri* (AY194150), *Mrakia frigida* (DQ831016), *Mycena monticola* (EU669336), *Mycena tenax* (EU669274), *Mycena plumbea*

(DQ470813), *Nolanea sericea* (AF223170), *Ochroconis gallopava* (AB125280), *Omphalina rivulicola* (ORU66451), *Ossicaulis lignatilis* (HE649954), *Ostropa barbara* (HM244773), *Pachyphloeus marroninus* (EU543209), *Panellus ringens* (AF347100), *Phacidiella eucalypti* (EF110620), *Phacidiopycnis* sp. ZLY-2010b (HM595597), *Phaeococcomyces chersonesos* (AJ507323), *Phaeomarasmius erinaceus* (AF261594), *Phialea strobilina* (EF596821), *Phlogicylindrium eucalypti* (DQ923534), *Pholiota gummosa* (AF195605), *Pholiota oedipus* (AF261649), *Pleiochaeta setosa* (EU167563), *Pleomassaria siparia* (AY004341), *Pleurotus eryngii* (EU365655), *Pluteus petasatus* (AF042611), *Pluteus romellii* (NG027625), *Protodontia piceicola* (DQ873660), *Psathyrella fagetophila* (AM712262), *Pseudoclitocybe cyathiformis* (EF551313), *Pseudoidriella syzygii* (JQ044441), *Psora decipiens* (AY756343), *Puccinia mariae-wilsoniae* (GU058022), *Puccinia violae* (GU058029), *Pycnora xanthococca* (AY853388), *Ramaria myceliosa* (JQ408230), *Ramaria rainierensis* (EU669412), *Ramaria* sp. 4 AK-2012 (JQ408229), *Ramaria stricta* (AF347098), *Retroconis fusiformis* (EU040239), *Rhizocladosporium argillaceum* (EU040240), *Rhodotorula psychrophenolica* (EF151256), *Rhodotorula* sp. FK.2.1 (FN400943), *Rhyzoscyphus ericae* (AM887699), *Sarcomyxa serotina* (EU365678), *Scleromitrula shiraiana* (AY789407), *Sclerotinia sclerotiorum* (AY789347), *Sistotrema biggsiae* (AM259217), *Sporobolomyces griseoflavus* (EF537895), *Stropharia albocrenulata* (AF195589), *Sympoventuria capensis* (DQ885904), *Syzygospora alba* (JN043616), *Tectonidula hippocrepida* (FJ617557), *Trechispora hymenocystis* (AF347090), *Tremella phaeophysciae* (JN043586), *Tremella polyporina* (JN043607), *Tricholoma apium* (AY586721), *Tricholoma orirubens* (DQ389734), *Trichosporon laibachii* (JN939451), *Trichosporon porosum* (JN939465), *Typhula phacorrhiza* (AY586724), *Udeniomyces puniceus* (DQ836005), Uncultured fungus clone (FJ040372), Uncultured Ascomycota clone asc07198 (HQ433120), Uncultured basidiomycete clone 4S1 D11 (EU489986), Uncultured Basidiomycota clone bas07010 (HQ433141), Uncultured Basidiomycota clone bas07088 (HQ433195), Uncultured fungus clone LSUTypeUS20 (FJ040366), Uncultured fungus clone LSUTypeUS22 (FJ040368), Uncultured soil fungus clone BPAGM2T0 1H (EU691365), *Veronaea botryosa* (EU041874)

APPENDIX B

OTU accession numbers and abundances

Table B1. Accession numbers and clone library abundance information for 99% sequence similarity OTUs. Clustering was performed on all Dikarya sequences recovered without subsampling. A representative clone sequence was selected from each 99% sequence similarity OTU and submitted to GenBank. Abundances are listed as the number of occurrences in clone libraries by site and N deposition treatment.

GenBank accession number	Representative clone name	Site			
		B		D	
		N deposition treatment			
		Ambient	Experimental	Ambient	Experimental
KC701765	B01A04	1	0	0	0
KC701766	B01A11	1	0	0	0
KC701767	B01B07	1	0	0	0
KC701768	B01B08	1	0	0	0
KC701769	B01B10	1	0	0	0
KC701770	B01B12	1	0	0	0
KC701771	B01E09	1	0	0	0
KC701772	B01E12	1	0	0	0
KC701773	B01F03	3	0	0	0

KC701774	B01F11	1	0	0	0
KC701775	B01G03	1	0	0	0
KC701776	B01H02	1	0	0	0
KC701777	B01H03	1	0	0	0
KC701778	B02B04	1	0	0	0
KC701779	B02C02	1	0	0	0
KC701780	B02C05	2	0	0	0
KC701781	B02C06	1	0	0	0
KC701782	B02D09	1	0	0	0
KC701783	B02F09	1	0	0	0
KC701784	B02G07	5	0	0	2
KC701785	B02G08	2	0	0	0
KC701786	B02H04	3	0	0	0
KC701787	B03A02	1	0	0	0
KC701788	B03A05	1	0	0	0
KC701789	B03A06	1	0	0	0
KC701790	B03A07	1	0	0	0
KC701791	B03B02	1	2	3	0
KC701792	B03B03	1	0	0	0
KC701793	B03C04	1	4	0	1
KC701794	B03E08	1	0	0	0
KC701795	B03E10	1	0	0	0
KC701796	B03F01	3	0	0	0
KC701797	B03F04	3	0	0	0
KC701798	B03F05	8	0	0	0

KC701799	B03F06	1	0	0	0
KC701800	B03F09	3	0	0	0
KC701801	B03G07	4	0	0	0
KC701802	B03H02	1	0	0	0
KC701803	B03H04	2	0	0	0
KC701804	B03H06	2	0	0	0
KC701805	B03H07	7	0	0	0
KC701806	B03H09	1	0	0	0
KC701807	B04A07	0	1	0	0
KC701808	B04B01	7	1	0	0
KC701809	B04B04	0	1	0	0
KC701810	B04B06	0	2	0	0
KC701811	B04B10	1	1	0	0
KC701812	B04C08	1	2	0	0
KC701813	B04C10	0	1	0	0
KC701814	B04C11	0	1	0	0
KC701815	B04D01	0	1	0	0
KC701816	B04D07	0	2	0	0
KC701817	B04D08	0	1	0	0
KC701818	B04E07	0	2	0	1
KC701819	B04E12	0	1	0	0
KC701820	B04F02	0	1	0	0
KC701821	B04F04	0	1	0	0
KC701822	B04F08	0	1	0	0
KC701823	B04F10	1	2	0	0

KC701824	B04G02	0	1	0	0
KC701825	B04G06	0	2	0	0
KC701826	B04H05	0	2	0	0
KC701827	B04H06	0	1	0	0
KC701828	B05A03	0	1	0	0
KC701829	B05A09	0	1	0	0
KC701830	B05A10	0	3	0	0
KC701831	B05B04	0	2	0	0
KC701832	B05B07	0	1	0	0
KC701833	B05B08	0	1	0	0
KC701834	B05B12	1	3	0	0
KC701835	B05C03	0	1	0	0
KC701836	B05C10	0	4	0	4
KC701837	B05D07	1	1	0	0
KC701838	B05D09	0	2	0	0
KC701839	B05D11	0	1	0	0
KC701840	B05E12	0	1	0	0
KC701841	B05F02	0	1	0	0
KC701842	B05G01	1	2	0	0
KC701843	B05G04	0	1	0	0
KC701844	B05G08	1	3	0	0
KC701845	B05G12	0	1	0	0
KC701846	B05H02	0	1	0	0
KC701847	B06A05	0	1	0	0
KC701848	B06A07	0	11	65	6

KC701849	B06B03	0	1	0	0
KC701850	B06C05	0	1	0	0
KC701851	B06C11	0	6	0	0
KC701852	B06D03	1	1	0	0
KC701853	B06D09	0	1	0	0
KC701854	B06F01	0	1	0	0
KC701855	B06F08	0	1	0	0
KC701856	B06G01	0	1	0	0
KC701857	B06G03	0	2	1	0
KC701858	B06G11	0	3	0	0
KC701859	B06H01	0	1	0	0
KC701860	B06H07	0	1	0	0
KC701861	D01A01	0	0	1	0
KC701862	D01A04	0	3	1	0
KC701863	D01B08	0	0	1	0
KC701864	D01C07	0	0	1	0
KC701865	D01D01	0	0	1	0
KC701866	D01D06	0	0	2	0
KC701867	D01D07	0	0	4	0
KC701868	D01D10	0	0	1	0
KC701869	D01E04	0	0	3	0
KC701870	D01F01	0	0	2	0
KC701871	D01F03	0	0	1	0
KC701872	D01F04	0	1	2	1
KC701873	D01F09	1	0	1	0

KC701874	D01G06	0	0	1	0
KC701875	D01H07	0	0	1	0
KC701876	D01H09	0	0	1	0
KC701877	D02A07	1	0	1	0
KC701878	D02A10	0	0	2	0
KC701879	D02B01	0	0	1	0
KC701880	D02C01	0	0	2	0
KC701881	D02H04	0	0	1	0
KC701882	D02H08	0	6	5	0
KC701883	D03A01	0	0	1	0
KC701884	D03A04	0	6	1	0
KC701885	D03A10	0	0	1	0
KC701886	D03B08	0	0	1	0
KC701887	D03C02	0	0	1	0
KC701888	D03C06	0	0	1	0
KC701889	D03D12	0	0	2	0
KC701890	D03E04	0	0	1	0
KC701891	D03E06	0	0	1	0
KC701892	D03F01	0	0	2	0
KC701893	D03F02	0	0	1	0
KC701894	D03F06	0	0	1	0
KC701895	D03F11	1	0	1	0
KC701896	D03F12	0	0	1	0
KC701897	D03G04	0	0	1	0
KC701898	D03G05	0	1	1	0

KC701899	D03G06	0	0	1	0
KC701900	D03H02	0	0	1	0
KC701901	D03H04	0	0	4	1
KC701902	D03H08	0	4	1	0
KC701903	D03H10	0	1	3	0
KC701904	D03H12	0	0	1	0
KC701905	D04A01	6	2	5	1
KC701906	D04B09	0	0	1	1
KC701907	D04B12	1	0	0	1
KC701908	D04C01	0	1	0	1
KC701909	D04C05	1	0	1	1
KC701910	D04C07	0	0	0	1
KC701911	D04C09	0	0	0	1
KC701912	D04D02	0	1	2	4
KC701913	D04E05	4	2	1	2
KC701914	D04E09	0	0	0	2
KC701915	D04E11	0	0	1	1
KC701916	D04G04	0	0	0	1
KC701917	D04G05	0	1	0	3
KC701918	D04G08	0	1	0	2
KC701919	D04H06	0	1	0	2
KC701920	D05A10	0	0	0	1
KC701921	D05B02	0	0	0	1
KC701922	D05D06	1	2	11	1
KC701923	D05D08	0	0	0	1

KC701924	D05D11	0	0	0	1
KC701925	D05E02	5	0	0	13
KC701926	D05F05	0	0	1	9
KC701927	D05F07	0	1	5	1
KC701928	D05F08	0	0	0	1
KC701929	D05F09	0	0	0	2
KC701930	D05G08	0	0	0	1
KC701931	D05G10	0	0	0	1
KC701932	D05H02	0	1	0	2
KC701933	D05H04	0	0	0	49
KC701934	D05H05	0	0	1	1
KC701935	D05H07	0	0	0	1
KC701936	D06A04	0	0	1	1
KC701937	D06A10	0	0	0	1
KC701938	D06A11	0	0	0	1
KC701939	D06B03	0	0	0	1
KC701940	D06B06	0	1	1	1
KC701941	D06B07	0	0	0	1
KC701942	D06C04	0	0	0	1
KC701943	D06C11	0	0	0	1
KC701944	D06C12	0	0	0	1
KC701945	D06D01	0	0	0	1
KC701946	D06D05	0	0	1	6
KC701947	D06D08	0	0	1	1
KC701948	D06D11	0	0	0	4

KC701949	D06E03	0	0	0	1
KC701950	D06E07	0	0	0	2
KC701951	D06E08	0	1	0	1
KC701952	D06E09	0	0	0	1
KC701953	D06F02	0	0	0	1
KC701954	D06F04	0	0	0	1
KC701955	D06F10	0	0	1	2
KC701956	D06F11	0	0	0	2
KC701957	D06G07	0	0	0	3
KC701958	D06G08	4	2	1	17
KC701959	D06G10	0	0	0	1
KC701960	D06G12	0	0	2	2
KC701961	D06H03	0	0	0	1
KC701962	D06H06	0	0	0	1
KC701963	D06H07	0	0	0	1
KC701964	D06H08	5	0	0	6
KC701965	D06H09	0	3	3	2

APPENDIX C

Fungal physiological categories for OTUs identified by SIMPER analysis

Table C1. Physiological categories to which we assigned OTUs identified in SIMPER analysis.

Physiology	Function	Taxa	Citations
White-rot and lignolytic litter decay[1]	Enzymatically decompose lignin	*Mycena*	(Worrall et al. 1997, Osono and Takeda 2002, Steffen et al. 2007, Liers et al. 2011, Cline and Zak 2015)
		Marasmius	(Osono and Takeda 2002, Steffen et al. 2007)
		Gymnopus	(Osono et al. 2003, Osono and Takeda 2006, Valaskova et al. 2007, Šnajdr et al. 2010, Cline and Zak 2015)
		Crepidotus	(Gutiérrez et al. 1999, Del Rıo et al. 2001, Martínez Ferrer et al. 2005)
		Sphaerobolus	(Robinson et al. 1993, Worrall et al. 1997, Baetsen 2013, Nagy et al. 2015)
		Hyphoderma	(Binder et al. 2013)
		Gomphales	(Ginns and Lefebvre 1993, Erden et al. 2009, Hibbett et al. 2014)
		Trechisporales	(Harkin et al. 1974, Nagy et al. 2015)
Soft-rot and	enzymatically	Cantharellales	(Boberg et al. 2011,

cellulolytic litter decay	decompose cellulose or hemicelluloses, but decay little to no lignin		Floudas et al. 2015, Nagy et al. 2015)
		Ascomycota (except Xylariales)	(Worrall et al. 1997, Osono et al. 2006, Osono and Takeda 2006, Boberg et al. 2011, Nagy et al. 2015)
Brown-rot	demethoxylate (“modify”) lignin but leave its phenolic and nonphenolic bonds intact	*Antrodia* spp.	(Binder et al. 2013)
		Anomoporia spp.	(Niemelä et al. 2007)
		Ceriporia reticulata	(Floudas and Hibbett 2015)
Weakly lignolytic	exhibit either high laccase but no - low peroxidase activity or relatively low laccase and peroxidase activity	Xylariaceae	(Osono and Takeda 2001, 2002, Stephen and Parungao 2003, Liers et al. 2011)
		Psathyrellaceae	(Ruiz-Duenas et al. 2009, Oliver et al. 2010, Liers et al. 2011)
		Entolomataceae	(Gramss 1997, Casieri et al. 2010)
		Tubariaceae	(Okino et al. 2000, Machado et al. 2005)
Mycorrhizal/ biotrophic	In a mycorrhizal or biotrophic association with a host	*Russula*	(Kirk et al. 2008)
		Tomentella	(Kirk et al. 2008)
		Hygrocybe	(Seitzman et al. 2011)
		Sebacinales	(Kirk et al. 2008)

1. Please note that our definition of “white-rot and lignolytic litter decay” fungi for this analysis is broader than our rather conservative definition of “highly lignolytic taxa” which we used for relative abundance analyses; white-rot and lignolytic litter decay fungi include any taxa which we have previously described as highly lignolytic, as well as well as some additional Agaricomycete taxa which we had excluded from previous analyses.

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APPENDIX D

Additional information regarding selected highly lignolytic taxa

Table D1. Additional information on the taxa we included in our compilation of highly lignolytic taxa used in our study. This includes the name of the taxa in our taxonomic summary file from mothur (Schloss et al. 2009), the taxonomic assignment for these taxa in the Ribosomal Database Project (RDP) v7 fungal 28S LSU classifier files (Liu et al. 2012), their current known taxonomic assignment, their ecology, and their morphology. Information including justifications for their inclusion and citations may be found in Table 3.2.

name	**assignment with RDP v7 classifier**	**current known taxonomic placement**	**ecology**	**morphology**
Marasmius	Agaricales/Marasmiaceae/*Marasmius*	Agaricales/Marasmiaceae/*Marasmius* (Matheny et al. 2006)	litter decay	gilled mushroom
Amyloflagellula	Agaricales/Tricholomataceae/ *Amyloflagellula*	Agaricales/Marasmiaceae/*Amylogflagellula* (Douanla-Meli and Langer 2008)	litter decay	gilled mushroom
Gymnopus	Agaricales/Tricholomataceae/*Gymnopus*	Agaricales/Marasmiaceae/*Gymnopus* (Matheny et al. 2006)	litter decay	gilled mushroom
Clitocybe	Agaricales/Tricholomataceae/*Clitocybe*	Tricholomataceae/*Clitocybe* (Matheny et al. 2006)	litter decay	gilled mushroom
Lepista	Agaricales/Tricholomataceae/*Lepista*	Tricholomataceae/*Lepista* (Matheny et al. 2006)	litter decay	gilled mushroom
Mycena	Agaricales/Tricholomataceae/*Mycena*	Agaricales/Mycenaceae/*Mycena* (Moncalvo et al. 2002)	litter decay	gilled mushroom
Poromycena	Agaricales/Tricholomataceae/*Poromycena*	Agaricales/Mycenaceae/*Poromycena* (Moncalvo et al. 2002)	litter decay	gilled mushroom

Crepidotaceae	Agaricales/Crepidotaceae	Agaricales/Crepidotaceae (Matheny et al. 2006)	white rot of wood and litter decay	gilled "oysterling"
Auriscalpiaceae	Polyporales/Auriscalpiaceae	Russulales/Auriscalpiaceae (Larsson and Larsson 2003, Miller et al. 2006)	white rot of wood and litter decay[a]	tooth and shelf fungi[a]
Lachnocladiaceae	Polyporales/Lachnocladiaceae	Russulales/Lachnocladiaceae (Kirk et al. 2008)[b]	white rot of wood	resupinate
Antrodiella	Polyporales/Coriolaceae/*Antrodiella*	Polyporales/Steccherinaceae/*Antrodiella* (Miettinen et al. 2012)	white-rot	
Ganodermataceae	Polyporales/Ganodermataceae	Polyporales/Ganodermataceae (Kirk et al. 2008)	White-rot saprotrophs and plant pathogen	shelf fungi
Aphyllophorales	Polyporales/Polyporales incertae sedis/Aphyllophorales	Aphyllophorales is a formerly used morphological taxonomic group for fungi without gills. However, molecular phylogenetics showed that Aphyllophorales to be comprised of distantly related taxa (Hibbett and Donoghue 1995). The sequences used to define this group in the RDP v7 classifier[c] now belong to several families in the Polyporales (Binder et al. 2013). In our data set, sequences classified as Aphyllophorales were members of the genera *Phanerochaete* and *Scopuloides*[d].		

a All Auriscalpiaceae sequences in our data set classified to the wood-rot genus *Lentinellus*, which are mushrooms with gills.

b Some authors include the genera contained in Lachnocladiaceae in Russulales/Peniophoraceae (Miller et al. 2006, Larsson 2007)

c Searches for the sequences used to define Aphyllophorales in the RDP v7 taxonomic classifier returned close matches for

sequences identified as *Antrodiella*, *Cerrena*, *Ceriporia*, *Phlebia*, *Bjerkandera*, *Rigidoporus*, *Phanerochaete*, and *Irpex* using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Tool (BLAST®) online portal.

d For sequences classified to Aphyllophorales, we obtained an alignment all of the unique sequences classified to this group using mothur 1.31.2 (Schloss et al. 2009). There were 637 unique sequences that had classified to Aphyllophoralles in our data set. We selected every 50th sequence in the alignment as well as the final two sequences in the alignment and conducted a search using NCBI BLAST®. *Phanerochaete laevis* (GenBank accession KJ668345) was the top BLAST® result with a 99% identity score for the 14 sequences which we selected from among the first 636 unique sequences in the alignment. S*copuloides hydnoides* (GenBank accession LN611118) was the top BLAST® match with 99% identity for the final sequence in the alignment. Therefore, we included Aphyllophorales among highly lignolytic taxa as we were confident that sequences classified to this group represented highly lignolytic white-rot Polyporales in our data set.

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APPENDIX E

Information regarding Agaricomycete taxa not selected as highly lignolytic

<table>
<tr><td colspan="3">Table E1. Agaricomycete taxa excluded from our list of “highly lignolytic taxa”. We excluded any classes, families, or genera that:
1) are largely or entirely not saprotrophic or for which nutritional modes are unknown,
2) for which we have no information regarding a role in lignin decay,
3) for which there is evidence that the taxa is nonlignolytic or weakly lignolytic,
4) are or may be lignolytic but for which there is insufficient evidence that they are highly lignolytic,
5) for which existing evidence regarding their capacity to decay lignin is contradictory,
6) which are represented in our data set by fewer than 20 sequences regardless of their physiology.
7) taxa that were unclassified at the order or class level (most not shown below).
8) taxa unclassified at the genus level (not shown below) if we had not included its family in our selection of highly lignolytic (see Table B1).
We list the current (to the best of our knowledge) taxonomic placement of any taxa we excluded. If this differed from the classification we obtained from the Ribosomal Database Project fungal LSU rRNA v7 classifier (Liu et al. 2012) files, we documented this in the notes column.</td></tr>
<tr><td>Taxa within Agaricomycetes</td><td>Reasons for exclusion with citations</td><td>Notes</td></tr>
<tr><td>Auriculariales</td><td>5. Inconsistent role in lignin decay. Observations of lignin removal range from none to very high for different species, strains, substrates, and studies (Worrall et al. 1997, Osono and Takeda 2006, Liers et al. 2011,</td><td></td></tr>
</table>

	Suhara et al. 2012). Species of this group are of interest for their role in lignin decomposition because they have high numbers of lignin-decomposing genes (Floudas et al. 2012, Nagy et al. 2015), including dye-decolorizing peroxidases (Liers et al. 2010). Despite their seemingly high potential for lignin decay, we have excluded Auriculariales because actual measurements for their role in this process show a high degree of variation.	
Agaricales/ Agaricaceae	4 & 5. Probably not highly lignolytic. *Agaricus* removed ~50% of lignin mass from straw over 70 days (Durrant et al. 1991), but the ability of *Agaricus* to decay fresh needle litter was observed to be weak (Osono et al. 2006). *Agaricus* species possess genes for lignolytic enzymes (Hildén et al. 2013, Floudas et al. 2015) and may be adapted for late-stage litter decay (Morin et al. 2012). *Lepiota*, the genus to which most of the Agaricaceae sequences in our data set belonged, is much less well-studied, in this regard. *L. cristata* was able to oxidize lignin model compound ABTS, but did not bleach humic acids nor oxidize Mn^{2+} (Steffen et al. 2000). No other information regarding the role of *Lepiota* in lignin decay could be found.	
Agaricales/ Amanitaceae	1. Largely mycorrhizal, although some species are saprotrophic litter decomposers with some lignin decomposing genes (Kohler et al. 2015)	
Agaricales/ Bolbitiaceae	4. Probably not highly lignolytic. *Agrocybe* comprised the preponderance of sequences from the Bolbitiaceae in our data set. *Agrocybe aegerita* produced low-molecular weight compounds, which are evidence of lignin decomposition, but caused a low amount of lignin loss on a mass basis compared to other fungi studied (Liers et al. 2011).	
Agaricales/ Clavariaceae	1. Includes saprotrophs and biotrophs, but the dominant genera in our data set (*Clavaria*, *Clavulinopsis* and *Ramariopsis*) are all biotrophic (Birkebak et al. 2013)	
Agaricales/ Cortinariaceae/ *Cortinarius*	1. Ectomycorrhizae	
Agaricales/	6. < 20 sequences	

Hymenogastraceae/ *Dermocybe*		
Agaricales /Cortinariaceae/ *Quadrispora*	6. < 20 sequences	
Agaricales/ Entolomataceae	2. Little is known about their role in decomposition or their capacity for lignin decay. Saprotrophs, especially of grasslands (Lynch and Thorn 2006, Griffith and Roderick 2008) and some mycorrhizal species (Smith et al. 2013).	
Agaricales/ Hydnangiaceae/ *Laccaria*	1. Ectomycorrhizae	*Laccaria* classified to Agaricales/Tricholomataceae , but belongs in Agaricales/Hydnangiaceae (Matheny et al. 2006, Kirk et al. 2008)
Agaricales/ Hygrophoraceae	1. *Hygrocybe*, the genus that dominates Hygrophoraceae sequences in our data set, is biotrophic (Seitzman et al. 2011). *Gliophorus* is also biotrophic (Seitzman et al. 2011).	*Gliophorus* classified to Agaricales/Tricholomataceae, but should be in the Agaricales/Hygrophoraceae (Seitzman et al. 2011)
Agaricales/ Hymenogastraceae/ *Flammula*	2. No information regarding lignin decay available. *Flammula alnicola* and *Flammula conissans* cause root and butt root, with ~2% mass loss on wood over a 6 month incubation. *F. alnicola* produces a rot described as vivid yellow to yellow brown (Denyer 1959).	classified to Agaricales/Cortinariaceae. In the past, *Flammula* was included in the Strophriaceae (Kirk et al. 2008), but others have found that is does not belong to the Strophariaceae (Moncalvo et al. 2002, Matheny et al. 2006) and is more appropriately placed in Agaricales/Hymenogastraceae (Matheny et al. 2006)
Agaricales/	4. Insufficient information regarding their role in lignin decay. *Galerina*	classified to

Hymenogastraceae/ *Galerina*	*marginata* is a white-rot organism with a high number of genes for lignin and crystalline cellulose decay (Floudas et al. 2015, Kohler et al. 2015) and other species in the genus *Galerina* are also wood decay fungi (Muraoka et al. 1999). A laccase from a *Galerina* species efficienty oxidize lignin model compound ABTS (Ibrahim et al. 2011) and decolorized synthetic dyes (Mendoza et al. 2014). *Galerina pseudomycenopsis* nor *Galerina mycenopsis* were able to utilize lignin in BIOLOG plates (Ibrahim et al. 2011); however, this is a poor means of assessing its lignolytic activity because fungi do not utilize lignin on its own. However, we were unable to locate measurements of lignin decay for this genus.	Agaricales/Cortinariaceae. This is correct according to some sources (Kirk et al. 2008), but others have placed it in the Agaricales/Hymenogastraceae using molecular phylogenetics (Matheny et al. 2006)
Agaricales/ Hymenogastraceae/ *Hebeloma*	6. < 20 sequences	classified to Agaricales/Cortinariaceae. This is correct according to some sources (Kirk et al. 2008), but molecular phylogentic studies have placed it in the Agaricales/Hymenogastraceae (Moncalvo et al. 2002, Matheny et al. 2006)
Agaricales/ Inocybaceae/ *Inocybe*	1. Ectomycorrhizae	*Inocybe* classified to Agaricales/Cortinariaceae, but should be in Agaricales/Inocybaceae (Matheny 2005, Kirk et al. 2008)
Agaricales/ Lycoperdaceae	6. < 20 sequences	
Agaricales/ Marasmiaceae/ *Moniliophthora*	1 & 6. < 20 sequences. *Moniliophthora* is a plant pathogen (Aime and Phillips-Mora 2005)	
Agaricales/ Pleurotaceae	6. < 20 sequences	
Agaricales/ Pluteaceae	3, 4, & 5. The straw mushroom, *Volvariella volvacea*, has a high number of genes for degradation of lignin and utilization of crystalline cellulose (Floudas et al. 2015). In a decomposition experiment, *V. volvacea* caused	

	a 20% mass loss of lignin in straw over a 21 day incubation, with the ratio of lignin-to-mass lost of 1.4, indicating some selectivity for lignin decomposition over other constituents of plant litter (Chang-Ho and Yee 1977). However, none of our sequences classified to the genus *Volvariella*. Instead of the Pluteaceae sequences classified to the genus *Pluteus*, for which there is very little known regarding its lignolytic abilities. One study found that *Pluteus petasatus* demonstrated no measurable laccase or MnP activity in enzyme assays and had low production of H_2O_2, a necessary cosubstrate for lignolytic peroxidases. Additionally, *P. petasatus* did not decolorize two aromatic compounds (Eichlerová et al. 2006). *Pluteus* has been described as a tertiary decomposer that colonizes after primary decomposers have broken down lignocellulose (Stamets 2004).	
Agaricales/ Psathyrellaceae	3 & 4. Psathyrellaceae also do not appear to cause high amounts of lignin decay. *Coprinellus radians* exhibited low oxidative enzyme activity, removed low amounts of lignin, and did not produce low molecular weight compounds during over 72 days on wood (Liers et al. 2011). When inoculated on wood that had already been rotted by other fungal species first, *Coprinopsis* species did not cause significant additional mass loss, but *Coprinellus* species did, suggesting that at least some Psathyrellaceae might be late stage decay fungi (Oliver et al. 2010). *Coprinopsis cinerea* possesses 17 laccases in its genome (Kilaru et al. 2006), but has only has a nonlignolytic general peroxidase and no lignolytic class II peroxidases (Ruiz-Duenas et al. 2009).	
Agaricales/ Schizophyllaceae	4. The Schizophyllaceae are wood-rot fungi, but are likely less lignolytic than many other white-rot fungi. *Schizophyllum commune* has fewer lignolytic genes than most white-rot species which may reduce its lignolytic capabilities compared to other white-rot species (Floudas et al. 2015). *S. commune* does attack the secondary cell wall which is the lignified part of plant cells. However, it leaves the middle lamella intact, much like a soft-rot species (Floudas et al. 2015). Additionally, *S. commune* appears to cause a relatively low mass loss in wood decay	

	studies (Martínez et al. 2000, Floudas et al. 2015).	
Agaricales/ Strophariaceae/ *Hypholoma*	6. < 20 sequences	
Agaricales/ Strophariaceae/ *Pholiota*	6. < 20 sequences	
Agaricales/ Tricholomataceae/ *Armillaria*	6. < 20 sequences	
" " *Cotobrusia*	6. < 20 sequences	
" "*Filoboletus*	6. < 20 sequences	
" "*Flagelloscypha*	6. < 20 sequences	
" " *Hemimycena*	6. < 20 sequences	
" " *Hydropus*	6. < 20 sequences	
" " *Hygroaster*	6. < 20 sequences	
" " *Hymenogloea*	6. < 20 sequences	
" " *Infundibulicybe*	6. < 20 sequences	
" " *Micromphale*	6. < 20 sequences	
" " *Mycenella*	6. < 20 sequences	
" " *Mycenoporella*	6. < 20 sequences	
" "*Omphalina*	6. < 20 sequences	
" " *Pseudobaeospora*	6. < 20 sequences	
" " *Ripartites*	6. < 20 sequences	
" "*Setulipes*	6. < 20 sequences	
" " *Tephrocybe*	6. < 20 sequences	
" " *Tricholoma*	1. Ectomycorrhizae	
Agaricales/ Tricholomataceae/*Tricholomella*	6. < 20 sequences	
Agaricales/ Tubariaceae/	2. *Tubaria* species have been isolated from lignin rich substrates like wood, needles, and dead roots (Martínez et al. 2000, Matheny et al. 2007),	Tubaria were classified to Agaricales/Strophariaceae, but now

Tubaria	but little is presently known regarding its ability to degrade lignin. *Tubaria furfuracea* was able to decolorize Remazol Brilliant Blue R (RBBR) dye, a substrate used to evaluate ligninolytic activity in studies examining fungi for biotechnological and bioremediation applications, in one study (Okino et al. 2000), but not in another (Machado et al. 2005).	have their own family, the Tubariaceae (Matheny et al. 2006, Vizzini 2008).
Amylocorticiales/ Amylocorticiacea/ *Anomoporia*	3. Brown-rot. Because the classifier includes both brown-rot and white-rot taxa in sequences used to define this genus (see note), we verified that sequences classified to *Anomoporia* represented brown-rot taxa by obtaining the unique sequences classified to this genus in mothur (Schloss et al. 2009) and conducting searches with these sequences using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Tool (BLAST®) online portal. All searches produced hits with 94-95% identity to *Anomoporia kamtschatica* (GenBank AY586630), a brown-rot species (Niemelä et al. 2007).	These sequences were classified to Polyporales/Coriolaceae/Anomoporia. It is now placed in the Amylocorticiales (Binder et al. 2010). Furthermore, the classifier includes both brown-rot and white-rot taxa in Anomoporia, but the genus has since been split into Anomoporia for brown-rot species and Anomoloma for white-rot species (Niemelä et al. 2007)
Boletales	1 & 3. Largely mycorrhizal with some brown-rot species (Floudas et al. 2012)	
Cantharellales	1, 3, & 4. Ectomycorrhizae, orchid mycorrhizae, lichen-associated fungi, plant pathogens, and saprotrophs. Saprotrophs from Cantharellales have been observed highly celluloytic but not lignolytic (Boberg et al. 2011). Rot has not been characterized as white-rot but as "uncertain" or ancestral soft-rot (Floudas et al. 2015, Nagy et al. 2015)	"Agaricomycetes incertae sedis/Tricellortus" and to "Polyporales/Polyporales incertae sedis/Aphyllophoralean" were also determined to represent Cantharellales. Sequence defining Tricellortus in classifier produced 93-99% NCBI BLAST matches to *Minimedusa*, *Sistotrema*, *Clavulinaceae*. Sequence defining Aphyllophoralean produced 96-97% identity matches in NCBI BLAST to *Sistotrema* and *Clavulina*.
Geastrales/ Geastraceae	6. < 20 sequences	

Geastrales/ Sphaerobolaceae/*Sphaerobolus*	4 & 5. Lack of information regarding their role in lignin decay. *S. stellatus* is often found as a saprotroph on mulch and is also responsible for turfgrass disease (Baetsen 2013). *S. stellatus* has a high number of oxidoreductases (Nagy et al. 2015), has been observed to decay lignin (Baetsen 2013), and has demonstrated high selectivity for lignin over other constituents of plant cells (Worrall et al. 1997). In media, *S. stellatus* strongly degraded lignin (Robinson et al. 1993). However, *Sphaerobolus* species on natural substrata have not caused high mass loss of lignin (Valmaseda et al. 1990, Worrall et al. 1997, Suhara et al. 2012), although this may be because *Sphaerobolous* are slow growing (Conway et al. 2000). While *Sphaerobolus* are lignolytic, data available are currently too limited and contradictory to determine if they are highly lignolytic.	
Gomphales/ Gomphaceae/ *Kavinia*	4. Lack of information regarding their ability to decay lignin. *Kavinia* are resupinate fungi often found on wood (Nordén and Paltto 2001, Kout and Hajšmanová 2015). *Kavinia* species possess strong laccase activity (Harkin et al. 1974) and have been described as white-rot (Ginns and Lefebvre 1993) or uncertain rot (Hibbett et al. 2014). However, no measurements of their ability to decay lignin presently exist in literature.	
Gomphales/ Gomphaceae/ *Phaeoclavulina*	1. Little known regarding their ecology	
Gomphales/ Gomphaceae/ *Ramiricium*	6. < 20 sequences	
Hymenochaetales	4. Lack of information regarding their ability to decay lignin. Hymenochaetales have unique manganese peroxidases (Morgenstern et al. 2010) and at least one species (*Fomitiporia mediterranea*) has a high number of genes for lignolytic enzymes (Floudas et al. 2012). However, we were unable to find measurements of their ability to decay lignin in the literature.	
Phallales	6. < 20 sequences	
Polyporales/	6. < 20 sequences	

Meripilaceae		
Polyporales/ Meripilaceae/ *Rigidoporus*	6. < 20 sequences	classified to Polyporales/Coriolaceae. Coriolaceae is no longer recognized as a family. *Rigidoporus* but is currently placed in the Meripilaceae (Kirk et al. 2008).
Polyporales/ Phanerochaetaceae/ *Ceriporia*	3. Many *Ceriporia* species are highly lignolytic white-rot fungi. However, *Leptoporus mollis* and *Ceriporia reticulata* are brown-rot species which were recently placed phylogenetically in the middle of this genus (Floudas and Hibbett 2015). In order to investigate whether the *Ceriporia* in our data set were more closely related to white-rot or brown-rot *Ceriporia* species, we obtained all of the unique sequences which were placed in this genus, using mothur 1.31.2 (Schloss et al. 2009). Of the 666 unique sequences classified to *Ceriporia* in our data set, we selected 10 and conducted a search through the NCBI BLAST® online portal. For all 10 sequences, the top BLAST hit was *Ceriporia reticulata* (99% identity, GenBank KP135204), a brown-rot species (Floudas and Hibbett 2015). Therefore, the *Ceriporia* sequences in our data set most likely represent brown-rot species.	classified to Polyporales/Coriolaceae/*Ceriporia*. However, Coriolaceae is no longer recognized as a family and *Ceriporia* belongs to the Phanerochaetaceae (Kirk et al. 2008, Floudas and Hibbett 2015)
" " *Ceriporiopsis*	6. < 20 sequences	classified Polyporales/Coriolaceae, but is currently placed in the Polyporales/Phanerochaetaceae (Kirk et al. 2008).
Polyporales/ Polyporaceae	6. < 20 sequences	
Polyporales/ Polyporaceae/ *Cerrena*	6. < 20 sequences	Classified Polyporales/Coriolaceae. Coriolaceae no longer recognized as a family. Now placed in the Polyporales/Polyporaceae (Kirk et

		al. 2008).
" " *Hapalopilus*	6. < 20 sequences	Classified Polyporales/Coriolaceae. Coriolaceae no longer recognized as a family. Now placed in the Polyporales/Polyporaceae (Kirk et al. 2008).
" " *Oligoporus*	6. < 20 sequences	Classified Polyporales/Coriolaceae. Coriolaceae no longer recognized as a family. Now placed in Polyporales/Fomitopsidaceae (Kirk et al. 2008).
Russulales/ Russulaceae	1. Ectomycorrhizal	
Sebacinales	1. Largely mycorrhizal	
Thelephorales	1. Ectomycorrhizal	
Trechisporales	7 & 4. Sequences associated with Trechisporales appear to have been classified only as far as class (Agaricomycetes) and were placed as unclassified at the order level with the RDP v. 7 classifier. Therefore, when we excluded unclassified Agaricomycetes, we excluded sequences in our data set from species in the Trechisporales. A recent genomic analysis (Nagy et al. 2015) has placed two members of this order among white-rot fungi. High laccase activity (Nobles 1958, Harkin et al. 1974, Kreetachat et al. 2016) and high peroxidase activity (Harkin et al. 1974) have been reported for some species from this group, but not from others (Harkin et al. 1974). Rot from species in the Trechisporales has previously been described as white-rot (Harkin et al. 1974, Gilbertson et al. 1975, Huckfeldt and Schmidt 2006) or as an unknown rot (Nobles 1958). However, we were unable to locate any studies measuring lignolysis by Trechisporales. Thus, Trechisporales are lignolytic, but we have insufficient data to be determine if they are highly lignolytic.	Some sequences that were classified as Agariomycetes/unclassified represent Agaricomycetes/Trechisporales

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APPENDIX F

SIMPER results

Table F1. Top ten SIMPER results for each type of sample for each collection date. These ten operational taxonomic units (OTUs) had the highest dissimilarity scores between fungal communities under ambient and experimental N deposition, respectively. The low-lignin (LL), high-lignin (HL), and wood (W) substrates were collected after 7 and 18 months of decomposition, while forest floor (FF) and soil (S) were co-collected during the 7-month sampling date. For each OTU, we list its average dissimiliarity score, its dissimilarity over its standard deviation (SD), and its contribution (%) to total dissimilarity; we additionally list its average proportional abundance under ambient and experimental N deposition, as well the difference between treatments with declines in mean abundance under experimental N deposition denoted in bold. Furthermore, we list the top BLAST® match to an identified species with outdated nomenclatures retained in parentheses. Based on our knowledge of the biology of these taxa, we gave each OTU a functional assignment: white-rot and lignolytic litter decay (WRL), soft-rot and celluloytic/hemicelluloytic litter decay (SRCH), brown-rot (BR), weakly lignolytic (WL) and mycorrhizal/biotrophic (MB), with a question mark (?) indicating that this assignment was tentative (Appendix C). Taxa were assigned to the phyla Basidiomycota (B) and Ascomycota (A), subphyla Agaricomycotina (Ag) and Pezizomycotina (Pez), with assignments to to class, order, and family abbreviated by leaving off the -mycetes, -ales, and -aceae, respectively.

Sample type	months of decomposition	OTU	Average dissimilarity	Dissimilarity/SD	contribution (%)	average proportional abundance of each OTU			functional assignment	Top BLAST match for an identified species				taxonomic placement for that species					
						ambient N	experimental N	change		name	GenBank ID #	sequence identity (%)	query coverage (%)	phylum	Subdivision	class	order	family	genus
F	-	4	1.5	1.5	4.8	0.101	0.030	**0.071**	WRL	*Mycena sanguinolenta*	AY207257	99	100	B	Ag	Agarico	Agaric	Mycen	*Mycena*
F	-	66	1.4	1.5	4.5	0.000	0.036	0.036	WRL (?)[1]	*Phaeoclavulina (Ramaria) abietina* [1]	JN649369	99	100	B	Ag	Agarico	Gomph	Gomph	*Phaeoclavulina*
F	-	19	1.3	1.5	4.1	0.059	0.101	0.042	SRCH	*Minimedusa polyspora*	KC176336	96	100	B	Ag	Agarico	Cantharell		*Minimedusa*
F	-	84	1.2	1.4	3.6	0.026	0.000	**0.025**	SRCH	*Sistotrema coroniferum*	AM259215	97	100	B	Ag	Agarico	Cantharell	Hydn	*Sistotrema*

F	-	13	1	1.5	3.0	0.100	0.172	0.072	SRCH	*Polyscytalum algarvense*	GQ303318	92	100	A	-	-	-	-	*Polyscytalum*
F	-	106	1	0.8	3.0	0.028	0.000	**0.028**	WRL	*Trechispora confinis*	AF347081	92	100	B	Ag	Agarico	Trechispor	Hydnodont	*Trechispora*
F	-	15	0.9	1	2.9	0.021	0.008	**0.014**	WRL	*Mycena plumbea*	DQ470813	99	100	B	Ag	Agarico	Agaric	Mycen	*Mycena*
F	-	20	0.8	3.4	2.5	0.003	0.020	0.017	WRL	*Mycena leptocephala*	HQ604773	99	100	B	Ag	Agarico	Agaric	Mycen	*Mycena*
F	-	42	0.8	1.4	2.4	0.014	0.001	**0.013**	WRL	*Marasmius pulcherripes*	FJ917601	99	96	B	Ag	Agarico	Agaric	Marasmi	*Marasmius*
F	-	79	0.7	2.3	2.3	0.016	0.004	**0.012**	WRL	*Mycena leaiana*	AF261411	98	100	B	Ag	Agarico	Agaric	Mycen	*Mycena*
LL	7	17	1.6	1.6	3.6	0.033	0.115	0.082	BR	*Antrodia infirma*	KC595895	91	100	B	Ag	Agarico	Polypor	Fomitopsid	*Antrodia*
LL	7	29	1.4	1.6	3.0	0.027	0.106	0.079	SRCH	*Phaeohelotium epiphyllum*	KJ472236	97	100	A	Pez	Leotio	Heloti	Heloti	*Phaeohelotium*

LL	7	8	1.2	1.6	2.8	0.060	0.016	**0.044**	MB	*Sebacina vermifera*	DQ983815	97	100	B	Pez	Agarico	Sebacin	Sebacin	*Sebacina*
LL	7	14	1.1	0.8	2.4	0.038	0.000	**0.038**	WL	*Anthostomella leucospermi*	EU552100	99	100	A	Pez	Sordario	Xylari	Xylari	*Anthostomella*
LL	7	77	1	2.6	2.3	0.026	0.023	**0.003**	SRCH	*Dactylella mammillata*	KT215290	100	100	B	Pez	Leotio	Orbili	Orbili	*Dactylella*
LL	7	100	1	1.7	2.2	0.024	0.019	**0.005**	SRCH (?)[2]	*Ceratosebacina calospora*	AF291304	98	83	B	Ag	Agarico	-	-	*Ceratosebacina*
LL	7	119	1	3.4	2.2	0.016	0.000	**0.016**	SRCH	*Hyalodendriella betulae*	EU040232	96	100	A	Pez	Leotio	Heloti		*Hyalodendriella*
LL	7	60	0.9	1.5	2.1	0.049	0.021	**0.028**	SRCH	*Arthrobotrys gephyropaga*	AY261168	99	97	A	Pez	Leotio	Heloti	Orbili	*Arthrobotrys*
LL	7	90	0.9	0.7	2.1	0.000	0.037	0.037	MB	*Piriformospora indica*	KF061284	97	100	B	Ag	Agarico	Sebacin	Sebacin	*Piriformospora*
LL	7	139	0.9	1	2.0	0.000	0.020	0.020	SRCH	*Craterocolla cerasi*	KF061265	94	100	B	Ag	Agarico	Sebacin	Sebacin	*Craterocolla*

HL	7	4	2.8	1.4	5.3	0.160	0.035	**0.125**	WRL	*Mycena sanguinolenta*	AY207257	99	100	B	Ag	Agarico	Agaric	Mycen	*Mycena*
HL	7	16	2.2	0.7	4.2	0.000	0.112	0.112	SRCH	*Minimedusa polyspora*	KC176336	98	100	B	Ag	Agarico	Cantharell	-	*Minimedusa*
HL	7	20	1.7	1.8	3.2	0.038	0.000	**0.038**	WRL	*Mycena leptocephala*	HQ604773	99	100	B	Ag	Agarico	Agaric	Mycen	*Mycena*
HL	7	30	1.5	0.7	2.9	0.000	0.052	0.052	BR[3]	*Ceriporia reticulata*[3]	KP135204	100	97	B	Ag	Agarico	Polypor	Phanerochaet	*Ceriporia*
HL	7	15	1.5	0.9	2.8	0.014	0.038	0.024	WRL	*Mycena plumbea*	DQ470813	99	100	B	Ag	Agarico	Agaric	Mycen	*Mycena*
HL	7	17	1.5	1.4	2.7	0.020	0.049	0.029	BR	*Antrodia infirma*	KC595895	91	100	B	Ag	Agarico	Polypor	Fomitopsid	*Antrodia*
HL	7	3	1.4	1.7	2.5	0.175	0.078	**0.097**	SRCH	*Hyaloscypha albohyalina* var. spiralis	AB546940	99	99	A	Ag	Leotio	Heloti	Hyaloscyph	*Hyaloscypha*
HL	7	8	1.3	1.6	2.4	0.025	0.022	**0.003**	MB	*Sebacina vermifera*	DQ983815	97	100	B	Ag	Agarico	Sebacin	Sebacin	*Sebacina*

HL	7	54	1.2	0.7	2.3	0.037	0.000	**0.037**	WRL	*Hyphoderma praetermissum*	DQ873597	99	100	B	Ag	Agarico	Polypor	Meruli	*Hyphoderma*
HL	7	18	1.1	3.3	2.0	0.024	0.022	**0.001**	SRCH	*Phaeohelotium epiphyllum*	KT876976	100	100	A	Pez	Leotio	Heloti	Heloti	*Phaeohelotium*
HL	18	10	3.1	1.1	5.0	0.115	0.120	0.004	WRL	*Fibrodontia brevidens*	KC928277	94	100	B	Ag	Agarico	Trechispor	Hydnodont	*Fibrodontia*
HL	18	56	2.3	1.7	3.7	0.043	0.000	**0.043**	WRL	*Trechispora alnicola*	AY635768	99	100	B	Ag	Agarico	Trechispor	Hydnodont	*Trechispora*
HL	18	4	2.3	1.5	3.7	0.044	0.045	0.001	WRL	*Mycena sanguinolenta*	AY207257	99	100	B	Ag	Agarico	Agaric	Mycen	*Mycena*
HL	18	11	2.2	1.8	3.4	0.137	0.123	**0.013**	SRCH	*Lachnellula willkommii*	KC492982	96	100	A	Pez	Leotio	Heloti	Hyaloscyph	*Lachnellula*
HL	18	55	2.2	1.6	3.4	0.038	0.000	**0.038**	WRL	*Trechispora alnicola*	AY635768	98	100	B	Ag	Agarico	Trechispor	Hydnodont	*Trechispora*
HL	18	72	1.8	1.3	2.9	0.031	0.000	**0.031**	WRL	*Trechispora confinis*	AY586719	97	100	B	Ag	Agarico	Trechispor	Hydnodont	*Trechispora*

HL	18	35	1.8	0.7	2.8	0.059	0.000	**0.059**	BR (?)[4]	*Ceraceomyces tessulatus*[4]	AY586642	99	100	B	Ag	Agarico	Amylocortici	Amylocoritici	*Ceraceomyces*
HL	18	64	1.8	0.7	2.8	0.052	0.000	**0.052**	WRL	*Sphaerobolus stellatus*	HQ604795	97	100	B	Ag	Agarico	Geastr	Geastr	*Sphaerobolus*
HL	18	12	1.7	0.7	2.8	0.000	0.048	0.048	WRL (?)[1]	*Kavinia himantia*[1]	AY586682	99	100	B	Ag	Agarico	Gomph	Lentari	*Kavinia*
HL	18	14	1.6	0.8	2.6	0.043	0.000	**0.043**	WL	*Anthostomella leucospermi*	EU552100	99	100	A	Pez	Sordario	Xylari	Xylari	*Anthostomella*
S	-	43	1.9	1.3	3.4	0.078	0.000	**0.078**	MB	*Hygrocybe parvula*	KF291189	98	100	B	Ag	Agarico	Agaric	Hygrophor	*Hygrocybe*
S	-	115	1.2	1.4	2.2	0.039	0.001	**0.038**	WL	*Entoloma (Inocephalus) murrayi*	GU384620	99	99	B	Ag	Agarico	Agaric	Entolomat	*Entoloma*
S	-	81	1.1	1.5	2.0	0.033	0.015	**0.018**	SRCH	*Geoglossum difforme*	KC222137	100	100	A	Pez	Geoglosso	Geogloss	Geogloss	*Geoglossum*
S	-	101	1.1	0.7	1.9	0.000	0.052	0.052	MB	*Russula aeruginea*	HQ604837	98	100	B	Ag	Agarico	Russul	Russul	*Russula*

S	-	156	0.9	1.2	1.7	0.021	0.000	**0.021**	MB	*Russula atropurpurea*	AF325296	99	100	B	Ag	Agarico	Russul	Russul	*Russula*
S	-	171	0.9	1	1.6	0.001	0.027	0.027	WL	*Entoloma sinuatum*	EU522771	100	100	B	Ag	Agarico	Agaric	Entolomat	*Entoloma*
S	-	157	0.9	1.4	1.6	0.000	0.018	0.018	MB	*Tomentella botryoides*	AY586717	97	100	B	Ag	Agarico	Thelephor	Thelephor	*Tomentella*
S	-	141	0.9	1.2	1.5	0.002	0.023	0.021	WL	*Entoloma sericellum*	GQ289190	97	100	B	Ag	Agarico	Agaric	Entolomat	*Entoloma*
S	-	10	0.8	2.2	1.5	0.012	0.041	0.030	WRL	*Fibrodontia brevidens*	KC928277	94	100	B	Ag	Agarico	Trechispor	Hydnodont	*Fibrodontia*
S	-	97	0.8	0.7	1.5	0.030	0.000	**0.030**	MB	*Russula vinacea*	KT933824	100	100	B	Ag	Agarico	Russul	Russul	*Russula*
W	7	5	5.3	1.2	9.7	0.232	0.000	**0.232**	WRL	*Crepidotus versutus*	AF205683	100	100	B	Ag	Agarico	Agaric	Crepidot	*Crepidotus*
W	7	1	3	1.4	5.5	0.118	0.138	0.020	WL	*Rosellinia abscondita*	KF719208	99	97	A	Pez	Sordario	Xylari	Xylari	*Rosellinia*

W	7	2	2.6	1.8	4.8	0.036	0.140	0.104	SRCH	*Herpotrichia vaginatispora*	KT934252	99	98	A	Pez	Dothideo	Pleospor	Melanommat	*Herpotrichia*
W	7	7	2.1	1.2	3.9	0.026	0.053	0.027	WRL	*Crepidotus fragilis*	AF367931	99	100	B	Ag	Agarico	Agaric	Crepidot	*Crepidotus*
W	7	9	2.1	1.4	3.8	0.005	0.052	0.048	SRCH	*Herpotrichia vaginatispora*	KT934252	96	98	A	Pez	Dothideo	Pleospor	Melanommat	*Herpotrichia*
W	7	4	2	1.5	3.6	0.042	0.042	0.000	WRL	*Mycena sanguinolenta*	AY207257	99	100	B	Ag	Agarico	Agaric	Mycen	*Mycena*
W	7	22	1.9	0.7	3.5	0.061	0.000	**0.061**	WRL	*Gymnopus dryophilus*	NG_027632	100	96	B	Ag	Agarico	Agaric	Marasmi	*Gymnopus*
W	7	8	1.9	1.2	3.4	0.079	0.054	**0.025**	MB	*Sebacina vermifera*	DQ983815	97	100	B	Ag	Agarico	Sebacin	Sebacin	*Sebacina*
W	7	15	1.9	1.1	3.4	0.039	0.000	**0.039**	WRL	*Mycena plumbea*	DQ470813	99	100	B	Ag	Agarico	Agaric	Mycen	*Mycena*
W	7	25	1.8	0.7	3.3	0.000	0.058	0.058	WL	*Coprinellus radians*	KM246027	99	100	B	Ag	Agarico	Agaric	Psathyrell	*Coprinellus*

W	18	1	3.6	1.3	6.5	0.070	0.145	0.075	WL	*Rosellinia abscondita*	KF719208	99	97	A	Pez	Sordario	Xylari	Xylari	*Rosellinia*
W	18	2	3.4	1.5	6.2	0.092	0.189	0.096	SRCH	*Herpotrichia vaginatispora*	KT934252	99	98	A	Pez	Dothideo	Pleospor	Melanommat	*Herpotrichia*
W	18	7	3.1	1.5	5.6	0.107	0.025	**0.082**	WRL	*Crepidotus fragilis*	AF367931	99	100	B	Ag	Agarico	Agaric	Crepidot	*Crepidotus*
W	18	4	3.1	1.3	5.6	0.077	0.000	**0.077**	WRL	*Mycena sanguinolenta*	AY207257	99	100	B	Ag	Agarico	Agaric	Mycen	*Mycena*
W	18	12	2.8	0.9	5	0.055	0.077	0.022	WRL (?)[1]	*Kavinia himantia*[1]	AY586682	99	100	B	Ag	Agarico	Gomph	Lentari	*Kavinia*
W	18	14	2.4	0.9	4.3	0.064	0.000	**0.064**	WL	*Anthostomella leucospermi*	EU552100	99	100	A	Pez	Sordario	Xylari	Xylari	*Anthostomella*
W	18	9	2.2	1.4	4.0	0.028	0.109	0.081	SRCH	*Herpotrichia vaginatispora*	KT934252	96	98	A	Pez	Dothideo	Pleospor	Melanommat	*Herpotrichia*
W	18	33	2	1	3.5	0.002	0.045	0.043	WL	*Psathyrella candolleana*	KM030175	99	100	B	Ag	Agarico	Agaric	Psathyrell	*Psathyrella*

W	18	6	1.9	1.6	3.4	0.095	0.039	**0.055**	SRCH	*Herpotrichia macrotricha*	GU38517 9	98	98	A	Pez	Dothide o	Pleospor	Melanomm at	*Herpotrichia*
W	18	28	1.8	0.7	3.2	0.000	0.045	0.045	WL	*Tubaria albostipitata*	EF051051	99	94	B	Ag	Agarico	Agaric	Tubari	*Tubaria*

[1] Isotopic analysis of *Phaeoclavulina (Ramaria) abietina* suggests it is a saprotroph (Agerer et al., 2012) and genomic analyses reveal it has dye-decolorizing peroxidases (Fernandez-Fueyo et al. 2015), which may be involved in lignin-decay (Liers et al. 2010). *Ramaria stricta*, a *Ramaria* species which grows on wood, has been observed to have high laccase and manganese-peroxidase activity (Erden et al. 2009). Gomphales species in the putatively saprotrophic genus *Kavinia* (Hosaka et al., 2006) are often found on wood (Kout & Hajšmanová, 2015; Nordén & Paltto, 2001), possess strong laccase activity (Harkin et al. 1974), and have been described as white-rot (Ginns & Lefebvre, 1993). Furthermore, the genus *Lentaria* has been described as white-rot (Hibbett et al. 2014). Therefore, we have tentatively considered all putatively saprotrophic Gomphales observed here to be white-rot.

[2] We have tentatively placed an OTU associated with *Ceratosebacina calospora* as SRCH. *C. calospora* could not be placed in any clade with confidence by Binder et al. 2005. *C. calospora* and two other species were placed in its own clade by Weiß & Oberwinkler (2001). Thus, *C. calospora* has not been definitively placed within any group subsequently determined to be white rot or soft-rot (Nagy et al., 2015). However, we could find no descriptions of it or its sister taxa (Weiß & Oberwinkler 2001) as white-rot. Because of this, we have tentatively described it as SRCH for the purposes of our study.

[3] Species in the genus *Ceriporia* are largely white-rot. However, brown-rot has been reported for the species *Ceriporia reticulata* (Niemelä, 1985). Additionally, *C. reticulata* was found to be closely related to another brown-rot species (*Leptoporus mollis*) in a recent phylogenetic analysis (Figure S5 *in* Floudas et al. 2015).

[4] *Ceraceomyces tessulatus* was phylogenetically placed in a clade with brown-rot species *Anomoporia bombycina*, *A. vesiculosa*, and *A. kamtschatica* (Niemelä et al., 2007). Additionally, this OTU had a high (94% sequenced identity) BLAST match for brown-rot species *Anomoporia kamtschatica* (GenBank AY586630).

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