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

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Abstract. 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 are 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 that decay lignin and other polyphenols in soil. In field studies, elevated rates of N deposition have reduced 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 fungal abundance 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 anthropogenic N deposition increases soil C storage.

Key words: Agaricomycetes; atmospheric N deposition; decomposition; forest; forest floor; fungal communities; fungi; lignin; nitrogen; soil C; soil organic matter.

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 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).

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Field studies have suggested that elevated rates of N deposition may increase soil C storage by depressing the rate of lignin decay in plant litter (Berg and Matzner 1997, Waldrop and Zak 2006). Lignin is a protective compound found in plant secondary cell walls that 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*b*), with Agaricomycete fungi being especially important for this process (Floudas et al. 2012). Changes in soil fungal communities, mediated by greater rates of N deposition, represent a 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 the forest floor, suggesting that high inorganic N availability also suppresses fungal lignolytic enzyme activity under field

conditions (Carreiro et al. 2000, DeForest et al. 2004*a*, Frey et al. 2004). 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 thereby decrease their abundance within the soil microbial community (DeForest et al. 2004*b*). As such, 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. 2004*a*, *b*).

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, Morrison et al. 2016) and to have no effect (Freedman et al. 2015) on fungal community composition. Furthermore, even when elevated N deposition has been found to alter fungal community composition, it has not been clear whether such a change is driven by a decline in lignolytic fungi. This is, in part, because past studies have often examined fungal communities at taxonomic levels that were too coarse to obtain sufficient insight into the physiology and ecology of the affected taxa (Edwards et al. 2011, Entwistle et al. 2013). However, such limitations can now be overcome by taking advantage of recent improvements in next-generation sequencing, automated tools for fungal sequence classification (Liu et al. 2012, Schloss et al. 2016), and knowledge of the phylogenomic distribution of fungal genes involved in lignin decay (Floudas et al. 2012, Nagy et al. 2015).

A direct comparison of fungal communities colonizing low- and high-lignin substrates under ambient and elevated rates of N deposition should also improve our understanding of the role of lignin and lignolytic fungi in ecosystem responses to anthropogenic N. 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 anthropogenic N deposition alters fungal community composition in a manner consistent with our proposed mechanism, we reasoned that these effects should be more evident in fungal communities colonizing lignin-rich substrates, relative to those colonizing low-lignin substrates. Furthermore, because cellulose and hemicellulose are decomposed more rapidly than lignin, making partially decayed materials relatively enriched in lignin compared to their initial condition, we similarly reasoned that the negative effects of anthropogenic N deposition on lignolytic fungi should become more apparent with time. Finally, if greater inorganic N availability suppresses lignin decay, undecayed lignin should protect larger proportions of cellulose and hemicellulose, making them inaccessible to the fungal community as an energy source. We hypothesized that, as a result, the overall size of the fungal community would be smaller under experimental N deposition, particularly on decomposing plant litter and substrates with intact lignocellulose (i.e., wood and high-lignin substrates, in this study).

To test our hypotheses, we allowed low-lignin, high-lignin, and wood substrates to be colonized by fungi 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. Last, we assessed fungal abundance on each of our substrates over time using quantitative PCR (qPCR). We also assessed fungal community composition and fungal abundance 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. It is one of four sites in the Michigan Gradient Experiment, a long-term elevated atmospheric N deposition experiment in Michigan, USA. Soils are well-drained sandy typic Haplorthods of the Kalkaska series. The forest floor (O_i) is dominated by sugar maple leaf litter.

The site contains six 30 × 30 m plots. Three plots receive ambient N deposition, whereas the other three have received ambient N deposition plus 3 g NO_3^--N·m^{-2}·yr^{-1} since 1994, an amount consistent with future levels expected in some 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 N deposition treatment. Experimental N addition 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 vary in their lignocellulose content and biochemistry; for example, newsprint is produced by mechanical pulping, with the resulting paper having a high lignin content (Ragnar et al. 2000). Kraft paper, by contrast, is subjected to chemical pulping, which reduces lignin content by 50% (Kleppe 1970) as well as breaks the bonds between the

remaining lignin, cellulose, and hemicellulose (Ragnar et al. 2000). Wood has not been subjected to physical or chemical alteration; therefore, it has both intact cell walls and intact lignocellulose. In our study, we use birch wood (*Betula* spp.; Woodsies, Loew Cornell, Cincinnati, Ohio, USA), a high-lignin paper substrate (newsprint, white 3401, Pacon Corporation, Appleton, Wisconsin, USA), and a low-lignin paper substrate (kraft paper; #60, Caremail, ShurTech Brands, Avon, Ohio, USA) in a field decomposition experiment to make comparisons between fungal communities targeting weakly and highly lignified substrates. To characterize 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, hemicellulose, and nitrogen content (Table 1).

Whereas wood is a natural product, the paper (high-lignin and low-lignin) substrates do not have precise analogues in nature. Our primary motivation for using high-lignin, low-lignin, and wood substrates was that it specifically allowed us to examine decomposition of products that are different from one another in terms of plant biochemistry. Beyond allowing us to easily manipulate plant litter biochemistry in our decomposition experiment, using these commercially available forest-products as our substrates held several advantages over using plant litter and wood collected from our study site. The substrates are homogenous in biochemical composition within samples, unlike field-collected leaf litter or woody debris, which contains tissues of varying biochemical composition within individual samples (e.g., leaf petioles, bark). Additionally, factory-processing of forest products results in homogeneity in physical and chemical characteristics between samples, unlike field-collected materials, which exhibit an unavoidable degree of biological variability. Furthermore, plant litter or woody debris from the field may have already experienced the very earliest phases of decomposition prior to collection; using forest industry products ensures we are starting with undecayed materials and removes another potential source of variation. Finally, by using materials that were not collected from our study sites, we avoid any potentially confounding effects of experimental N deposition on initial plant biochemistry (Zak et al. 2004, Xia et al. 2015).

In preparation for deploying substrates to the field, bags of nylon screen with a mesh size of 1.13 × 1.30 mm were filled with ~6 g of either kraft paper or newsprint, which consisted of four pieces of kraft paper or eight pieces of newsprint (10.8 × 13.97 cm), in order to give the low- and high-lignin paper substrates similar initial mass and surface area. The wood substrate (15 × 2 × 0.15 cm) was not placed in a litter bag. Processed forest industry products are subjected to drying at elevated temperature for sterilizing purposes (Ross 2010); because of this, we did not conduct an additional sterilization step prior to assembling litter bags or deploying substrates to the field.

After recording the initial mass of each substrate sample, we tethered one substrate of each type (high-lignin, low-lignin, wood) to each stake, using <0.5 m of monofilament. In November 2011, one stake for each collection date was placed at five points within each plot, specifically 3 m from each corner of the rectangular plot and within 1 m of the plot center. This design enabled us to collect decomposing substrates of each type over two dates from the same locations within plots, thus minimizing spatial variability between samples. When placing substrates in the field, we removed the O horizon, placed the substrates on top of the mineral soil, and then covered the substrates by returning the O horizon.

Field collection

We collected substrates (30 of each type per collection date) from the field after 7 and 18 months of decay. During our first collection date, we also collected the forest floor (Oi, Oe, Oa horizons) from a 100-cm^2 area and a 5 cm deep mineral soil core from within 1 m of each substrate location in order to assess the composition and abundance of the forest floor and soil fungi. Samples were transported to the lab on ice, and stored at −80°C prior to DNA extraction.

Sample preparation and characterization

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

Table 1. Summary of results of biochemical analysis of low-lignin, high-lignin, and wood substrates.

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

Notes: Measurements were performed by the Soil & Forage Lab, UW-Madison. Results are reported as a percentage of dry mass.
†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.

DNA extraction

We extracted DNA from each sample in triplicate using a PowerLyzer PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, California, USA). 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 Powerlyzer Glass Bead Tube with 0.1 mm glass beads. We bead-beat the material for extraction on a PowerLyzer 24 homogenizer for 45 s at 2,500 rpm for soil, 1 min at 3,500 rpm for forest floor, 1 min at 4,500 rpm for wood, or 45 s at 4,500 rpm for the low-lignin and high-lignin substrates. We followed manufacturer's instructions with several modifications. Specifically, we increased 4°C incubation times to 10 min and added two centrifugations and a 10-min ethanol evaporation step in order to remove residual wash buffer and ethanol prior to DNA elution. In order to maximize DNA yield at the elution step, we incubated the molecular biology grade water on the membrane for one minute before centrifuging; we then repeated this process using the eluted DNA. The quality of the extracted DNA was measured on a NanoDrop spectrophotometer (ThermoScientific, Waltham, Massachusetts, USA). 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′; *available online*).[5] 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 (PacBio) RS II sequencer (barcodes *available online*).[6] Reactions contained 2.5 μL dNTPs (2 mmol/L each), 2.5 μL Expand High Fidelity 10× Buffer (Roche, Indianapolis, Indiana, USA) with 15 mmol/L $MgCl_2$, 0.5 μL bovine serum albumin (20 mg/mL), 0.5 μL of barcoded LR0R (20 μmol/L), 0.5 μL barcoded LR3 (20 μmol/L), 16 μL molecular biology-grade water, 0.5 μL Expand™ High Fidelity DNA polymerase, and 2 μL of 10× diluted target DNA. For samples that amplified weakly, PCR was repeated with template DNA using 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 MinElute PCR Purification Kit (Qiagen, Valencia, California, USA). The quality of purified PCR products was measured on a NanoDrop spectrophotometer, while the concentration of PCR products was measured fluorometrically using a Quant-IT PicoGreen dsDNA kit (Life Technologies, Carlsbad, California, USA) on a Synergy HT microplate reader (BioTek Instruments, Winooski, Vermont, USA). We combined 10 barcoded samples in equimolar concentrations for multiplexing on a PacBio SMRT chip. Samples were sequenced at the University of Michigan sequencing core on a PacBio RS II sequencer with P6-C4 chemistry. We obtained circular consensus sequencing files with 5× coverage; these files have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) as described in the Data Accessibility section of this manuscript.

28S rDNA sequence data processing

We processed the 28S rDNA sequences in mothur v.1.31.2 (Schloss et al. 2009). We removed sequences that had quality scores lower than 25 over windows of 50 bp, >1 mismatch to the primer or barcode, >1 ambiguous nucleotide, homopolymers of >8 nucleotides, or lengths that were outside of our expected amplicon size of 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 and aligned (Freedman et al. 2015) in Clustal Omega (Sievers et al. 2011) according to default parameters (training set *available online*).[7] Chimeric sequences 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 (see footnote 7) 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) at 97% similarity using the cluster.split command with sequences split for clustering at the class level. The most abundant sequence in each OTU was considered the representative sequence for that OTU and identified using NCBI BLAST. Our complete pipeline and all associated files used in sequence processing are available with the archived data associated with this publication. To assist in subsequent data interpretation of our fungal community composition analyses, we initially explored the overlap of fungal OTUs occurring on substrates, soil, and the forest floor and examined the effects of sample type, time, and experimental N deposition on fungal OTU richness. For the examination of overlap of fungal OTUs among substrates, soil, and forest floor, we present values averaged across substrates for simplicity, as the trends for each type of substrate were similar in this regard.

[5] https://sites.duke.edu/vilgalyslab/rdna_primers_for_fungi/

[6] https://github.com/PacificBiosciences/Bioinformatics-Training/blob/master/barcoding/pacbio_barcodes_paired_nopadding.fasta

[7] http://mothur.org/wiki/RDP_reference_files

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 two-factor permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) with 9,999 permutations and the default options for sum of squares and permutation of residuals. Relative OTU abundances, calculated after removing singleton OTUs, were used for analyses. PERMANOVA analyses were performed in PRIMER-E (version 6) with the PERMANOVA+ add-on (Clarke and Gorley 2006). We restricted analysis to OTUs that comprised ≥0.5% of at least one sample. OTU abundances were square-root transformed, and pairwise distances among samples were calculated as Bray-Curtis similarity.

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 two types of substrates individually with N deposition treatment and time as factors. However, we had only one time point for forest floor and soil, which were only collected once, and for the low-lignin substrate, which decayed too rapidly in the field for a second collection date. We were unable to perform individual PERMANOVA comparisons for sample types obtained from 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; we followed PERMANOVA with pairwise tests to further examine differences in fungal community composition between sample types.

We performed similarity percentages (SIMPER) analyses (Clarke 1993) in PRIMER-E (Clarke and Gorley 2006) to determine whether the changes in fungal community composition we detected in response to experimental N deposition were driven by shifts from strong lignin decomposers to weaker decomposers of lignin. SIMPER analyses were one-factor, with each sample type and collection date considered independently; all other conditions for SIMPER analyses were the same as those previously used for PERMANOVA. From the results for each of these comparisons, we report the 10 OTUs accounting for the highest amount of dissimilarity between fungal communities under ambient and experimental N deposition. We subsequently identified the closest known species for each reported OTU using a megablast search through the NCBI BLAST portal. Based on our knowledge of the biology of these species, we subsequently classified each OTU as either a white-rot and lignolytic litter decay fungus, a soft-rot and cellulolytic/hemicelluloytic litter decay fungus, a brown-rot fungus, a weakly lignolytic fungus, or a mycorrhizal/biotrophic fungus, as summarized in Appendix S1: Table S1. Although some ectomycorrhizal (ECM) fungi may be lignolytic (Shah et al. 2015), a paucity of data on the extent and distribution of this ability among ECM fungi led us to classify them as mycorrhizal/biotrophic fungi rather than include them in either the white-rot and lignolytic litter decay fungi or weakly lignolytic fungi categories. 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. Therefore, we examined the relative abundances of two groups of fungi colonizing our substrates as well as residing in soil and forest floor: (1) the class Agaricomyetes, a class that contains many lignin-decomposing species, and (2) a selected subset of Agaricomycete taxa that had been identified as having highly lignolytic physiologies. We identified this suite of highly lignolytic Agaricomycete fungi a priori (Table 2; Appendix S2: Table S1) based on the criteria that they consistently demonstrated high lignin loss and/or selectivity for lignin in published studies and were represented by >20 sequences across the data set, i.e., >0.006898% of all sequences in our data set. A list of taxa that we excluded from consideration and our justifications for doing so are also provided in Appendix S3: Table S1. These highly lignolytic taxa were treated as a group, rather than as individual taxa, for the purposes of relative abundance comparisons. 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. Considering Agaricomycetes as a class and "highly lignolytic taxa" as a group allowed us to make comparisons across substrates, soil, and forest floor, which may have different fungal communities from each other (Lindahl et al. 2007, Edwards and Zak 2010); for example, wood and forest floor may harbor different lignolytic taxa from each other, as some species are specialists for either leaf-litter or wood (Floudas et al. 2015, Nagy et al. 2015).

Fungal ITS1 qPCR

To test the hypothesis that the overall size of the fungal community would be smaller on substrates with intact lignocellulose (i.e., the wood and high-lignin substrate) as well as in in soil and forest floor under experimental N deposition, we conducted quantitative PCR (qPCR) of the fungal ITS1 region to measure fungal abundance. We calculated ITS1 copy number for each sample as both

Table 2. Agaricomycete taxa selected as highly lignolytic.

Order and family or genera included from this order	Citations documenting lignolytic activity
Agaricales	
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)
Mycena§	Worrall et al. (1997), Osono and Takeda (2002), Steffen et al. (2007), Liers et al. (2011), Cline and Zak (2015)
Clitocybe¶	Osono et al. (2003, 2011)
Crepidotaceae	Gutiérrez et al. (1999), Del Río et al. (2001), Martínez Ferrer et al. (2005)
Polyporales#	
Ganodermataceae	Blanchette (1984)
Antrodiella	Patel and Rao (1993)
Phanerochaete‖	Kirk and Farrell (1987), Hatakka (1994), Del Río et al. (2002)
Scopuloides‖	Kuuskeri et al. (2015)
Russulales††,‡‡	
Lachnocladiaceae	Cline and Zak (2015)
Auriscalpiaceae	Miller and Stewart (1971), Miller and Methven (2000)

Notes: All taxa are represented by >20 sequences in our data set. More details on taxa selected below are in Appendix S2: Table S1; excluded taxa are described in Appendix S3: Table S1.

†Also included sequences that were assigned to the closely related genus *Amyloflagellula* (Douanla-Meli and Langer 2008).

‡Some *Gymnopus* species were formerly *Collybia*.

§Also included sequences assigned to closely related genus *Poromycena* (Moncalvo et al. 2002).

¶Also included sequences classified to closely related genus *Lepista* (Matheny et al. 2006).

#White-rot Polyporales are broadly highly lignolytic (Campbell 1932, Ander and Eriksson 1977, Ruiz-Duenas et al. 2013). The selected Polyporales taxa listed above are the white-rot Polyporales present in our data set (Binder et al. 2013).

‖Sequences assigned to the "Aphyllophorales" by the RDP v7 classifier represent these genera in our data set (see Appendix S2: Table S1 for more details). "Aphyllophorales" is no longer a recognized taxonomic group (Hibbett and Donoghue 1995, Binder and Hibbett 2002).

††These families are 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.

‡‡Saprotrophic Russulales are white-rot fungi that have broadly been observed to be highly lignolytic (Blanchette 1984, Otjen and Blanchette 1984, Del Río et al. 2002, Speranza et al. 2009, Floudas et al. 2012, Nagy et al. 2015). The selected Russulales taxa listed above are the saprotrophic Russulales present in our data set.

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.

Quantification of fungi with qPCR can be influenced by other variables besides the amount of fungi present including interspecific variation in either the genomic copy number or the length of the selected marker, as well as amplification biases from the selected primers. To minimize these potential problems, we selected the fungal ITS1 region for fungal qPCR because it is both short (~300 bp) and does not vary significantly in length among fungal phyla (Bellemain et al. 2010, Toju et al. 2012). Furthermore, we selected fungal primers ITS1f (5′ CTTGGTCATTTAGAGGAAGTAA 3′) and 5.8s (5′ CGCTGCGTTCTTCATCG 3′) to amplify this region (Gardes and Bruns 1993, Vilgalys 2018); prior to employing this primer set in qPCR, we verified that it was both specific for fungi and had broad coverage across fungal taxa (data not shown). Although the number of ITS copies within fungal genomes varies both within and between species (Vilgalys and Gonzalez 1990), interspecific variation in genomic ITS copy number did not prevent accurate assessment of fungal ITS copy number in qPCR of mixed template samples in a previous study (Manter and Vivanco 2007); hence, we do not have any reason to think variation in copy number between species in our template DNA is likely to be an impediment to quantifying fungal ITS1 copy number in the soil, forest floor, and substrates here.

Standard DNA for qPCR was prepared by cloning the ITS1 region of *Agaricus bisporus* with a TOPO TA pCR 2.1 Cloning Kit (Life Technologies). The insert was subsequently amplified with M13 primers to create a linear target appropriate for qPCR (Hou et al. 2010). The DNA concentration of the qPCR standard was quantified fluorometrically, in the manner previously described for 28S rDNA PCR products, and its copy number was calculated according to manufacturer instructions (*available online*).[8] Quantitative PCR was calibrated with a standard curve ranging from 1.71×10^3 to 1.03×10^6 copies/μL. Prior to qPCR, sample DNA was also quantified fluorometrically and diluted as needed in order to obtain an appropriate target concentration.

Each 25-μL qPCR consisted of 0.625 μL of each primer (20 μmol/L), 0.375 μL ROX reference dye (500× dilution),

[8] http://www6.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf

0.5 μL bovine serum albumin (20 mg/mL), 9.375 μL molecular biology-grade water, 12.5 μL Brilliant III Ultra-Fast SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, California, USA; catalogue number 600882), and 1 μL target DNA. Reactions for samples contained 0.8–11 ng DNA. Quantitative PCR assays were performed on a Stratagene Mx3000P qPCR System (Agilent Technologies) using the following thermocycling program: initial denaturation (5 min, 95°C), followed by 40 cycles of denaturation (45 s, 95°C), annealing (30 s, 53°C), and extension (30 s, 72°C), and 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 and collection date) in a single 96-well run. Each run was performed three times for quality control. Efficiencies of all qPCR runs were ≥80%. The R^2 values for each run ranged from of 0.991 to 0.999. More detailed quality control information and all qPCR data is available in the Dryad data archive associated with this paper.

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. Fungal OTU richness analyses were performed in the R statistical environment (R Core Team 2017) with the R commander interface (Fox 2005). Prior to analysis, values for substrates, soil, and forest floor were averaged across each plot. No data transformations were applied to mass loss, fungal richness, or relative abundance data, but qPCR data were $\log_{10}$-transformed so that the data would meet the assumption of homoscedasticity required for ANOVA. 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

High mass loss and evidence of faunal grazing (e.g., distinct holes in substrate) were visually apparent on low-lignin substrates after 7 months of decay. By contrast, the high-lignin substrate was entirely intact after 7 months of decay, with visible evidence of fungal colonization, but no apparent damage by soil fauna. Wood samples were intact at 7 months, 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 the 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.

When mass loss was examined across both of the N deposition treatments, the percentage of mass lost after 7 months of decay from the low-lignin (–61%), high-lignin (–26%), and wood substrates (−10%) varied significantly among substrates of different types (two-way ANOVA; $F_{1,\ 16} = 218.947$, $P < 0.001$). This result confirmed that our selection of substrates, which varied in their initial lignocellulose content, represented a range of decomposability under field conditions. 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.

After 7 months, substrates decomposed under experimental N deposition exhibited no significant differences in the amount of mass lost compared to those decomposed under ambient N deposition (Fig. 1). Similarly, mass loss of the high-lignin substrate after 18 months of decay under experimental N deposition (28%) was still not significantly different from the amount of mass lost under ambient N deposition (32%) over that same time period. We were unable to accurately assess mass loss of wood after 18 months of decay, because several wood substrate samples were fragmented prior to or during collection of samples at this time point.

Fungal 28S sequences and OTUs

Sequencing produced a total of 575,339 reads with 5× circular consensus coverage. Forty-seven percent of sequences were removed during quality-control steps, a percentage of sequence loss that is similar to those that

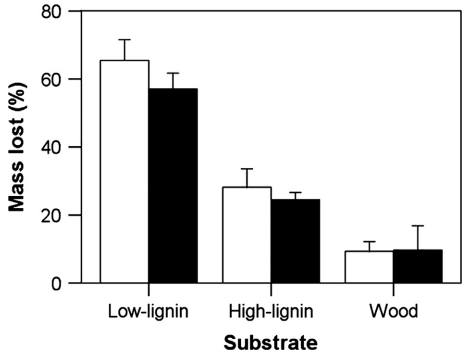

Fig. 1. Mass loss of wood, high-lignin, and low-lignin substrates under ambient (open bars) and experimental N deposition (solid bars) after 7 months of decomposition in the field. Error bars represent 2 SE. Experimental N deposition did not significantly alter mass loss for any substrate (independent sample *t* tests).

have been previously reported for targeted gene sequencing of soil microbial communities with the PacBio RS II sequencing platform (Freedman and Zak 2014, 2015). 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 initially clustered to 9,115 operational taxonomic units (OTUs) at the 97% sequence similarity level; of these, 2,589 OTUs remained after removal of singletons. Most OTUs were of low abundance with only 33% of OTUs represented in the data set by more than 10 sequences and only 10% of OTUs represented in the data set by more than 100 sequences. Mean Good's coverage for 97% sequence similarity OTUs was 94% for substrates and 88% for soil and forest floor samples.

Most of the fungi occurring on our substrates were also observed in the surrounding soil and forest floor fungal communities. The percentage of OTUs shared between substrate fungal communities and those of the soil and forest floor communities were similar across substrate types (i.e., low-lignin, high-lignin, and wood). When we compared fungal communities occurring on substrates that had decayed in the field for 7 months to those of the forest floor and mineral soil, approximately one-half of the fungal OTUs that colonized each type of substrate were also found in the forest floor, while approximately one-third of the OTUs occurring on each type of substrate were also detected in the mineral soil. More specifically, for each type of substrate, ~26% of its OTUs were additionally detected in both the soil and forest floor fungal communities, ~25% of its OTUs were observed in the forest floor, but not in the soil, and ~8% of its OTUs occurred in soil, but not in the forest floor, respectively. However, a remaining ~40% of the OTUs on each type of substrate were observed only on substrates and were not detected in the surrounding soil and forest floor fungal communities. Interestingly, most OTUs did not colonize more than one type of substrate. Among fungi occurring on substrates that had decayed for 7 months in the field, 57% of observed OTUs were unique to only one type of substrate, 26% of OTUs occurred on two different types of substrate, and only 17% of fungal OTUs were found on all three substrates.

Experimental N deposition did not reduce fungal richness and fungal richness was similar between substrates and over time in our experiment. Experimental N deposition did not alter fungal OTU richness either on substrates decayed for 7 months in the field (two-way ANOVA, $F_{1,\ 16} = 1.73$, $P = 0.21$), in the surrounding soil or forest floor collected on the same date (two-way ANOVA, $F_{1,\ 10} = 2.58$, $P = 0.15$), or on high-lignin substrate across 7 and 18 months of decay (two-way ANOVA, $F_{1,\ 10} = 0.21$, $P = 0.66$); however, experimental N deposition significantly increased fungal OTU richness on wood substrates (+40%, two-way ANOVA, $F_{1,\ 10} = 6.24$, $P = 0.04$) when we considered substrates decayed at both 7 and 18 months.Fungal OTU richness was not different among wood, high-lignin, or low-lignin substrates after 7 months of decay (two-way ANOVA, $F_{2,\ 15} = 1.73$, $P = 0.21$). However, when we compared fungal OTU richness among substrates, forest floor, and soil collected during the first collection date, observed fungal OTU richness was significantly different between sample types (one-way ANOVA, $F_{4,\ 25} = 14.3$, $P < 0.0001$); this response was driven by the significantly higher fungal OTU richness of the forest floor in comparison to either mineral soil or substrate fungal communities (Tukey tests, $P \leq 0.001$). Furthermore, time (i.e., 7 or 18 months of decay) did not alter fungal OTU richness on either high-lignin (two-way ANOVA, $F_{1,\ 10} = 0.38$, $P = 0.55$) or wood (two-way ANOVA, $F_{1,\ 10} = 0.06$, $P = 0.81$) substrates.

Fungal community composition

Experimental N deposition significantly altered fungal community composition on both wood and the high-lignin

Table 3. PERMANOVA results for comparisons of fungal communities under ambient and experimental rates of N deposition.

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 × 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 × Time	1	1362	1362	0.92149	0.6118
Low-lignin, high-lignin, and 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 × Sample type	4	4807.8	1202	1.0647	0.2909

Notes: 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)). Significant comparisons are indicated in bold.

substrate (Table 3). Whereas fungal community composition significantly changed over time on the high-lignin substrate (Table 3), time was not a significant factor for wood fungal community composition ($P = 0.055$, Table 3). There was no significant interaction of N deposition treatment and time for fungal community composition on either wood or the high-lignin substrate (Table 3).

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). Unsurprisingly, all substrates, as well as soil and forest floor, harbored distinct fungal communities that varied significantly from each other (Table 3; all pairwise tests for sample type, permutational $P < 0.01$); however, there were no significant interactions between N deposition treatment and sample type (Table 3), indicating 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 altered 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 S4: Table S1). Many of the OTUs on the high-lignin substrate, wood, and forest floor were white-rot and lignolytic litter decay taxa (Table 4) and these fungi were less abundant under experimental N deposition (Fig. 2). This response was significant on wood after 7 months of decay, whereas a negative but non-significant trend was observed for wood after 18 months of decay as well as for the high-lignin substrate and forest floor (Fig. 2). In contrast, no white-rot or lignolytic litter decay fungi were among the top 10 SIMPER results for the low-lignin substrate (Table 4, Fig. 2). The only positive response of white-rot and lignolytic litter decay taxa occurred in mineral soil, in which a single OTU (Table 4) slightly, but significantly, increased in response to experimental N deposition (Fig. 2).

Experimental N deposition appears to stimulate fungi that decompose cellulose and hemicellulose, but not lignin. For example, the relative abundances of OTUs associated with soft-rot as well as cellulolytic and hemicelluloytic litter decay fungi significantly increased on wood and in forest floor under experimental N deposition (Fig. 2). Other comparisons displayed apparent increases in brown-rot, soft rot and cellulolytic and hemicellulolytic, or weakly lignolytic fungi; however, these were not significant (Fig. 2).

In soil, most of the OTUs identified by SIMPER as the greatest contributors to community dissimilarity were associated with biotrophic or mycorrhizal species (Table 4, Fig. 2); overall, their abundance in soil declined under experimental N deposition, but not significantly so (Fig. 2).

Relative abundance of Agaricomycetes

Experimental N deposition reduced the relative abundance of Agaricomycetes on wood across both collection dates (−46%, two-way ANOVA, $F = 18.368$, $P = 0.003$, Fig. 3A). However, experimental N deposition had no effect on the relative abundance of Agaricomycetes on the

TABLE 4. SIMPER results for the 10 operational taxonomic units (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.

Sample type	Cumulative contribution to dissimilarity (%)	Number of OTUs assigned to each fungal physiology				
		White-rot and lignolytic litter decay	Soft-rot and cellulolytic/ hemicellulolytic litter decay	Brown-rot	Weakly lignolytic	Mycorrhizal/ biotrophic
Substrates						
7 months of decomposition						
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 months of decomposition						
High-lignin	33.18	7	1	1	1	0
Wood	47.19	3	3	0	4	0
Environmental samples						
Forest floor	33.11	7	3	0	0	0
Soil	18.92	1	1	0	3	5

Notes: Detailed results for individual OTUs can be found in Appendix S4: Table S1.

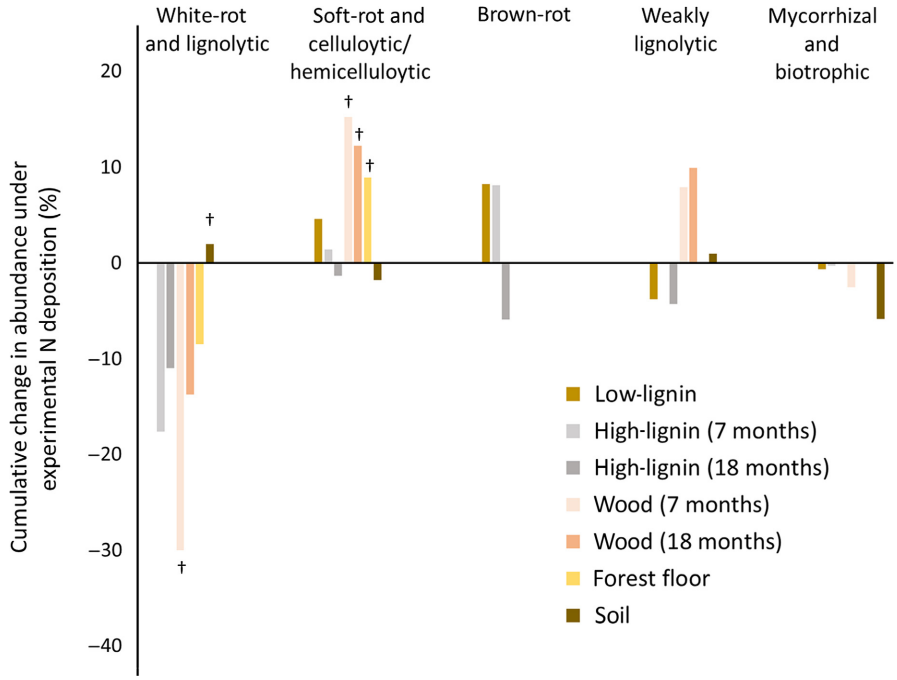

FIG. 2. The response (percent change in relative abundance) of fungi of different physiologies to experimental N deposition among the 10 operational taxonomic units (OTUs) with the largest dissimilarity scores according to SIMPER analysis. Significant comparisons (marked with a dagger †) are those for which the 95% confidence intervals for relative abundance under ambient and experimental N deposition did not overlap.

high-lignin substrate (Fig. 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 (Fig. 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 (Fig. 4A, C).

We had further hypothesized that reductions in Agaricomycete abundance under experimental N deposition would be enhanced with time. Instead, Agaricomycete relative abundance was significantly higher (+37%) on wood at 7 months of decay relative to 18 months of decay (two-way ANOVA, $F = 10.757$, $P = 0.011$). Agaricomycete abundance did not change over time on the high-lignin substrate. There also was no interaction between N deposition and time with a two-way ANOVA. Thus, the negative effect 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, and that this effect would be most evident on substrates in which energy-rich organic compounds are protected by lignin. Accordingly, experimental N deposition significantly reduced the proportion of highly lignolytic fungi present on wood across both collection dates (Fig. 3B, two-way ANOVA, $F = 8.254$, $P = 0.021$), with no significant treatment 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 (Fig. 3D), but there was a significant interaction between 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 not after 18 months of decomposition (Fig. 5). By contrast, highly lignolytic taxa were neither abundant (~9% of Agaricomycetes) nor significantly affected by experimental N deposition on the low-lignin substrate (Fig. 3F). Consistent with our hypothesis, experimental N deposition reduced the abundance of highly lignolytic taxa on both lignin-rich substrates, albeit in a time-dependent manner, 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 declined (−20%) under experimental N deposition, although not significantly (Fig. 4B). Taxa that we had identified as highly lignolytic were uncommon in mineral soil (~6% of Agaricomycetes,

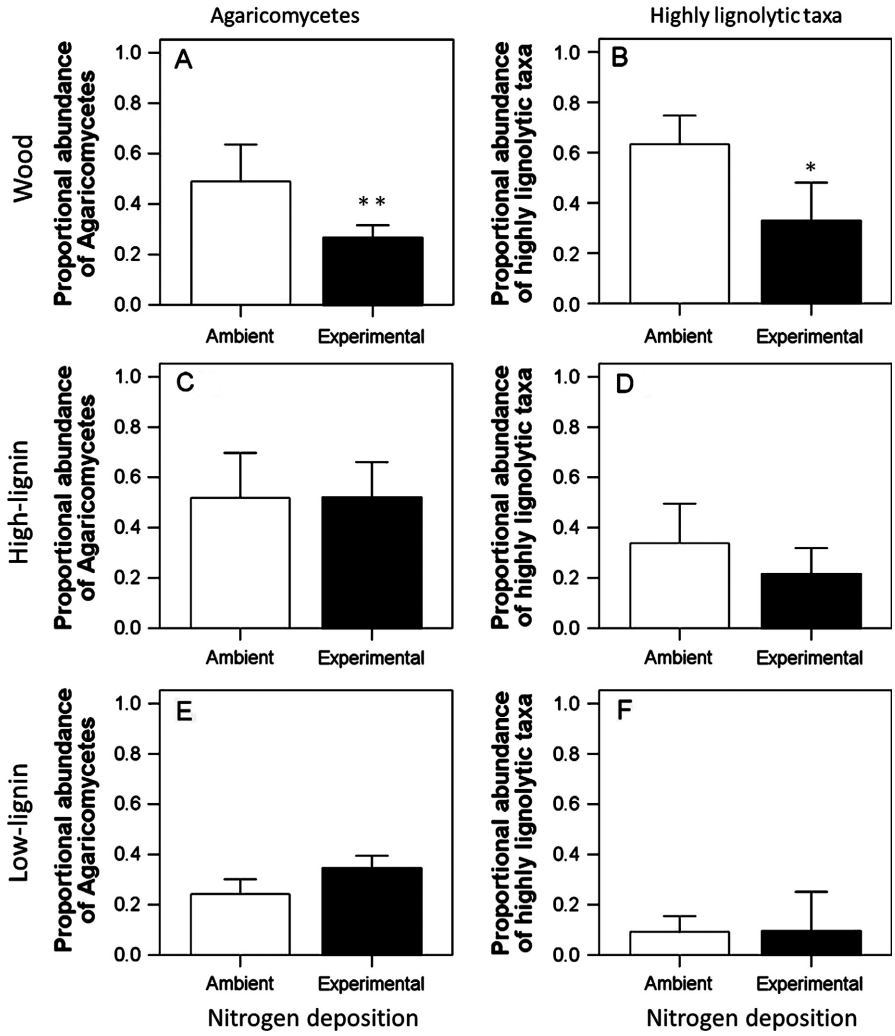

FIG. 3. Relative abundance of Agaricomycetes (left) and highly lignolytic taxa (right) in fungal communities on a wood (top), high-lignin (middle), and low-lignin (bottom) substrates after 7 and 18 months of decomposition in the field. Relative abundance of Agaricomycetes was calculated as a proportion of fungi. Relative abundance of highly lignolytic taxa was calculated as a proportion of Agaricomycetes. Error bars represent 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 $*P < 0.05$, $**P < 0.01$.

~3% of all fungi), and their relative abundance was not significantly altered by experimental N deposition (Fig. 4D).

We had further hypothesized that experimental N deposition would most negatively impact highly lignolytic taxa later in decay when substrates become enriched in lignin relative to their initial state. However, our results did not support this hypothesis. Instead, experimental N deposition reduced the abundance of highly lignolytic fungi colonizing the high-lignin substrate early in decay (i.e., after 7 months), but not after 18 months (Fig. 5). Additionally, the negative effects of experimental N deposition on the highly lignolytic taxa occurring on wood were not greater after 18 months of decay than they were after 7 months of decay, as neither time effects nor time-by-treatment interactions were statistically significant.

Fungal abundance

We reasoned that experimental N deposition should reduce energy flow to the fungal community as a consequence of diminished fungal decay of lignin. Consistent with this hypothesis, experimental N deposition reduced fungal ITS1 copy number per μg of extracted DNA on wood (Fig. 6A, two-way ANOVA, $F = 8.309$, $P = 0.020$). This trend was the same for wood when we calculated fungal ITS1 copy number per g of substrate, although no longer statistically significant (Fig. 6B). However, experimental N deposition did not alter fungal ITS1 copy number on the high-lignin substrate (Fig. 6C, D). On the low-lignin substrate, experimental N deposition significantly increased fungal ITS1 copy number per g of substrate (Fig. 6F, independent samples *t* test, $P = 0.003$), while

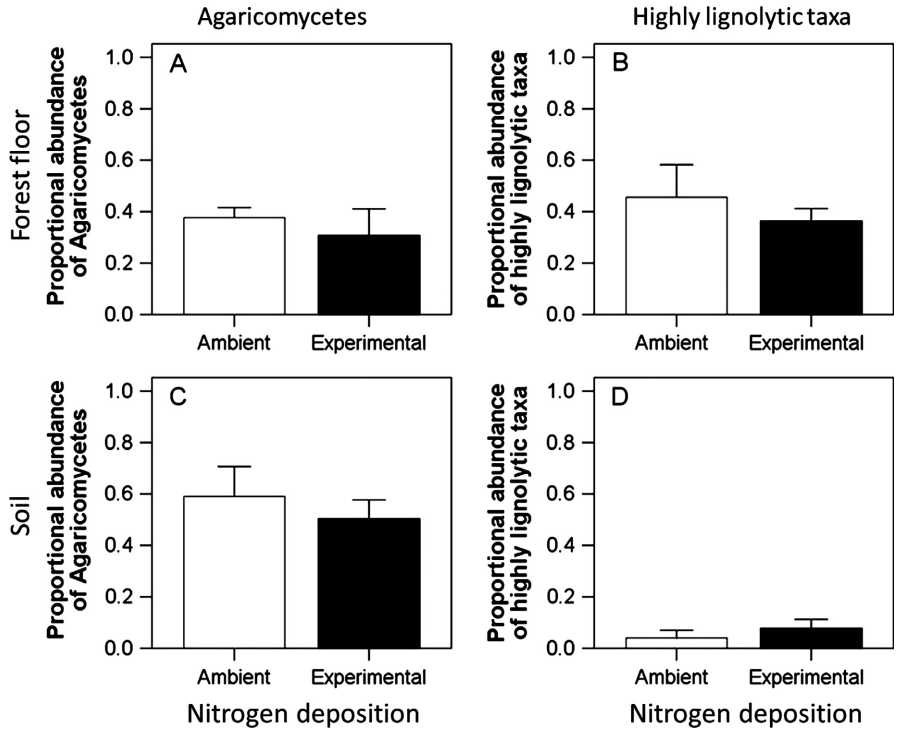

FIG. 4. Relative abundance of Agaricomycetes (left) and highly lignolytic taxa (right) of fungal communities in forest floor (top) and soil (bottom). Relative abundance of Agariomycetes was calculated as a proportion of fungi, while relative abundance of highly lignolytic taxa was calculated as a proportion of Agaricomycetes. Error bars indicate 2 SE.

having no significant effect on fungal ITS1 copy number per μg of extracted DNA (Fig. 6E). In contrast to our expectations, experimental N deposition did not significantly reduce fungal ITS1 copy number in either forest floor or soil (Fig. 7), although there were nonsignificant trends toward lower ITS1 copy numbers under experimental N deposition in soil (Fig. 7C, D) and forest floor (per g of sample, Fig. 7B).

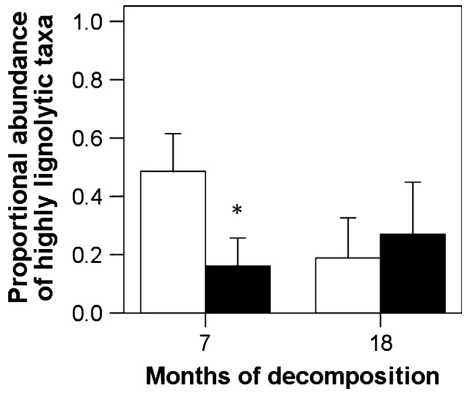

FIG. 5. Relative abundance of highly lignolytic taxa on the high-lignin substrate collected after 7 and 18 months of decomposition under ambient (open bars) and experimental (solid bars) rates of N deposition. Relative abundance of highly lignolytic taxa was calculated as a proportion of Agaricomycetes. Error bars represent 2 SE. Each collection date was analyzed with independent samples t tests, for which significance is indicated with $*P < 0.05$.

Fungal ITS1 copy number (per μg of extracted DNA) on wood was greater at 7 months than after 18 months of decomposition in the field (two-way ANOVA, $F = 16.263$, $P = 0.004$). Time did not significantly affect fungal ITS1 copy number on the high-lignin substrate, nor was the interaction of N deposition treatment and time a factor significantly affecting ITS1 copy number on either wood or the high-lignin substrate.

DISCUSSION

We have found evidence that greater soil C storage under chronic N deposition arises from a decline in the abundance of soil fungi with lignolytic physiologies. In this study, experimental N deposition, at rates predicted for midcentury (Galloway et al. 2004), reduced the relative abundance of fungi with lignolytic capacity on both wood and a high-lignin substrate. Furthermore, experimental N deposition induced overall changes in fungal community composition, including a shift toward cellulolytic fungi on decomposing wood and in the forest floor. Finally, we found that experimental rates of N deposition decreased fungal abundance on wood, but increased fungal abundance on a low-lignin, cellulose-rich substrate. Thus, our observations are consistent with those of previous studies, wherein elevated rates of N

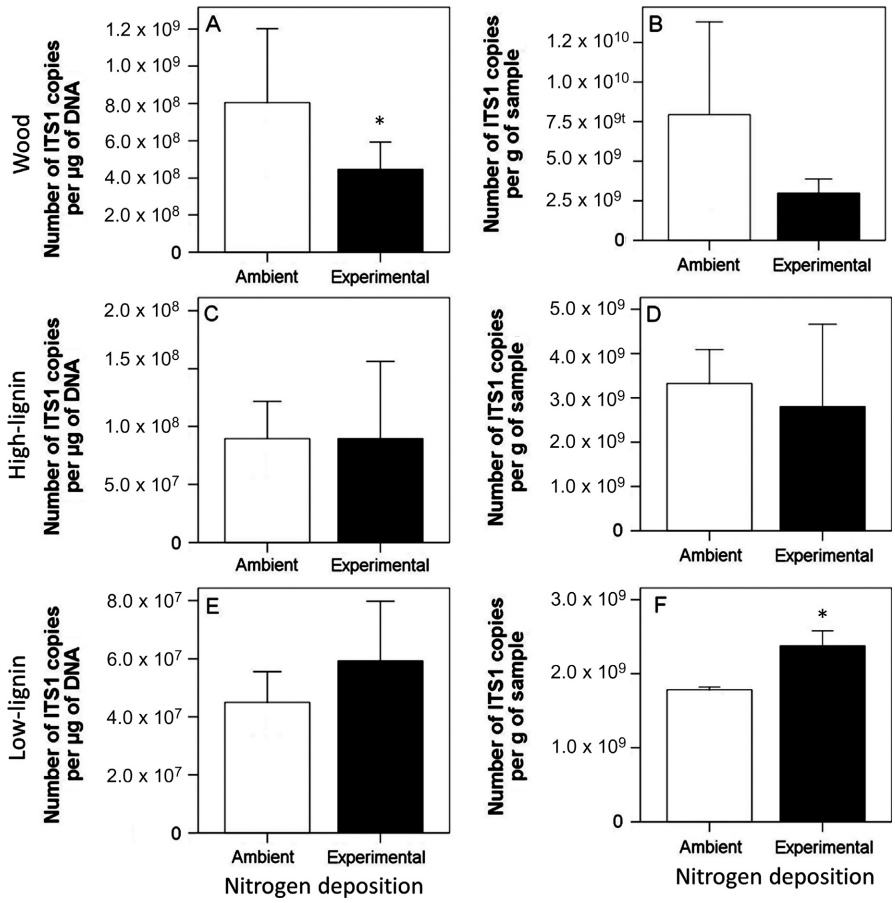

FIG. 6. Fungal abundance as measured by quantitative PCR and presented as ITS1 copies/µg DNA (left) and ITS1 copies/g sample mass (right) on decomposing substrates of varying recalcitrance (wood, high-lignin, low-lignin) collected after 7 and 18 months of decomposition in the field. We compared ITS1 copy number on the wood (top) and high-lignin (middle) substrates between time points and 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 $*P < 0.05$.

deposition suppressed lignin decay, but stimulated cellulose decay (Berg and Matzner 1997, Carreiro et al. 2000, Talbot and Treseder 2012). Although a decline in the relative abundance of lignolytic fungi has long been postulated as a response to elevated N deposition (Fog 1988, DeForest et al. 2004*b*), to our knowledge, this is the first study to document shifts in fungal community composition that are physiologically consistent with observations of reduced lignin decay under experimental N deposition.

Previous work in both our long-term field study and elsewhere has provided evidence that experimental N deposition increases soil C storage by altering microbial decomposition of lignin, not by changing plant litter production or biochemistry. For example, experimental N deposition has reduced decay of leaf and root-litter (Zak et al. 2008, Xia et al. 2017*a*) and increased retention of lignin in decaying leaf and root-litter (Xia et al. 2017*b*), while increasing neither the production of plant litter nor the lignin content of plant tissues (Burton et al. 2004, Frey et al. 2014, Xia et al. 2015). Furthermore, experimental N deposition has decreased soil respiration without altering root respiration (Burton et al. 2004, 2012), suggesting that reduced soil microbial respiration underlies this response. Moreover, stable isotope tracing with ^{13}C-labelled plant litter has revealed that experimental N deposition diminishes the amount of C transferred from plant litter into the soil microbial community (Gan et al. 2013). Finally, long-term experimental N deposition has negatively affected fungal lignocellulolytic potential (Eisenlord et al. 2013), fungal lignocellulolytic gene expression (Edwards et al. 2011, Hesse et al. 2015), and fungal lignolytic enzyme activity (DeForest et al. 2004*a*, *b*, Sinsabaugh 2010, Freedman and Zak 2014), as well as fungal capacity for litter decomposition (van Diepen et al. 2017). Our results thus add to a growing body of evidence that experimental N deposition negatively affects lignolytic soil fungi, organisms that mediate the rate-limiting step of organic matter decay.

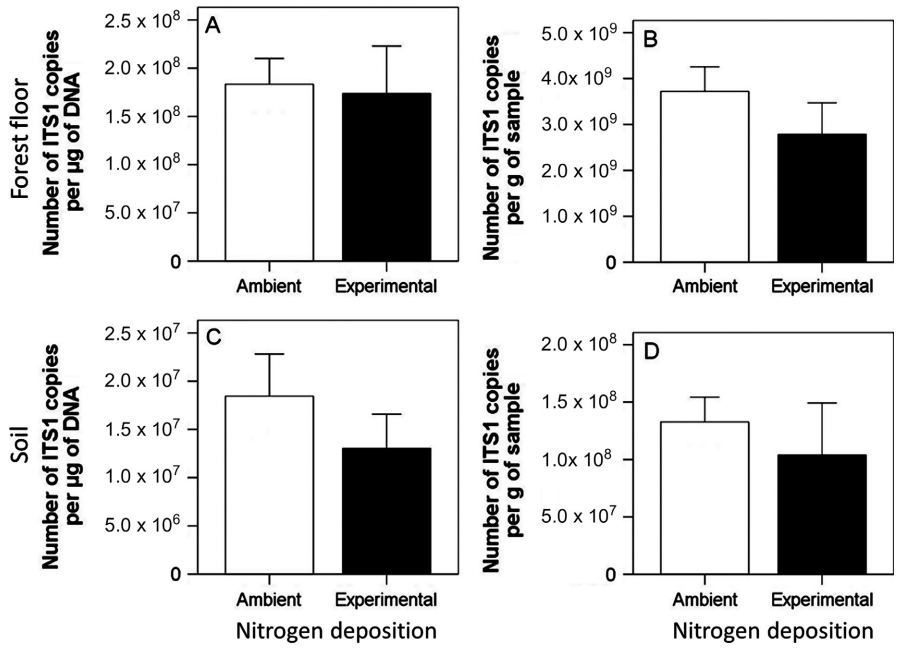

FIG. 7. Fungal abundance as measured by quantitative PCR and presented as ITS1 copies/µg DNA (left) and ITS1 copies/g sample mass (right) in forest floor (top) and soil (bottom). Error bars indicate 2 SE. ITS1 copy numbers under ambient and experimental N deposition were compared with independent samples *t* tests. No comparisons were significant.

However, lignin-decay is not strictly limited to fungi, because some bacterial decomposers also possess lignolytic activity (McCarthy et al. 1986, Bugg et al. 2011*a*, *b*) In our study system, experimental N deposition has had an overall positive effect on the abundance of bacterial genes mediating plant cell-wall decay (Freedman et al. 2016). Furthermore, experimental N deposition has increased the abundance of bacterial laccase-like multicopper oxidase (LMCO) genes (Freedman and Zak 2014), which may play similar roles to fungal laccases in lignin decay (Ridge et al. 2007, Reiss et al. 2013, Lu et al. 2014). However, this expanded bacterial role in lignin-decay has apparently not offset reductions in fungal lignin decay, given the reductions in lignin decay occurring under experimental N deposition in our field experiment. This may be 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) that bacteria do not, giving fungi an overall greater oxidative capacity to decay lignin than that of bacteria (Kirk and Farrell 1987, Floudas et al. 2012).

Our working model for conceptualizing these responses predicts that higher N availability, resulting from increased rates of inorganic N deposition, leads to lower rates of fungal lignin decay (DeForest et al. 2004*b*). This idea originates from the observation that the suppression of lignolytic activity appears to be a physiological response to high inorganic N concentrations for some species of lignolytic fungi (Leatham and Kirk 1983, Commanday and Macy 1985), with the highly lignolytic wood decay fungus *Phanerochaete chrysosoporium* being the most extensively studied species in this regard. For example, lignolytic activity in *P. chrysosoporium* cultures is suppressed by the additions of high amounts of inorganic N (Jeffries et al. 1981) and stimulated by conditions of N starvation (Keyser et al. 1978). Interestingly, we observed the strongest and most consistent negative effects of experimental N deposition on lignolytic fungi on the wood substrate. It is plausible that high N availability may have similar negative effects on the activity of the lignolytic wood-decomposing species colonizing our wood substrates, as it does on *P. chrysosporium*.

Although it is not clear why saprotrophic lignolytic fungi would reduce lignin decay in response to the presence of high inorganic N, several physiological explanations for this phenomenon have been proposed. For example, low N conditions in culture may stimulate lignin and polyphenol decay because those conditions are similar to those of its natural environment, i.e., wood (Keyser et al. 1978), making it possible that inorganic N concentration serve as a signal for environmental conditions appropriate for lignin and polyphenol decay. In culture, lignin decay occurs as part of fungal secondary metabolism; Keyser et al. (1978) suggest that the sensitivity of fungal lignolytic activity to N availability may be related more broadly to the role of N in secondary metabolism, as N limitation has been observed to induce expression of some other fungal secondary metabolites (Gold et al. 1973, Bu'Lock et al. 1974). Additionally, Fog (1988) suggested that nitrogenous compounds may react with the

intermediate products of lignin decay, thereby preventing effective fungal decay of lignin under high N conditions and making low N conditions ideal for this activity. While N suppression of fungal lignolytic activity is consistent with our results and those of previous studies in our long-term field experiment, not all fungal species appear to reduce lignin decay in response to increased N availability (Boyle et al. 1992, Kaal et al. 1995) and many of the lignolytic taxa (Table 2, Appendix S2: Table S1, Appendix S4: Table S1) in our study system have not yet been studied in this regard. Direct observation of the taxa in question under laboratory culture could provide further insight as to whether this mechanism underlies the responses of lignolytic fungi to experimental N deposition that we have observed in the field.

Because anthropogenic N deposition is also known source of acid-deposition (Liechty et al. 1993), that, on poorly-buffered soils inherently low in base cations, reduces soil pH and thereby increases leaching of cationic nutrients (Long et al. 1997, Bowman et al. 2008, Lieb et al. 2011), the question often arises as to whether soil microbial responses to anthropogenic N deposition result from the effects of increased N availability or, instead, reflect the effects of reduced soil pH or cationic nutrient availability. For example, reduced soil pH could reduce the activity of lignolytic enzymes (Sinsabaugh et al. 2008) or induce the loss of cations (e.g., Mn^{2+}) required by fungal lignolytic enzymes (e.g., manganese peroxidases; Hofrichter 2002, De Santo et al. 2009). However, our study sites are located on well-buffered calcareous sands (Zak et al. 2008). Our long-term experimental N deposition treatment has not altered soil pH, base saturation of the cation exchange complex, nor the concentrations of exchangeable Ca^{2+} in soil (Zak et al. 2008, Patterson et al. 2012). By extension, it also is unlikely that our N deposition treatment has differentially affected the loss of Mn^{2+} from soil in our experiment. Therefore, cation loss is unlikely to explain the declines in lignolytic fungi we have documented in our study.

While the mechanisms we have discussed thus far focus on the physiological effects of anthropogenic N deposition on lignolytic fungal physiology, ecological explanations have also been proposed to explain reduced fungal lignolytic activity under experimental N deposition. For example, fungi can use the same oxidative enzymes involved in lignin decay to "mine" N that is either bound in soil organic matter (SOM-N) or protected by lignin in plant litter. It has been thought that fungi may reduce their production of lignolytic enzymes under experimental N deposition because mining for N in SOM becomes less necessary when inorganic N availability is high (Craine et al. 2007). However, reduced fungal assimilation of N that is protected by lignin is unlikely to explain the reduced abundance of lignolytic fungi on high-lignin and wood substrates observed in our study, as these substrates are energy-rich, but N-poor (Table 1). Similarly, a reduction in fungal metabolism of SOM-N also seems insufficient to explain the negative effects of experimental N deposition on lignolytic saprotrophs in our study, as SOM-N mining is an activity typically attributed to some ectomycorrhizal fungi in the mineral soil (Lindahl et al. 2007, Talbot et al. 2013); by contrast, we observed a decline in lignolytic saprotrophic decomposer fungi in the forest floor and on decomposing high-lignin and wood substrates. In a decomposition experiment conducted in a boreal forest, experimental N deposition reduced lignin decay but not N loss in plant litter, suggesting that reduced fungal foraging for plant litter N did not underlie this observation. Our conclusions here are consistent with those of Talbot and Treseder (2012).

While all of these aforementioned mechanisms focus on the direct effect of high N availability on lignolytic fungi, an alternative mechanism proposes that experimental rates of N deposition negatively affects lignolytic fungi indirectly, through increased competition with fast-growing fungal decomposers of energy-rich organic substrates (Fontaine et al. 2003, Talbot and Treseder 2012). According to this mechanism, experimental N deposition removes N-limitation of cellulose decay thereby stimulating the growth of fungi that efficiently decompose cellulose but inefficiently decompose lignin. Indeed, elevated rates of N deposition have been documented to stimulate cellulose decay, while slowing lignin decay (Berg and Matzner 1997, Carreiro et al. 2000, Talbot and Treseder 2012). Furthermore, experimental N deposition in a boreal forest slowed lignin decomposition, while simultaneously increasing both cellulase activity and fungal antagonism, leading the authors to conclude that increased competition between cellulolytic and lignolytic fungi was responsible for slowed lignin decay (Talbot and Treseder 2012).

In our study, experimental N deposition increased fungal biomass on a cellulose-rich, low-lignin substrate (Fig. 6A), suggesting that a similar stimulation of cellulolytic fungi may have occurred here. At the same time, we also observed increases in cellulolytic fungi in wood and in forest floor under experimental N deposition (Fig. 2), a result that we attributed to decreased competition from the suppressed lignolytic fungi. Nevertheless, we cannot rule out the alternate possibility that higher N availability instead directly stimulated growth of cellulolytic fungi, thus increasing competition for energy-yielding substrates between cellulolytic and lignolytic fungi, and that increased competition from cellulolytic fungi led to the decline in lignolytic fungal relative abundance that we observed. However, if experimental N deposition directly enhanced cellulose decay, this should also increase mass loss of the high-cellulose, low-lignin substrate, an effect that we did not observe (Fig. 1). Furthemore, a recent study (Xia et al. 2017*a*) revealed that experimental N deposition did not change the amount of plant cell-wall carbohydrate fraction that was lost during decay (Xia et al. 2017*b*). Nevertheless, future studies should additionally assess both fungal competition and the rate of cellulose loss in order to bring further clarity to our understanding of the mechanism through which experimental N deposition alters fungal community composition and lignin decay.

We had additionally hypothesized that we would observe the largest reductions in lignolytic fungi later in decay when substrates are more enriched in lignin relative to their initial state, a time when lignin-decomposing fungi should be more abundant. Surprisingly, we observed the greatest suppression of lignolytic fungi under experimental N deposition at our first time point, not later in the decay process as we had hypothesized. Our results suggest experimental N deposition suppresses lignolytic fungi early in decay when the labile C compounds that may "prime" lignin decay are most abundant (Fontaine et al. 2003), while having a neutral or even positive effect on cellulolytic and hemicellulolytic fungi (Fig. 2). Therefore, plant litter may be entering later stages of decay with higher lignin-to-cellulose ratios under experimental N deposition than under ambient N deposition. Because plant litter decay plateaus as the ratio of lignin-to-cellulose increases (Melillo et al. 1989), it is possible that reduced abundance of lignolytic fungi early in decomposition process ultimately exerts a large influence on both the lignin content of decaying litter and the rate and extent of plant litter decay. We note, however, it is also possible that we were able to most strongly detect declines in lignolytic fungi early in decay, because our selection of lignolytic taxa is biased towards early-colonizing species. Most published studies of fungal decomposition are of relatively short duration and involve inoculating a single species on fresh litter or wood, which may have led us to underrepresent late-stage decomposers that may grow slowly or prefer to colonize substrata that are already partially decomposed.

Interestingly, a recent experiment (Xia et al. 2017*a*, *b*) in our study sites found that experimental N deposition retarded lignin decomposition in sugar-maple leaf litter after 3 months of decomposition, but not at later stages of decomposition; by contrast, lignin decay of sugar maple root litter was retarded after 3 yr of decomposition under experimental N deposition, but not at earlier time points examined (Xia et al. 2017*a*). At the stage in decomposition at which experimental N deposition increased lignin retention in sugar-maple plant litter (i.e., 3 months for leaf litter, 3 yr for root litter), 35–49% of plant litter mass had been lost, respectively (Xia et al. 2017*a*), an extent of decay that exceeds those of our high-lignin and wood substrates here. This suggests it is plausible that the biological response to experimental N deposition (i.e., decline in lignolytic fungi) might precede the chemical response (i.e., reduced lignin decay), although we presently lack data on lignin-loss in our substrates, data from later points in decomposition of our substrates, or microbial data from the root and leaf litter in the experiment conducted by Xia et al. (2017*a*, *b*), which would allow us to confirm whether this is true. Moreover, in contrast to our expectations, we did not observe significant declines in high-lignin or wood substrate mass loss under experimental N deposition, which we would anticipate given the reduced forest floor mass loss documented in our study system (Zak et al. 2008); we cannot rule out that this may not be strongly evident until later in decomposition, as has been observed for root litter (Xia et al. 2017*b*).

In this experiment, the most consistent and significant negative responses to experimental N deposition were observed among fungi colonizing 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 (Fig. 3) and the size of the fungal community (Fig. 6) on wood, elevated rates of N deposition led to a reduction in the absolute abundance of lignolytic fungi on wood. 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*). Although the dominant overstory tree in our system is sugar maple (*Acer saccharum*), the results we obtained with birch (*Betula* spp.) substrates are likely broadly reflective of responses of wood-decay fungi to experimental N deposition at our field sites, as birch and maple are both hardwoods with similar lignin and cellulose contents (Pettersen 1984). 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.

Conclusions

Increased soil C storage may be a widespread response to anthropogenic N deposition in temperate forests (Nave et al. 2009, Janssens et al. 2010, Frey et al. 2014). Negative effects on lignin-decomposing fungi, like those documented here, may explain soil C accumulation under elevated rates of N deposition. It is possible that increases in anthropogenic N deposition that have occurred over the past century and a half may have already had widespread effects on fungal biodiversity, as well as elicited increases in soil C in temperate forests. Our results indicate that further increases in anthropogenic N deposition, which have been predicted for some parts of Earth, could both increase soil C storage and have consequences for fungal biodiversity in temperate forests.

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Supporting Information

Additional supporting information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecm.1288/full

Data Availability

Circular consensus sequencing files for this manuscript have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession numbers SAMN06475267–SAMN06475287 in BioProject PRJNA222775. All other underlying data files are available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.5bv7p.