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Are Basidiomycete Laccase Gene Abundance and Composition Related to Reduced Lignolytic Activity Under Elevated Atmospheric NO_3^- Deposition in a Northern Hardwood Forest?

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Abstract Anthropogenic release of biologically available N has increased atmospheric N deposition in forest ecosystems, which may slow decomposition by reducing the lignolytic activity of white-rot fungi. We investigated the potential for atmospheric N deposition to reduce the abundance and alter the composition of lignolytic basidiomycetes in a regional network of four northern hardwood forest stands receiving experimental NO_3^- deposition (30 kg NO_3^-–N ha^{-1} $year^{-1}$) for a decade. To estimate the abundance of basidiomycetes with lignolytic potential, we used PCR primers targeting laccase (polyphenol oxidase) and quantitative fluorescence PCR to estimate gene copy number. Natural variation in laccase gene size permitted use of length heterogeneity PCR to profile basidiomycete community composition across two sampling dates in forest floor and mineral soil. Although past work has identified significant and consistent negative effects of NO_3^- deposition on lignolytic enzyme activity, microbial biomass, soil respiration, and decomposition rate, we found no consistent effect of NO_3^- deposition on basidiomycete laccase gene abundance or community profile. Rather, laccase abundance under NO_3^- deposition was lower (−52%), higher (+223%), or unchanged, depending on stand. Only a single stand exhibited a significant change in basidiomycete laccase gene profile. Basidiomycete laccase genes occurring in mineral soil were a subset of the genes observed in the forest floor. Moreover, significant effects on laccase abundance were confined to the forest floor, suggesting that species composition plays some role in determining how lignolytic basidiomycetes are affected by N deposition. Community profiles differed between July and October sampling dates, and basidiomycete communities sampled in October had lower laccase gene abundance in the forest floor, but higher laccase abundance in mineral soil. Although experimental N deposition significantly suppresses lignolytic activity in these forests, this change is not related to the abundance or community composition of basidiomycete fungi with laccase genes. Understanding the expression of laccases and other lignolytic enzymes by basidiomycete fungi and other lignin-decaying organisms appears to hold promise for explaining the consistent decline in lignolytic activity elicited by experimental N deposition.

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Introduction

Chronic atmospheric NO_3^- deposition has the potential to directly affect microbial communities and initiate physiological changes that could alter the cycling and storage of C in soil. Lignin degradation, a rate-limiting step in the decom-

position of plant detritus, is carried out by a subset of the soil microbial community, which includes certain actinobacteria [34], some ascomycota (especially within Xylariaceae) [1, 58], and many species of basidiomycota [28, 55]. The lignolytic activity of basidiomycete fungi is a key determinant of decomposition and soil C cycling, and represents one of the few naturally occurring enzymatic systems capable of completely mineralizing lignin to CO_2 [28, 55]. Many basidiomycetes carry genes for enzymes that mediate lignin degradation, including laccase, Mn-peroxidase, and lignin peroxidase [4, 28]. Laboratory studies have frequently identified a negative relationship between inorganic N availability and lignolytic enzyme synthesis in cultures of wood-rotting basidiomycete fungi [10, 32, 41]. However, comparatively few studies have examined the effects of inorganic N on basidiomycetes common in the soil and forest floor, which are N-rich substrates compared to wood [55]. If a negative inorganic N response is common among basidiomycetes, then the widespread and increasing phenomenon of chronic atmospheric N deposition could slow lignin decomposition and increase soil organic matter (SOM) [6, 24].

Nitrogen deposition frequently alters biogeochemical processes in a manner consistent with reduced or incomplete lignin decomposition by basidiomycete fungi [14]. Nitrogen deposition can slow soil C cycling and increase soil organic matter content [22, 54, 55, 59], particularly where plant litter is high in lignin [19, 75]. Several studies have also reported lower polyphenol oxidase activity under experimental N deposition, tying decomposition changes more closely to microbial enzyme expression [14, 18, 25, 68]. Furthermore, observations of increased dissolved organic carbon (DOC) export under N deposition [29, 50, 60, 76] suggest that N deposition can shift microbial activity toward incomplete lignin depolymerization and solubilization, an observation consistent with reduced lignin mineralization by soil basidiomycetes.

High N concentrations could reduce the production of lignolytic enzymes by basidiomycetes by reducing their abundance, inhibiting enzyme expression without a negative effect on abundance, or by both mechanisms simultaneously. With regard to enzyme production and decomposition, various basidiomycete species exhibit negative or neutral lignolytic responses to increasing inorganic N concentration [38, 79], but examinations of sporocarp abundance generally find neutral or positive effects of N deposition on saprotrophic basidiomycete species abundance and diversity [56, 67, 73]. Studies examining ectomycorrhizal (ECM) basidiomycetes frequently observe negative N deposition effects on ECM species abundance, as well as on total ectomycorrhizal biomass and infection rates [2, 43, 57, 77, 78]. However, our current understanding of ECM basidiomycetes suggests that negative effects of N on their abundance should not alter organic matter turnover rates, as ECM fungi are widely regarded as minor agents of lignin decomposition [16, 44, but see 17, 62]. Arbuscular mycorrhizal (AM) fungi are similarly regarded as incapable of significant ligninolytic activity [74].

We examined the effects of atmospheric N deposition on the community of basidiomycete fungi with genes encoding laccases in a series of sugar maple (*Acer saccharum* Marsh.) dominated forests experiencing ambient and experimental NO_3^- deposition in the Upper Great Lakes region. Although forest floor saprotrophs deploy a variety of exoenzymes during lignin decomposition, laccases are most frequently associated with organic matter decomposition in the forest floor [4, 55]. We have been able to demonstrate that experimental NO_3^- deposition has significantly decreased forest floor decomposition, thereby increasing the organic matter content of forest floor and surface mineral soil [59, 80]; this response paralleled significant declines in phenol oxidase activity and fungal biomass (Table 1) [18]. Therefore, we expected that experimental NO_3^- deposition had lowered the abundance of basidiomycete laccase genes in forest floor and surface mineral soil. We further hypothesized that experimental NO_3^- deposition had altered the composition of the basidiomycete community, causing the relative proportions and occurrence of some basidiomycete laccase genes to decline. We tested these hypotheses using quantitative fluorescence polymerase chain reaction (QPCR) to estimate the abundance of basidiomycete laccase genes, and length heterogeneity PCR (LH-PCR) to profile the community composition of these organisms.

Methods

Study Areas

The Michigan Gradient Experiment [49, 60, 82] provides a regional-scale laboratory for examining the effects of NO_3^- deposition on northern hardwood forests, a dominant forest ecosystem in the northeastern United States and Canada.

Table 1 Significant effects of NO_3^- deposition treatments observed in the Michigan gradient, relative to ambient deposition controls

Variable	% Change	Citation
Oe/a horizon organic matter	+51	[80]
Mineral soil organic matter (0–10 cm)	+18	[59]
Leached DOC (annual C flux)	+300	[60]
Leached DON (annual N flux)	+80	[82]
Phenol oxidase activity (forest floor)	−35	[18]
Total microbial biomass (PLFA)	−18	[18]
Soil respiration (annual CO2 flux)	−15	[13]

The experiment consists of four forest stands (designated A through D) located along a climatic gradient across Michigan, USA (Fig. 1). The stands are situated on sandy, well-drained Spodosols classified as Kalkaska sand (Typic Haplorthod) and are dominated by sugar maple (*A. saccharum* Marsh), a species with an AM mycorrhizal association. The average organic matter in the O horizon is 1767 g m^{-2}, while the organic matter content of mineral soil (0–10 cm) is 4068 g m^{-2} [80]. All stands are floristically and edaphically similar, but differ in mean annual temperature, ambient atmospheric N deposition, and soil N availability [60].

Beginning in 1994, three 30×30-m plots in each stand have received experimental NO_3^- deposition of 30 kg N ha^{-1} $year^{-1}$ as solid $NaNO_3$ granules, applied in six equal individual increments over the growing season. A 10-m-wide buffer zone around each NO_3^- deposition plot also receives this treatment. Three 30×30-m plots in each stand receive only ambient atmospheric N deposition, which, over the last decade has ranged from ~4 kg N ha^1 $year^{-1}$ at stand A to as high as 12 kg N ha^1 $year^{-1}$ at stand D [11, 52]; 60% of this N is deposited as NO_3^-.

In July and October 2004, forest floor and mineral soil were collected from all plots receiving ambient and experimental N deposition. Within each plot, forest floor (Oi/e/a horizons) was collected from ten randomly located 10×10-cm areas. A mineral soil sample was collected in the center of each 10×10-cm area using a 1.9-cm diameter soil probe to a depth of 10 cm; this sample extended through the A and E horizons. The ten forest floor and mineral soil samples from each plot were composited in the field and stored on ice for transport to the University of Michigan. Mineral soil was homogenized by passing it through a 2-mm mesh; forest floor was subsampled and pulverized inside sterilized vessels on a SPEX CertiPrep 8000M mixer/mill (Spex Certiprep, Metuchen, NJ). All sample materials were stored at −80° C prior to DNA extraction and analyses.

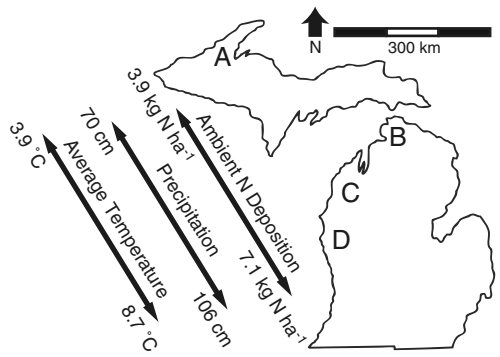

Figure 1 Locations of Michigan Gradient experimental stands (*A* to *D*) within Michigan, USA *Arrows* indicate approximate directionality of temperature, precipitation, and ambient N deposition gradients; *values* describe the ranges of these factors from stand *A* (northern end) to stand *D* (southern end) in 2004. Data were obtained from the National Atmospheric Deposition Program (2005) and the National Oceanic and Atmospheric Administration (2005)

Extraction of Community DNA

Community DNA was extracted from all samples using MoBio PowerSoil DNA kits, following the manufacturer's instructions. For soil samples, ~0.5 g of field-moist soil (~0.23 g dry weight) were extracted; approximately 0.1 g (~0.06 g dry weight) of field-moist material was used to extract DNA from forest floor. Three replicate extractions (analytical replicates) were performed for each of the 96 composite forest floor and mineral soil samples to provide improved estimates of plot means. Extractions yielded whole-community DNA dissolved in 50 μL Tris–HCl; success of DNA extraction was confirmed by 1% agarose gel electrophoresis and ethidium bromide staining.

Quantitative Fluorescence PCR of Basidiomycete Laccases

We estimated basidiomycete laccase copy number using quantitative fluorescence PCR (QPCR) with basidiomycete laccase PCR primers Cu1F (5′-CAT(C) TGG CAT(C) GGN TTT(C) TTT(C) CA-3′) and Cu2R (5′-G G(A)CT GTG GTA CCA GAA NGT NCC-3′) [46]. Quantitative estimates of laccase gene concentration were based on the Ct method, which assumes a negative linear relationship between the log of initial PCR template concentration and the cycle number (Ct) at which reaction fluorescence rises above background fluorescence [30].

The relationship between Ct and initial template concentration was calibrated within each run using standards generated from laccase PCR products pooled across samples within a stand. Hence, each standard represented a mixture of laccase genes naturally occurring in a stand. Concentrations (ng μL^{-1}) of pooled PCR products were measured on an fMax fluorimeter (Molecular Devices Corporation, Sunnyvale, CA) using PicoGreen quantitative dsDNA dye (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. We then used serial dilutions to create laccase standards ranging from 10^{-4} to 10^{-10} ng template DNA μL^{-1}.

The region amplified by primers Cu1F/Cu2R spans ~140 exon bases between conserved regions for essential copper-binding histidine residues in the laccase enzyme [46]; however, the region may be punctuated by one or more introns within the coding sequence. Published laccase gene sequences reveal at least 16 probable intron sites within this short span (Supplemental Figure S1). Predicted introns in the target sequence vary in length between ~46 and ~68 bp; up to two or more introns may occur in the same amplicon (Supplemental Table S1). Additional, as-yet-unidentified environmental laccase-like sequences contain up to three

introns in the Cu1F/Cu2F target region, yielding total amplicon lengths of ~350+ bp [J. Hassett, unpublished data; 9, 46].

In order to convert mass-based estimates of laccase concentrations (nanogram laccase amplicons gram $soil^{-1}$) to copy number (laccase gene copies gram $soil^{-1}$), it was necessary to collect information on the distribution of different-length laccase amplicons within samples. We therefore determined weighted average amplicon length for each sample using LH-PCR (described below), and converted DNA mass to copy number assuming a molecular weight of 660 g mol^{-1} bp^{-1}. QPCR reactions were performed on the Stratagene MX3000P real-time PCR system using Brilliant SYBR Green Master Mix (Stratagene, La Jolla, CA). Each run contained template DNA from samples taken on the same date in one stand; runs also included two replicate sets of standards, and five replications of a negative control (reaction components without DNA template) to screen for contamination. All samples were run twice to ensure concordance between analytical runs.

Quantitative fluorescence polymerase chain reaction volumes were 25 μL; reaction mixtures contained 12.5 μL of 2× Brilliant SYBR Green Master Mix, 30 nM ROX reference dye, 0.5 μM of each PCR Primer (Cu1F and Cu2R), and 2.5 μL of template DNA. Optimal template concentrations were achieved by screening QPCR products diluted five times, 20 times, or 100 times; optimal dilution ratios minimized heterodimer formation and achieved Ct within the range of analytical standards. Thermocycling parameters followed [9] and consisted of initial denaturation at 94°C for 5 min, 40 amplification cycles (1 min denaturation at 94°C, 2 min annealing at 50°C, 5 min extension at 72°C), and final extension at 72°C for 10 min. A double-stranded DNA (dsDNA) dissociation curve was generated immediately following thermal cycling to screen PCR products for primer heterodimer contamination [65]; reactions exhibiting heterodimer formation were subsequently re-optimized and re-amplified. Variation in PCR efficiency was determined by measuring efficiency (E) in the log-linear phase of each reaction using the difference method of Liu and Saint [45] along a four-cycle log-linear segment of the amplification curve, anchored at Ct according to the recommendations of Kontanis and Reed [36].

Genomic laccase copy number is known to vary from none [40] to up to 17 genes among white-rot basidiomycetes [33]; laccase gene copy number in an environmental sample is therefore a function of both community size (number of fungal nuclei) and relative community composition (ratios of organisms containing different numbers of laccase genes). In order to distinguish between these independent mechanisms, QPCR must be performed in parallel with an analysis of community composition, which, in our case, was accomplished by comparison of length heterogeneity profiles.

Laccase Length Heterogeneity Profiling of Basidiomycete Communities

The variable length of the basidiomycete laccase PCR target sequence permits gene composition to be examined by comparison of amplicon-length profiles using length heterogeneity PCR (LH-PCR) [9]. Because the set of genes amplified in PCR is a function of the composition of genes in environmental samples, changes in observed length-heterogeneity profiles can serve to indicate changes in community composition [66, 71; Supplemental Figure S2]. This method is similar in principle to other PCR-based microbial community profiling methods (e.g., automated ribosomal intergenic spacer analysis [ARISA] and terminal restriction fragment length polymorphism [TRFLP]). However, the individual length classes resolved by laccase LH-PCR are not necessarily analogous to taxonomic species: different taxa can potentially contribute equal-length amplicons to the profile, and a single genome may contain loci of different lengths [9, 46; Supplemental Table S1]. For these reasons, LH-PCR profiles of laccase genes cannot be used to provide estimates of laccase gene diversity, much less taxonomic diversity.

Reaction mixtures for LH-PCR analysis were identical to those used in our QPCR reactions, with the exception that the forward primer (Cu1F) was labeled with a fluorophore (FAM) to permit amplicon detection by capillary gel electrophoresis. Thermal cycling was performed on a Stratagene Robocycler Gradient 96-well thermocycler (Stratagene Inc.); thermal cycling parameters were the same as in QPCR, but were repeated for 36 amplification cycles to bring all samples near the point of Ct.

Polymerase chain reaction products were diluted 1:100, 1:20, or 1:5 (depending on PCR yield) with high-performance liquid chromatography (HPLC)-grade water, and loaded onto bar-coded ABI Prizm 96-well optical reaction plates (Applied Biosystems, Foster City, CA). Each well contained 1 μL diluted PCR product, 12 μL Hi-Di formamide, and 0.02 μL Genescan 500 LIZ internal size standard (Applied Biosystems). Plates were submitted to the University of Michigan DNA Sequencing Core, where PCR products were separated and quantified by capillary gel electrophoresis on an Applied Biosystems Model 3730xl DNA Analyzer. PCR amplicon patterns in the resulting electropherograms were aligned, baselines were subtracted, and amplicon peaks were screened using GeneMarker 1.5 Software (SoftGenetics, State College, PA). Peaks achieving less than 50 fluorescence units were excluded. Samples yielding a total fluorescence <5000 units above baseline for peaks 130 bp to 400 bp in length were re-amplified and again submitted for electrophoresis. Peak-matching and filtering operations were performed using GelCompar II version 4.0 (Applied Maths, Austin, TX); peaks were matched with a position tolerance of 0.4%

(±1 bp). Peaks contributing less than 1% of the total fluorescence in a profile were excluded to control for variation in detection sensitivity between runs, and peaks appearing once among all profiles were excluded as probable artefacts. Resulting peak-distribution tables were standardized to express peak heights as proportional to total peak height in a profile (relative intensity). We determined mean amplicon length (bp) within each sample as the intensity-weighted average of all peak lengths in the profile.

Statistical Analyses

Values used in statistical analyses were plot means, representing analytical averages across replicate sample extractions and instrument runs. We calculated the mean coefficients of variation (CV) for replicate extractions and analytical replications of sample batches to compare sampling and analytical variability. Statistical comparisons of laccase gene abundance were performed separately by each soil horizon.

We expected experimental NO_3^- deposition would decrease the abundance of laccase-containing basidiomycetes; to test this hypothesis, factors associated with significant variation in laccase gene copy number were identified by analysis of variance (ANOVA). Stand, NO_3^- deposition treatment, and sampling date were fixed factors in this analysis; gravimetric water content was tested as a potential covariate. An ANOVA model was also used to examine the effects of experimental N deposition, stand, and sampling date on QPCR efficiency (E), with Ct as a covariate. Where significant variation was identified by ANOVA, multiple comparisons were performed using Fisher's protected LSD. Statistical significance was accepted at $\alpha=0.05$.

Our second hypothesis predicted that laccase amplicon-length heterogeneity profiles from forest floor and mineral soil receiving NO_3^- deposition treatments would differ significantly from those under ambient N deposition. To test this hypothesis, we compared frequency distributions of amplicon length classes among all samples using redundancy analysis (RDA) with Hellinger transformation [8]. The analysis was performed using Canoco 4.5 (Microcomputer Power, Ithaca, NY). Statistical significance ($\alpha=0.05$) of correlations within data sets were tested by Monte Carlo comparison of observed *F*-statistics with 999 random permutations of the community composition data.

Results

Laccase Gene Copy Number

Laccase abundance (gene copy number) averaged $2.24\times10^7\pm3.93\times10^6$ copies per gram (mean ± standard error) in the forest floor and $1.01\times10^6\pm1.59\times10^5$ copies per gram in mineral soil. The abundance in forest floor was influenced by a significant interaction between N deposition and stand (Fig. 2A; $P=0.028$, $F=3.439$, $df=3$). For example, stand B had lower laccase abundance under experimental NO_3^- deposition, whereas laccase genes were more abundant under experimental NO_3^- deposition in stand C. In stands A and D, there was no significant effect of experimental NO_3^- deposition on laccase abundance. In mineral soil, there was no interaction between NO_3^- deposition and stand (Fig. 2B; $P=0.849$, $F=0.267$, $df=3$. However, there was a significant stand-by-sampling date interaction in mineral soil. October samples displayed a 61% decrease in laccase abundance at site C relative to July, whereas laccase abundance increased in sites A (353%), B (175%), and D (247%) from July to October ($P=0.008$, $F=4.724$, $df=3$). NO_3^- deposition treatments had no significant main effect on laccase gene abundance (copy number) in either mineral soil ($P=0.820$, $F=0.052$, $df=1$) or the forest floor ($P=0.801$, $F=0.064$, $df=1$).

Across the four forest stands, sampling date had a significant effect on laccase gene copy number in the forest floor (Fig. 3), wherein laccase abundance in October was 27% of that observed in July ($P=0.000$, $F=18.513$, $df=1$). In mineral soil, laccase gene abundance in July was 34% lower than in October ($P=0.043$, $F=4.449$, $df=1$).

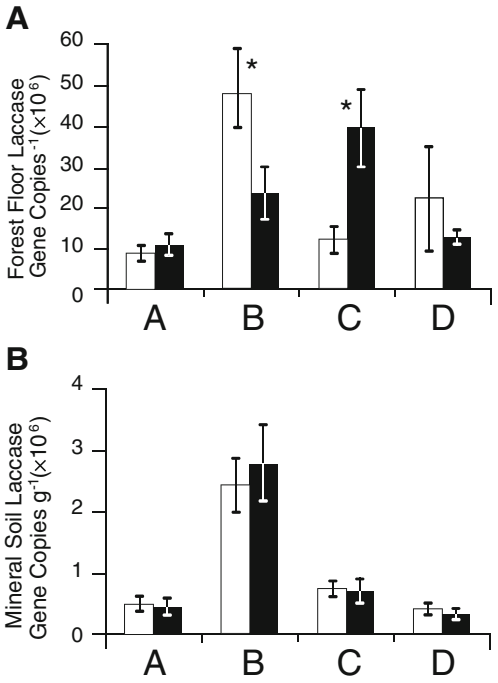

Figure 2 N deposition effects on basidiomycete laccase gene abundance in the Michigan gradient experiment. The top panel (**A**) depicts laccase abundance in the forest floor; the bottom panel (**B**) provides abundance in mineral soil. *Bars* are paired by stand (*A* to *D*); *open bars* represent mean laccase abundance in the ambient treatment, *filled bars* are mean values for the experimental NO_3^- deposition treatment. For each *bar*, $n=6$. *Error bars* are ± std error. *Paired bars* marked with an *asterisk* exhibited significant N-deposition effects at $\alpha=0.05$

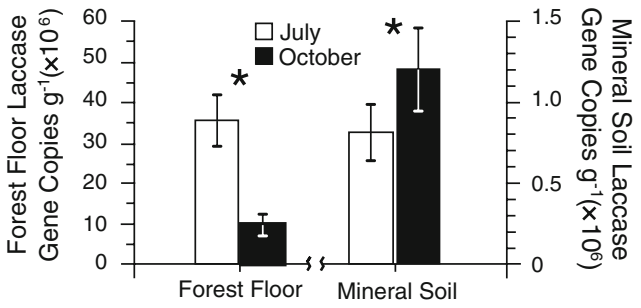

Figure 3 Average basidiomycete laccase gene abundance in forest floor (*left side*) and mineral soil (*right side*), on two dates, across stands. *Open bars* indicate mean values of July samples, *filled bars* represent means from October samples. For each *bar*, n=24. *Error bars* are ± std error. The *asterisk* indicates a significant difference in laccase gene abundance between dates at α=0.05

Stand had a significant main effect on laccase gene abundance in both the forest floor (P=0.024, F=3.616, df=3) and mineral soil (P=0.000, F=28.251, df=3). The overall pattern of differences between stands was similar in mineral soil and forest floor, with copy number following the general trend stand B ≥ stand C ≥ stand D ≥ stand A (Fig. 4). Gravimetric water content was not a significant covariate for laccase gene abundance (P=0.541, F=0.382)

Mean coefficients of variation were smaller between replicate analytical runs (38.2±3.2%) than between replicate

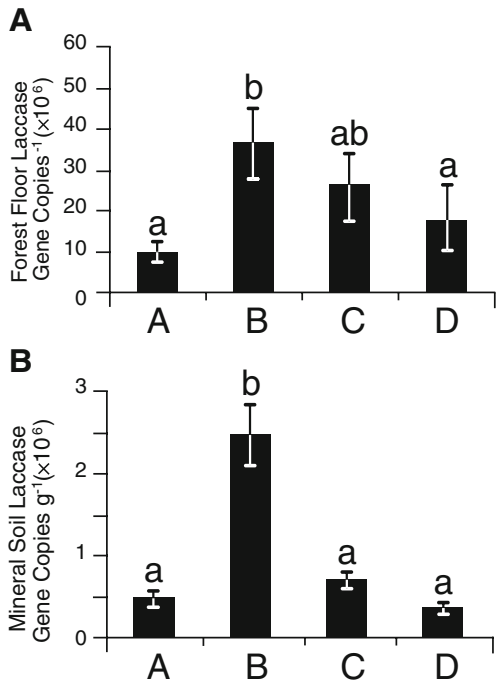

Figure 4 Mean basidiomycete laccase gene abundance averaged across all plots (ambient + N deposition) in each of the four forest stands (*A* to *D*). The top panel (**A**) depicts laccase abundance in the forest floor; the bottom panel (**B**) provides abundance in mineral soil. For each *bar*, n=12. *Error bars* are ± std error. *Bars* marked with the same *lowercase letter* are not significantly different at α=0.05

extractions within plots (45.1±2.9%). Replicate analytical runs were also positively linearly correlated (r^2>0.88), indicating that relative differences between samples within runs were largely unaffected by analytical variation. Coefficients of variation between replicate extractions within plots were greater in the forest floor (50.6±4.4%) compared to mineral soil (39.5±3.8%).

Experimental NO_3^- deposition had no effect on PCR amplification efficiency (E) in samples from forest floor (P=0.751, F=0.102, df=1) or mineral soil (P=0.170, F=1.944, df=1). Efficiency was similar between forest floor and mineral soil samples (79.7±0.4% in forest floor, 80.6±0.4% in mineral soil). Amplification efficiency was significantly influenced by sample date, decreasing in the forest floor from 80.5±0.4% in July to 79.2±0.6% in October (P=0.032, F=5.020) and increasing in mineral soil from 79.7±0.3% in July to 81.4±0.8% in October. Stand also had a significant effect on E in both horizons (forest floor: P=0.006, F=5.329; mineral soil: P=0.000, F=8.405). In the forest floor, E was significantly higher in stand A (81.4±0.7%); there were no significant differences between stands B (79.1±1.0%), C (78.9±1.0%), and D (79.4±0.8%). In mineral soil, E was significantly higher in stands A (82.1±1.5%) and B (81.6±0.7%) relative to stands C (79.7±0.8%) and D (79.0±0.4%). Both horizons also exhibited a significant stand-by-sample date interaction effect on E (forest floor P=0.001, F=7.437, df=1; mineral soil P=0.000, F=17.199, df=1). In the forest floor of stand A, E increased 2.4% from July to October, whereas the other stands showed decreases or no change. In mineral soil, E increased 7.7% from July to October at site A, and 1.5% at Site B; sites C and D exhibited no significant change in E. Threshold cycle (Ct) was not a significant covariate for E in forest floor (P=0.236, F=1.466, df=1) or mineral soil (P=0.120, F=2.567, df=1).

Basidiomycete Community Laccase Length Profiles

A total of 89 amplicon length classes were identified in LH-PCR profiles. Of these, 85 occurred in mineral soil and 62 occurred in the forest floor. Mean amplicon length in mineral soil was 176±2 bp, whereas the average was 192±3 bp in forest floor. Environmental axes in redundancy analysis were significantly correlated with changes in gene length profiles, and all canonical axes accounted for 41% of the variation in gene length (P=0.001, F=2.039; Table 3). However, NO_3^- deposition had no significant main effect on length heterogeneity profiles across the four stands. (P=0.329, F=0.777). Factors exhibiting significant main effects were sampling date, horizon, and stand, which together with their interactions accounted for 28.5% of the variation in profile composition (Table 2). Clusters of samples occurring in different horizons or on different dates

Table 2 Comparisons of basidiomycete laccase gene copy number across the Michigan gradient

			Laccase Gene Copy Number g^{-1} ($\times 10^6$)									
Horizon	Date	Treatment	All Stands		Stand A		Stand B		Stand C		Stand D	
Forest Floor			22.39	(5.56)	9.81	(2.52)^{a}	36.44	(8.75)^{b}	25.84	(8.14)^{abc}	17.46	(8.91)^{ac}
	July		**35.32**	**(6.39)**	**16.54**	**(3.04)^{a}**	**49.90**	**(13.79)^{a}**	**45.80**	**(11.24)^{a}**	**29.03**	**(16.82)^{a}**
		Ambient	*36.60*	*(10.49)*	15.80	(2.34)^{a}	*64.95*	*(21.18)^{a}*	*21.56*	*(5.12)^{a}*	44.08	(34.4)^{a}
		+ NO_3^-	*34.04*	*(7.77)*	17.28	(6.33)^{a}	*34.86*	*(16.6)^{a}*	*70.03*	*(4.23)^{b}*	13.99	(2.15)^{a}
	October		**9.45**	**(2.79)**	**3.07**	**(0.72)^{a}**	**22.98**	**(8.62)^{b}**	**5.87**	**(2.48)^{a}**	**5.88**	**(3.56)^{a}**
		Ambient	*9.70*	*(5.35)*	*1.97*	*(0.48)^{a}*	33.70	(15.61)^{b}	2.83	(0.92)^{a}	0.30	(0.13)^{a}
		+ NO_3^-	*9.20*	*(1.98)*	*4.17*	*(1.08)^{a}*	12.26	(3.55)^{a}	8.92	(4.55)^{a}	11.47	(5.68)^{a}
Mineral Soil			1.01	(1.10)	0.48	(0.34)^{a}	2.48	(1.29)^{b}	0.70	(0.38)^{a}	0.37	(0.24)^{a}
	July		**0.81**	**(0.18)**	**0.21**	**(0.03)^{a}**	**1.80**	**(0.46)^{b}**	**1.01**	**(0.06)^{c}**	**0.21**	**(0.08)^{a}**
		Ambient	*0.75*	*(0.24)*	0.21	(0.04)^{a}	1.71	(0.68)^{b}	*0.92*	*(0.08)^{ab}*	0.15	(0.03)^{a}
		+ NO_3^-	*0.87*	*(0.27)*	0.21	(0.04)^{a}	1.90	(0.75)^{b}	*1.11*	*(0.05)^{ab}*	0.27	(0.16)^{a}
	October		**1.20**	**(0.26)**	**0.75**	**(0.11)^{a}**	**3.15**	**(0.47)^{a}**	**0.39**	**(0.11)^{a}**	**0.53**	**(0.08)^{a}**
		Ambient	*1.22*	*(0.31)*	*0.80*	*(0.12)^{a}*	*2.92*	*(0.34)^{b}*	0.50	(0.22)^{a}	*0.67*	*(0.05)^{a}*
		+ NO_3^-	*1.18*	*(0.44)*	*0.69*	*(0.21)^{a}*	*3.38*	*(0.96)^{b}*	0.28	(0.02)^{a}	*0.39*	*(0.09)^{a}*

Table rows are levels of main effect in the analysis; columns contain analytical mean (±SE) laccase gene copy number across all stands (first column) and within each stand (A–D). Significant date and treatment effects within each horizon are indicated by boldface type; significant treatment effects within dates are indicated using italics. Within each row, stands sharing the same superscript did not differ significantly. For all comparisons, significance is accepted at α=0.05

occupied different regions of the ordination plot using the first two canonical axes (Fig. 5).

Factors with significant interaction effects were N deposition-by-stand, stand-by-horizon, stand-by-date, and stand-by-horizon-by-N deposition (Table 3). RDA ordinations conducted separately for the individual stands revealed a significant effect of NO_3^- deposition on gene length distribution in the forest floor, but not the mineral soil of stand C (P=0.035, F=2.020). Within stand C, N deposition treatments accounted for 16.8% of the total

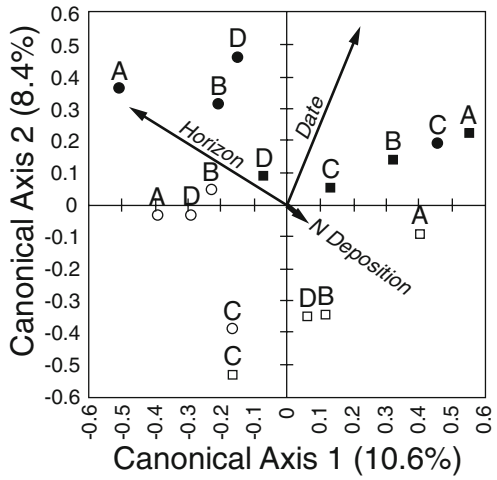

Figure 5 Redundancy analysis ordination diagram showing average positions of laccase LH-PCR profiles from Michigan gradient experimental stands (labeled *A* through *D*) on normalized component axes. Each point represents the average of all observations in a stand from a single horizon and date (n=6). *Squares* represent mineral soil profiles, *circles* are profiles from the forest floor. *Open shapes* indicate community profiles from July, *filled shapes* are October profiles. Component *axis labels* indicate the percent of total variation in profiles accounted for by each axis. *Arrows* indicate the magnitude and directionality of environmental vectors along the ordination

Table 3 Significant environmental factors affecting laccase community profiles across Michigan gradient forest stands, as determined by Monte Carlo comparisons of the observed RDA ordination to 999 permutations of profiles and environmental factor combinations

	F	P value	Variation Explained (%)[a]
Main effects:			
Stand	2.714	0.005	5.3
Horizon	6.867	0.001	5.7
Sampling Date	6.646	0.001	5.5
Interactions:			
Horizon × Stand	2.869	0.001	7.2
Treatment × Stand	1.340	0.045	3.4
Sampling Date × Stand	1.464	0.009	3.7
Horizon × Treatment × Stand	1.371	0.030	3.4
Model (All Factors[b]):	2.039	0.001	41
(Significant Factors Only):	2.378	0.001	34.1
(Excluding all N Treatment Factors:)	2.753	0.001	28.5

[a] Variation is that explained using the indicated factor alone. Due to covariation of some environmental factors, the variation explained by each is non-additive

[b] Significance of the model is tested including all environmental factors, whether or not their individual effects on community profiles are significant

profile variation profile in the O horizon. No other stand or horizon exhibited a significant effect of N deposition on LH-PCR profile composition.

Discussion

Deposition of biologically available N forms in terrestrial ecosystems has the potential to suppress the lignin-degrading activity of basidiomycete fungi in forest floor and mineral soil. In our long-term study, experimental NO_3^- deposition has reduced fungal biomass and altered ecosystem processes in a manner consistent with this model: organic matter in forest floor and surface mineral soil has increased, the activity of phenol oxidase has decreased, and rates of litter decay have slowed [18, 59, 60, 80] (Table 1). Therefore, we expected that basidiomycete genes coding for enzymes, which mediate lignin depolymerization, would be less abundant under experimental NO_3^- deposition, and that changes in basidiomycete species composition would be apparent in profiles of laccase gene length heterogeneity. Contrary to these expectations, our examination of basidiomycete laccase genes found no consistent effect of experimental NO_3^- deposition on laccase gene abundance or gene length heterogeneity, indicating that the changes in decomposition observed in previous work in these forest stands are not directly related to changes in the size or composition of this community.

Our failure to identify a relationship between altered OM decomposition and laccase gene abundance may result from the simple fact that correlations between the abundance of functional genes and their observed activities can occur only when genes are used to form functional products in the limiting step of gene expression. Reduced lignin degradation need not result from reduced abundance of organisms with lignolytic genes, and inhibition of lignin decomposition does not imply negative effects on fungal growth. In an instructive example of this distinction, laboratory incubations of some wood-decaying basidiomycetes have demonstrated that although adding inorganic N decreased lignin decomposition and slowed substrate mass loss, the growth of some species with decreased lignolytic activity was unaffected or even enhanced [10, 41]. In our experimental system, previous efforts have assayed decomposition rates, SOM pools, and the active pool of lignolytic enzymes in soil; this study adds to our understanding of lignin decomposition by assaying the "genetic potential" for laccase enzyme production; however, the transcriptional expression of basidiomycete laccase genes remains to be examined. Although high inorganic N is known to negatively affect the transcriptional regulation of laccases in some basidiomycetes in culture [4, 15], assessing gene expression in environmental samples remains technically challenging [68]. Recent analyses of co-extracted DNA and RNA in soil from a European mixed oak–beech forest suggested that only 30% of the basidiomycete laccase genes in the community were actually expressed [48], further emphasizing the distinction between gene abundance and gene expression. A similar response may be underlying our observations, but confirmation of this would require further investigation.

Aside from possible changes in basidiomycete laccase gene expression, it is plausible that the enzymatic responses we previously documented may have resulted from N-concentration-related changes in the activity or abundance of organisms other than litter-decaying basidiomycetes [71]. Actinobacteria and ascomycete fungi are also capable of lignin decomposition, but their physiological responses to high N availability have been poorly characterized. Biederbeck et al. [7] found decreased abundance of actinobacteria under N deposition, suggesting that the contributions of those organisms to lignin decomposition may decline. White-rot ascomycetes like *Xylaria* sp. might also use lignin decomposition as a means to access organic N in a manner parallel to basidiomycetes, and therefore decrease lignolytic activity when N is freely available [55]. These points highlight the importance of examining responses within the broader lignolytic community, regardless of how N deposition affects basidiomycetes.

The significant stand-by-NO_3^- deposition interaction we observed suggests that compositionally, basidiomycete communities may be somewhat idiosyncratic in their responses to NO_3^- deposition. In our study, communities in different stands contained different laccase genes, and exhibited positive, negative, and neutral responses to NO_3^- deposition. Although the profiles do not contain sufficient information to identify species, our LH-PCR results support the hypothesis that NO_3^- deposition significantly altered basidiomycete community composition in stand C. Furthermore, experimental NO_3^- deposition in stand C substantially increased laccase copy number in forest floor. This result was contrary to our expectations because stand C also exhibited increased soil organic matter and DOC export [60, 80, 82], although an observed ~25% decline in phenol oxidase activity in this stand was not statistically significant [18]. In stand A, laccase gene abundance and community composition were unaffected by NO_3^- deposition, but that stand previously exhibited ~40% lower phenol oxidase enzyme activity in the forest floor under experimental NO_3^- deposition [18]. Only the forest floor at stand B exhibited the expected pattern of laccase gene abundance, but with no change in community composition. Taken collectively, these results suggest that the abundance and variety of basidiomycetes with laccase genes in these forests may be altered by N deposition, but in a manner that is determined uniquely by the particular microbial community in a stand. More complete interpretation of community differences, including

identification of species, might be achieved by obtaining the nucleotide sequences of PCR-amplified basidiomycete laccase sequences [9, 46, 47], which overcomes some limitations of the length heterogeneity method. Sequence libraries from our study sites would also permit meaningful exploration of gene diversity, following the example of Luis et al. [47], who observed correlation between measured extracellular laccase activity and basidiomycete laccase gene diversity in a mixed beech–oak ecosystem.

Although our experimental NO_3^- deposition treatment takes the form of solid $NaNO_3$ granules scattered on the forest floor, it is unlikely the microbial and biogeochemical responses we have documented result from the accumulation of Na^+. Although exchangeable Na^+ in surface soil (0 to 10 cm depth) is 52% higher under our NO_3^- deposition treatments (35±2.4 mmol/kg vs 23+2.3 mmol Na^+ per kilogram; $P<0.001$; W.E. Holmes, unpublished data), this concentration falls below levels presently documented to negatively affect soil microbial activity and litter decomposition (ca. 80 mmol Na^+ per kilogram; [42]). Moreover, the average concentration of Na^+ in soil water in these forests (0.45±0.000 mMol; W.E. Holmes, unpublished data) is two orders of magnitude lower than concentrations known to alter microbial community composition and decrease its biomass, respiration, and extracellular enzyme activity (40 mMol Na^+ as NaCl; [27]). Finally, Na^+ composes a small fraction of the cation-exchange capacity in both ambient (3.2+0.80%) and experimental NO_3^- deposition treatments (3.6±1.75%; W. Holmes, unpublished data), well below the percent Na^+ concentrations of saline soils in which microbial activity is negatively affected by Na+ accumulation [64]. Inasmuch, the responses we observed to experimental NO_3^- deposition do not result from an accumulation of Na^+.

Our results provide evidence that laccase-containing basidiomycete laccases are distributed differently in mineral soil and forest floor horizons, perhaps due to differences in the distributions of ectomycorrhizal (ECM) and saprotrophic basidiomycetes between these horizons. LH-PCR profiles from the forest floor contained a subset of the amplicon classes occurring in surface soil. Luis et al. [47], observing basidiomycete laccase gene distribution in beech–oak forests, found that relatively few of the laccase genes in mineral soil could be attributed to saprotrophs, whereas organic horizons contained similar numbers of ECM and saprotrophic species. Although our analysis was performed in a forest ecosystem in which ECM-forming trees comprise less than 30% of the total basal area, we frequently noted ECM root tips in mineral soil samples, and Oe/Oa horizons contained a discontinuous but dense mat of fine roots and mycelium, perhaps indicating that ECM hyphae do exploit the forest floor as well.

Seasonal fluctuations in substrate input and climate have a profound influence on saprotrophic microbial communities in soil, and these effects are apparent in our analysis. In forest floor, basidiomycete laccase gene abundance in July was more than three times the abundance in October, whereas we observed a 34% decrease in mineral soil over the same period of time. In October, recently abscised leaves dominate the forest floor, and basidiomycetes have had little opportunity to colonize and exploit this new material. By July, decomposition has presumably depleted unlignified substrates, and lignin-degrading basidiomycetes should be much more abundant [5, 21]. In contrast, fine root turnover delivers dead plant tissues to mineral soil at a more or less constant rate over the growing season [12, 31]. Additionally, temperatures are cooler and soil water potential is less negative in autumn [13], which may facilitate fungal growth [20] and permit basidiomycetes with laccase genes to more easily exploit diffusible substrates and nutrients in soil [83].

Our QPCR and LH-PCR assays share the limitations of other PCR-based characterizations of microbial communities [35, 72], including DNA extraction bias, PCR amplification bias, and variability of PCR efficiency between samples. Although unknown biases in DNA extraction efficiency clearly have the potential to confound quantitative analyses of gene copy number, our experimental design reduces the impact of such bias because we used the identical procedure in both ambient and experimental NO_3^- deposition treatments, which were replicated within each of our four study sites. Our four research sites are edaphically similar, especially in regard to soil texture and pH, which are known to affect DNA recovery [26, 39, 69]. The sandy soil texture in our study (~85–90% sand [81])) and low mineral soil organic matter content (~5% [59]) also favor the efficient extraction of DNA relative to soils with higher clay content and high SOM [69]. Additionally, procedures that rely on bead-beating to achieve cell lysis (as in the Mo-Bio kits used in this study) produce greater recovery efficiencies relative to alternative lysis methods, such as freezing, sonication, and enzymatic lysis, [39], particularly for the extraction of mycelial DNA [23, 63]. Looking beyond the present work, methods involving the introduction of internal DNA standards such as transformed bacterial vectors [51] or non-native mycelial tissue [3] may permit quantitative calibration of real-time PCR results using known DNA extraction efficiencies, insofar as the inoculants accurately simulate the extraction of DNA from target organisms.

The efficiency of PCR amplification is an important consideration during QPCR, as small differences in PCR efficiency (E) can generate divergent estimates of Ct [36, 61]; however, in our analyses overall variation in E was low, and NO_3^- deposition had no significant influence on E. We

observed substantial run-to-run variation in QPCR results; however, this variation was surpassed in both horizons by the variation between replicate extractions within plots, which may reflect the patchy spatial distribution of laccase genes in soil [47]. From this, we infer that our gene abundance calculations have not been significantly compromised by analytical error or artifactual variation in PCR efficiency.

The information we collected on basidiomycete laccase amplicon length heterogeneity was essential for the interpretation of QPCR results in meaningful units of gene copy number, but as a community profiling method, LH-PCR of basidiomycete laccase is limited by the broad distribution of equal-length targets among species (Supplemental Table S1). Because of this, we contend that while the probability of type II error with this method is high, the probability of type I error is correspondingly low: barring PCR bias issues (which affect all community composition methods, including clone-and-sequence methods), we cannot conceive of a scenario in which a significant change in the relative occurrence of a particular-length product does not correspond to an actual change in community composition.

Although heterogeneity in target length may well have given rise to PCR amplification bias in our real-time QPCR analyses, this conjecture is difficult to test. We have observed no apparent suppression of laccase amplification in multi-species mixtures derived from DNA extraction of sporocarp tissues, but this alone does not rule out a length-related bias effect. In general, we recommend that investigators wishing to perform quantitative analyses of gene abundance in environmental samples should select amplification targets that do not exhibit length heterogeneity. Given the ubiquity of introns in the functional genes of many eukaryotes, however, this may not always be practical or possible, and examination of product length heterogeneity as described here may prove informative.

Conclusion

We suspect that many of the significant responses to experimental NO_3^- deposition in our experiment, including decreased microbial biomass, increased soil organic matter content, and greater DOC production, are related to the broad suppression of basidiomycete lignolytic activity by high inorganic N availability. This study has attempted to link these observed effects to basidiomycete communities by quantifying and profiling basidiomycete laccase, a functional gene with an important role in the oxidation of polyphenolics in litter and soil. Depending on the stand, NO_3^- deposition had positive, negative, or no effect on laccase gene abundance in the forest floor. Therefore, we conclude that while NO_3^- deposition has the potential to dramatically alter the abundance of basidiomycete fungi, these effects are not related to the general suppression of organic matter decomposition that occurs as a significant effect across all four stands in our experiment. Rather, it is more likely that the primary effect of high NO_3^- concentrations on decomposition in these ecosystems occurs at the level of laccase gene transcription and expression, although lignolytic organisms other than basidiomycetes may also shape ecosystem responses to increasing deposition of anthropogenic N [14, 55, 71].

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