Molecular analysis of fungal communities and laccase genes in decomposing litter reveals differences among forest types but no impact of nitrogen deposition

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Summary

The fungal community of the forest floor was examined as the cause of previously reported increases in soil organic matter due to experimental N deposition in ecosystems producing predominantly high-lignin litter, and the opposite response in ecosystems producing low-lignin litter. The mechanism proposed to explain this phenomenon was that white-rot basidiomycetes are more important in the degradation of high-lignin litter than of low-lignin litter, and that their activity is suppressed by N deposition. We found that forest floor mass in the low-lignin sugar-maple dominated system decreased in October due to experimental N deposition, whereas forest floor mass of high-lignin oak-dominated ecosystems was unaffected by N deposition. Increased relative abundance of basidiomycetes in high-lignin forest floor was confirmed by denaturing gradient gel electrophoresis (DGGE) and sequencing. Abundance of basidiomycete laccase genes, encoding an enzyme used by white-rot basidiomycetes in the degradation of lignin, was 5–10 times greater in high-lignin forest floor than in low-lignin forest floor. While the differences between the fungal communities in different ecosystems were consistent with the proposed mechanism, no significant effects of N deposition were detected on DGGE profiles, laccase gene abundance, laccase length heterogeneity profiles, or phenol oxidase activity. Our observations indicate that the previously detected accumulation of soil organic matter in the

high-lignin system may be driven by effects of N deposition on organisms in the mineral soil, rather than on organisms residing in the forest floor. However, studies of *in situ* gene expression and temporal and spatial variability within forest floor communities will be necessary to further relate the ecosystem dynamics of organic carbon to microbial communities and atmospheric N deposition.

Introduction

Human activity has substantially increased atmospheric N deposition across large portions of North America and Europe (Holland *et al.*, 2005), a widespread environmental change that will alter the biogeochemical cycling of C and N at local, regional and global scales (Driscoll *et al.*, 2001; Fenn *et al.*, 2003; Pregitzer *et al.*, 2004). Although much attention has been paid to the response of terrestrial vegetation, a mounting body of evidence suggests that microbial degradation of plant litter and soil organic matter mediates the effects of N deposition on C storage in soil (Burton *et al.*, 2004; Waldrop *et al.*, 2004a,b). Moreover, it appears that atmospheric N deposition can either stimulate or suppress microbial activity depending on plant litter biochemistry (Berg and Matzner, 1997; Knorr *et al.*, 2005), giving rise to an increase or decrease in soil C storage (Waldrop *et al.*, 2004b).

The primary biochemical constituent of litter determining the response of decomposition to N deposition is lignin. The microorganisms most efficient in the complete degradation of lignin are the white-rot fungi in the phylum *Basidiomycota* (Hatakka, 2001; Rabinovich *et al.*, 2004). High N availability can suppress decomposition of lignaceous material by a variety of white-rot basidiomycetes in culture (Worrall *et al.*, 1997). Therefore, lignin content in plant detritus and the abundance of white-rot fungi appear to be two important factors by which atmospheric N deposition could directly influence decomposition and soil organic matter formation. Laccase is an extracellular enzyme which is a major contributor to polyphenol oxidase activity (Baldrian, 2006). It appears to be secreted by most white-rot species and is necessary for efficient lignin breakdown (Hatakka, 2001; Rabinovich *et al.*, 2004). Basidiomycete laccase genes can be used

Table 1. Effects of ecosystem type on forest floor mass and biochemical composition.

	Forest floor mass (g m^{-2})		Forest floor biochemical composition (%)				
	June 2004	October 2003/2004	Lignin	Cellulose	Polar extractives	Non-polar extractives	Ash
BOWO	505 (113) a	377 (80) a	34 (6) a	44 (6) a	16 (3) a	4 (1) ab	3 (1) a
SMRO	563 (85) a	494 (60) a	30 (4) b	44 (2) a	17 (4) a	6 (3) a	3 (1) a
SMBW	394 (103) a	267 (152) b	26 (3) b	50 (3) b	18 (3) a	3 (2) b	3 (1) a

Means are shown with standard deviation in parentheses. Means shown with different letters in the same column are significantly different ($P < 0.05$). In addition, a significant ecosystem–N treatment interaction effect on October forest floor mass is shown in Fig. 1 and discussed in the text.

to directly assay the abundance and composition of this functional group, which can then be linked to the ecosystem process of litter decomposition.

The observation that high N availability suppresses white-rot basidiomycete activity has formed the basis for a mechanism proposed to explain why decomposition of different litter types can have opposing responses to N deposition (Carreiro *et al.*, 2000; Sinsabaugh *et al.*, 2002). In these studies, N deposition depressed decomposition rate of high-lignin litter, a response correlated with a decline in phenol oxidase activity. In contrast, N deposition stimulated decomposition of low-lignin litter, which was correlated with an increase in cellulase activity. Similar observations have also been made in N deposition experiments examining soil organic matter and soil enzyme activity (Frey *et al.*, 2004; Waldrop *et al.*, 2004b), with the implication being that N deposition may significantly affect soil C sequestration depending on the response of the resident microbial community. The mechanism proposed to explain these opposing responses of litter decomposition to N deposition is that white-rot basidiomycetes are more important decomposers in high-lignin litter than in low-lignin litter (Carreiro *et al.*, 2000; Sinsabaugh *et al.*, 2002). In addition, in high-lignin litter, white-rot basidiomycetes are the primary organisms responsible for phenol oxidase activity, and they are suppressed (in abundance and/or expression of ligninolytic enzymes) by added N. In low-lignin litter, N addition stimulates the broader group of cellulose-decomposing fungi and bacteria by relieving N limitation for growth on cellulose-rich litter. Production of laccase and other phenol oxidases in low-lignin litter has been attributed to saprophytic microorganisms which can outcompete white-rot fungi in low-lignin litter, but which inefficiently degrade lignin (Carreiro *et al.*, 2000; Gallo *et al.*, 2004; Waldrop *et al.*, 2004a). This could explain why changes in phenol oxidase activity in low-lignin litter due to N deposition have been inconsistent (Carreiro *et al.*, 2000; DeForest *et al.*, 2004; Gallo *et al.*, 2004).

Previous work suggested that the mechanism described above may be driving response of mineral soil C to experimental N deposition (Waldrop *et al.*, 2004b).

Our experiment includes three forest ecosystems which are representative of native deciduous forests throughout the Great Lakes region of the United States. Leaf litter from the sugar maple-basswood (SMBW) ecosystem is expected to have the lowest lignin content, whereas litter from the black oak-white oak (BOWO) ecosystem is expected to have the highest, and sugar maple-red oak (SMRO) litter is intermediate. Ecosystem stands are replicated at the landscape scale (separated by ~8 km over a ~1400 km^2 area) and were chosen by a random draw from a pool of stands of each ecosystem type (Zak and Pregitzer, 1990). Atmospheric N deposition treatments include ambient deposition, ambient +30 kg N ha^{-1} year^{-1} (low-N), and ambient +80 kg N ha^{-1} year^{-1} (high-N). Organic C increased and phenol oxidase activity decreased due to N deposition in BOWO, whereas the reverse was true in SMBW (Waldrop *et al.*, 2004b).

In this study, we examine whether the mechanism described above resulting in differential responses of litter decomposition to experimental N deposition could explain the changes in mineral soil C we previously observed. We test the specific hypotheses that (i) forest floor mass accumulates in the BOWO ecosystem (and possibly in SMRO) and declines in the SMBW ecosystem due to experimental N deposition, and that these patterns are consistent with observed responses of soil organic matter; (ii) the forest floor fungal community in BOWO (and possibly SMRO) has a larger population of white-rot basidiomycetes resulting in greater phenol oxidase activity; (iii) white-rot basidiomycete population size drives phenol oxidase activity in the forest floor, such that if white-rot basidiomycetes are suppressed by N deposition, then phenol oxidase activity will also decline.

Results

Forest floor mass and biochemistry

In October of 2003 and 2004, forest floor mass (g m^{-2}) was significantly lower in the SMBW ecosystem than in SMRO and BOWO (Table 1). There was also a significant ecosystem type by N treatment interaction ($P < 0.05$), with
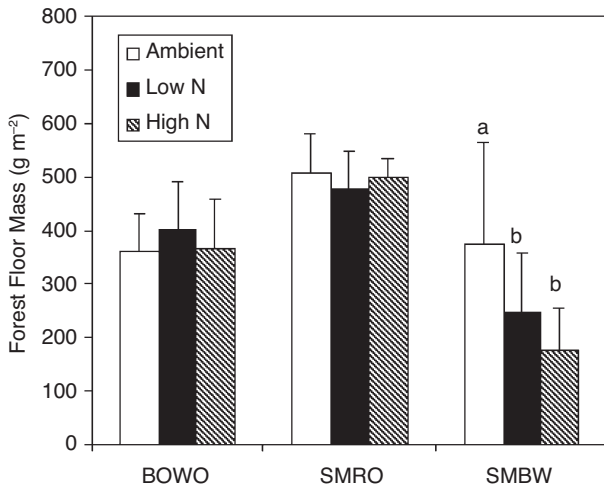
Fig. 1. Forest floor mass in October averaged over 2003 and 2004. Forest floor mass differs significantly between ecosystems as described in text. Letters indicate significant differences between N treatments within SMBW which resulted in the significant N treatment–ecosystem interaction described in text. Error bars show one standard deviation.

forest floor mass significantly reduced by N deposition in the SMBW ecosystem (Fig. 1). Average SMBW forest floor mass in the high-N and low-N treatments was reduced by 53% and 34%, respectively, relative to the ambient treatment. This significant reduction in SMBW forest floor mass in October was consistent across both sampling years. Forest floor mass in other ecosystems was not affected by N deposition treatment. Treatments did not significantly affect forest floor mass in June 2004. Considerable variability was present in forest floor mass (and other biological parameters) due to replication of ecosystem stands at the landscape scale (Table 1). This

variability was taken into account, however, by explicitly nesting N treatment plots within stands and using the appropriate mean square error and degrees of freedom according to a split-plot experimental design (Hinkelmann and Kempthorne, 1994).

Lignin content was significantly higher in BOWO forest floor than in SMBW or SMRO forest floor, whereas cellulose content was significantly higher in SMBW than in BOWO or SMRO (Table 1). SMRO forest floor had a biochemical makeup intermediate to that of BOWO and SMBW forest floor, as predicted. Experimental N deposition did not affect forest floor biochemistry.

General fungal community structure

Denaturing gradient gel electrophoresis (DGGE) was used to examine differences in fungal community composition between ecosystems and N treatments. Fungal DGGE profiles were significantly affected by ecosystem type on both sampling dates ($P < 0.001$). Ecosystem accounted for 21% of total variation in Hellinger-transformed DGGE profiles in 2003, and 19% in 2004. N treatment (main effect) and the ecosystem by N treatment interaction were not significant. In canonical ordination plots, BOWO and SMBW profiles appear to be quite different, with no overlap in error bars (Fig. 2). SMRO profiles are differentiated from BOWO and SMBW by the second ordination axis, but SMRO error bars do overlap with the other ecosystems. Large error bars are associated with the BOWO ambient N deposition treatment in 2003 because two of these profiles had a very distinct pattern compared with all other samples. These patterns were confirmed by repeating the PCR and DGGE.
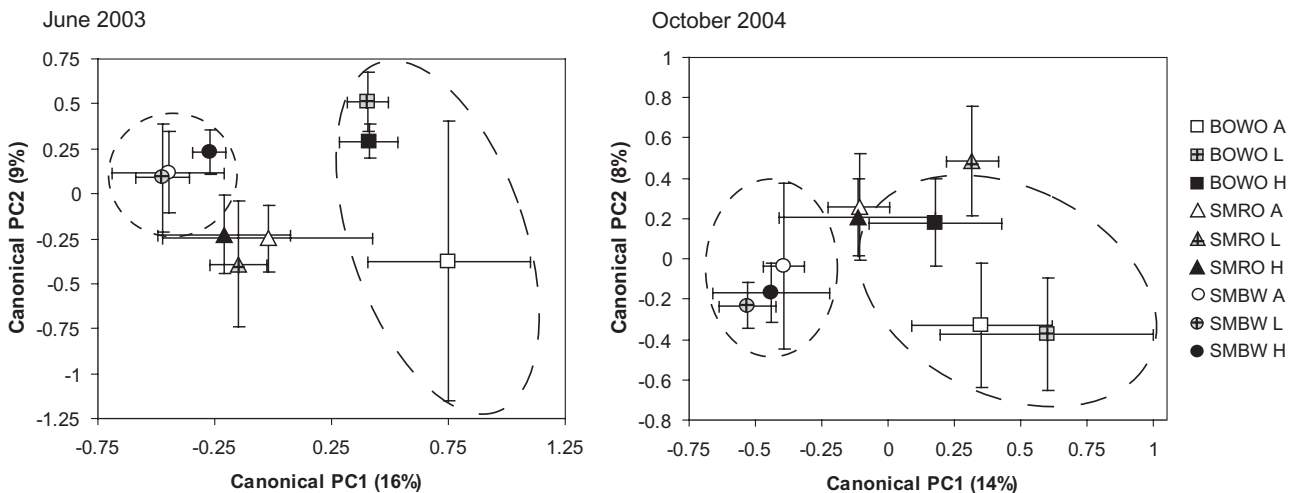

Fig. 2. Canonical ordination plot showing differences between fungal DGGE profiles due to ecosystem and N treatment. Error bars show standard deviation. Ovals enclosing BOWO and SMBW error bars show no overlap. Redundancy analysis indicated that DGGE profiles of different ecosystems were significantly different ($P < 0.001$). A, ambient; L, low N treatment; H, high N treatment.

Fig. 3. Phylogenetic tree of fungal ribosomal sequences obtained from DGGE bands affiliated with an ecosystem type (in boxes) and reference sequences. Symbols on the right indicate treatment that DGGE band is affiliated with (open squares, BOWO; filled squares, BOWO 2003 outliers; circles, SMBW; triangles, SMRO). Symbols connected by a line show sequences which were obtained from clone libraries when clean sequences could not be directly obtained from DGGE bands due to co-migration of multiple sequences. BOWO outliers are ambient plots in 2003 which had unique DGGE profiles unrelated to any other samples.

Ten DGGE bands were excised and sequenced because they strongly influenced the canonical ordination generated by redundancy analysis, and therefore were affiliated with a particular ecosystem on one or both sampling dates (20–73% of their variance explained by treatment on at least one date). Sequences from three of the four bands affiliated with BOWO clustered with basidiomycetes, including potential white-rot basidiomycetes such as *Auricularia polytricha* and *Multiclavula mucida* (Hibbett and Donoghue, 2001) (Fig. 3). A clone library was generated from the fourth band affiliated with BOWO, and one of the three dominant sequences in this library also clustered with white-rot basidiomycetes. In contrast, sequences from the six bands affiliated with SMBW and SMRO were predominantly clustered with functionally diverse organisms in *Ascomycota*. Three other bands were affiliated with the ambient BOWO 2003 outliers mentioned above. These bands yielded sequences which clustered in the *Ascomycota* (near *Aspergillus*, a genus

commonly found in soil, and *Ophioceras*, an aquatic wood-decay fungus) and the *Zygomycota* (near *Piptocephalis*, a mycoparasitic fungus frequently found in dung) (Fig. 3).

Phenol oxidase activity and abundance of laccase-producing basidiomycetes

Phenol oxidase activity was not significantly affected by ecosystem type (Table 2) or experimental N deposition on either sampling date.

Abundance of laccase-producing basidiomycetes was measured by a quantitative PCR (QPCR) assay as number of basidiomycete laccase genes in forest floor (copy number g^{-1}). Laccase gene abundance was lower in SMBW forest floor than in BOWO and SMRO forest floor on both dates; this was significant for BOWO in 2003 and for SMRO on both dates ($P < 0.05$) (Table 2). Experimental N deposition did not significantly affect laccase

Table 2. Effects of ecosystem type on phenol oxidase activity and laccase gene abundance in forest floor.

	Phenol oxidase activity (μmol h^{-1} g^{-1})		Laccase gene abundance (copies g^{-1} $\times 10^6$)	
	June 2003	October 2004	June 2003	October 2004
BOWO	10.6 (3.2) a	11.3 (5.6) a	4.5 (3.6) a	7.7 (6.6) ab
SMRO	10.7 (3.6) a	5.0 (5.1) a	5.9 (5.5) a	5.7 (3.9) a
SMBW	7.3 (3.4) a	7.0 (1.4) a	0.8 (0.5) b	1.0 (0.6) b

Means are shown with standard deviation in parentheses. Means with different letters in the same column are significantly different ($P < 0.05$).

Table 3. Mixed linear model analysis of phenol oxidase activity for June 2003 samples.

	Degrees of freedom			
Independent variable	Numerator	Error	F statistic	*P*-value
Ecosystem type	2	6	2.51	0.16
Laccase gene abundance	1	15	6.45	0.022
Ecosystem type × laccase gene abundance interaction	2	15	7.13	0.0067

The same analysis was performed for October 2004 samples and laccase gene abundance did not significantly affect phenol oxidase activity.

gene abundance. Standard curves were generated for each QPCR run, with $R^2 > 0.99$ and amplification efficiency > 0.87. Correlation between replicate runs was 0.96. A range of amplicon sizes was obtained in the QPCR assay (primarily from 141 to 330 bp). Length heterogeneity of the PCR product was determined by electrophoresis for each sample so that gene copy number could be calculated. Laccase gene abundance was log-transformed prior to hypothesis testing to reduce the effect of increasing variance with mean.

The ability to predict phenol oxidase activity from laccase gene abundance was tested using a mixed linear model analysis of covariance, which also tested potential differences in this relationship among ecosystems. Laccase gene abundance and its interaction with ecosystem type significantly affected phenol oxidase activity in 2003 (Table 3). Phenol oxidase activity was linearly related to laccase gene abundance in BOWO forest floor, but not in SMBW or SMRO forest floor (Fig. 4). Phenol oxidase activity was not significantly affected by laccase gene abun-

dance or ecosystem type in 2004 (Fig. 4). Moisture content was not a significant covariate in any analyses.

Laccase-producing basidiomycete community composition

Laccase length heterogeneity (LH)-PCR profiles were only marginally affected by ecosystem type ($P = 0.02$ in 2003 and $P = 0.06$ in 2004), and not affected by N treatment. Ordination plots (not shown) also indicated that the effects of ecosystem on laccase-producing basidiomycete LH-PCR profiles were weak.

Laccase clone libraries were analysed from BOWO and SMBW ambient N treatment plots by restriction profiling. Redundancy analysis found no differences in composition of laccase clone libraries due to ecosystem type ($P > 0.1$), with little overlap in restriction fragment length polymorphism (RFLP) profiles between samples. There were also no differences in clone library diversity due to ecosystem as measured by the Chao1 index (data not shown). One
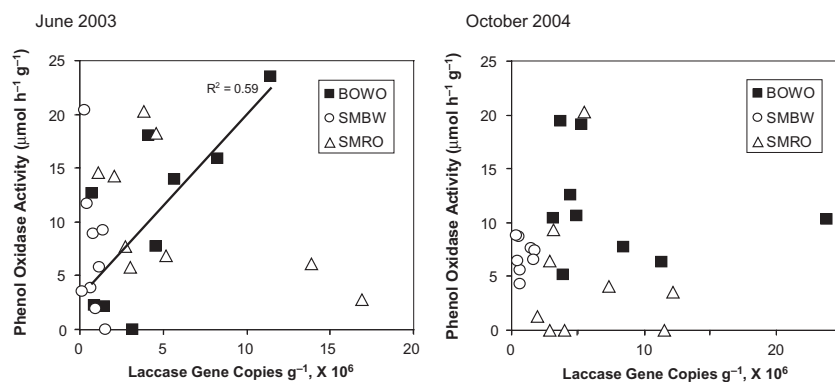

Fig. 4. Relationship between basidiomycete laccase abundance and phenol oxidase activity. Regression line shown is for BOWO samples only ($P < 0.05$).

Table 4. GenBank laccase sequences that forest floor sequences obtained in this study were affiliated with by multiple phylogenetic reconstruction methods.

Order	Species	Accession	BOWO	SMBW
Agaricales	*Coprinellus congregatus (Coprinus congregatus)*	AJ542532	0	1
Agaricales	*Lepista nebularis (Clitocybe nebularis)*	AJ542626	0	1
Agaricales	*Macrotyphula juncea*	AJ542615	0	1
Agaricales	*Macrotyphula juncea*	AJ542615	0	1
Agaricales	*Macrotyphula juncea*	AJ542616	0	2
Agaricales	*Mycena cinerella*	AJ542607	2	1
Agaricales	*Mycena crocata*	AJ542585	1	0
Agaricales	*Mycena cinerella*	AJ542608	0	1
Ceratobasidiales	*Thanatephorus cucumeris (Rhizoctonia solani)*	Z54215	0	1
Heliotales	*Botryotinia fuckeliana*	AY047482	1	0
Russulales	*Lactarius subdulcis*	AJ542650	1	1
Unknown	Uncultured basidiomycete	AJ420338	1	1
Unknown	Uncultured basidiomycete	AJ540301	1	0
Unknown	Uncultured basidiomycete	AJ580835	1	1

The number of ambient-N plots where affiliated laccase OTUs were found is shown under BOWO and SMBW columns. An additional five OTUs were found in BOWO and two in SMBW that did not cluster consistently with existing laccase sequences, and represented novel laccase lineages. Taxonomy according to Kirk and colleagues (2001).

clone library contained greatly reduced diversity compared with other samples. In addition, the LH-PCR profile for this sample contained only a single band.

Laccase sequence analysis

Several representative clones from the dominant RFLP patterns in each clone library were sequenced, resulting in 58 total sequences (8–11 per sample). Clones representative of the entire range of amplicon lengths were included. Following the suggestions of Robertson and colleagues (2005), we examined the phylogenetic affiliations of uncultured laccase sequences by placing them into a backbone tree of 192 full-length sequences. Closely related sequences (distance < 23%) were consistently clustered together, and we subsequently used 23% as a cut-off to define sequence operational taxonomical units (OTUs). This resulted in 21 total OTUs among sequences obtained in this experiment. Ten of these represented novel laccase lineages or were only affiliated with other uncultured basidiomycete sequences, whereas eight OTUs were consistently clustered with existing white-rot sequences from the order *Agaricales* (i.e. *Mycena*, *Macrotyphula*, *Lepista* and *Coprinellus* sp.) (Table 4). One OTU clustered with a sequence from an ectomycorrhizal

species (*Lactarius subdulcis*), and two OTUs clustered with laccases from plant pathogenic species, one from within the *Basidiomycota* (*Thanatephorus*) and one from the *Ascomycota* (*Botryotinia*). Due to the limited sequence information present in Cu1F-Cu2R amplicons (only 141 protein-coding nucleotide positions), the tree topology was not stable at higher-level clades (i.e. very short internal branch lengths, little bootstrap support, and differences between methods of sequence addition).

Results of chi-square tests indicated that Cu1F-Cu2R amplicon length was significantly congruent with OTUs defined by sequence similarity (Table 5). Cramer's V values in the range of 0.8–0.9 indicate that amplicon length can be used as a rough approximation of sequence OTUs. However, there are some OTUs that are divided between amplicon length categories, and others that are lumped together by amplicon length. Detected diversity will be reduced using amplicon length heterogeneity, although this is not as severe if only sequences obtained from a given site are considered (Table 5).

Discussion

While we have not found significant effects of experimental N deposition on the fungal community which

Table 5. Congruence between laccase PCR amplicon length and OTUs defined by protein-coding nucleotide sequence similarity.

Sequences	# sequences	# OTUs	Non-clustered amplicon length		Clustered amplicon length	
			Cramer's V	# of categories	Cramer's V	# of categories
All	354	178	0.83	65	0.88	27
This study	58	21	0.81	23	0.88	7

Operational taxonomical units were determined by sequence similarity of protein-coding nucleotides in the region amplified by primers Cu1F-Cu2R, using a distance cut-off of < 23%. Amplicon length is calculated from the genomic amplicon (including introns). Under 'Non-clustered amplicon length' each amplicon length is considered a unique category, while under 'Clustered amplicon length' amplicons were clustered into 5 bp windows.

resides in forest floor, the patterns that we have seen support the central assumption of the mechanism proposed to explain opposing responses of ecosystems to N deposition: that white-rot basidiomycetes are more important in the decomposition of litter with relatively high-lignin content. To our knowledge, data presented here represent the first molecular evidence to support this assumption. Analysis of fungal DGGE profiles indicated that overall forest floor-inhabiting fungal communities differed among forest ecosystems, and that basidiomycetes (including white-rot basidiomycetes) formed a larger part of the community in the BOWO ecosystem. White-rot basidiomycetes are the most efficient lignin degraders (Hatakka, 2001; Rabinovich *et al.*, 2004), and these lignin specialists are likely to be better competitors under BOWO where lignin content is the highest. However, white-rot basidiomycetes are a functionally defined, polyphyletic group. To gain further insight into how they respond to ecosystem differences, we used PCR primers targeting laccase genes of basidiomycetes. Quantitative PCR revealed that basidiomycete laccase gene abundance was 5–10 times greater in BOWO (and SMRO) forest floor than in SMBW forest floor. This clearly confirms that plant litter tissue biochemistry is an important driver of the functional and taxonomic composition of decomposer communities, and provides strong evidence for the reduced importance of white-rot basidiomycetes in decomposition of sugar maple leaf litter where lignin content is lower than in oak litter.

Laccases are well-characterized as extracellular enzymes secreted by fungi to degrade lignin through phenol oxidase activity (Baldrian, 2006), particularly within the families *Agaricales* and *Polyporales*. Laccase genes have also been detected in several ectomycorrhizal species, and have been implicated in potential lignin degradation by these organisms (Chen *et al.*, 2003; Luis *et al.*, 2004). However, we should note that laccases can have a variety of other functions in fungi as well, including in morphogenesis and biochemical interaction with other organisms (Baldrian, 2006). In soil and decaying litter, we expect that basidiomycete laccases are primarily involved in lignin degradation because lignocellulose is a major source of C and energy for fungi in these environments. Furthermore, we found only two laccase OTUs which clustered with plant pathogenic species, which may not be involved in saprophytic lignin degradation at all (Schouten *et al.*, 2002).

Surprisingly, there was no correspondence between ecosystem type and white-rot basidiomycete community composition detected either by LH-PCR or by RFLP analysis of clone libraries. Instead, community composition of white-rot basidiomycetes in forest floor appeared to be highly variable and idiosyncratic to particular stands.

This is similar to the high spatial variability of laccase sequences found by Luis and colleagues (2005a). The implication is that, while plant litter tissue biochemistry is important in determining the success of this functional group, community composition within the group is likely regulated by other factors such as dispersal and pre-emptive colonization.

The heterogeneity in the length of the laccase fragment amplified from genomic DNA is due to the presence of multiple introns. We demonstrated with chi-square tests that there is good, but not perfect, correspondence between amplicon length and OTUs based on DNA sequences. We also found that the correspondence between RFLP groups and OTUs was not perfect (data not shown). This situation is similar to what is observed when profiling methods are used on ribosomal PCR amplicons (Anderson and Cairney, 2004). Laccase LH-PCR is therefore a useful method to rapidly screen laccase community composition of a large number of samples, to be followed up by more targeted sequencing efforts. Length heterogeneity-PCR is also necessary to correct for variable lengths of PCR products which would otherwise confound SybrGreen-based QPCR results. While we could associate over half of the laccase sequences we obtained with existing laccase sequences in GenBank, this analysis was hampered by unstable global topology of a phylogenetic tree (e.g. low bootstrap support for large clades), similar to previously published trees based on this very short laccase fragment (Luis *et al.*, 2004; 2005a). Sequence analysis will be improved as additional full-length laccase sequences become available and identification algorithms are improved.

We expected that white-rot basidiomycetes control phenol oxidase activity because they are specialized lignin degraders. We found that, for the samples taken during mid-summer season, there was a significant linear relationship between laccase-producing basidiomycete abundance and phenol oxidase activity in BOWO forest floor samples. This relationship, however, was not present at a mid-autumn sampling when microbial growth was possibly slowed by reduced temperature. We believe that this is due to seasonal changes in the factors that regulate enzyme activity, with abundance of the relevant functional groups being an important driver of enzyme activity during periods of active growth, and other factors such as adsorption onto surfaces being important later in the season. Luis and colleagues (2005b) have shown that presence of a genomic laccase sequence does not necessarily imply transcription in the environment, but we would expect this correspondence hold up the best when organisms are most active. Phenol oxidase activity in SMBW, where complete degradation of lignin may not be required to gain access to

carbohydrates, is likely a product of the metabolism of other organisms which solubilize lignin but do not mineralize it, such as *Streptomyces* sp. (e.g. Mason *et al.*, 1988). In the SMRO system, variability is likely to be the highest because there is a mixture of litter types with high- and low-lignin contents, potentially resulting in different organisms regulating decomposition depending on the substrate in question.

Our results provide support for some, but not all, of the hypotheses derived from the mechanism where the importance of white-rot basidiomycetes in different ecosystems causes different responses to N deposition. There are a variety of factors which could explain why this is the case. A reduction of soil organic matter in SMBW due to N deposition, and an increase in BOWO, was previously observed at this experimental site (Waldrop *et al.*, 2004b). The reduction in soil organic matter in SMBW could be due in part to the more rapid loss of forest floor observed here. However, this is likely to be driven by organisms other than white-rot basidiomycetes, and the activity of other enzymes such as cellulases may be more important than phenol oxidases in driving the rate of decomposition in SMBW. In BOWO, we did not find an accumulation of forest floor or a reduction in phenol oxidase activity, unlike the pattern observed previously in soil organic matter and soil phenol oxidase activity (Waldrop *et al.*, 2004b). Hence, while our data confirm that white-rot fungi are important in the degradation of BOWO litter, the abundance of these organisms does not appear to be reduced by experimental N deposition, at least on the dates we sampled. The biological mechanisms causing accumulation of soil organic matter may therefore be unrelated to slowing of decomposition by fungi in forest floor (thereby increasing organic matter input to the soil), instead being a consequence of reduced decomposition within the mineral soil itself. Alternatively, N deposition may alter the expression of ligninolytic genes by white-rot basidiomycetes without altering their abundance or community composition. We may also miss effects of experimental N deposition due to our current lack of understanding of temporal and spatial variability within microbial communities, and how this natural variability within the community relates to ecosystem activity. This is illustrated by changes in SMBW forest floor mass being manifest in October after at least a year of decomposition, but resulting from biological processes occurring earlier in the season. The finding that decomposition of SMBW forest floor is enhanced by N deposition, whereas decomposition of other forest floors is not, is further evidence that N deposition alters biogeochemical cycling of C in an ecosystem-specific manner and depends on characteristics of the microbial community residing in the ecosystem in question.

Experimental procedures

Field experiment and sample collection

Samples were obtained from an N deposition experiment in Northern Lower Michigan described by Waldrop and colleagues (2004a). Briefly, experimental plots were established in three replicate stands of each ecosystem type (BOWO, SMRO and SMBW) described in the *Introduction*. In each stand, three 10 m $\times$ 30 m plots were randomly located, and plots were randomly selected to receive one of three experimental N deposition treatments: ambient, ambient + 30 kg N ha^{-1} year^{-1} (low-N), and ambient + 80 kg N ha^{-1} year^{-1} (high-N). Beginning in 2001, experimental N deposition treatments were imposed by monthly addition of dry NaNO$_3$, beginning in April and ending in September.

Forest floor mass was determined in June and October 2004, and October 2003. October samples were obtained before the current canopy abscised. Four forest floor samples were taken in each plot using a 0.25 m^2 quadrangle placed at a predetermined random location. The four samples were composited into a large bin, and branches and stems (diameter > 7 mm) were removed. Bins were then weighed (wet), and a subsample of the litter was dried for 2–5 days in a forced air oven (70°C) to determine moisture content.

Forest floor was also collected in June 2003 and in October 2004 for DNA extraction and measurement of enzyme activity. Samples of forest floor were pooled from five locations within each plot. Samples were transported on ice to the laboratory, where they were ground and stored at −20°C. Moisture content was determined by drying subsamples overnight at 65°C.

Forest floor biochemistry

For samples collected in October 2003, biochemical constituent analysis was performed using a series of extractions modified from Ryan and colleagues (1990). Two grams of forest floor was mixed with 30 ml of dichloromethane (CH$_2$CL$_2$), sonicated for 1 h, centrifuged for 10 min at 2000 r. p.m., and filtered through a G8 glass fibre filter to remove the non-polar component of the sample (fats, waxes and other organic soluble materials). This procedure was repeated once with 10 ml dichloromethane. The non-polar fraction was dried under N$_2$ and quantified gravimetrically. Polar extractives were removed from insoluble residue retained on the filter by boiling for 3 h in 30 ml water with caps tight, centrifuging, and then filtering the supernatant through a second G8 glass fibre filter. Polar extractives in filtrate were dried in a 65°C forced air oven and then weighed. Residue retained on the filter after removal of polar extractives was lyophilized. Lignin and cellulose content of this residue was determined by a modified wood-products chemistry procedure (Ryan *et al.*, 1990). A 0.3 g subsample was digested in 1.5 ml 72% sulfuric acid for 2 h in a 30°C water bath stirring every half hour. The solution was then diluted with 42 ml water. Secondary hydrolysis was carried out by autoclaving for 2 h at 121°C. At the end of this process, the sample was passed through a G8 glass fibre filter. Residual tissue was washed with hot water, dried at 65°C, cooled in a dessicator, and weighed. Residual tissue was considered lignin and mass

lost during digestion was considered cellulose. A subsample of the final residue was ashed at 450°C for 8 h in a pre-weighed crucible to determine ash free dry mass.

Phenol oxidase activity assay

Phenol oxidase activity was measured following the procedure of Saiya-Cork and colleagues (2002). Briefly, 0.5 g of ground litter was homogenized in acetate buffer (pH 5.0). This suspension was then inoculated into 16 replicate microplate wells containing 50 μl of 25 mM L-3,4-dihydroxyphenylalanine (DOPA). Sample and substrate controls were also run for each microplate. Microplates were incubated in the dark at 20°C for up to 24 h. Oxidation of DOPA was measured as optical density at 460 nm using a spectrophotometer (Bio-Tek Instruments, Winooski, VT). Phenol oxidase activity is expressed as μmol DOPA oxidized $g^{-1} h^{-1}$.

Genomic DNA extraction

Genomic community DNA was extracted from approximately 0.15 g ground forest floor samples using the PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's instructions. To facilitate lysis of hyphae after addition of cell lysis solution (C1), samples were incubated at 70°C for 5 min, vortexed 10 min, and incubated at 70°C for 5 min.

General fungal DGGE

A fragment of the fungal 18S ribosomal gene was amplified from community DNA by PCR using general fungal primers FR1 (with a GC-clamp) and FF390 (Integrated DNA Technologies, Coralville, IA) (Vainio and Hantula, 2000). PCR was conducted in 25 μl reactions using final concentrations of 0.1 μM each primer, 0.2 mM each dNTP, 0.1 μg μl⁻¹ BSA, 1.5 mM $MgCl_2$, 1× PCR buffer, and 0.02 U μl⁻¹ Expand High-Fidelity DNA polymerase (Roche Diagnostics, Indianapolis, IN); 5 μl of diluted genomic DNA was added to each PCR. Samples were diluted (1:50–1:200) to achieve optimal PCRs for each sample (strong PCR product but no background smear of DNA in an agarose gel stained with ethidium bromide). Reactions were performed in a Robocycler 96 (Stratagene, LaJolla, CA) using the following program: 5 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at 50°C, and 2 min at 72°C, followed by 10 min at 72°C. Optimal PCRs were run in triplicate and pooled.

DNA concentration was quantified in pooled PCRs using a microplate PicoGreen assay following the manufacturer's instructions (Invitrogen, Carlsbad, CA). 100 ng of PCR product was subjected to DGGE in a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA) according to Vainio and Hantula (2000). An aliquot of 100 bp ladder (100 bp PCR Molecular Ruler) (Bio-Rad) was also run in three lanes of each gel. Gels were stained with SybrGold (Invitrogen) and digitally photographed with a MiniDarkroom (UVP, Upland, CA) with illumination provided by a Dark Reader Transilluminator (Clare Chemical, Dolores, CO). Denaturing gradient gel electrophoresis was performed

twice, using different sample combinations in the second set of gels to facilitate aligning lanes between gels. Gel images were analysed in GelCompar II (Applied Maths, Austin, TX). Duplicate DGGE profiles were used to verify band presence and alignments. A matrix of relative band intensity was exported from GelCompar II, with samples as rows and band categories as columns.

Denaturing gradient gel electrophoresis bands were excised from several lanes using a sterile razor and mixed with 500 μl H_2O and ~100 μl glass beads (1 mm diameter). Polyacrylamide slices were ground using a FastPrep bead-beater (Qbiogene, Irvine, CA) and allowed to soak overnight at 4°C. Denaturing gradient gel electrophoresis bands were then re-amplified using 25 cycles of the PCR program described above. PCR product was sequenced at the University of Michigan DNA Sequencing Core. Amplicons which did not yield clean sequences were cloned into *E. coli* JM109 using the pGEM-T Vector System (Promega, Madison, WI) following the manufacturer's instructions. Inserts were screened by restriction digestion with MspI and RsaI, and a representative insert from each dominant restriction pattern was sequenced.

Laccase-producing basidiomycete abundance and community composition

Concentration of basidiomycete laccase genes was determined by QPCR using primers Cu1F and Cu2R (Luis *et al.*, 2004). Quantitative PCRs were run in 25 μl reactions with final concentrations of 0.5 μM each primer (Integrated DNA Technologies), 1× Rox reference dye, and 1× Brilliant SybrGreen QPCR Master Mix (Stratagene). 2.5 μl of diluted genomic DNA was added to each PCR using the same dilution factor previously found to be optimal for FR1-FF390 PCRs. Quantitative PCR was performed in an MX3000 thermocycler (Stratagene) using the following program: 4 min at 94°C, followed by 40 cycles of 1 min at 94°C, 2 min at 54°C, and 5 min at 72°C, followed by 10 min at 72°C. SybrGreen fluorescence was measured at the end of each extension step. Size of PCR product was checked by generation of a dissociation curve after PCR (Ririe *et al.*, 1997).

A standard curve relating C_t to ng μl⁻¹ of basidiomycete laccase genes was generated by mixing together Cu1F-Cu2R PCR product that had previously been obtained from each ecosystem type. The DNA concentration in this mixture was then determined using a PicoGreen assay, and serial dilutions from 6.14×10^{-4} to 6.14×10^{-10} ng μl⁻¹ were used as standards. Quantitative PCR was performed on all samples in duplicate runs, and each run included two sets of standards and no-template controls.

Laccase was also amplified from all samples using the above conditions except that the SybrGreen QPCR Core Reagent kit (Stratagene) was used without addition of SybrGreen or a reference dye. PCR product from these reactions was quantified using PicoGreen and then used for LH-PCR and cloning. For LH-PCR, 10 ng of DNA was electrophoresed in a 3.5% NuSieve 3:1 agarose gel in TBE buffer at 90 V for 2 h 15 min. Two lanes of 100 bp ladder were run as size standards in each gel. Gels were stained with SybrGold and gel images were analysed in GelCompar II, producing a matrix of relative band intensity.

To normalize the effect of different-size PCR amplicons, laccase concentration (in ng DNA g^{-1} dry forest floor) was partitioned among different size classes based on relative band intensities in LH-PCR profiles. This concentration was then converted to number of gene copies g^{-1} for each band class by assuming a molecular weight of 660 g mol^{-1} bp^{-1}. Copies per gram was then summed across band classes to obtain a measurement of total population density in each sample. Relative abundance of each band class, to be used in community analysis of laccase-producing basidiomycetes, was also calculated from copies pre gram.

Amplicons from the ambient BOWO and SMBW plots were cloned and sequenced as described above for DGGE bands.

Statistical analyses

This experiment was treated as a split-plot design (Hinkelmann and Kempthorne, 1994) in all statistical tests, with ecosystems represented by replicate stands across the landscape ($n = 3$). Stands were whole-plots, and N treatment plots within stands were the subplots. Forest floor mass, phenol oxidase activity, and laccase gene abundance were analysed using mixed linear models by Proc Mixed in SAS version 8 (SAS Institute, Cary, NC) with N treatment plots nested within stands. October 2003 and 2004 forest floor mass data were analysed together by a repeated-measures mixed linear model in Proc Mixed. The relationship between phenol oxidase activity and laccase abundance was assessed by analysis of covariance in Proc Mixed in order to account for the nested plot design.

Denaturing gradient gel electrophoresis, LH-PCR, and clone library restriction analysis profiles were analysed by redundancy analysis of Hellinger distances between profiles in Canoco version 4 (Microcomputer Power, Ithaca, NY) (Blackwood *et al.*, 2003). Significance of treatment effects was assessed using 9999 random permutations of sample identity. Diversity of laccase clone libraries was compared using accumulation curves of the Chao1 diversity estimator on RFLP patterns in EstimateS (Colwell, 2005).

DNA sequence analyses

Denaturing gradient gel electrophoresis band sequences were aligned to the ARB 2004 small-subunit ribosomal database using the integrated aligner (Ludwig *et al.*, 2004). Alignments were checked manually and then sequences were inserted into the phylogenetic tree using the Parsimony insert function. The tree was reduced for display to include experimental sequences, representative named sequences closely related to experimental sequences, and several reference sequences.

Laccase exons were identified and aligned by comparison to an ARB laccase database (C.B. Blackwood, unpubl. results). The alignment was performed using CLUSTALW followed by manual adjustment. Laccase sequences obtained in this study were then added to a pre-existing 'backbone tree' using several methods which were compared. The backbone tree was a maximum likelihood tree consisting of 192 nearly full-length fungal laccases and bacterial multicopper oxidases (1289 aligned protein-coding nucleotides) (C.B.

Blackwood, unpubl. results). The sequence addition methods included construction of a maximum likelihood tree in PAUP* with the backbone tree as a topological constraint, bootstrap neighbour-joining in PAUP* with the same constraint, and the Parsimony insertion function in ARB.

Chi-square tests were used to test the hypothesis that Cu1F-Cu2R amplicon length and OTUs based on sequence similarity resulted in congruent categorizations of sequences. Pearson and likelihood ratio chi-square tests were implemented by Monte Carlo approximation of exact tests in Proc Freq in SAS. Cramer's V was also calculated to describe the degree of association between amplicon length and sequence OTUs, with 1 implying perfect congruence (potentially with nesting of categories) and 0 implying complete independence.

Sequences obtained in this study have been deposited in GenBank under accession numbers DQ520014-DQ520091.

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