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Simulated nitrogen deposition favors stress-tolerant fungi with low potential for decomposition

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ABSTRACT

Global changes such as atmospheric nitrogen (N) deposition can alter the structure of microbial communities, but a mechanistic understanding of the linkages between community structure and ecosystem function is lacking. Here we apply a trait-based framework to an analysis of litter fungal communities in a temperate forest exposed to > 20 years simulated N addition in order to develop hypotheses regarding the mechanisms underlying community responses and resulting changes in ecosystem function. We performed metabarcoding of the total and active fungal communities and measured extracellular enzyme activity in leaf litter after it decomposed for approximately two years in a long-term simulated N deposition experiment. We found that N fertilization decreased the relative abundance of six species in the active community that were highly correlated with lignin decomposing enzyme activity. Four of these species have currently unknown taxonomic identity and should be targets for isolation and further characterization. Nitrogen fertilization also increased species richness and relative abundance of yeasts in the total community and decreased their relative activity levels, suggesting these species may be dormant or otherwise inactive. Together these responses may contribute to accumulation of organic matter in soils by favoring yeasts that are not strong enzyme producers and by disfavoring the fungal species that are most active in litter decomposition.

1. Introduction

Fungi, as the primary agents of plant litter decomposition in temperate forest soils (Schneider et al., 2010, 2012), are likely to mediate changes in ecosystem carbon (C) and nutrient cycling that occur with global change. The various species of fungi involved in decomposition of plant litter have a wide array of potential functions – ranging from lignin-decomposers (e.g., white-rot fungi) that can produce a variety of cellulose- and lignin-decomposing extracellular enzymes (Floudas et al., 2012) to yeast species that are better-adapted for rapid consumption of sugar monomers and other simple compounds (Botha, 2011; Treseder and Lennon, 2015). A change in the distribution of species in these different guilds is therefore likely to alter ecosystem functioning in important ways, but evidence for causal relationships between taxonomic composition of communities and ecosystem function has remained elusive (Nemergut et al., 2013; Crowther et al., 2014). This is partly due to the lack of a cohesive framework to describe the relationship between microbial community structure and ecosystem functioning – taxonomic composition is often assessed independently of microbial functional traits that control ecosystem processes like litter decomposition (Crowther et al., 2014). However, traits are not independent of species, and fungi in particular can be defined in terms of functional groups for which a suite of traits is consistently present (Treseder and Lennon, 2015), providing a means to link taxonomic identity to functioning in the environment. To this end, a response-effect trait framework (*sensu* Lavorel and Garnier, 2002) has been proposed to understand how environmental perturbations cause changes in fungal community composition that then feed back to ecosystem functioning (Crowther et al., 2014; Koide et al., 2014; Treseder and Lennon, 2015). In this framework, a response trait determines how an organism *responds* to its environment (e.g., change in fitness) while an effect trait determines how an organism *affects* ecosystem processes. When response and effect traits are linked (e.g., the traits consistently occur in the same groups of organisms), then an environmental

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perturbation that affects species abundance will also alter ecosystem processes.

Long-term simulated N deposition experiments provide a particularly cogent study system within which to apply a response-effect trait framework, because of the breadth of research on fungal community and ecosystem responses to chronic N addition. For example, N addition to temperate forest ecosystems consistently causes an accumulation of C in soils and reduces organic matter decomposition rates (Pregitzer et al., 2008; Liu and Greaver, 2010; Zak et al., 2011; Lovett et al., 2013; Weber et al., 2013; Frey et al., 2014), depresses activity of lignin decomposing enzymes (Carreiro et al., 2000; Sinsabaugh et al., 2002; DeForest et al., 2004; Frey et al., 2004), and causes accumulation of lignin compounds in soil and litter (Magill and Aber, 1998; Frey et al., 2014). Despite that fungal community responses are relatively well studied (e.g., Allison et al., 2007; Osono, 2007; Entwistle et al., 2013; Eisenlord et al., 2013; Weber et al., 2013; Mueller et al., 2014; Freedman et al., 2015; Hesse et al., 2015; Morrison et al., 2016; Entwistle et al., 2017) it is still not clear whether there is a general response of the fungal community that underlies the observed ecosystem responses.

There are several examples of fungal groups or specific fungal traits that fit a response-effect framework in the context of long-term N deposition and that could contribute to the ecosystem responses observed (e.g., soil organic matter accumulation). We focus on traits such as potential for stress resistance and production of extracellular decomposition enzymes that are broadly correlated with different fungal body forms, specifically single-celled yeasts versus filamentous body forms (Treseder and Lennon, 2015), and use these groupings of traits to circumscribe fungal functional groups. For example, yeasts, defined here as fungi within Ascomycota and Basidiomycota with a single-cell body form (*sensu* Kurtzman et al., 2011), can be an important component of the forest litter and soil fungal community in terms of both relative abundance in the community and the number of species present (i.e. species richness; Masinova et al., 2017). Yeasts have greater genetic capacity for inorganic N uptake than filamentous fungi suggesting that they may be favored in N-rich environments, but have less capacity to produce cellulose and lignin-degrading enzymes (Treseder and Lennon, 2015). Yeasts are also enriched in traits conferring stress resistance (Singaravelen et al., 2008; Kurtzman et al., 2011; Treseder and Lennon, 2015) suggesting they may be favored in high N environments in which total fungal biomass is often suppressed (Frey et al., 2004; Wallenstein et al., 2006; Treseder, 2008). Potential mechanisms explaining suppression of fungal biomass in high-N soil environments include formation of reactive nitrogen compounds in soils (e.g., nitric oxide, nitric acid) and subsequent nitrosative or oxidative stress (Brown et al., 2009; Cánovas et al., 2016), osmotic stress induced by added N salts (Averill and Waring, 2018), lower availability of lignin-complexed cellulose and hemicellulose resulting from transcriptional down regulation of genes encoding lignin-degrading enzymes (Edwards et al., 2011; Zak et al., 2011), and/or reduced exudation by plant roots resulting in lower availability of labile soil C (Carrara et al., 2018), though the relative contributions of these or other mechanisms is unknown. Yeasts therefore represent a functional group that may exhibit a positive response to chronic N deposition and have the potential to effect an increase in soil organic matter accumulation by competing with other fungi that are stronger decomposers of plant litter.

Filamentous saprotrophic fungi represent a second functional group with key traits that may be important in determining ecosystem responses to chronic soil N enrichment. Filamentous fungi generally have a lower genetic capacity for uptake of inorganic N and show lower stress-tolerance than yeasts (Treseder and Lennon, 2015); they may therefore be disfavored relative to yeasts in high-N environments, though to our knowledge the responses of yeasts relative to filamentous fungi have not been specifically examined in N-enriched soils. Filamentous fungi also have a higher capacity to produce plant cell wall degrading enzymes than yeasts overall (Treseder and Lennon, 2015), and some groups of filamentous fungi, especially those typically classified as "white-rot," harbor high capacity for production of ligninases (Floudas et al., 2012; Kohler et al., 2015). White-rot fungi have been shown to have reduced relative abundance with N addition in high-lignin substrates like wood, but have increased relative abundance in N-amended soil (Entwistle et al., 2017), and their response to N addition in leaf litter is yet unclear. In general, high soil N availability may disfavor filamentous saprotrophs relative to yeasts, thereby reducing extracellular enzyme production and decomposition rates.

In the present study we applied a response-effect trait lens to an examination of the fungal communities of decomposing leaf litter in a long-term simulated N deposition experiment in a temperate hardwood forest. We used fungal species traits inferred from previously published literature (e.g., morphology and trophic guild categories) to classify fungal species into broad functional groups. Because traits may be variable within groups and may display plasticity in different environments we performed more direct measurements of two key traits – extracellular enzyme production and relative activity levels. First, we compared taxon relative abundance to extracellular enzyme activities measured in the same litter samples as an indication of extracellular enzyme production by individual taxa, and as a means to confirm the distribution of traits inferred from functional group classification. We also performed measurements of relative activity levels as an indication of dormancy by comparing ratios of ribosomal RNA (rRNA) to ribosomal RNA genes (rDNA) (i.e. rRNA:rDNA ratio, *sensu* Jones and Lennon, 2010), expecting that groups with higher levels of dormancy and lower activity would be enriched in general stress-tolerance traits (Lennon and Jones, 2011; Treseder and Lennon, 2015). General stress-tolerance is likely to be an important response trait in high-N conditions where total fungal biomass is reduced as is the case at our study site (Frey et al., 2004; Wallenstein et al., 2006). Specifically, we focused our analyses on filamentous saprotrophic fungi, filamentous saprotrophs identified as white-rot fungi, and yeasts. We expected these functional groups to have strong responses to N addition because of high stress-tolerance and inorganic N uptake potential of yeasts, and the association of filamentous saprotrophs and white-rot fungi with extracellular enzyme production (Treseder and Lennon, 2015), especially ligninases (Floudas et al., 2012) which are often reduced by N addition (Carreiro et al., 2000; Sinsabaugh et al., 2002; DeForest et al., 2004; Frey et al., 2004).

2. Methods

2.1. Site description, experimental design, and sample collection

This research was performed at the Chronic Nitrogen Amendment Study at the Harvard Forest Long-term Ecological Research (LTER) site in Petersham, Massachusetts, USA (42° 30′ N, 72° 10′ W). The experiment, which is described in full elsewhere (Aber et al., 1989; Aber and Magill, 2004; Frey et al., 2014), is situated in a hardwood forest stand dominated by *Quercus rubra* and *Q. velutina* (red and black oak) with lesser contributions of *Fagus grandifolia* (American beech) and *Acer rubrum* (red maple). The soils are Typic Dystrudepts of the Gloucester series (Peterjohn et al., 1994), with no significant variation amongst N treatments in pH or concentrations of the base cations Ca and Mg after 20 years of N addition (Turlapati et al., 2013). The experiment is divided into three 30 × 30 m megaplots that have received one of three N addition treatments since the inception of the experiment in 1988: ambient (control) N deposition (hereafter, N0), which is currently 7–9 kg N ha^{-1} yr^{-1} at this site (Schwede and Lear, 2014); 50 kg N ha^{-1} yr^{-1}, representing current (Vet et al., 2014) or future (Galloway et al., 2008; Reay et al., 2008) rates of N deposition in areas of the world that are susceptible to high N deposition; or 150 kg N ha^{-1} yr^{-1}, a space-for-time substitution meant to push the ecosystem toward N saturation in order to examine the long-term consequences of high cumulative rates of N deposition (J. Aber, pers. comm.). Nitrogen is applied to soils

in the N treatment plots monthly throughout the growing season with an aqueous solution of ammonium nitrate. Each treatment plot is subdivided into 36 5 × 5 m subplots, which are treated as experimental replicates with the outer plots excluded to reduce edge effects. Although the experiment is sometimes criticized as pseudo-replicated, previous work at the site has confirmed clear treatment differences among plots not caused by random variation (Aber and Magill, 2004; Frey et al., 2014).

Leaf litter was collected at senescence from control plots by use of mesh baskets suspended above the forest floor. Collected litter was air-dried, separated and weighed by species. Litter (10 g) was cut into ~3 × 3 cm pieces and homogenized, and placed into 0.3-mm mesh bags (20 × 20 cm) in a species mix representative of the tree species composition at the site (85% oak, 7.5% beech and 7.5% red maple). Litterbags were separated into two compartments, with one compartment designated for enzyme and biochemical assays and the other for DNA and RNA extraction. This separation was necessary so that litter designated for DNA/RNA analyses could be preserved immediately in the field at harvest. Litterbags were weighed, and then ten randomly selected bags were placed on the forest floor in each experimental treatment (November 2010), by first removing the layer of freshly fallen leaves, pinning the litterbags in place, and then replacing the leaves to cover the litterbags. Litterbags were harvested after approximately two years of decomposition (October 2012). At harvest, litterbags were weighed for calculation of mass loss, then the litter from the DNA/RNA compartment was removed using sanitized forceps, weighed, homogenized and flash frozen in liquid N in the field. These samples were transported to the University of New Hampshire in liquid N and stored at −80 °C until DNA and RNA extractions were performed. The remaining litter was transported on ice, stored at 4 °C, and processed within three days of harvest.

2.2. Litter mass loss, chemistry, and enzyme activities

Fresh litter was cut into ~1 × 1 cm pieces and homogenized. Moisture content was measured by drying a 1 g subsample at 60 °C for 48 h. Mass loss was determined as the difference between initial dry weight of leaf litter and dry weight at harvest. Litter cellulose and lignin (acid unhydrolyzable residue, AUR) contents were measured by the acid detergent fiber procedure (Goering and Van Soest, 1970).

We evaluated potential activities of a suite of extracellular enzymes involved in the decomposition of organic C, N, and P substrates. The methods used were described in full by van Diepen et al. (2015) and followed the protocols as outlined by Saiya-Cork et al. (2002) and DeForest (2009). Briefly, a subsample (0.5 g) of field-moist litter was homogenized in 125 ml sodium acetate buffer at pH 4.7, representing the average pH of the sampled litter. Activities of the hydrolytic enzymes leucine aminopeptidase (LAP), *N*-acetyl-glucosaminidase (NAG), acid phosphatase (PHOS), β-glucosidase (BG) and cellobiohydrolase (CBH) were measured fluorometrically using MUB-linked substrates, or AMC-linked L-leucine in the case of LAP. Activities of the oxidative enzymes phenol oxidase (PPO, ABTS) and peroxidase (PER, TMB) were measured colorimetrically using two substrates for each enzyme. Phenol oxidase activity was measured using L-3,4-dihydroxyphenylalanine (L-DOPA) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), while peroxidase was measured using L-DOPA + H_2O_2 and 3,3′,5,5′-Tetramethylbenzidine (TMB) + H_2O_2.

2.3. Fungal community characterization

We co-extracted DNA and RNA from subsamples (0.5–0.75 g) from five of the ten litterbags from each N treatment. DNA and RNA were extracted using a MoBio Powersoil Total RNA Extraction kit and DNA elution accessory kit following the factory protocols. Nucleic acid samples were purified using a Zymo One-Step PCR Inhibitor Removal column. RNA was subjected to a DNAse treatment (Ambion DNA*free*, DNA Treatment and Removal) following the standard factory protocol then reverse transcribed to complementary DNA (cDNA) using a Promega GoScript Reverse Transcription System kit following the factory protocol. The fungal internal transcribed spacer 2 (ITS2) region of the ribosomal operon was amplified from DNA and cDNA by PCR with the primers fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990), which were modified to include 10 bp molecular identification indices and the Roche 454 Lib-L adaptor A (ITS4) or B (fITS7) following the protocols described in Morrison et al. (2016). Electrophoresis was performed on a 1.5% agarose gel to confirm fragment size. PCR products were purified using AMPure paramagnetic beads to remove fragments less than 200 bp. DNA concentration in the purified PCR products was quantified by fluorometry using a Qubit fluorometer, then PCR products were combined into an equimolar amplicon library and submitted for sequencing on a ¼ plate Roche 454 FLX Titanium sequencing run at the Roy J. Carver Biotechnology Center at the University of Illinois.

2.4. Sequence data processing

Sequence data were demultiplexed, denoised, and quality filtered using AmpliconNoise (Quince et al., 2011) in QIIME 1.7.0 (Caporaso et al., 2010). Sequences less than 200 bp in length were removed, and then the ITS2 region was extracted using FungalITSextractor (Nilsson et al., 2010). ITS2 sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using usearch (Edgar, 2010) with the most abundant sequence in each OTU taken as a representative sequence. We use these OTUs as a proxy for species though it is unlikely that a single OTU clustering threshold can delineate fungal species universally (Kõljalg et al., 2013). Chimeras were identified using the uchime *de novo* method (Edgar, 2010). Representative sequences were identified taxonomically using the blast implementation of the assign_taxonomy.py script in QIIME 1.7.0 (Caporaso et al., 2010) with the dynamically clustered UNITE fungal ITS database (Kõljalg et al., 2013) used as a reference. Representative sequences that did not have a satisfactory match in the UNITE database were compared to the NCBI non-redundant nucleotide database using blastn. Blast results from NCBI were processed using the LCA algorithm in MEGAN v4.70.4 (Huson et al., 2011) with a minimum bit score of 150, low complexity filter of 0, minimum support of 3, and top 5% of matches. Chimeric OTUs, OTUs with a single sequence (global singletons), and OTUs that were assigned to taxa other than fungi were removed and excluded from further analysis, resulting in 964 total OTUs. The results of sequence processing steps and quality control measures are reported in Table S1. Samples were rarefied to a depth of 900 sequences per sample to achieve equal sequencing depth across samples, resulting in 815 of the original 964 OTUs remaining in the analysis.

2.5. Data analyses

Morphological and trophic classifications were assigned to OTUs by comparing genus names to the morphological and trophic traits assigned to fungal genera by Tedersoo et al. (2014). We assigned traits to OTUs at the family level when genus names could not be identified. This was achieved by determining the proportion of genera within a family that shared a trophic status according to Tedersoo et al. (2014). If the proportion of genera was over 95% we assigned traits to the family as a whole; if over 75%, we assigned a “putative” trait classification. Assignments were checked manually for accuracy. If OTUs could not be identified to the family level they were assigned a function of unknown (i.e., OTUs either had no taxonomy assigned or the lowest level of taxonomic assignment was above the family level). Our approach is similar to FUNGuild (Nguyen et al., 2016) in that it is based on the same primary database (derived from Tedersoo et al., 2014), but allows classification at the family level, whereas FUNGuild primarily considers genus-level assignments. However, we also performed

assignments using FUNGuild to test whether our approach gave similar results, and found that similar numbers of annotations were made using either approach. Four hundred twenty-eight OTUs were assigned a morphological status (filamentous or yeast) and 387 OTUs were assigned a trophic status (e.g., saprotroph, ectomycorrhizal, etc.) using our approach excluding "putative" assignments, which constituted 52.5% and 47.5% of 815 total OTUs, respectively, after subsampling to equal sampling depth per sample, whereas 383 classifications (47.0% of total) were made using FUNGuild (FUNGuild database accessed online at www.stbates.org on 02/23/18).

We determined the effects of simulated N deposition on litter mass loss and potential enzyme activities using ANOVA in R v3.1.0 (R Core Team, 2015). Model residuals were checked for normality and heteroscedasticity. When the assumption of normality was violated data were log-transformed, and when the assumption of heteroscedasticity was violated mean comparison was performed using a generalized least squares model allowing for heterogeneous variance structure in R package nlme (Pinheiro et al., 2015). To examine the effects of N addition on the full suite of enzyme activities and fungal community composition, we calculated Bray Curtis distance after relativization by the maximum value for each enzyme or log + 1 transformation of OTU sequence abundances and performed NMDS, adonis, and PERMDISP tests (Oksanen et al., 2015). We used Monte-Carlo randomizations and pairwise t-tests to make pairwise comparisons between treatments (Caporaso et al., 2010).

We used multiple approaches to examine how individual fungal OTUs responded to N treatment, and performed correlations of OTU relative abundance and enzyme activity as a measure of the contribution of each OTU to total enzyme activities, which is a primary fungal effect trait in regards to litter decomposition. We limited these analyses to OTUs that had at least 25% frequency across all samples in an attempt to focus on common OTUs that were more important contributors to functional processes (Magurran and Henderson, 2003; Unterseher et al., 2011). First, in order to visualize taxonomic relationships we calculated taxonomic distance (Pienkowski et al., 1998; Clarke and Warwick, 2001) based on categorical taxonomic ranks for each OTU, performed hierarchical agglomerative clustering, and plotted the resulting dendrogram. We then used Indicator Species Analysis (ISA; de Cáceres and Legendre, 2009, de Cáceres et al., 2010) to test for fungal OTUs within the total (DNA) or active (RNA) community that had consistently elevated abundance and/or frequency in one of the treatments. We also calculated Cohen's *d* effect size of the change in OTU relative abundance between the control and each fertilized treatment, calculated Euclidean distance of the effect size observations, and performed hierarchical agglomerative clustering of the matrix using Ward's d clustering method. The resulting dendrogram was pruned to achieve the smallest number of interpretable response groups which were assigned to small, medium, and large positive and negative responses as recommended by Cohen (1992). Finally, we compared the structure of the two dendrograms, describing taxonomic relationships and Cohen's d effect sizes, respectively, using the cophenetic correlation coefficient (Sokal and Rohlf, 1962) with 999 random permutations of the effect size tree to test for significance.

In order to understand how OTUs related to enzyme activity we categorized enzymes into groups based on the types of substrates they decompose – cellulases (BG, CBH), ligninases (PPO, PER, ABTS, TMB), and nutrient acquiring enzymes (LAP, NAG, PHOS) – performed principal components analysis (PCA; Oksanen et al., 2015) on each group of enzymes, and then performed linear regression using the log10 + 1 transformed sequence abundance of individual OTUs as the predictor variable and the first axis of each of the PCAs as the response variable.

Comparisons of rRNA:rDNA ratios of OTU relative abundance and species (OTU) richness in the total and active community were used to gauge activity levels of different functional groups. These two metrics are complementary as they combine presence/absence and relative abundance based metrics (Nguyen et al., 2015) – rRNA:rDNA ratios are a more direct measure of activity based on relative sequence abundance (Jones and Lennon, 2010), while species richness provides a measure of the success of different groups and does not rely on sequence relative abundance which can bias interpretation of fungal community data (Amend et al., 2010). In addition, relative sequence abundance of fungi in soils is correlated with independent measures of relative abundance in some systems, such as colonization of root tips by ectomycorrhizal fungi (Morrison et al., 2016), suggesting that community sequence data can provide a good indicator of changes in relative abundance within groups of fungi that are broadly similar morphologically.

Ribosomal RNA has been criticized as a poor indicator of active bacteria because functional ribosomes can be found in dormant bacterial cells (Blazewicz et al., 2013). However, the same is not likely true of the fungal ITS region, which is not present in mature ribosomes and is degraded within ~100 s of the initiation of transcription in *Saccharomyces cerevisiae* (Woolford and Baserga, 2013), so that ITS2 RNA is indicative of fungi that are actively producing ribosomes. To determine effects of N treatment on fungal activity, we performed linear regression with N treatments as factorial predictor variables, total (DNA) OTU sequence counts as a continuous predictor variable, and with active (RNA) OTU sequence counts as the response variable. Sequence counts were log10 + 1 transformed before performing the analysis. We tested for effects of N treatment on the whole community or within specific functional groups (filamentous saprotrophs, white-rot fungi, yeasts) on both the intercept and slope of the relationship, and when significant differences were found in the overall model we performed post-hoc tests by running pairwise models of each combination and using a Bonferonni corrected p-value to determine significance ($P < 0.0167$ was considered significant after correction for three pairwise comparisons). In this analysis we considered a change in slope to indicate a change in the activity levels of the more dominant or rare portion of the community. This analysis is similar to that performed by Jones and Lennon (2010) where, for example, a decreased slope indicates lower activity levels in the more dominant portion of the community and increased investment in dormancy as a competitive strategy. To test for differences in richness of different functional groups we used linear mixed effects models (Pinheiro et al., 2015) with N treatment, nucleic acid pool (i.e. DNA or RNA), and their interaction as main effects, and litterbag sample as a random effect to account for lack of independence arising from sampling DNA and RNA pools from the same litterbag. Assumptions of normality and homoscedasticity were met as described above.

A p-value < 0.05 was considered significant in all statistical tests. The Benjamini and Hochberg FDR procedure (Verhoeven et al., 2005) was used to control for multiple comparisons in ANOVA, linear mixed effects models and ISA analyses.

2.6. Accession numbers

Sequence data has been deposited in the NCBI Sequence Read Archive at accession number PRJNA400908.

3. Results and discussion

3.1. Litter mass loss, chemistry, and enzyme activities

Long-term N additions decreased litter mass loss by 8–17%, resulting in 20–40% more litter remaining in the N fertilized compared to control plots after two years of decay (Table 1). The proportion of cellulose remaining in the decomposed litter was not significantly different among N treatments; however, the proportion of AUR-lignin increased by 7–12% in N-amended plots. More litter remaining in N-amended plots that was enriched in recalcitrant relative to labile compounds resulted in the absolute amount of AUR-lignin being 29–58% higher in N-amended compared to control plots (Table 1). These N-induced litter mass loss and chemistry responses were

Table 1
Biochemical responses of decomposing plant litter to simulated N deposition. Standard errors are presented in parentheses, with significant differences highlighted in bold. Different letters indicate significant differences between treatments.

	Nitrogen addition level (kg N ha^{-1} yr^{-1})		
	0	50	150
Mass loss (%)	**70.3 (1.8)**[a]	**64.5 (2.4)** [ab]	**58.2 (3.2)**[b]
Total C (%)	54.06 (0.39)	54.82 (0.29)	54.97 (0.39)
Total N (%)	2.18 (0.05)	2.19 (0.03)	2.19 (0.04)
Moisture content (%)	59.61 (2.13)	56.93 (2.56)	58.60 (4.32)
AUR-lignin (%)	**41.10 (0.51)**[a]	**44.26 (1.38)** [ab]	**46.21 (1.32)**[b]
Cellulose (%)	18.41 (0.73)	16.62 (1.00)	17.37 (1.28)
Lignin remaining (g)[a]	1.22	1.57	1.93
Beta-glucosidase[b]	**1.48 (0.41)**[b]	**3.34 (0.42)**[a]	**2.94 (0.42)**[a]
Cellobiohydrolase	0.54 (0.18)	1.02 (0.15)	1.02 (0.19)
Polyphenol oxidase (L-DOPA)	**22.21 (3.84)**[a]	**16.24 (2.01)** [ab]	**11.26 (2.56)**[b]
Peroxidase (L-DOPA)	**24.50 (2.71)**[a]	**11.21 (2.18)**[b]	**7.38 (1.30)**[b]
Polyphenol oxidase (ABTS)	**76.96 (13.28)**[a]	**18.31 (4.44)**[b]	**19.73 (3.93)**[b]
Peroxidase (TMB)	**77.32 (8.98)**[a]	**41.63 (4.88)**[b]	**39.54 (6.68)**[b]
Acid phosphatase	3.47 (8.94)	3.20 (0.63)	3.74 (0.59)
N-acetyl glucosaminidase	0.76 (0.14)	1.00 (0.13)	0.70 (0.08)
Leucine aminopeptidase	10.20 (3.82)	16.53 (12.08)	20.25 (5.62)

[a] Lignin remaining was calculated from the average mass remaining of the original 10 g of litter and the average proportion AUR-lignin of remaining litter in each treatment. Statistics were not performed on this derived value.
[b] Enzyme activities are presented in units of μmol hr^{-1} g^{-1} litter except leucine aminopeptidase which is presented in units of nmol hr^{-1} g^{-1} litter.

Table 2
Relative sequence abundance and OTU richness of morphological and functional groups associated with litter after two years of decomposition. Values represent total of all OTUs, including both DNA and RNA, across experimental treatments. Values in parentheses are the percentages represented by each category based on total assigned OTUs. OTUs that could not be identified to the family level were assigned a function of "unassigned".

Morphological/ functional group	Relative sequence abundance (%)	Number of OTUs
Filamentous fungi	62.8 (94.6)	310 (72.4)
Saprotrophs[a,b]	39.4 (59.3)	145 (33.9)
white-rot fungi	4.0 (6.0)	16 (3.7)
animal parasites	0.1 (0.2)	6 (1.4)
mycoparasites	0.1 (0.2)	6 (1.4)
plant pathogens	8.2 (12.3)	65 (15.2)
lichen associates	0.1 (0.2)	4 (0.9)
ectomycorrhizal fungi	7.8 (11.7)	27 (6.3)
Unassigned[c]	3.1 (4.7)	41 (9.6)
Yeasts	3.6 (5.4)	118 (27.6)
Total assigned	66.4 (100)	428 (100)
Total unassigned	33.6	387
unknown taxonomy	14.5	210
taxonomy insufficient[d]	19.1	177

[a] White-rot fungi excluded.
[b] Includes a single OTU within the Dothioraceae family that was assigned a "putative" filamentous status but was confirmed as a saprotroph (Tedersoo et al., 2014).
[c] Includes OTUs that were assigned a filamentous morphology but either had unknown trophic status or were assigned a "putative" trophic status.
[d] Taxonomy was either not sufficiently well resolved to assign function (e.g., phylum assignment only), or function of taxon unknown.

associated with different extracellular enzyme activities; the activity of oxidative enzymes was more closely associated with litter decomposing in the control treatment while that of hydrolytic enzymes was more closely associated with N-amended plots (Fig. 1A). More specifically, long-term N enrichment significantly reduced oxidase and peroxidase enzyme activities and elevated cellulase activity (Table 1). These results are consistent with previous work at Harvard Forest showing that greater than two decades of soil N enrichment is associated with declines in soil respiration, fungal biomass, proteolytic and lignin-degrading enzyme activities, the decay of plant litter and wood, and the accumulation of soil C (Magill and Aber, 1998; Frey et al., 2004, 2014; Wallenstein et al., 2006; van Diepen et al., 2015), results that have been observed at other sites (Pregitzer et al., 2008; Zak et al., 2008; Nave et al., 2009; Janssens et al., 2010; Lovett et al., 2013).

3.2. Fungal community responses to simulated N deposition

Decomposed litter from N-amended plots had significantly different fungal communities relative to that collected from the control treatment (Fig. 1B). The total (DNA) and active (RNA) communities within treatments were also significantly different from each other; however, the N treatment effect was the dominant driver of fungal community composition. To evaluate the response of fungal taxa and functional groups to N enrichment, sequences were clustered into 815 OTUs of which 74.2% (605 OTUs) were identified taxonomically, 52.5% (428 OTUs), representing 66.4% of sequences, were assigned a morphological status, and 47.5% (387 OTUs), representing 63.3% of sequences, were assigned a trophic status (Table 2, Table S1). Morphologies assigned were filamentous fungi or yeasts, while trophic categories assigned were saprotrophs, white-rot fungi, animal parasites, mycoparasites, plant pathogens, lichen associates, and ectomycorrhizal fungi. These morphological and trophic groupings are thought to be predictive of response and effect traits that determine how fungal taxa respond to environmental changes like N addition and affect ecosystem processes such as decomposition. (Treseder and Lennon, 2015). Filamentous saprotrophs (other than white-rot fungi) dominated the litter fungal community, representing 39.4% relative abundance of all sequences (i.e., including assigned and unassigned OTUs across total (DNA) and active (RNA) communities); however, this is likely a conservative estimate given that at least some of the OTUs that could not be assigned a taxonomic group using existing databases are likely saprotrophs. Of those OTUs that could be assigned to a functional group (i.e., excluding unassigned OTUs), filamentous saprotrophs represented 59.3% relative

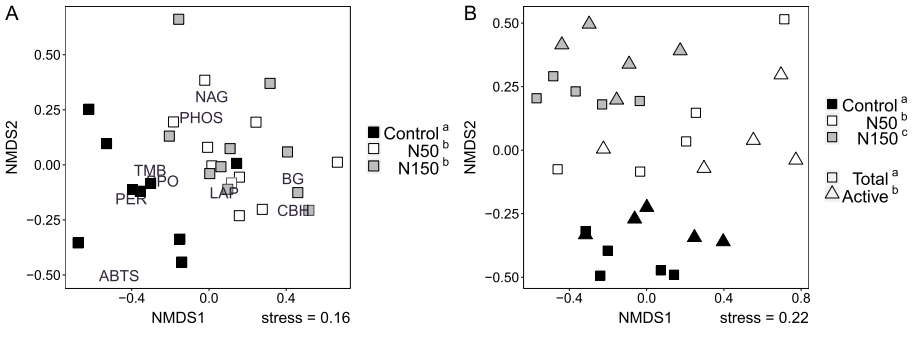

Fig. 1. Comparison of extracellular enzyme activities (A) and fungal communities (B) associated with litter decomposing for nearly two years in control and N amended plots. The NMDS was performed on log+1 transformed sequence abundances of the 815 OTUs found across N treatments. Total indicates community composition within DNA samples while Active indicates community composition within RNA samples. Different letters in the legends indicate significant differences among treatments. Abbreviations: Control: ambient N deposition; N50: 50 kg N ha^{-1} yr^{-1}; N150: 150 kg N ha^{-1} yr^{-1}; BG: beta-glucosidase; CBH: cellobiohydrolase; PPO: polyphenol oxidase (L-DOPA); PER: peroxidase (L-DOPA + H_2O_2); ABTS: polyphenol oxidase; TMB: peroxidase; LAP: leucine aminopeptidase; NAG: *N*-acetyl glucosaminidase; PHOS: acid phosphatase.

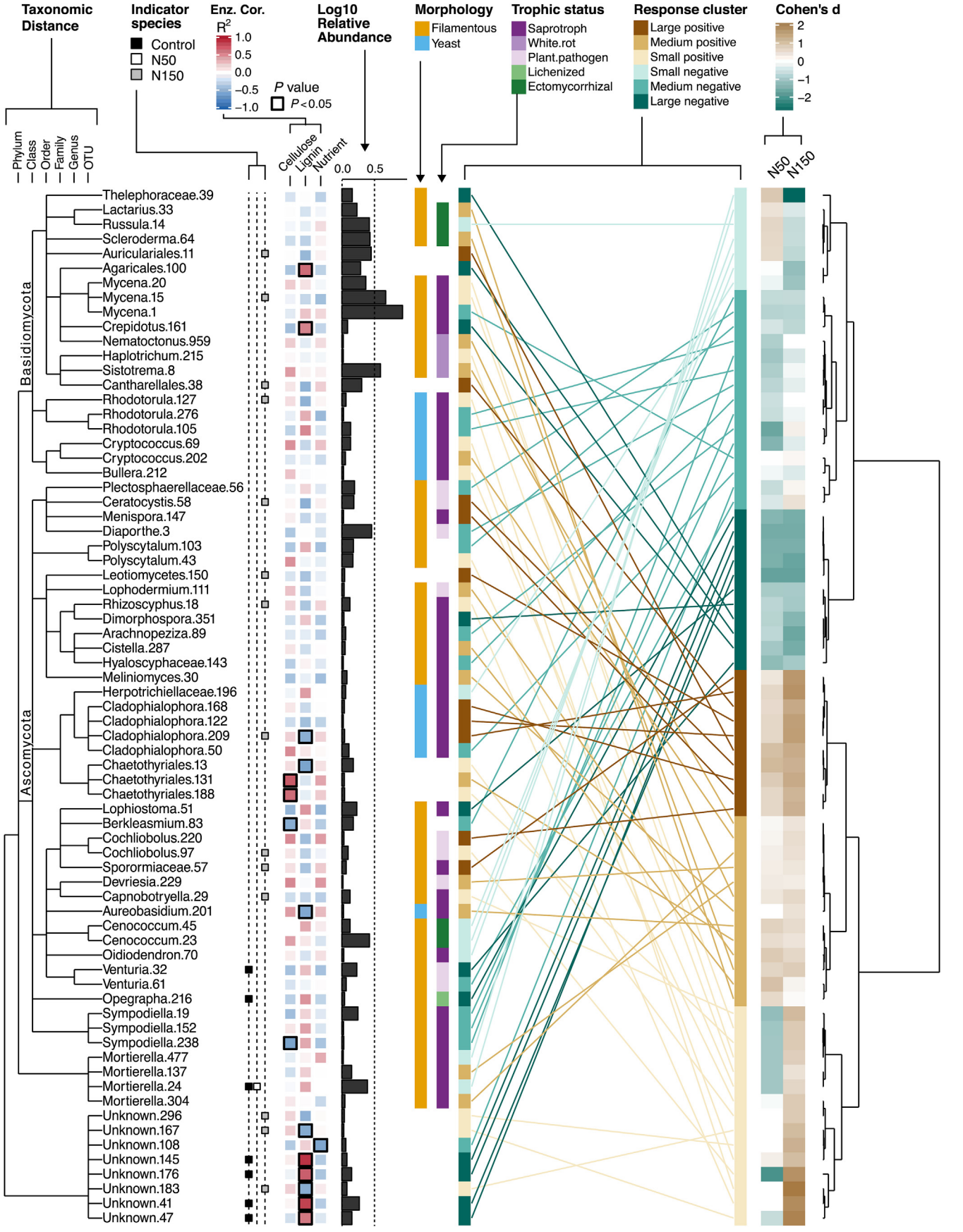

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Fig. 2. Relationships among individual fungal taxa, their respective morphological and functional groupings, enzyme activities, and N treatment response for the active (RNA) community. Those OTUs identified as indicator species of an N treatment are shown in column "indicator species". "Enz. Cor." represents the correlation between individual OTU relative abundances and the first PCA axis for cellulose (BG, CBH), lignin (PPO, PER, ABTS, TMB), and nutrient-acquiring (LAP, NAG, PHOS) enzyme activities (PCAs shown in Supplemental Fig. S4). Significant positive (red) or negative (blue) correlations are bolded. Global OTU relative abundance was $\log_{10}$ transformed for readability. Morphology (filamentous vs. yeast) and trophic status classifications were assigned according to Tedersoo et al. (2014). "Response Cluster" shows the direction (positive or negative) of OTU relative abundance in response to N treatment based on agglomerative hierarchical clustering of the N response effect size (Cohen's d). For the sake of brevity this figure only includes OTUs that had either significant statistical results in terms of ISA or enzyme activity correlations, or had morphological or functional classifications; the full figure is provided in Supplemental Fig. S1. The same analysis for the total (DNA) fungal community is shown in Supplemental Fig. S2.

sequence abundance and 33.9% of OTUs. Taxa identified as plant pathogens or ectomycorrhizal fungi each represented about 8% relative sequence abundance including assigned and unassigned OTUs, while white-rot fungi represented 4%, and animal parasites, lichenized taxa and mycoparasites each represented 0.1% relative abundance. Because of our focus on plant litter decay, we targeted three main decomposer groups for further analysis—yeasts, white-rot fungi, and "other saprotrophs" (filamentous saprotrophs other than white-rot fungi). We expected these functional groups to have strong responses to N addition because of high stress-tolerance and inorganic N uptake potential of yeasts and the association of filamentous saprotrophs and white-rot fungi with extracellular enzyme production (Treseder and Lennon, 2015), especially ligninases (Floudas et al., 2012) which are often reduced by N addition (Carreiro et al., 2000; Sinsabaugh et al., 2002; DeForest et al., 2004; Frey et al., 2004). Although the yeast morphological grouping is not comprised entirely of saprotrophic taxa and included 16 OTUs classified as mycoparasites (14% of yeast OTUs) and a single OTU classified as an animal parasite, the majority of the yeast OTUs were classified as saprotrophs (Fig. 2). We should also note that comparing relative abundances across groups is problematic, especially with regards to comparing filamentous fungi to yeasts since it is unclear how sequence abundance translates to biomass due to potential differences in rRNA gene copy number and/or ploidy (Baldrian et al., 2013). Therefore, we only directly compare relative abundances among N treatments for a specific group.

We observed no taxonomic signal in response to N addition based on cophenetic correlation analysis (active (RNA) community test statistic = −0.004, $P > 0.05$; total (DNA) community test statistic = 0.013, $P > 0.05$; indicating no correlation between taxonomic and Cohen's d trees) and as indicated by the many crossing lines between the leaves of the dendrogram describing taxonomic relationships among OTUs and the dendrogram describing clustering of taxa by response to N (Fig. 2, S1 and S2). That is, closely related taxa often had dissimilar responses to N addition, suggesting that traits controlling fungal responses to soil N enrichment are conserved at shallow taxonomic levels (e.g., genus or species) rather than at deeper taxonomic groupings (e.g., class or order) (Martiny et al., 2015). Similarly, functional group (filamentous saprotroph, white-rot fungi, or yeast) was not a strong predictor of N response except in the case of yeasts (Fig. S3). Neither filamentous saprotrophs nor white-rot fungi as a whole had significant responses to N in terms of relative abundance. Yeast relative abundance in the total community increased in the N plots, but there was no response in the active community. Yeasts, therefore, appear to share a response trait, currently unknown, that allows them to persist in high-N conditions, whereas the distribution of traits controlling responses of filamentous saprotrophs and white-rot fungi is more divergent. Such a shallow conservation of response traits within fungal species that are taxonomically and functionally similar has been previously observed in the disparate responses to soil N enrichment of ectomycorrhizal species in the genus *Russula* (Avis, 2012; Morrison et al., 2016).

Given divergent taxonomic and functional group responses we also examined responses at the level of individual taxa (OTUs). Thirty-three taxa, representing 14.5% relative sequence abundance in the active (RNA) community, showed a medium to large positive response to N addition (Fig. S1) including taxa classified as filamentous saprotrophs, white-rot fungi, and yeasts. Six taxa showing the strongest positive responses were determined to be indicator species for N enrichment (column "Indicator species" in Fig. 2). A larger number of active community taxa (48 OTUs), representing 27.5% relative sequence abundance, showed a medium to large negative response to N addition including filamentous saprotrophs and yeasts. Six of these taxa were indicator species for the control treatment and showed a large negative N response (i.e., relative abundance declined with N addition).

3.3. Correlation of taxa with enzyme activity

To examine the linkage between the relative abundance responses of individual fungal taxa and their effects on litter decomposition through extracellular enzyme production (i.e. decomposition effect traits), we grouped measured enzymes into cellulases (BG, CBH), ligninases (PPO, PER, ABTS, TMB), and nutrient acquiring enzymes (LAP, NAG, PHOS), performed principal components analysis (PCA), and regressed OTU relative abundance against the first PCA axis for each enzyme group (column "Enz. Cor" in Fig. 2). The first axis of the enzyme PCAs explained 93.2%, 75.6% and 42.8% of variation in the activities of cellulases, ligninases, and nutrient acquiring enzymes, respectively (Fig. S4). We focused this analysis on the active (RNA) community because we previously observed a strong correlation between active community composition and enzyme activities, but no correlation for the total (DNA) community (unpubl. data). Only four taxa in the active community had significant correlations with cellulase activity–two positive and two negative–and all four exhibited moderate to small responses to N addition (see "Response cluster" in Fig. 2). In contrast, 11 OTUs had significant correlations with ligninase activity, six of which showed positive correlations and a strong negative N response in terms of relative abundance. That is, taxa that correlated positively with lignin-degrading activity declined in relative abundance with N addition. These included *Crepidotus* OTU 161, which was classified as a filamentous saprotroph, Agaricales OTU 100, and four taxa with unknown taxonomy (OTUs 41, 47, 145, 176) that were all indicator species of the control treatment. These six taxa represented 3% relative sequence abundance in the active community combined. Interestingly, those taxa that showed significant correlations with enzyme activity were not always aligned with those taxa identified as indicator species, and not all taxa that declined in relative abundance in the N treatments were positively correlated with ligninase activity. A notable exception and one of the more interesting observations is that the four taxa of unknown identity showed a strong negative N response, were significantly positively correlated with lignin-degrading enzyme activity, and were indicator species for control litter. The poor characterization of fungal lignin decomposers occurring in non-woody substrates appears to be a more general phenomenon in that the majority of peroxidase genes in a maple-dominated humic soil were only distantly related to peroxidase genes from well-characterized species (Entwistle et al., 2018). The unknown taxa described here should be high priority for taxonomic identification and future analysis as they appear to play an important role in litter decay in the low N (control) environment and are strongly negatively impacted by long-term N enrichment.

Nitrogen addition appears to reduce ligninase enzyme activity by reducing the abundance of enzyme producers in the community though

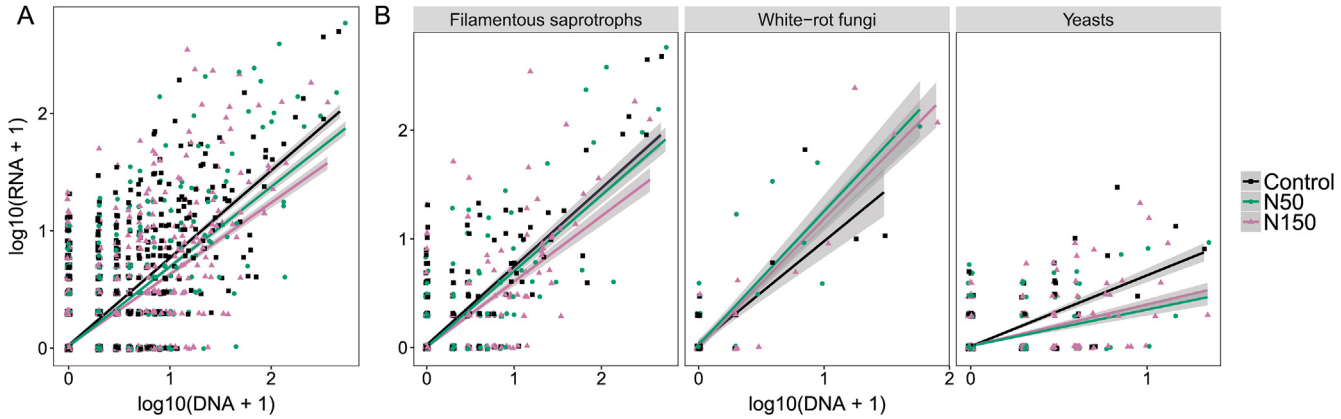

Fig. 3. Fungal activity among N treatments for (A) the whole fungal community and (B) decomposer functional groups. Points represent the sequence abundance of individual OTUs and lines represent the relationship between total (DNA) and active (RNA) community OTUs in each treatment as estimated by linear regression, with the gray band representing the 95% confidence interval as estimated by the R package ggplot2 (Wickham, 2009). Overplotting of points was avoided by allowing the points to move 0.015 units along the x- or y-axis using the position_jitter function in ggplot2.

the mechanism for this response is yet unclear. This observation is consistent with previous work showing reduced transcription of genes encoding ligninases such as laccase (Edwards et al., 2011; Hesse et al., 2015), where transcription is taken to be an indicator of activity in enzyme production. Our observation that ligninase enzyme activity was only correlated with a small subset of the fungal species that likely house ligninase genes may help explain previous observations of low correlation between total laccase gene abundance and laccase enzyme activity in soil (Hofmockel et al., 2007), where gene abundance is commonly interpreted as an indicator of potential for production of an enzyme but may not directly correlate with activity. That is, not all potential lignin decomposers responded similarly to N addition and not all were associated with ligninase activity *in situ*.

Notably, the species most highly correlated with ligninase activity were not species of white-rot fungi, which did not have a strong correlation with measured enzyme activities and tended to be negatively correlated with ligninase activity, but positively correlated with cellulase activity (Fig. 2; OTUs Nematoctonus 959, Haplotrichum 215, Sistotrema 8). White-rot species are often considered to be the strongest lignin decomposers in forest systems, and have been shown to decline in relative abundance with N addition in woody substrates while increasing in relative abundance in soil (Entwistle et al., 2017). However, white-rot fungi are more commonly associated with wood than litter, and would be more likely to forage in soil and decomposing litter secondarily (Boddy, 1999) potentially explaining the low correlation of white-rot species with ligninase activity in litter observed in the present study. Instead, the species that were most strongly associated with ligninase activity were a *Crepidotus* species, which is a common litter and organic soil-inhabiting basidiomycete (Edwards et al., 2011; Gates et al., 2011), an unidentified species within the Agaricales that may also be a filamentous saprotroph, and several previously unidentified species as noted above. We note that the filamentous saprotroph functional group was not a strong predictor of extracellular enzyme production in that species in this group had variable correlation to enzyme activity (Fig. 2, S1 and S2). The level of variation within this group suggests that though these fungi generally have a higher genetic potential for enzyme production than other functional groups (Treseder and Lennon, 2015), classifying taxa at this level in ITS barcoding studies may not be a useful predictor for functioning in decomposition processes. Litter- and organic soil-associated saprotrophic fungi have historically received less attention than other groups such as ectomycorrhizal fungi and wood-inhabiting white-rot fungi (Osono, 2007; Morin et al., 2012; Baldrian, 2016), but appear to be important drivers of ecosystem functioning in temperate forests. A better understanding of the ecology of these species is needed to predict their responses to global change.

3.4. Relative activity levels of functional groups

Dormancy is thought to maintain the viability of microbial populations by allowing them to persist through suboptimal conditions (Lennon and Jones, 2011), and may therefore be an important fungal response trait determining viability in high N conditions which reduce fungal biomass (Frey et al., 2004; Wallenstein et al., 2006; Treseder, 2008). One metric for microbial dormancy, and relative activity levels more generally, is the ratio of rRNA:rDNA, where dormancy is indicated by low ratios of rRNA:rDNA of dominant OTUs in a community (i.e. indicating a “seed bank” of dormant or quiescent cells). Cellular rRNA concentration as measured in 16S barcoding studies has been criticized as a poor indicator of active bacteria because dormant cells may contain high concentrations of mature ribosomes, and cellular ribosome content does not generally correlate with growth phase (Blazewicz et al., 2013). However, these same criticisms are largely ameliorated by the use of the fungal ITS region as a barcode, which is an intron and not a part of mature ribosomes, and should therefore be indicative of the level of rRNA transcription (i.e., ribosome production) and not the concentration of ribosomes in a cell. Here we use two metrics of activity where we first compared the relative abundance of OTUs in the active and total communities, similar to the rRNA:rDNA ratio calculated by Jones and Lennon (2010), and also compared the number of OTUs found in the total versus the active community as an indicator of the number of dormant versus active taxa.

Overall the activity of the whole fungal community was reduced in N-amended plots relative to the control treatment (Fig. 3). However, individual functional groups showed differential responses to N enrichment. The activity of saprotrophs other than white-rot fungi was significantly reduced by N addition, but only at the highest N level (N150), whereas, white-rot fungi were more active in N-amended compared to the control treatment (Fig. 3B). In contrast, the activity of yeasts was significantly reduced by N addition, with changes in activity being weighted towards the more dominant (higher relative abundance) OTUs, suggesting that yeasts employed dormancy (*sensu* Jones and Lennon, 2010) as a strategy to persist in the high-N environments.

We observed an increase in OTU richness in the highest N treatment in the total (DNA) community but not the active (RNA) community (Fig. 4A). The total community results mirrored the richness response to N addition found previously in the organic soil horizon at this site (Morrison et al., 2016) and for a temperate pine forest (Weber et al., 2013). In contrast to our finding of increased richness in the total community, in a maple-dominated temperate forest stand richness of active fungi decreased with simulated N deposition (Freedman et al., 2015). Despite this difference in the sign of the response, these findings are consistent in that N addition appears to increase the ratio of total:active

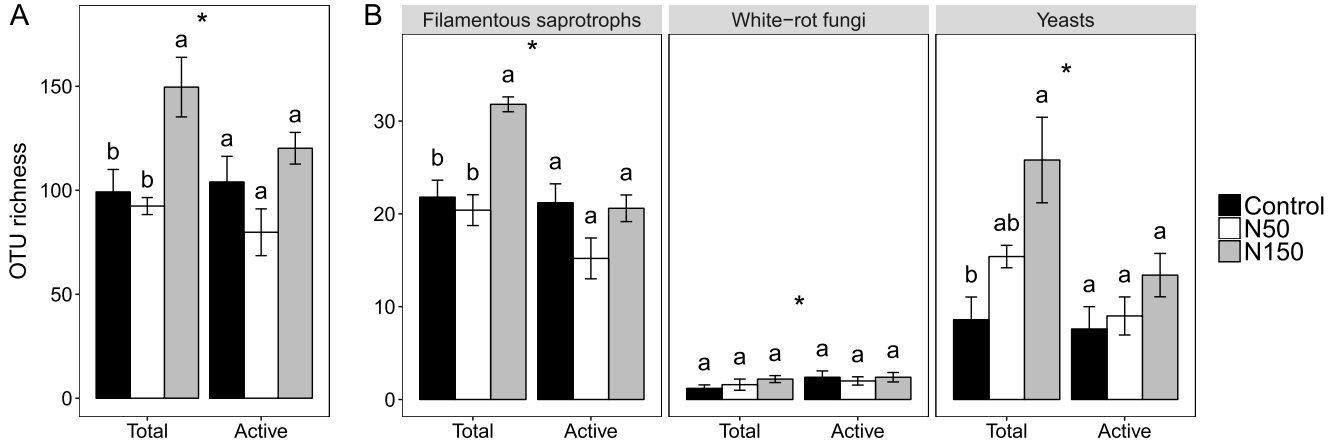

Fig. 4. OTU richness in the whole fungal community (A) and of filamentous saprotroph, white-rot fungi, and yeast functional groups (B) in the total (DNA) and active (RNA) community across N treatments. The y-axes in panels A and B have different scales. Error bars represent standard errors, and different letters indicate significant differences among treatments. An asterisk indicates a significant difference between the total and active communities. The filamentous saprotroph grouping excludes white-rot fungi.

community OTUs – whether by decreasing active community richness (Freedman et al., 2015) or increasing total community richness (present study) – suggesting that N addition consistently increases the number of dormant or otherwise inactive fungal taxa in the community overall.

Responses of OTU richness within functional groups (Fig. 4B) generally confirmed comparisons of rRNA:rDNA ratios (Fig. 3B). Filamentous saprotrophs had elevated richness only in the highest N treatment in the total community and had higher average richness in the total community overall, indicating a greater number of dormant or inactive taxa in the highest N treatment. White-rot fungi had no significant responses to N addition, but had higher richness in the active versus the total community on average. Yeasts as a group showed a strong N response, with richness increasing significantly with N addition in the total community but not the active community.

Increased yeast richness and/or relative abundance under N fertilization (Fig. 4 and Fig. S3) appear to be common responses across diverse forest ecosystems. For example, yeasts responded positively to N addition in a temperate pine forest (Weber et al., 2013) and in a boreal spruce forest (Allison et al., 2007). Together with decreased relative activity suggested by rRNA:rDNA ratios (Fig. 3), these results suggest that yeasts employ dormancy (Jones and Lennon, 2010) as a means to persist in high-N environments, a finding that is congruent with the observation that yeasts are more enriched in traits that confer stress resistance than filamentous fungi (Treseder and Lennon, 2015). This finding may help to explain the reduction in relative abundance of enzyme producers, where traits conferring stress resistance in yeasts represent a trade-off with potential for extracellular enzyme production – allowing yeasts to flourish in disturbed or stressful environments where enzyme producers are disfavored. Indeed, yeast OTUs generally had low correlation with enzyme activity, and where there were significant correlations, the majority were negative (Fig. 2, S1 and S2). In general, dormancy could facilitate competition with filamentous fungi for labile C or other resources for which yeasts are normally poor competitors by allowing them to maintain large population sizes until conditions are favorable for rapid resource consumption. Added N often reduces the contribution of plant root exudation to labile soil C pools (Carrara et al., 2018), such that stress induced by C limitation may play an important role in limiting filamentous saprotrophs. Excess N may encourage this strategy by providing excess substrates for growth – yeasts are thought to be better competitors for inorganic N because of greater genomic concentrations of inorganic N transporters (Treseder and Lennon, 2015). Alternatively, similar to observations that N fertilization increases bacterial participation in decomposition when fungi are disfavored (Freedman and Zak, 2014), general stress tolerance may favor yeasts in high-N environments where total fungal biomass is typically reduced (Frey et al., 2004; Wallenstein et al., 2006; Treseder, 2008).

4. Conclusions

We identified changes in the fungal community associated with decomposing leaf litter that can partly explain slowed decomposition rates and accumulation of organic matter in soils exposed to long-term simulated N deposition. We used a response effect trait scheme (*sensu* Lavorel and Garnier, 2002, Treseder and Lennon, 2015) to understand how changes in fungal community composition were linked with the observed ecosystem responses (Fig. 5). These responses include decreased relative abundance of the fungi most strongly associated with ligninase production, partly explaining reduced decomposition rates and lignin accumulation. However, not all fungi that were classified as filamentous saprotrophs were associated with enzyme activity *in situ*, and in particular those classified as white-rot fungi had low correlation with ligninase activity in leaf litter. These classifications, therefore, appear to be poor predictors of the function of fungal taxa in litter decomposition, suggesting that more intensive efforts in species-level characterization of the physiology of litter- and humic soil-inhabiting fungi are needed in order to accurately predict the effects of global change on community functioning. Nitrogen addition caused an increase in the species richness and relative abundance of yeasts that form a relatively inactive or dormant fungal "seed bank" (*sensu* Lennon and Jones, 2011) as a strategy for persistence in high-N soils, contribute little to enzymatic decomposition processes, and appear to employ a ruderal type strategy (Grime, 1977) that allows them to be successful in high-disturbance, high resource availability environments. We show that the success of yeasts in high-N environments was associated with declines in overall extracellular enzyme production and relative

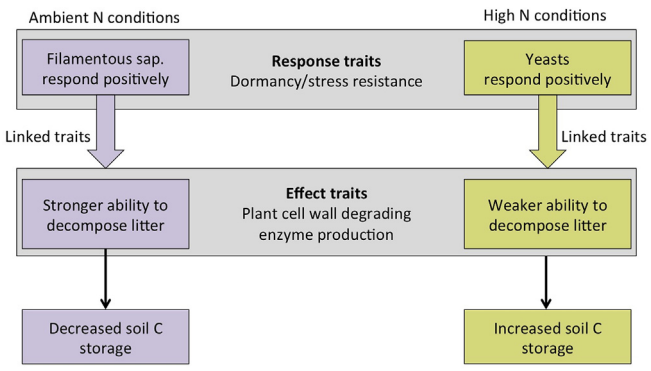

Fig. 5. Conceptual understanding of fungal responses to soil N enrichment and feedbacks to soil C storage (adapted from Treseder and Lennon, 2015).

abundance of fungal species that typically are the most productive extracellular enzyme producers. It is yet unclear whether these responses are direct effects of N addition, or whether competition between these groups for labile C or other resources plays a role in the overall community response. However, both the increased proportion of yeasts in the community (Allison et al., 2007; Weber et al., 2013) and decreased relative abundance of ligninase producers (Osono et al., 2007; Edwards et al., 2011; Hesse et al., 2015; Entwistle et al., 2017) appear to be common responses to simulated N deposition that contribute to organic matter accumulation in different types of forest ecosystems. These changes in the fungal community, in turn, were associated with reduced litter and lignin decomposition, an observation that helps explain previous research showing that organic matter accumulates in N-fertilized temperate forests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.soilbio.2018.06.027, and https://github.com/ewmorr/fungal_functional_DB.

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