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MANGANESE LIMITATION AS A
MECHANISM FOR REDUCED
DECOMPOSITION IN SOILS UNDER
LONG-TERM ATMOSPHERIC NITROGEN
DEPOSITION

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MANGANESE LIMITATION AS A MECHANISM FOR REDUCED DECOMPOSITION IN
SOILS UNDER LONG-TERM ATMOSPHERIC NITROGEN DEPOSITION

BY

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THESIS

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This thesis has been examined and approved in partial fulfillment of the requirements for the degree of Master of Science in Natural Resources by:

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ABSTRACT

MANGANESE LIMITATION AS A MECHANISM FOR REDUCED DECOMPOSITION IN SOILS UNDER LONG-TERM ATMOSPHERIC NITROGEN DEPOSITION

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University of New Hampshire, December, 2017

Long-term atmospheric nitrogen (N) deposition has been shown to reduce leaf litter and lignin decomposition in forest soils, leading to an accumulation of soil carbon. Reduced decomposition has been accompanied by altered structure and function of fungal communities, the primary decomposers in forest ecosystems; however, a mechanistic understanding of fungal responses to chronic N enrichment is lacking. A reduction in soil and litter manganese (Mn) concentrations under N enrichment (i.e., Mn limitation) may explain these observations, because Mn is a cofactor and regulator of lignin-decay enzymes produced by fungi. We conducted a 6-month incubation study to evaluate the effect of Mn availability on decomposition dynamics in chronically N-enriched soils. We measured ligninolytic enzyme activities, mass loss and lignin (% change) in litter, and characterized the whole litter fungal community by ITS2 metabarcoding. We show a significant positive correlation between Mn availability and ligninolytic enzyme activities in litter. In addition, we demonstrate an increase in the relative abundance of ‘weak’ decomposers (e.g., yeasts) under long-term N enrichment, and a reversal of this response with Mn amendment. Our results suggest that higher Mn availability may promote fungal communities better adapted to decompose lignin. We conclude that Mn limitation plays an important role in decomposition dynamics under long-term atmospheric N deposition and may represent a mechanism that explains reduced decomposition and soil C accumulation under this global change factor.

1. Introduction

Human activities have greatly increased the release of nitrogenous compounds (NO_x) to Earth's atmosphere. As a result, atmospheric nitrogen (N) deposition has risen by 200% since the start of the industrial revolution, and current rates of deposition are projected to double by 2050 in many parts of the world (Galloway et al. 2004; 2008). Long-term N deposition has been shown to slow leaf litter and lignin decomposition in forest soils, resulting in an accumulation of soil carbon (Berg & Matzner 1997; Vitousek et al. 1997; Magill & Aber 1998; Knorr et al. 2005; Pregitzer et al. 2008; Zak et al. 2011; Lovett et al. 2013; Frey et al. 2014). A long history of research has attempted to pinpoint the underlying cause of this C accumulation and has focused much attention on the microbes that regulate decomposition processes in soils, namely fungi (Frey et al. 2004; Allison et al. 2007; Freedman et al. 2015; Morrison et al. 2016). Through various field studies, simulated N deposition has been shown to reduce fungal biomass (Frey et al. 2004; Wallenstein et al. 2006; Treseder 2008), alter fungal community composition (Allison et al. 2007; Freedman et al. 2015; Morrison et al. 2016), repress lignin-decay enzyme activity (Carreiro et al. 2000; DeForest et al. 2004; Frey et al. 2004) and down-regulate the expression of genes encoding these enzymes (Edwards et al. 2011; Hesse et al. 2015). Despite extensive study, a mechanistic understanding of this repression of the soil fungal community is still lacking.

Biological processes in temperate forests are limited by N. The addition of bioavailable N via atmospheric deposition has lifted N restrictions on these processes, but has generated novel nutrient limitations (Ollinger et al. 1993; Aber et al. 1998; Crowley et al. 2012). Nitrogen-induced reductions in soil base cation (e.g., calcium) and phosphorus concentrations have received considerable attention (Gilliam et al. 1996; Peterjohn et al. 1996; Vitousek et al. 1997; Currie et al. 1999; Naples & Fisk 2010; Lovett et al. 2015; Fatemi et al. 2016). Meanwhile,

biologically essential soil metals like manganese (Mn) have largely been ignored. Despite this, Mn limitation appears to be common across simulated N deposition studies. In a meta-analysis of leaf litter chemistry across such experiments in temperate forests, van Diepen et al. (2015) showed that litter Mn concentrations are reduced by an average of 24% under N enrichment. Berg et al. (2015) synthesized the results of two N deposition experiments in boreal forests and showed a significant decline in needle litter Mn with increasing litter N concentrations. In addition, others have measured reductions in soil (Turlapati et al. 2013) and foliar (Minocha et al. 2015) Mn under long-term N enrichment.

Myriad studies have demonstrated a strong positive relationship between litter Mn concentrations and the rate and extent of decomposition (Berg 2000; Berg et al. 2007; 2010; Davey et al. 2007; Trum et al. 2015; Keiluweit et al. 2015). After synthesizing the results of 56 decomposition studies, Berg et al. (2010) concluded that Mn was the “single main factor” influencing litter decomposition rates in forests, even when compared to other litter components linked to decomposition (e.g., N, P, K, Ca and Mg). The importance of Mn in decomposition is thought to derive from its role in lignin-decay enzyme production (Perez & Jeffries 1992; Steffen et al. 2002; Hofrichter 2002). Because of this function, Mn is particularly crucial to the decomposition of lignin, a chemically complex, recalcitrant plant biopolymer comprising up to ~25% of leaf litter (Taylor et al. 1989; Berg et al. 2007; 2010). The rate at which lignin decomposes greatly influences total litter mass loss (Berg et al. 1993; Berg 2000). Thus, lignin accumulation has been attributed as the process driving C accumulation across simulated N deposition experiments (Whittinghill et al. 2012; Eisenlord et al. 2013; Frey et al. 2014). By repressing lignin decomposition, reductions in Mn availability (i.e., Mn limitation) under N deposition may thereby contribute to soil C accumulation.

Manganese influences lignin decay via two distinct mechanisms: (1) enhancing the activity of lignin-decay enzymes, and (2) oxidizing lignin via redox reactions (i.e., Mn (III/IV) oxidizes lignin and is reduced). The latter mechanism depends on the former, because lignin-decay enzymes oxidize bioavailable Mn (II), thereby generating Mn (III/IV) oxides. Enzymatic Mn oxidation is performed primarily by fungi (Hofrichter 2002; Hansel et al. 2012), the dominant decomposers in forest ecosystems (Schneider et al. 2012). The most common lignin-decay (i.e., ligninolytic) enzyme produced by wood and litter-decomposing fungi is Mn peroxidase, which depends on Mn for its activity (Hofrichter 2002). The activities of other ligninolytic enzymes are enhanced by Mn, including heme peroxidases (lignin peroxidase; versatile peroxidase) and phenol oxidase (e.g., laccase; Dashtban 2010; Hatakka & Hammel 2010). Manganese peroxidase and laccase influence lignin decay by oxidizing Mn (II) to Mn (III/IV; Hofrichter 2002; Schlosser & Hofer 2002). Manganese (III/IV) oxides are some of the strongest oxidants in nature, and can rapidly depolymerize phenolic components of lignin (Sunda & Kieber 1994; Hansel et al. 2012; Remucal & Ginder-Vogel 2014). Lignin peroxidase and versatile peroxidase can decompose lignin directly by opening its ring structures, and this activity is stimulated by Mn (Archibald & Roy 1992; Perez & Jeffries 1992; Dashtban 2010; Hatakka & Hammel 2010).

Due to its heterogeneous and chemically complex structure, lignin tends to accumulate during late-stage decomposition, and its decay is considered the rate-limiting step of the decomposition process (Berg 2000; 2014). To accomplish efficient lignin decomposition, fungi use a suite of enzymes (discussed above) that target distinct components of the lignin polymer (Rayner & Boddy 1988; Dashtban 2010; Hatakka & Hammel 2010). The only group of fungi known to produce the entire suite of enzymes, and thus capable of complete lignin

decomposition to CO₂, are white-rot fungi (Hatakka 1994; Hofrichter 2002; Dashtban 2010). Therefore, white-rot fungi are considered the most efficient ('strong') lignin decomposers in the fungal community. In addition, white-rot fungi are members of the Agaricomycetes within the Basidiomycota, and the ability to produce class II peroxidases (manganese peroxidase; lignin peroxidase; versatile peroxidase) is restricted to the Agaricomycetes (Floudas et al. 2012; Bodeker et al. 2014). Although the Agaricomycetes encompass ectomycorrhizal (Bodeker et al. 2009; 2014) and pathogenic (Yakolev et al. 2013) fungal taxa, genes encoding the class II peroxidases are most abundant (i.e., highest copy numbers) in white-rot fungi (Floudas et al. 2012; Kohler et al. 2015), contributing to the efficiency of lignin decay by these organisms.

In contrast, other fungal functional groups are unable (e.g., yeasts) or less efficient (e.g., pathotrophs with the capacity for facultative saprotrophy) at decomposing lignin (Eastwood et al. 2011; Aguilar-Trigueros et al. 2014; Kohler et al. 2015; Treseder & Lennon 2015). Yeasts are a group of unicellular saprotrophs associated with the decomposition of highly labile C compounds (e.g., sugars; Botha et al. 2010; Treseder & Lennon 2015). Yeasts do not possess genes encoding lignin-decay enzymes and are thus considered 'weak' decomposers. Recently, Morrison et al. (in prep) demonstrated an increase in the relative abundance of yeasts under long-term N deposition, concluding that this increase in 'weak' decomposers may explain reductions in ligninolytic enzyme activity and lignin decay. Due to the fundamental role of Mn in the production of ligninolytic enzymes, Mn limitation under N deposition could be an important factor shaping the relative proportion of 'strong' vs. 'weak' decomposers in the fungal community.

We hypothesize that (1) Mn limitation contributes to reduced lignin and leaf litter decomposition under long-term N deposition; and (2) Mn limitation is a factor underlying shifts in fungal community composition that have been observed in long-term simulated N deposition

experiments (e.g., Morrison et al. 2016; in prep). To test these hypotheses, we (1) quantified Mn concentrations in leaf litter at the Harvard Forest Chronic Nitrogen Amendment Study, hereafter CNAS (Petersham, MA, USA); and (2) conducted an incubation experiment in which we applied Mn amendments to soils from this site. After ~6 months of incubation, we evaluated litter mass loss, the percent change in litter lignin, and the potential activities of ligninolytic enzymes (peroxidase and phenol oxidase). Further, we characterized fungal community composition by ITS2 metabarcoding of the whole litter fungal community. We link our community data to recent work characterizing the total and active litter fungal communities at CNAS (Morrison et al. in prep) with the goal of describing the role that Mn plays in structuring fungal communities.

2. Materials and methods

2.1. Experimental site

Samples were collected from the Chronic Nitrogen Amendment Study at the Harvard Forest Long-Term Ecological Research (LTER) site (Petersham, MA, USA; 42° 30'N, 72°10'W). This experiment was established in 1988 and had received 27 consecutive years of fertilization prior to our sampling (June 2015). Soils are classified as Typic Dystrudepts of the Gloucester series (Peterjohn et al. 1994) and are stony-to-sandy loam in texture. Experimental plots are in a mixed-oak forest dominated by black and red oak (*Quercus velutina* and *Quercus rubra*, respectively), with some interspersed black birch (*Betula lenta*), red maple (*Acer rubrum*) and American beech (*Fagus grandifolia*). Understory vegetation includes seedlings/saplings of striped maple (*Acer pensylvanicum*) and American beech as well as some herbaceous shrubs. Mean annual precipitation is 110 mm and mean temperatures range from 20°C in July to -7°C in January. Ambient atmospheric N deposition averages 8-10 kg N ha⁻¹ yr⁻¹ (Schwede and Lear

2014). This experiment is comprised of three 30 m x 30 m megaplots that receive one of the following N treatments as liquid NH_4NO_3 fertilizer: N0 (control; no N addition), N50 (50 kg N $\text{ha}^{-1} \text{yr}^{-1}$), and N150 (150 kg N $\text{ha}^{-1} \text{yr}^{-1}$). Each megaplot is divided into thirty-six 5 m x 5 m subplots. The outermost row of subplots is excluded from each megaplot to prevent the influence of an edge effect, leaving sixteen active 5 m x 5 m subplots per megaplot (N treatment). Nitrogen treatments were established to evaluate the effects of N enrichment on ecosystem processes. Globally, N deposition rates are projected to double by 2050 with some areas experiencing up to 60 kg N $\text{ha}^{-1} \text{yr}^{-1}$ (Galloway et al. 2008). Thus, CNAS uses chronic N amendments as a space-for-time substitution to simulate future impacts of elevated N (N50 treatment) and N saturation (N150 treatment; Aber et al. 1998; Aber & Magill 2004) on ecosystem processes.

2.2 Sample collection

We conducted a two-factor factorial incubation experiment with samples collected from CNAS that we amended with Mn (N x Mn factorial). We first describe sample collection for this incubation experiment. Then, we describe the collection of soils and leaf litter that were analyzed to determine the initial chemistry of the soils and litter we used in our incubations.

In preparation for the incubation experiment, intact soil cores (5 x 10 cm) were collected from six randomly selected subplots of each N treatment at CNAS. These cores contained approximately 2/3 organic horizon soil, and 1/3 mineral soil, although the exact proportions varied based on the depth of the O horizon at each sampling location. These cores were transported in 10 oz. plastic cups on ice to the University of New Hampshire where they were stored at 4°C until incubation initiation. In addition, newly shed leaf litter was collected from each subplot to serve as the litter layer in the incubation ('undecomposed litter'). This leaf litter

was later analyzed to evaluate initial chemistry of these litter inputs.

To evaluate the starting chemistry of soils in our incubation, additional organic horizon and mineral soil samples were collected from each N treatment. Organic soils (O_e/O_a) were retrieved by removing 20 x 20 cm forest floor squares to the depth of the mineral soil. Leaf litter (O_i) was carefully removed from the top of each square. Mineral soil was collected from beneath each forest floor square using a 5 cm diam. x 10 cm deep corer. Samples were transported to the lab where organic and mineral soils were sieved (<2 mm) to remove coarse woody debris, roots and rocks.

2.3 Leaf litter and soil chemistry

To characterize the initial chemistry of litter inputs to our incubation, undecomposed litter nutrient (Ca, Mn, Mg, P, K, Al, B, Cu, Fe and Zn) concentrations were determined via ICP-AES. Samples were prepared for analysis by dry ashing and acid digestion in a solution of 50% hydrochloric acid (Kalra and Maynard 1989). Litter C and N were assessed via dry combustion using a Perkin-Elmer CHN Series II 2400 Elemental Analyzer (Perkin Elmer Inc., Waltham, MA). Litter organic matter chemistry was characterized using pyrolysis gas chromatography and mass spectrometry (py-GCMS) to determine the relative abundance of lignin, phenols and other aromatic compounds. Briefly, samples were pyrolyzed at 600°C on a CDS Pyroprobe 5150 pyrolyzer (CDS Analytical Inc., Oxford, PA) and decomposed products were transferred to a Thermo Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, Austin, TX) and subsequently to a Polaris Q mass spectrometer (Thermo Fisher Scientific). Here, products were ionized and detected using an Automated Mass Spectral Deconvolution and Identification System (AMDIS, V 2.69). Recorded peaks were classified using the National Institute of

Standards and Technology (NIST; accessed: March 2017) compound library and the relative percentages of organic matter compounds were calculated as in Grandy et al. (2009) and Wickings et al. (2011).

Soil pH was quantified in distilled water (1:10 wt/vol) using a digital pH meter. Soil moisture was determined by oven drying organic horizon material at 60°C for 48 hours and mineral soils at 105°C for 24 hours. Exchangeable soil acidity was evaluated by soil extraction with 1M KCl and subsequent titration with dilute NaOH. Cation exchange capacity (CEC) was calculated thereafter using the equation, $CEC = \text{exchangeable acidity (meq)} + \text{exchangeable base cations (meq; Ca, Mg, K, Na)}$.

2.4 Extracellular Enzyme Activity

Within 24 hours of sampling, leaf litter collected from each forest floor square was assessed for the activities of two ligninolytic enzymes. Fresh leaf litter was homogenized, subsampled and analyzed using colorimetric assay techniques outlined by Saiya-Cork et al. (2002). Peroxidase activity was assessed with the substrate 3,3'-5,5'-Tetramethylbenzidine (TMB + 0.3% hydrogen peroxide [H₂O₂]; Johnsen and Jacobsen 2008). Phenol oxidase activity was evaluated using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; Floch et al. 2007). For assays, ~0.5 g fresh leaf litter were homogenized in 125 mL of 50 mM sodium acetate buffer (pH = 4.7) in a blender for 30 seconds to form leaf litter slurries. Litter slurries were transferred to 96-well microplates along with enzyme-specific substrates (TMB, ABTS). Microplates were incubated at 25°C for 15 minutes (ABTS) or 20 minutes (TMB + H₂O₂), representing the time necessary to elicit maximal potential enzyme activity (substrate-specific; determined by Vmax test). Following incubation, absorbance was determined using a BioTek

Synergy™HT Multi-detection Microplate Reader with emission wavelengths set at either 420 nm (ABTS) or 450 nm (TMB + H₂O₂). Enzyme activities were standardized by litter moisture contents, which were determined by drying litter at 60°C for 48 hours. Final enzyme activity values were calculated following methods outlined by DeForest (2009) and are reported as $\mu\text{mole substrate per hour per gram dry litter } (\mu\text{mol h}^{-1} \text{ g}^{-1})$.

2.5 Incubation assembly

Intact soil cores, which included both organic and mineral soil, were incubated in 10 oz. plastic cups set inside one-gallon glass pickle jars. Each soil core was topped with a mesh compartment (0.3 mm pores) containing 1.6 g of dry, undecomposed oak leaf litter (2 cm x 2 cm pieces), representing the dominant litter type at CNAS (~85% of trees in the plots are oak). The use of intact soil cores was intended to simulate a realistic medium for leaf litter decomposition (a mini soil profile) and mesh was used to facilitate the complete removal of litter at the end of the incubation. The quantity of oak litter added represents approximately two-times the average litter mass found at CNAS on an area basis. Based on previous CNAS decomposition studies, we estimated that we would need ~1.6 g litter to ensure that decomposition could continue for six months and enough litter would remain for our planned analyses.

Soil cores (n = 48) were incubated at 25°C for ~6 months (167 days) to evaluate the role of Mn in mid- to late-stage litter decomposition. Before incubation, soil moisture was standardized to 60% water-holding capacity and 60% field capacity across the forest floor (O_e/O_a) and mineral soil components of each core, respectively. Briefly, water-holding capacity of forest floor material was determined for six replicates of each N treatment as in Kittredge (1955) and Naeth et al. (1991) and mineral soil field capacity was assessed following standard

methods (*sensu* Veihmeyer & Hendrickson 1949). Once soil cores were assembled for incubation, Mn amendments were surface applied to leaf litter. Mn amendments were added using liquid solutions of manganese sulfate tetrahydrate ($\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$; $223.05 \text{ g mol}^{-1}$). We anticipated that it would take a long time for Mn amendments to reverse the effects of ~30 years of N fertilization, especially in the strongly acid soils of CNAS; thus, we chose to repeatedly apply Mn via once monthly amendments (0.5 ml applications; 6 applications total). These amendments served as a space-for-time substitution (like that of N enrichment at CNAS) to simulate conditions where Mn was no longer limiting. One of three Mn amendments was applied: ambient (no additional Mn, only native litter Mn); low Mn; or high Mn. The ‘low’ rate of Mn amendment was based on initial Mn concentrations in control N litter ($\sim 3 \text{ mg g}^{-1}$ litter; Table 1). The high Mn amendment rate was 2x that of the low Mn rate. Over the course of the incubation, more Mn was added to chronically N-fertilized samples than control N samples (Table 3) because these samples were assumed to be more Mn-deficient due to lower initial litter Mn concentrations (Table 1). Due to an error in our Mn application scheme, we have excluded the control N, low Mn treatment pair. As such, all of our analyses are based on 8 treatment pairs (3 Mn treatments x 3 N treatments – 1 = 8 treatment pairs).

2.6 Incubation harvest and analysis

Following nearly 6 months of incubation, mesh dividers were removed and remaining litter was weighed to determine mass loss. Litter was then homogenized and subsampled for analysis. A subsample was immediately lyophilized to determine litter moisture at time of harvest. This subsample was later analyzed via ICP-AES to determine total litter Mn concentrations as described in section 2.3. A separate ~0.5 g subsample of fresh litter was

collected for extracellular enzyme analysis. Litter samples were slurried with 50 mM sodium acetate buffer and the activities of peroxidase and phenol oxidase were assayed as described in section 2.4. Final enzyme activity values were standardized on a sample by sample basis using the dry weight equivalent of litter at harvest. Lastly, a third subsample of fresh litter (~0.75 g) was collected in microcentrifuge tubes, flash frozen with liquid nitrogen, and stored at -80°C for downstream DNA extraction.

2.7 DNA extraction and amplification

Following incubation, DNA was extracted from decomposed leaf litter (0.25 g) using the DNeasy PowerMax Soil Kit (Qiagen Sciences Inc., Germantown, MD). The ITS2 region (Schoch et al. 2012) was amplified using fungal specific primers *fITS7* (Ihrmark et al. 2012) and *ITS4* (White et al. 1990) which contained an Illumina adaptor sequence, an 8 bp pad sequence, a 2 bp linker sequence and one of 48 (n = 48) unique 8 bp index sequences (Morrison et al. 2016). Triplicate PCR reactions were conducted under the conditions outlined by Caporaso et al. 2011: 10 µM *fITS7* (0.5 µL), 10 µM *ITS4* (0.5 µL), Five Prime Hot Master Mix (10 µL), PCR grade water (13 µL) and template DNA (1 µL). DNA was amplified in a Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA) following the temperature cycles used by Caporaso et al. 2011. Successful DNA amplification and fragment size were confirmed on a 1.5% agarose gel. PCR products were purified using the AxyPrep MAG PCR Clean-up Kit (Corning, Tewksbury, MA). Cleaned PCR products were assessed fluorometrically with a Qubit® 3.0 Fluorometer to quantify DNA concentrations (Life Technologies, Grand Island, NY). An equimolar amplicon library was generated and DNA sequencing was conducted by Illumina MiSeq (v2; 2 x 250 bp chemistry) at the Indiana University Center for Genomics and

Bioinformatics (Bloomington, IN). Sequences were de-multiplexed during data extraction from the sequencer.

2.8 Bioinformatics

Sequencing adaptors and PCR primers were removed from sequence reads using Trimmomatic v. 0.36 (Bolger et al. 2014) prior to downstream filtering. Paired end reads were merged using the 'UPARSE' command in USEARCH v. 10 ('usearch10'; Edgar 2013) and bases with Phred scores < 3 removed (with specification 'fastq_minqual=3'). Merged sequences were quality filtered with the 'fastq_filter' function in usearch10 (maximum expected error rate setting of 0.5) and all sequences < 150 bp were removed. Filtered sequences were dereplicated using the usearch10 'derep_fulllength' command and input into ITSx (Bengtsson-Palme et al. 2013) for ITS2 region extraction. Extracted ITS2 sequences were clustered based on a similarity threshold of 97% using the usearch10 cluster_otus algorithm to generate operational taxonomic units (OTUs; Edgar 2010). Fungal taxonomy was assigned in QIIME (Caporaso et al. 2010) by comparing the representative sequence of each OTU to the UNITE database (Kõljalg et al. 2013). Abundant representative sequences without a match in the UNITE database were blasted against the NCBI non-redundant nucleotide database using the 'blastn' search option. Blast searches were performed manually for representative sequences with >500 sequences per OTU in our dataset and hits were accepted if they met the following standards: a minimum bit score of 200; E value < 0.0001 (although, ours ranged from $10e^{-70}$ to $10e^{-50}$); and query coverage $\geq 98\%$ (E-value based on minimum accepted E-value in QIIME; Caporaso et al. 2010; see also Pearson 2013). Singletons (OTUs with a single sequence) and non-fungal OTUs were removed, and samples were rarefied to a depth of 9,278 sequences, representing the minimum number of

sequences in any sample. This left 569 fungal OTUs in our dataset for analysis. Relative sequence abundance of each OTU was calculated and this metric was used for comparisons of community composition across treatments. Because DNA extraction efficiency varies from sample to sample, absolute sequence abundance is not an accurate metric by which to compare OTU abundance, and calculating relative abundances is thus necessary. Thereafter, OTUs with known taxonomies were parsed into functional groups using database curation by Tedersoo et al. (2014). For the purpose of comparison to Morrison et al. (in prep), a functional analysis of litter fungal communities across N treatments at CNAS, we parsed our community data into the following functional groups: filamentous saprotrophs, yeasts, white-rot fungi, plant pathogens and ectomycorrhizal fungi following criteria outlined in this paper. Classification of OTUs as yeasts vs. non-yeasts is readily accomplished using the Tedersoo et al. (2014) database, which is why we selected this classification approach over more recent fungal functional classification tools such as FunGUILD (Nguyen et al 2015).

2.9 Statistical analyses

All statistical analyses were performed in R 3.3.1 (R Core Team 2016). The effects of field N treatments on soil C and N stocks, pH, exchangeable acidity, litter micro and macronutrient concentrations, total “aromatics” (lignin, phenols, and aromatic compounds) and ligninolytic enzyme activities were evaluated using one-way ANOVA and post-hoc Tukey HSD tests. Levene’s test of homogeneity of variances and the Shapiro-Wilk normality test were used to assess homoscedasticity and the normality of residuals, respectively. When homoscedasticity was not achieved, data were evaluated using non-parametric Kruskal-Wallis and post-hoc Dunn tests.

In the two-factor factorial laboratory experiment, the effects of Mn and N treatments on mass loss, percent change in lignin and ligninolytic enzyme activities were assessed using two-way ANOVA. Ligninolytic enzymes were evaluated as the summation of peroxidase (PER) and phenol oxidase (POX) activities, i.e., PER + POX. Enzyme activity values were square root transformed to meet assumptions of normality and homoscedasticity of residuals. The relative abundance of lignin, phenols and aromatics were summed and were analyzed as one common term, total “aromatics”. The percent change in total aromatics was calculated and differences across Mn and N treatments were evaluated with two-way ANOVA.

To visualize the effects of Mn and N treatments on fungal community composition, we performed non-metric multidimensional scaling (NMDS) of Bray-Curtis distances calculated from the $\log(x+1)$ -transformed OTU relative abundance values (‘vegan’ package; Oksanen et al. 2015). To confirm the statistical significance of the NMDS ordination, we conducted permutational multivariate analysis of variance (PerMANOVA; Anderson 2001). To assess the influence of Mn and N treatments on individual OTUs or fungal functional groups, we conducted univariate two-way ANOVA on OTU or functional group relative abundance. Prior to analysis, data were arcsine-transformed to meet homogeneity of variance assumptions. If the analysis resulted in a significant interaction between Mn and N, the simple effects of Mn and N were evaluated independently using Tukey HSD tests. If there was no significant interaction term, only the main effects were tested. We evaluated the relative abundance of individual OTUs for significant differences across treatments if they represented $\geq 10\%$ of OTUs in any given treatment pair. This cutoff encompassed OTUs with a minimum average relative abundance across all treatments of 0.068% (i.e., we evaluated down to a very low presence across the entire dataset).

3. Results

We conducted a two-factor factorial incubation experiment with samples from a long-term simulated N deposition experiment that we amended with Mn (N x Mn factorial). We first describe the effects of the chronic N field treatments on *in situ* leaf litter and soil chemistry to give context for how these varied in our incubation based on the N treatment they were sourced from. Then, we present the results of our incubation experiment, in which we describe the effects of Mn amendments on decomposition dynamics and fungal community characteristics. We also describe how the legacy effects of chronic N enrichment influenced these variables.

3.1 *In situ* litter and soil chemistry

Chronic N increased litter N concentrations by 25%, but significantly reduced litter Mn, as well as the base cations Ca, Mg, and K (Table 1). Manganese was reduced by ~57% and ~72% under N50 and N150 conditions, respectively, and was one of the elements most affected by N. In addition, chronic N significantly reduced the abundance of aromatic compounds, representing the sum total of lignin, phenols and other aromatics. Aromatics declined by ~44% under chronic N enrichment, from 28.6% (N0) to 16.1% (N150) of litter components (Fig. S3a). We assayed leaf litter from the field N treatments and found a suppression of phenol oxidase activity ($P = 0.0004$) with N addition, but no difference in peroxidase activity (Table 1).

Soil pH of the organic horizon declined from approximately 4.03 in the N0 treatment to 3.38 in N150 ($P = 0.009$; Table 2). Mineral soil pH followed a similar trend, decreasing from 4.66 (N0) to 3.93 (N150; $P = 0.0005$). Correspondingly, exchangeable soil acidity increased up to ~29% in the organic horizon and 65% in mineral soil with N addition ($P = 0.005$ and $P =$

0.001, respectively). Soil CEC also increased in the mineral horizon ($P = 0.002$; Table 2). These data represent initial litter and soil chemistry prior to incubation initiation.

3.2 Incubation experiment

3.2.1 Mn accumulation in decomposed litter

To confirm that Mn amendments elevated litter Mn concentrations as intended, we analyzed the total Mn concentration in leaf litter at harvest (i.e., after 6 months of decomposition). All litter accumulated Mn, and Mn-amended samples accumulated the most (Fig. S1). There was a relationship between the rate of Mn accumulation and field N enrichment level: samples from the highest N treatment accumulated and/or retained less Mn than control and intermediate N (N50) treatments.

3.2.2 Litter mass loss and ligninolytic enzyme activities

Long-term N enrichment reduced total litter mass loss and lignin loss ($P = 0.003$ and $P = 0.001$, respectively; Table 3). Counter to expectations, Mn amendments did not significantly increase decomposition. However, there was a trend towards increased mass loss with increasing Mn ($P = 0.07$; two-way ANOVA). This trend was visually apparent in the N0 and N50 treatments (Fig. S2); but, there was no apparent change in mass loss with Mn in the N150 treatment. Lignin percent change varied by N treatment, but not by Mn treatment (Table 3). Chronic N enrichment increased lignin retention in the highest N treatment ($P = 0.001$; two-way ANOVA).

Long-term N enrichment repressed ligninolytic enzyme activity, particularly in the highest N (N150) treatment. However, Mn amendments significantly elevated the activity of

these enzymes ($P < 0.0001$; two-way ANOVA; Fig 1a), releasing N-induced limitations on their activity. There was a significant correlation between enzyme activity and total Mn ($P < 0.0001$; $R^2 = 0.481$), where total Mn represents the initial litter Mn concentration plus the cumulative amount of Mn added over the course of the incubation. Overall enzyme activity levels were lowest in the highest N treatment (N150); however, this treatment experienced the greatest percent increase in ligninolytic enzyme activity in response to the high Mn amendment (relative to ambient Mn levels; Table 3). We present the ligninolytic enzyme data as the sum of peroxidase (PER) and phenol oxidase (POX) activities (Fig. 1a; 1b) because PER and POX responded similarly to Mn amendments.

3.2.3 Fungal community composition

During incubation, both long-term N enrichment and Mn amendments restructured the litter fungal community (Fig. 2; N: $P = 0.001$; Mn: $P = 0.002$; PerMANOVA). There was a significant interaction between the effects of N and Mn on filamentous saprotroph relative abundance, the most dominant fungal functional group in our dataset (Fig. 3a). Thus, we have analyzed the simple effects of N and Mn separately. Chronic N enrichment reduced the relative abundance of filamentous saprotrophs from ~94% to ~68% (ambient Mn samples; Tukey HSD test; Fig. 3a). Manganese amendments compounded this effect in the highest N treatment (N150), further decreasing filamentous saprotroph relative abundance from ~68% (ambient Mn) to ~37% (high Mn; Tukey HSD test; Fig. 3a).

After filamentous saprotrophs, the second most abundant functional group was comprised of taxa for which there was no functional annotation (i.e., no assigned trophic status; Fig. 3b). While filamentous saprotrophs declined in response to high N and Mn availability, the “no

functional annotation” category increased in relative abundance, reaching its highest point in the highest N, highest Mn treatment, where almost 60% of taxa had no functional annotation. Notably, one OTU comprised the majority of this “no functional annotation” group, *Coccinonectria rusci* (OTU2). We present the simple effects of N and Mn on the relative abundance of this organism due to a significant interaction in the two-way ANOVA (Fig. 4). The relative abundance of *C. rusci* increased under chronic N enrichment from ~0.14% (N0) to ~6.13% (N150) across ambient Mn samples (Tukey HSD test). *C. rusci* responded even more strongly to the combined effects of added N and Mn, increasing from ~6.13% in ambient Mn samples to 53.3% in highest N, highest Mn samples (Fig. 4).

Chronic N addition increased the relative abundance of pathotrophs from ~3% (N0) to ~16% (N150) of the fungal community ($P = 0.008$; two-way ANOVA; Fig. 3c). In contrast, Mn amendments reduced pathotroph abundance from 16% to 5% relative abundance ($P = 0.023$; two-way ANOVA). This decline with Mn addition returned pathotroph relative abundance to levels comparable to those of N0 and N50 treatments (Fig. 3c). There was no difference in pathotroph abundance between N0 and N50 treatments. All described pathotrophs were classified as Ascomycetes.

Similar to the pathotroph response, chronic N addition increased the relative abundance of yeast taxa in the highest N treatment (ambient Mn samples; note significant N*Mn interaction; Fig. 3d). Yeast relative abundance was ~0.27% in N0 samples (ambient Mn), compared to ~1.50% in N150 samples (ambient Mn). Adding Mn counteracted this effect, reducing the average relative abundance of yeasts back to 0.27% in the highest N, highest Mn treatment. The relative abundance of white-rot fungi was elevated in the highest N treatment ($P = 0.001$), but was unaffected by Mn amendment (Fig. S6).

4. Discussion

We show that long-term simulated N deposition reduces the concentration of Mn available to support fungal decomposition of leaf litter. Further, we demonstrate that the addition of bioavailable Mn significantly increases the activities of the ligninolytic enzymes peroxidase and phenol oxidase. Concomitant with this change in microbial function, we show a shift in fungal community composition with Mn addition, which helps to explain the functional response we observed.

In a recent meta-analysis of leaf litter chemistry under long-term N enrichment, van Diepen et al. (2015) demonstrated a decline in litter Mn concentrations with increasing N fertilization in temperate forests. Here, we show the same trend in litter Mn at Harvard Forest. Others have shown that Mn is reduced in soils (Turlapati et al. 2013) and foliage (Minocha et al. 2015) at this site. We hypothesize that reduced Mn availability is a result of N-induced leaching of Mn from soils. Nutrient (i.e., Mn) recycling in organic horizon soils is the primary source of plant-available Mn (Graham et al. 1988); Mn leaching from soils may thereby reduce foliar and litter Mn concentrations.

Manganese leaching may be driven by reductions in soil pH. We documented a decline in both organic horizon and mineral soil pH with chronic N additions. Acidification is a common effect of simulated N deposition, because microbial uptake and nitrification of ammonium from fertilizers generates hydrogen ions, thereby decreasing pH (Vitousek et al. 1997). As soil pH declines, Mn becomes solubilized (Mn^{2+}) in soil solution, making it more bioavailable (Sims 1986; Kogelmann & Sharpe 2006). However, at very low pH, like those observed in our study (pH 3-4), soil cation exchange sites are commonly occupied by strongly-bound Al^{3+} cations (Bowman et al. 2008; Rengel 2015) and Mn^{2+} is susceptible to leaching. Reductions in soil pH

below pH 4 have been shown to induce Mn leaching (Ha et al. 2011). Furthermore, nitrate (NO_3^-) leaching is common in long-term N enrichment studies (Peterjohn et al. 1996; Vitousek et al. 1997; Aber et al. 2003; Lovett & Goodale 2011), including at Harvard Forest (Aber et al. 1998; Currie et al. 1999), and NO_3^- typically binds with a cation (e.g., Mn^{2+}) when leaching (Peterjohn et al. 1996; Vitousek et al. 1997; Aber et al. 2003). Thus, both of these N-induced leaching mechanisms may contribute to reductions in Mn availability for decomposer fungi.

Elevating bioavailable Mn concentrations for six months did not significantly influence total litter mass loss; however, given our understanding of the role of Mn in litter decomposition, this is not entirely unexpected. Manganese is known to play an important role in lignin decay, which dominates during late-stage litter decomposition (Berg & Matzner 1997; Berg & McClaugherty 2014). Litter mass loss proceeded to a greater extent in the control and intermediate N treatments than in the highest N (N150) treatment. In accordance with this trend, N150 samples retained more lignin (i.e., slower rates of lignin decomposition). Because greater lignin decay occurred in control and intermediate N samples, more Mn was likely required, which explains the trend towards increased mass loss with Mn amendment in these samples (Fig. S2). In contrast, there was no effect of Mn on mass loss in the N150 samples, which can likely be explained by lower rates of lignin decay and thus lower fungal requirements for Mn.

While mass loss was unaffected, ligninolytic enzyme activities increased significantly following Mn amendment. The role of Mn in the production of ligninases like Mn peroxidase (MnP) is well known; however, our understanding of this relationship comes primarily from culture work with model taxa (e.g. *Phanaerochaete chrysosporium*; Glenn et al. 1983; Tien & Kirk 1983; Perez & Jeffries 1992; Hatakka 1994; Steffen et al. 2002). To the best of our knowledge, this is the first direct evidence of the relationship between Mn availability and

ligninase activities from natural soil microbial communities. Further, we are only aware of two studies which have used Mn amendments to directly evaluate the role of Mn in decomposition (Trum et al. 2011; 2015), neither of which were conducted in the context of atmospheric N deposition. Thus, to the best of our knowledge, this is also the first paper to demonstrate the importance of Mn availability in decomposition processes under chronic N enrichment.

Given that ligninolytic enzyme activities increased to such a great extent with Mn amendment, we expect that a longer incubation period (1+ years) would have captured a positive effect of Mn on mass loss, regardless of N treatment. Overall ligninase activities were lowest in the highest N treatment; however, relative to ambient conditions (no Mn amendment), the highest N, highest Mn treatment experienced the greatest increase in ligninase activity. This suggests that there is a legacy effect of high chronic N enrichment (N150 treatment) which suppressed the upper limit of enzyme activity; but, Mn amendment began to release this limitation. As decomposition progresses and lignin decay comes to dominate (Berg & Matzner 1997; Berg & McLaugherty 2014), the strength of this Mn effect will likely grow.

In addition to the clear response in ligninolytic enzyme activities, we observed a significant shift in fungal community composition with Mn amendment, which helps to explain the enzyme response we observed. Previous studies evaluating the effects of long-term N enrichment on fungal community composition have demonstrated shifts in the relative abundance of fungal functional groups. Specifically, N enrichment has been shown to increase the relative abundance of yeasts in leaf litter (Morrison et al. in prep) and pathogenic fungi in soils (Morrison et al. 2016). We show a reversal of these N effects on fungal community composition with Mn amendment, suggesting that Mn plays an important role in structuring litter fungal communities under long-term N enrichment.

In our study, the relative abundance of yeasts increased from 0.27% to 1.5% relative abundance with chronic N enrichment (under ambient Mn conditions). Morrison et al. (in prep) demonstrated a ~200% increase in yeast abundance under long-term N enrichment in the field at our site, from 2.0% to 5.8% relative abundance in the highest N treatment. Although yeasts represented a smaller proportion of the fungal community in our incubations, we show a 455% increase in yeast relative abundance between the control and highest N treatments (under ambient Mn conditions). Yeasts do not possess genes encoding lignin-decay enzymes, and are better adapted to decompose simple carbon compounds (e.g., sugar monomers; Botha 2011; Treseder & Lennon 2015). For this reason, an increase in the relative abundance of yeasts has been suggested as a mechanism for reduced decomposition and increased soil C storage (Treseder & Lennon 2015), which may explain soil C accumulation under long-term N enrichment (Morrison et al. in prep). We show that the addition of bioavailable Mn reduces the relative abundance of yeasts to control N levels (N150 treatment), suggesting that Mn limitation underlies this increase in yeasts under long-term N enrichment.

In addition, we show an increase in the relative sequence abundance of pathotrophic fungi under chronic N enrichment, which is consonant with the results of Morrison et al. (2016). Consistent with the response we observed in yeast taxa, adding Mn reduced pathotroph relative abundance, again suggesting that Mn limitation may have contributed to previously observed shifts in the fungal community under long-term N enrichment. It is important to note that we excluded pathotroph hosts from our incubation; thus, the functional role of so-called fungal ‘pathotrophs’ (e.g., plant pathogens) is not explicit. It is likely that many taxa in this category were acting as saprotrophs because facultative saprotrophy is a well-established alternate function of ‘pathotrophs’ (Jumpponen & Trappe 1998; Promputtha et al. 2007; Rodriguez et al.

2009). Although certain facultative pathotroph-saprotrophs may be classified as ‘intermediate’ or ‘strong’ decomposers (e.g., the white-rot pathogen *Heterobasidion irregulare*), there appear to be drawbacks to having dual-ecologies. These drawbacks include possessing genes encoding only one, rather than the entire suite of class II peroxidases (e.g., MnP in the case of *H. irregulare*; Yakolev et al. 2013), and possessing low copy numbers of such genes (Kohler et al. 2015). These patterns are suggestive of less efficient lignin decomposition in these organisms.

In our study, all documented pathotrophs were classified as Ascomycetes. Since the capacity to produce class II peroxidases is limited to the Agaricomycetes in the Basidiomycota (Floudas et al. 2012), these facultative pathotroph-saprotrophs likely fall into the weak or intermediate decomposer categories. As support for this, we show a negative correlation between pathotroph relative abundance and ligninolytic enzyme activity (Fig. S5a). We interpret these findings as evidence that Mn limitation under chronic N enrichment leads to an increase in the relative abundance of ‘weak’ decomposers (e.g. yeasts and pathotrophs). Since Mn amendments reduced the abundance of such groups, we hypothesize that Mn promotes fungal communities with greater capacity for leaf litter decomposition (i.e., capacity to produce ligninases).

In our incubation experiment, we showed a significant reduction in the relative abundance of filamentous saprotrophs in the highest N treatment (N150). In the study by Morrison et al. (in prep), there was a similar trend towards reduced filamentous saprotroph relative abundance with long-term N enrichment, albeit insignificant. In contrast to the yeast and pathotroph responses, filamentous saprotroph relative abundance declined further with Mn amendment in the N150 treatment. This response was driven by a decrease in the relative abundance of ascomycetous and zygomycetous saprotrophs. The relative abundance of basidiomycetous saprotrophs was unaffected by Mn amendment. Ascomycetous and

zygomycetous saprotrophs do not possess class II peroxidase genes and are associated with low to no copy numbers of genes encoding other ligninases (Floudas et al. 2012; Kohler et al. 2015); thus, they can be classified as weak to intermediate decomposers. Declines in ascomycetous and zygomycetous saprotrophs with Mn amendment may therefore be consistent with the phenomenon we observed with yeast and pathotroph taxa, wherein Mn amendment reduces the relative abundance of weak and intermediate decomposers. To support this, we created a synthetic ‘weak’ decomposer category in which we summed the average relative abundance of yeasts, pathotrophs and zygomycetous filamentous saprotrophs and regressed this group against ligninase activity. This model explained slightly more variation in enzyme activity than the pathotroph-only model (Fig. S5b). We excluded ascomycetous filamentous saprotrophs from the model because the relative abundance of this group was not correlated with enzyme activities.

We have discussed reductions in the relative abundance of fungal functional groups with Mn amendment, but have yet to explore fungi that increased in dominance with Mn, namely those OTUs without functional annotations in the Tedersoo et al. (2014) database (Fig. 3b). Interestingly, there was a synergistic effect of the highest levels of N and Mn on this group, where the relative abundance of OTUs with no annotation increased to >50% of the fungal community. This suggests that high combined resource availability of these nutrients (N, Mn) generates novel communities of fungi for which we do not have adequate ecological information. Due to the shortage of information about the ecologies of these organisms, it is impossible to say whether Mn promotes an increase in the relative abundance of strong decomposers in the fungal community. However, our enzyme data suggest that Mn affects fungal community function by increasing the potential for lignin decay. Therefore, Mn either (1) increased the lignin-decay

activities of strong decomposers already present in the community; or (2) increased the relative abundance of taxa performing this function.

Because we observed clear reductions in the relative abundance of taxa that are associated with weak to intermediate decomposition following Mn amendment, we might expect the OTUs that increased in relative abundance to have greater capacity for ligninase production. However, *C. rusci*, the OTU that dominated the ‘no functional annotation’ category, is classified as an ascomycete, and as such would not be expected to have a high capacity for ligninase production (Floudas et al. 2012). Further, the relative abundance of this OTU was only weakly positively correlated with ligninolytic enzyme activities. It is important to note, however, that DNA-based measurements of the fungal community (i.e., our study) often fail to reveal patterns that RNA-based measurements of active fungi capture. For instance, Morrison et al. (in prep) showed a strong correlation between enzyme activities and the relative abundance of active fungi (RNA), whereas no correlation was found between these enzymes and DNA-based metrics of relative sequence abundance. Thus, we believe that the taxa without known ecologies, particularly *C. rusci*, deserve further study. RNA-based measurements of the active fungal community and surveys of functional genes encoding ligninase production in these taxa may be particularly useful.

Lastly, contrary to expectations, the relative abundance of white-rot fungi increased in the highest N treatment (Fig. S6), a finding consistent with Morrison et al. (in prep). Because long-term N enrichment has been shown to repress ligninase activity and lignin decomposition, it has long been assumed that N enrichment suppresses white-rot fungi (Waldrop & Zak 2006; Hofmockel et al. 2007; Treseder et al. 2008). Evidence is emerging to suggest this may not be the case. Instead, lower rates of lignin and litter decomposition may be driven by an increase in

the relative abundance of ‘weak’ decomposers. Our data suggest that this release of the weak decomposer community may be triggered by Mn limitation.

5. Conclusion

We conclude that Mn limitation is a notable mechanism reducing ligninolytic enzyme activity and altering fungal community composition under long-term atmospheric N deposition. Our results suggest that Mn limitation may be an important control on decomposition and soil C storage under soil N enrichment. We applied Mn amendments to chronically N-fertilized soils to demonstrate the relationship between Mn availability and ligninolytic enzyme activities. We show the first evidence of a strong positive correlation between these two parameters for natural microbial communities in leaf litter and soils. We also demonstrate a shift in fungal community composition with Mn addition that helps to explain the enzyme response we observed. Specifically, we show that elevated Mn reduces the relative abundance of fungi thought to be ‘weak’ decomposers, referring to their poor to intermediate ability to decompose lignin (relative to white-rot fungi, which are considered ‘strong’ decomposers). This decline in weak decomposer relative abundance with Mn was only observed in the highest N treatment (N150), which was the only N treatment to experience a significant increase in the relative abundance of these organisms. Incidentally, this treatment was also the most Mn-deficient. This suggests that Mn played the strongest role in shaping fungal communities where fungi were most affected by chronic N enrichment and/or most limited by Mn. Further, the finding that Mn amendments reduce the relative abundance of ‘weak’ decomposers suggests that higher Mn availability promotes fungal communities that have greater capacity for lignin decay. Taken together, our results suggest that Mn limitation plays a critical role in decomposition dynamics under long-

term atmospheric N deposition and represents a mechanism that may help explain reduced decomposition and soil C accumulation under this global change factor.

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TABLES AND FIGURES

Table 1. Initial litter chemistry, representing the starting quality of litter inputs to the incubations. Mean concentrations (n=6) of total aromatics (sum of the %lignin, %phenols and %aromatics), C and N and litter macro and micronutrients are presented with standard errors in parentheses. Means that do not share a letter are significantly different ($P < 0.05$). The percent change from control levels was calculated for each parameter and significant increases/decreases are denoted with asterisks ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$; one-way ANOVA). Baseline oxidative enzyme (PER, POX) activities were also evaluated. We present these data here to demonstrate common reductions in oxidative enzymes induced by chronic N enrichment.

Component	Litter origin			Percent change from N0	
	N0	N50	N150	N50	N150
Aromatics (%)	28.6 (1.51) ^a	26.5 (2.96) ^a	16.1 (2.70) ^b	-7.57	-43.68 ^{**}
N (%)	1.50 (0.07) ^a	1.73 (0.05) ^{ab}	1.88 (0.07) ^b	14.75	24.83 ^{**}
C:N	33.0 (2.19) ^a	29.6 (0.85) ^a	27.9 (1.03) ^a	-10.32	-15.27
Mn (mg/g)	2.96 (0.06) ^a	1.28 (0.08) ^b	0.83 (0.06) ^c	-56.57 ^{***}	-71.87 ^{***}
Ca (mg/g)	5.74 (0.11) ^a	4.16 (0.12) ^b	3.08 (0.11) ^c	-27.53 ^{***}	-46.35 ^{***}
K (mg/g)	1.32 (0.09) ^a	1.19 (0.05) ^a	1.00 (0.04) ^b	-9.71	-24.09 ^{**}
Mg (mg/g)	1.04 (0.04) ^a	0.93 (0.03) ^{ab}	0.83 (0.03) ^b	-10.91	-20.55 ^{**}
P (mg/g)	1.06 (0.04) ^{ab}	1.13 (0.03) ^a	0.93 (0.04) ^b	6.78	-11.83
Al (mg/kg)	515 (180) ^a	197 (19.6) ^a	111 (11.0) ^b	-61.73	-78.48 ^{***}
B (mg/kg)	15.3 (0.58) ^a	12.1 (0.42) ^b	11.9 (0.22) ^b	-20.87 ^{***}	-22.50 ^{***}
Cu (mg/kg)	6.29 (0.34) ^a	6.13 (0.60) ^{ab}	5.08 (0.27) ^b	-2.62	-19.36 ^{**}
Fe (mg/kg)	579 (223) ^a	222 (24.7) ^a	131 (12.7) ^b	-61.64	-77.33 ^{**}
Zn (mg/kg)	55.7 (2.21) ^a	47.2 (3.10) ^b	30.1 (0.88) ^c	-15.18 [*]	-45.93 ^{***}
PER	5.12 (1.07) ^a	3.66 (0.77) ^a	3.41 (1.45) ^a	-28.52	-33.40
POX	284 (40.2) ^a	112 (10.1) ^b	69.6 (32.5) ^{bc}	-60.56 ^{**}	-75.49 ^{***}

Table 2. *In situ* soil characteristics showing the effect of chronic N on pH, exchangeable acidity and cation exchange capacity (CEC) of the O (organic) and A (mineral) soil horizons. Average values (n=6) are presented with standard errors in parentheses. Means that do not share a letter are significantly different ($P < 0.05$). The percent change from control levels was calculated for each parameter and significant increases/decreases are denoted with asterisks ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$; one-way ANOVA).

Component	Soil origin			Percent Change from N0	
	N0	N50	N150	N50	N150
pH (O)	4.03 (0.10) ^a	3.82 (0.18) ^{ab}	3.38 (0.07) ^b	-5.21	-16.13 ^{**}
pH (A)	4.66 (0.07) ^a	4.45 (0.06) ^{ab}	3.93 (0.16) ^b	-4.44	-15.59 ^{**}
Acidity (O)	10.5 (0.67) ^a	13.5 (0.73) ^b	13.5 (0.45) ^b	28.57 [*]	28.57 [*]
Acidity (A)	5.70 (0.51) ^a	6.24 (0.58) ^a	9.42 (0.72) ^b	9.47	65.26 ^{**}
CEC (O)	15.2 (1.15) ^a	18.0 (0.97) ^a	18.9 (1.30) ^a	18.18	24.23
CEC (A)	6.22 (0.54) ^a	6.79 (0.61) ^a	9.94 (0.75) ^b	9.20	59.77 ^{**}

Table 3. Mn amendment levels and enzyme and decomposition responses to combined N and Mn treatments following 6 months of decomposition. The cumulative amount of Mn added to leaf litter (mg g⁻¹) over the course of the 6-month incubation is included. We list these data here, as it is important to note that more Mn was added to N-fertilized (N50, N150) samples than to control (N0) samples because these samples were assumed to be more Mn-deficient. Average litter mass loss (%) and percent change in lignin are presented with standard deviations in parentheses. Lignin percent change represents the average % increase or % decrease in lignin relative to the average starting litter lignin concentration for each N treatment pre-incubation. The percent change in oxidative enzyme activity relative to control levels (ambient Mn) is presented last. The control N, low Mn treatment pair was excluded from the experiment; we represent this with a dashed line. The dashed lines in the 'Cumulative Mn' category signify that no Mn was added (ambient Mn). The dashed lines in the enzyme (% change) category are present because values represent percent increases above the ambient Mn category.

N treatment	N0			N50			N150		
	Ambient	Low Mn	High Mn	Ambient	Low Mn	High Mn	Ambient	Low Mn	High Mn
Cumulative Mn	-----	-----	16.2	-----	7.8	24.0	-----	10.9	27.2
Mass loss (%)	64.7 (2.03)	-----	68.7 (3.01)	54.3 (4.58)	56.1 (5.11)	64.7 (4.07)	54.1 (5.09)*	51.6 (2.62)*	52.3 (2.17)*
Lignin (% change)	1.61 (9.67)	-----	4.95 (25.2)	2.04 (20.4)	-8.84 (18.9)	-30.6 (15.5)	45.9 (35.4)*	66.7 (28.3)*	57.4 (21.5)*
Enz. (% change)	-----	-----	205 (45.5)*	-----	244 (77.7)*	390 (82.0)*	-----	259 (52.7)	671 (178)*

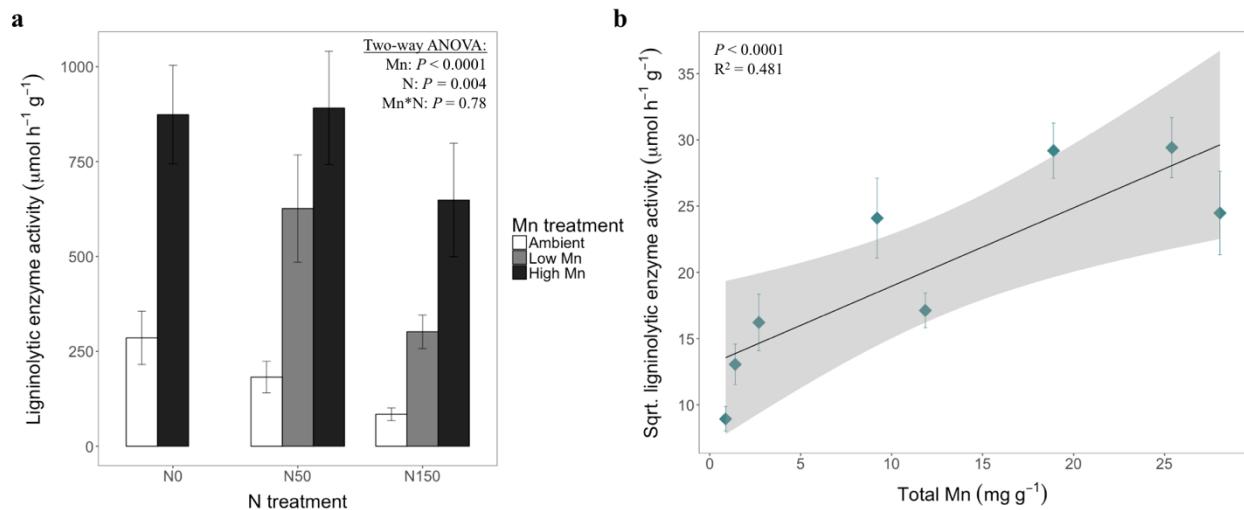


Figure 1. (a) Ligninolytic enzyme activity ($\mu\text{mol h}^{-1} \text{g}^{-1}$) across N and Mn treatments. The activities of peroxidase and phenol oxidase have been summed (*sensu* Ng et al. 2014) because responses to Mn amendment (% change from ambient) were highly similar. Bar color is representative of Mn treatments, where white is ambient, gray is low Mn, and black is high Mn. Two-way ANOVA results are presented for the square-root transformed enzyme data. (b) Linear regression showing the effect of total Mn (mg g^{-1}) on ligninolytic enzyme activity (square-root transformed), where total Mn represents the initial litter Mn concentration plus the cumulative amount of Mn added over the course of the incubation. The p-value and R^2 presented are for a linear model which includes all observations ($n = 48$), not average values.

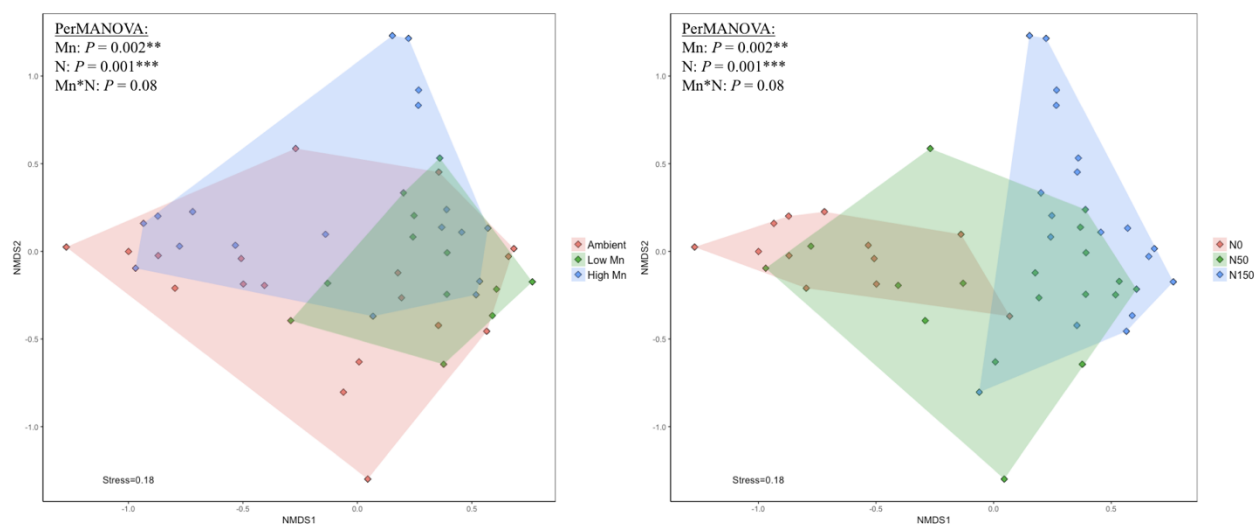


Figure 2. NMDS ordinations of fungal ITS2 data (stress = 0.18). Panel (a) represents the effects of Mn amendments on litter fungal community composition evaluated after 6 months of decomposition and panel (b) shows the effects of long-term N enrichment on fungal communities (evaluated in litter after 6 mo. of decomposition). Polygons outline the bounds of samples within each Mn (a) or N (b) treatment group; they are not representative of any statistical parameter. Polygon color represents the level of Mn or N application: control/ambient (red), intermediate (green), or high (blue) levels of each nutrient. PerMANOVA with a two-way interaction was used to test for significant differences in community composition across treatments; the same PerMANOVA results are shown in the Mn and N panels.

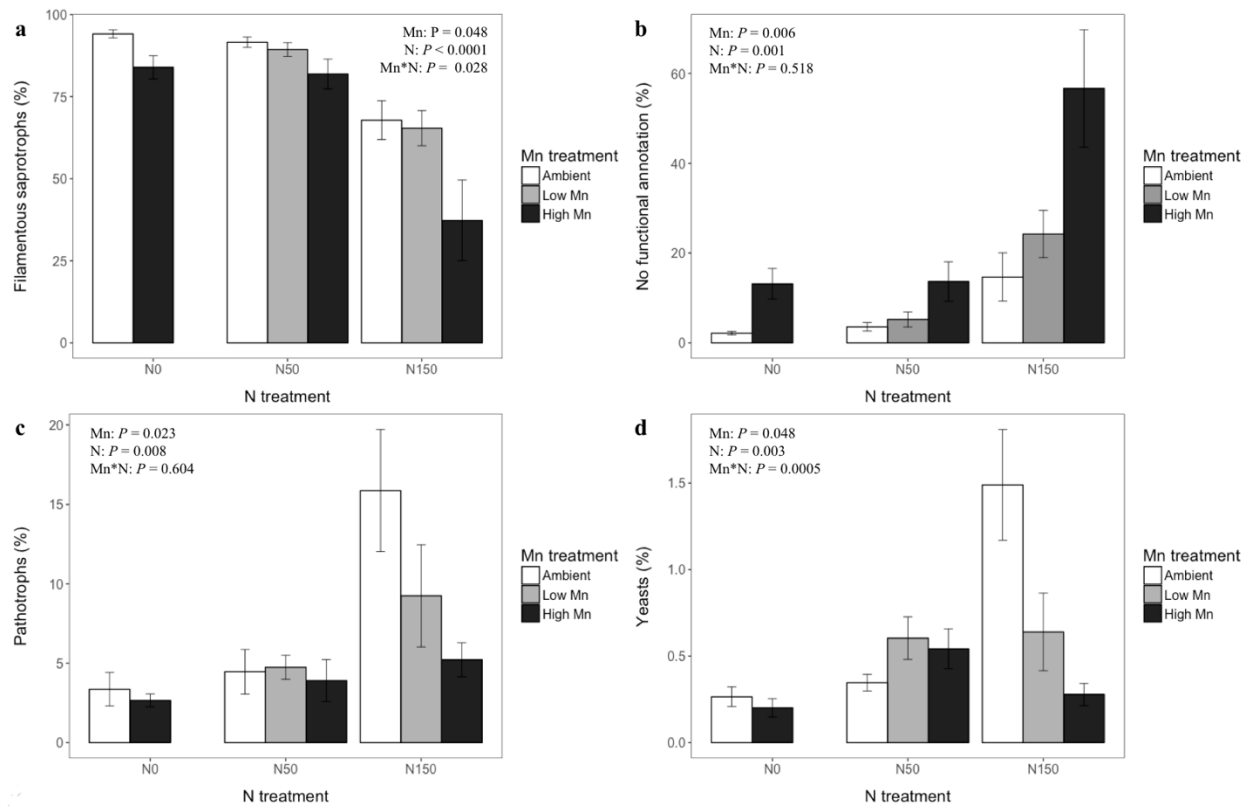


Figure 3. Relative abundance (%) of fungal functional groups (filamentous saprotrophs (a), no functional annotation (b), pathotrophs (c) and yeasts (d)) with significant responses to Mn treatments. Bar color is representative of Mn treatment, where white is ambient, gray is low Mn, and black is high Mn. Two-way ANOVA results (arcsine-transformed data) are presented for each functional group.

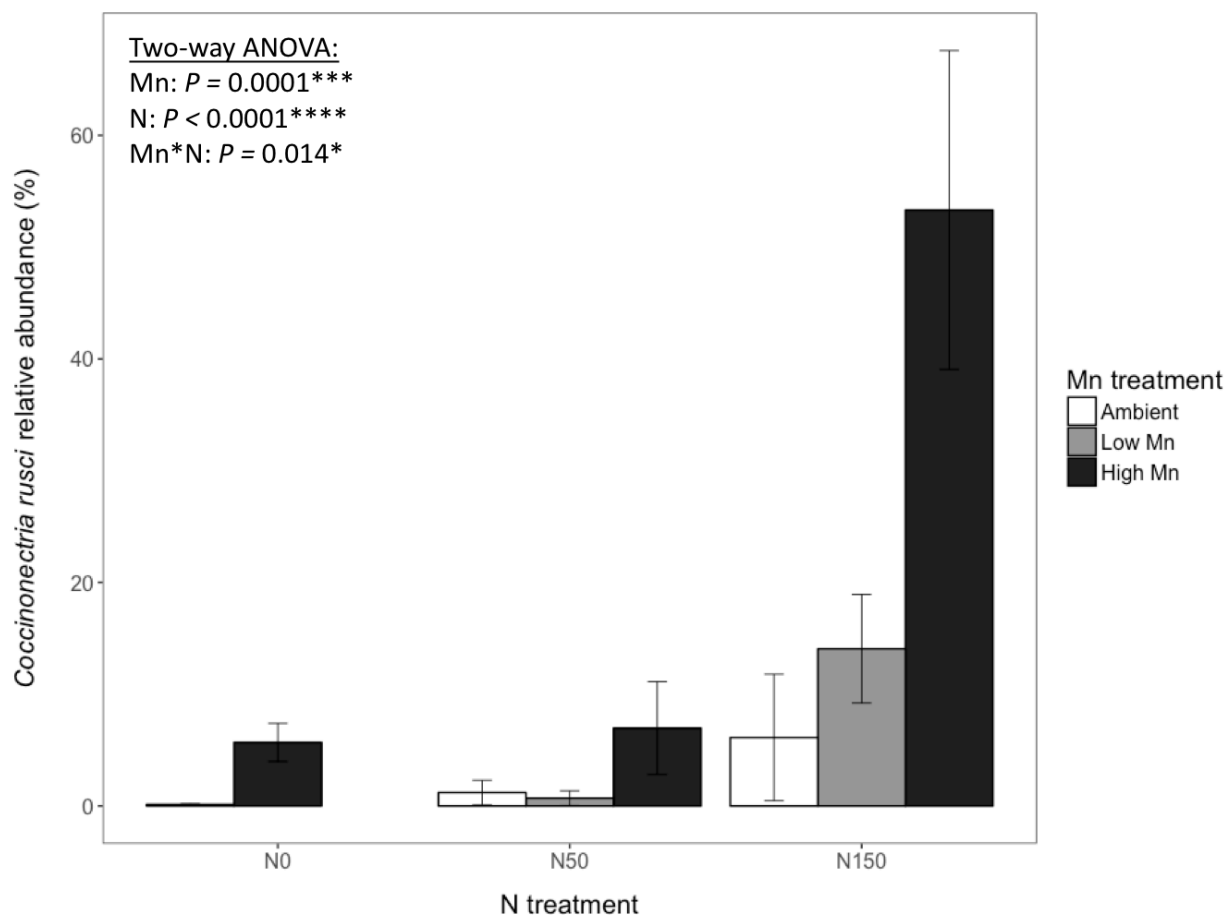


Figure 4. Relative abundance (%) of *Coccinonectria rusci*, the most abundant organism lacking a functional annotation in the Tederso et al. (2014) database. Bar color is representative of Mn treatment, where white is ambient, gray is low Mn, and black is high Mn. Two-way ANOVA results for the response of *C. rusci* to Mn and N amendments are presented.

APPENDIX A: SUPPLEMENTARY FIGURES

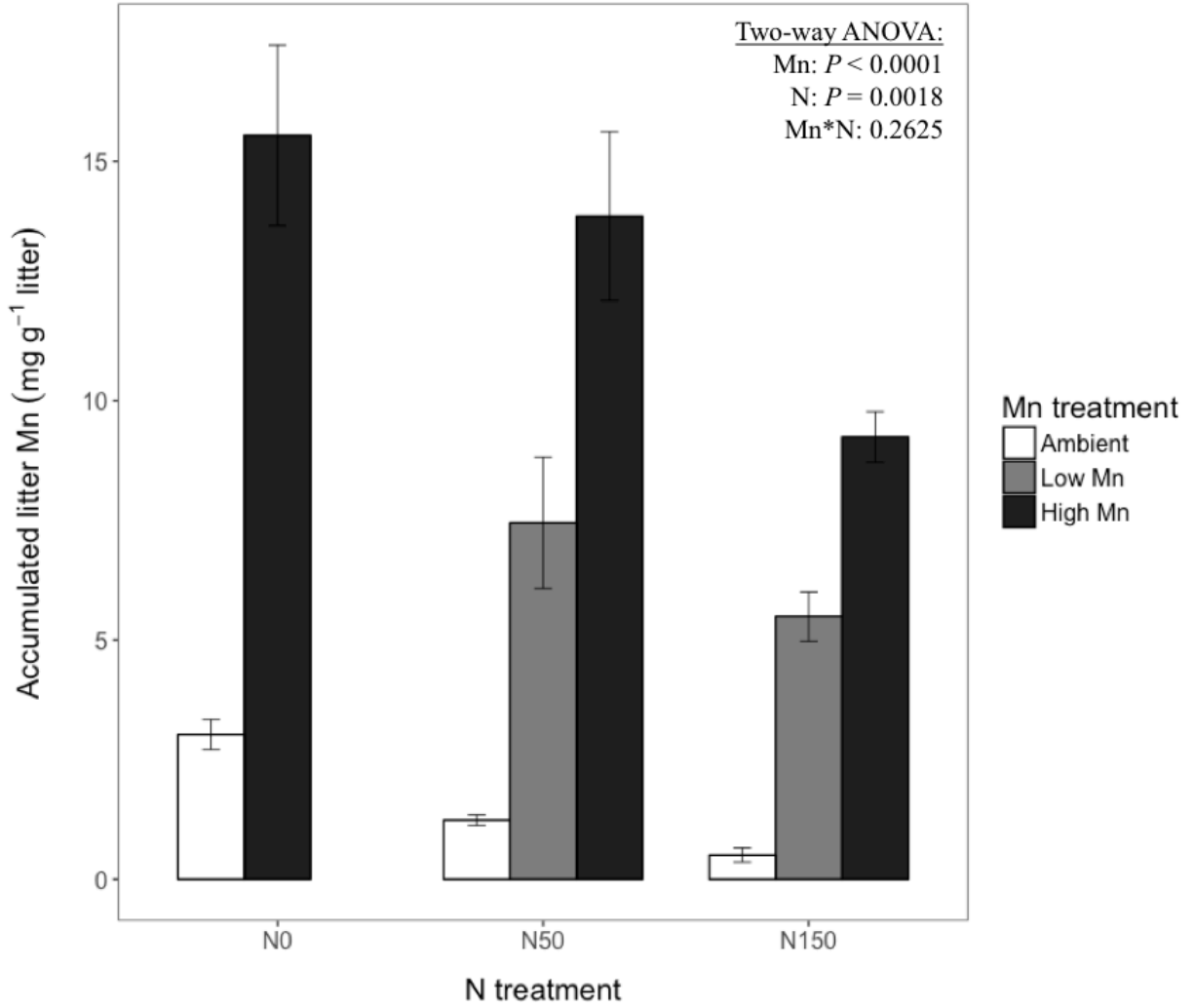


Figure S1. Amount of Mn accumulated in leaf litter throughout the 6-month incubation. Accumulated Mn was calculated as the difference between the final litter Mn concentration and the starting litter Mn concentration (mg g^{-1}).

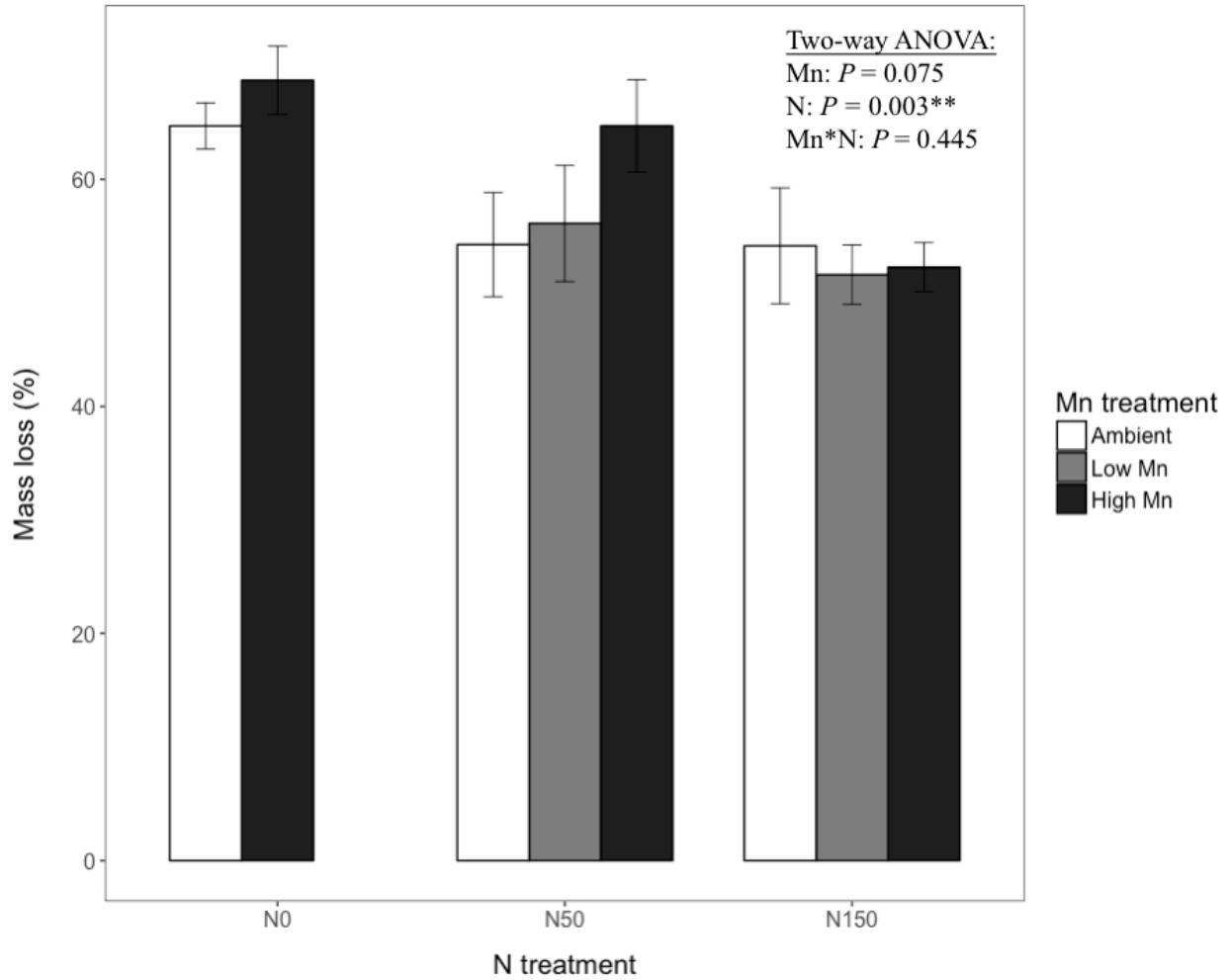


Figure S2. Total litter mass loss (%) across N and Mn treatments. Bar color is representative of Mn treatment, where white is ambient, gray is low Mn, and black is high Mn. Differences in mass loss were evaluated using two-way ANOVA.

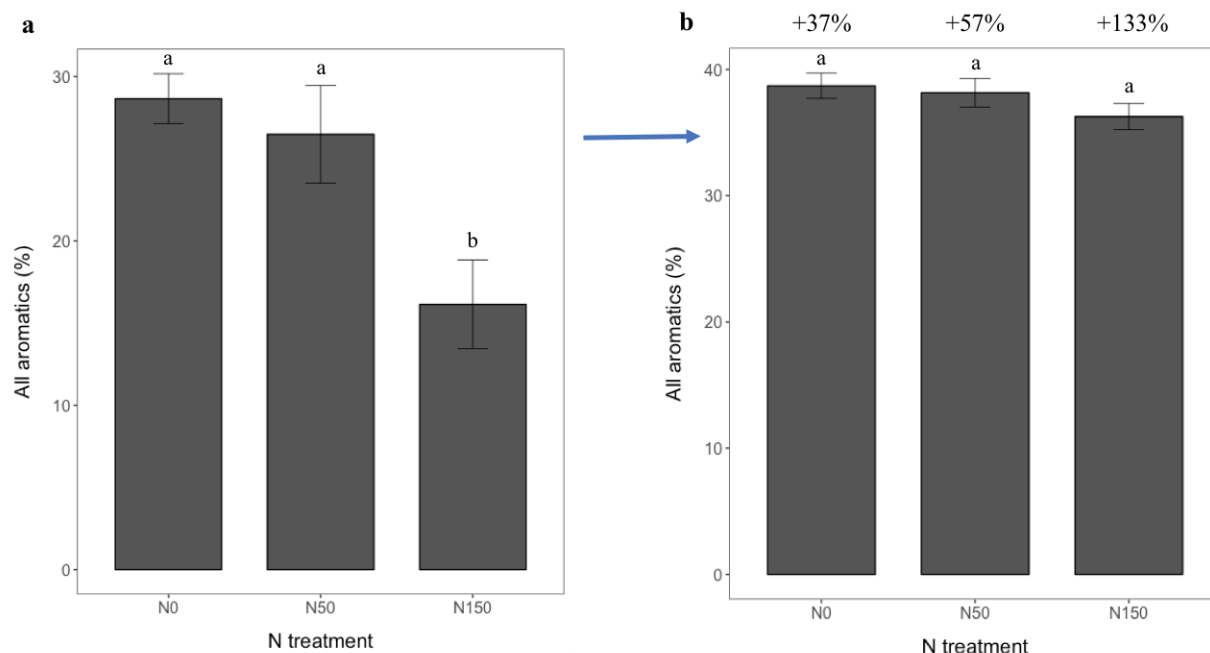


Figure S3. Total aromatics (%lignin + %phenols + %aromatics) in undecomposed litter **(a)** and litter after 6 months of decomposition **(b)**. Significant differences between N treatments in panels **(a)** and **(b)** are indicated with lower-case letters. Within each panel, bars that do not share a letter are significantly different. Percentages at the top of panel **(b)** represent the percent increase in litter aromatics within each N treatment throughout decomposition. More lignin and other aromatics were retained (i.e., slower lignin decomposition) in the N150 treatment than N0 and N50. In panel **(a)** we show lower initial aromatic/lignin concentrations in undecomposed litter in the highest N treatment. We believe this may suggest that oxidative enzyme activity (phenol oxidase and peroxidase) is suppressed in plants as well. Plants use oxidative enzymes to synthesize lignin. Lower litter lignin concentrations are suggestive of reduced lignin synthesis by plants. Lignin synthesis may be suppressed via an N-induced repression of oxidative enzymes in plants, mirroring the effect of N on microbial oxidative enzyme production. If oxidative enzyme activities are reduced in plants, Mn limitation may explain this observation.

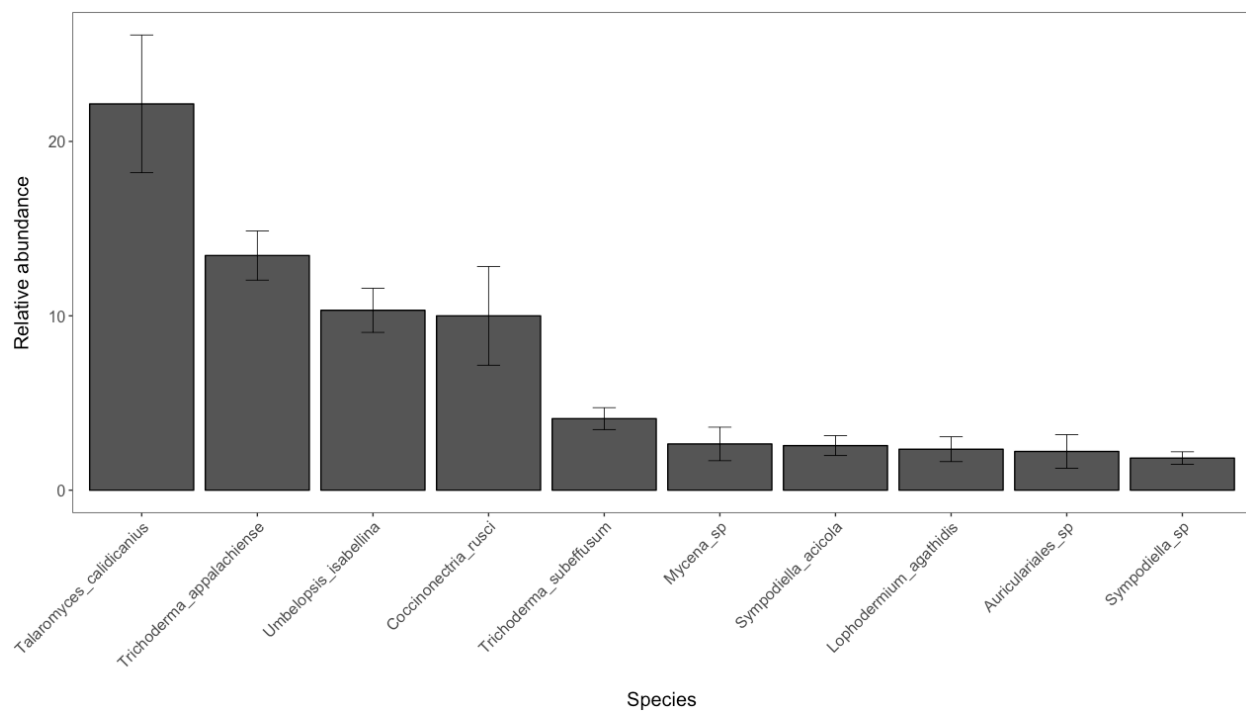


Figure S4. Relative abundance (%) of the top 10 most abundant OTUs in our dataset. *C. rusci*, the fourth most abundant species is explored in more detail in the main text (Fig. 4).

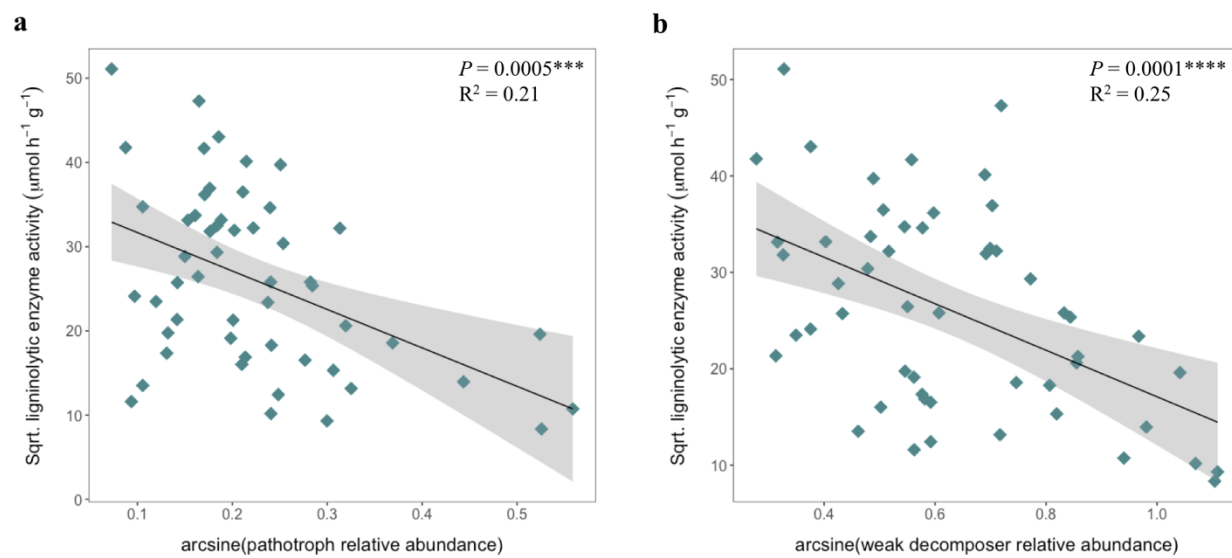


Figure S5. Linear regressions between ligninolytic enzyme activity (square-root transformed) and the arcsine-transformed relative abundance of ‘weak’ decomposers (pathotrophs **(a)** and yeasts, pathotrophs and zygomycetous filamentous saprotrophs **(b)**).

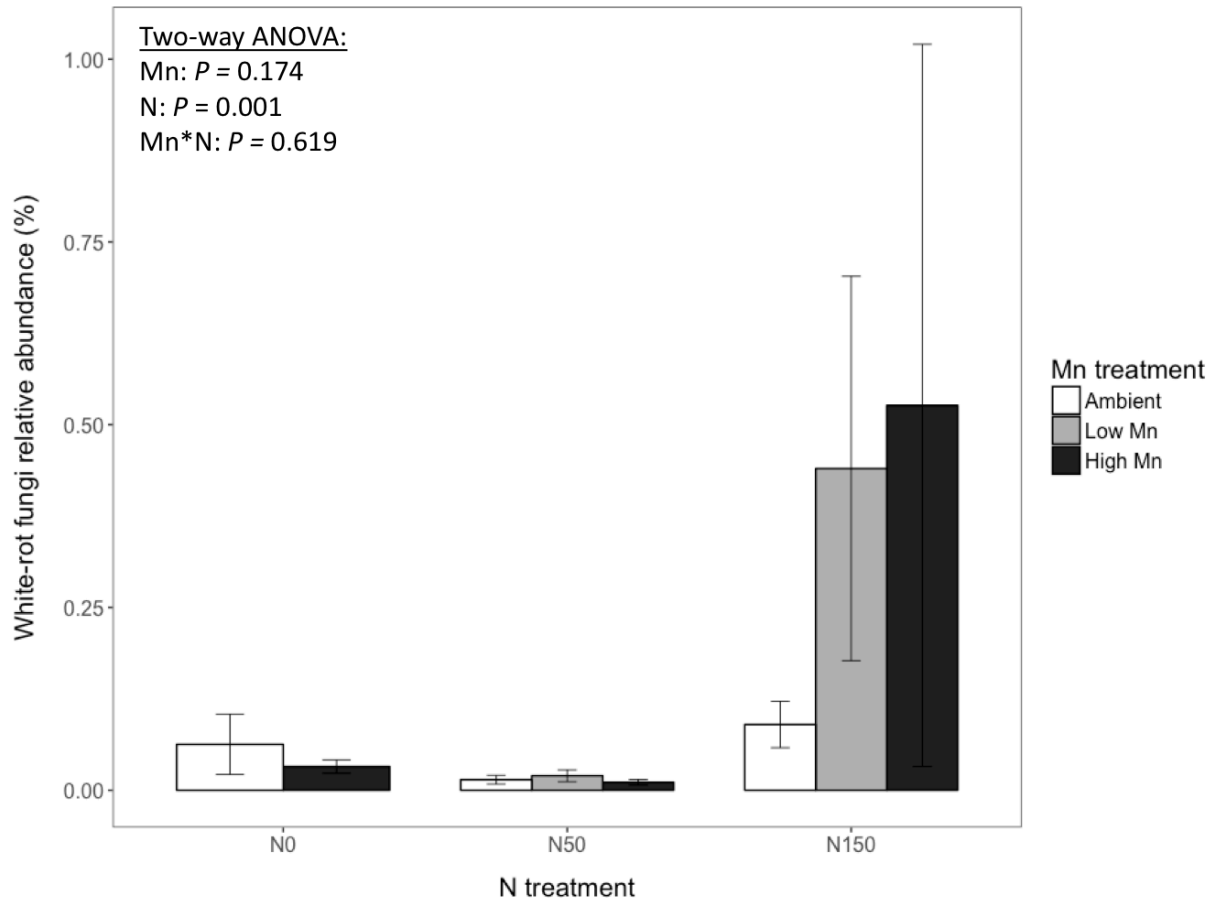


Figure S6. Relative abundance (%) of white-rot fungi across N and Mn treatments. Bar color is representative of Mn treatment, where white is ambient, gray is low Mn, and black is high Mn. Two-way ANOVA and post-hoc Tukey HSD show a significant increase in white-rot relative abundance in the highest N treatment (N150), albeit highly variable.

APPENDIX B: PHOTOS OF INCUBATION EXPERIMENT



Photo 1. Individual incubation cups with visible fungal colonization of leaf litter



Photo 2. Incubation jars in the environmental chamber (25°C). Sealed glass pickle jars with wetted paper towels were used to maintain soil and litter moisture. Large pickle jars were selected to maximize headspace, thus minimizing the times jars needed to be aerated.