Manganese limitation as a mechanism for reduced decomposition in soils under atmospheric nitrogen deposition

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ABSTRACT

Long-term atmospheric nitrogen (N) deposition has been shown to reduce leaf litter and lignin decomposition in temperate forest soils, leading to an accumulation of soil carbon (C). 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 help explain these observations, because Mn is a cofactor and regulator of lignin-decay enzymes produced by fungi. We conducted a laboratory study to evaluate the effect of Mn availability on decomposition dynamics in chronically N-enriched soils. We measured litter mass loss, lignin relative abundance, and lignin-decay enzyme activities, and characterized the litter fungal community by ITS2 metabarcoding. We observed a significant positive correlation between Mn availability and lignin-decay enzyme activities. In addition, long-term (28 years) N enrichment increased the relative abundance of 'weak' decomposers (e.g., yeasts), but this response was reversed with Mn amendment, suggesting that higher Mn availability may promote fungal communities better adapted to decompose lignin. We conclude that Mn limitation may represent a mechanism to explain shifts in fungal communities, reduced litter decomposition, and increased soil C accumulation under long-term atmospheric N deposition.

1. Introduction

Human activities have greatly increased the release of nitrogenous compounds (NOx) 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 (>5 years) N deposition has been shown to slow leaf litter and lignin decomposition in temperate forest soils, resulting in an accumulation of soil carbon (C; Berg and Matzner, 1997; Frey et al., 2014; Knorr et al., 2005; Lovett et al., 2013; Magill and Aber, 1998; Pregitzer et al., 2008; Zak et al., 2008, 2011). 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 (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; Treseder, 2008; Wallenstein et al., 2006), 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) 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 typically limited by low supplies of available N. The addition of bioavailable N via atmospheric deposition has lifted N restrictions on these processes, but has generated novel nutrient limitations (Aber et al., 1998; Crowley et al., 2012; Perakis et al., 2013). Nitrogen-induced reductions in soil base cation (e.g., calcium) and phosphorus concentrations have received considerable attention (Currie et al., 1999; Fatemi et al., 2016; Gilliam et al., 1996; Lovett et al., 2016; Naples and Fisk, 2010; Peterjohn et al., 1996; Vitousek et al., 1997). Meanwhile, biologically essential soil metals like manganese (Mn) have largely been ignored. Despite this, Mn depletion in soils and plant tissues 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 litter decomposition (Berg, 2000; Berg et al., 2007, 2010; Davey et al., 2007; Keiluweit et al., 2015; Trum 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). Similarly, Stendahl et al. (2017) found that Mn was the strongest predictor of soil C storage in boreal forests. The importance of Mn in decomposition is thought to derive from its role in the production and regulation of enzymes targeting lignin (Hofricher, 2002; Perez and Jeffries, 1992), a chemically complex, recalcitrant plant biopolymer that comprises up to ∼25% of leaf lignin (Hofricher, 2002; Perez and Jeffries, 1992).

2.1. Experimental site and sample collection

Samples were collected from the Chronic Nitrogen Amendment Study (CNAS) 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 soils received 28 consecutive years of N addition prior to our sampling (2016). Soils are classified as Typic Haplaquept with a sandy loam texture. The site was established in 1988 and soils received 28 consecutive years of N addition prior to our sampling (2016). Soils are classified as Typic Haplaquept with a sandy loam texture. Each megaplot is divided into thirty-six 5m x 5m plots, with the goal of describing the role that Mn plays in structuring fungal communities.

2. Materials and methods

2.1. Experimental site and sample collection

Samples were collected from the Chronic Nitrogen Amendment Study (CNAS) 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 soils received 28 consecutive years of N addition prior to our sampling (2016). Soils are classified as Typic Haplaquept with a sandy loam texture. Experimental plots are in a mixed-oak forest (Quercus velutina) and include understory vegetation (e.g., Quercus rubra, Betula lenta, Acer negundo, Fagus grandifolia). Soil samples were collected from the A horizon (0-15 cm) at each megaplot, with the goal of describing the role that Mn plays in structuring fungal communities.
5 m subplots. The outermost row of subplots is excluded to prevent the influence of an edge effect, leaving sixteen 5 m × 5 m subplots per N treatment. The N50 (50 kg N ha⁻¹ yr⁻¹) treatment is representative of an influence of an edge effect, leaving sixteen 5 m × 5 m subplots per N treatment. The N50 (50 kg N ha⁻¹ yr⁻¹) treatment is representative of N deposition scenarios for 2050, at which time, some parts of the world are expected to receive up to 50 kg N ha⁻¹ yr⁻¹ (Galloway et al., 2008). The N150 treatment was established as a space-for-time substitution simulating N saturation (c.f. Aber et al., 1998; Aber and Magill, 2004).

In preparation for the incubation experiment, intact soil cores (5 × 10 cm) were collected from six randomly selected subplots of each of the three N addition treatments (N0, N50, N150). Fresh, undecomposed leaf litter was also collected from each subplot to serve as the litter layer in the incubations. Collected soil and litter samples were transported to the University of New Hampshire where soil samples were stored at 4 °C until analysis and initiation of the incubation study (described below). Small subsamples (~0.5 g) of fresh litter were weighed for enzyme analysis, and remaining litter was dried at 60 °C for 48 h to standardize litter moisture content for the incubation study.

2.2. Background leaf litter and soil chemistry

To characterize the chemistry of litter prior to incubation, nutrient (Ca, Mn, Mg, P, K, Al, B, Cu, Fe and Zn) concentrations of fresh, undecomposed leaf litter were determined via ICP-AES. Samples were prepared for analysis by dryashing and acid digestion in a solution of 50% hydrochloric acid (Kalra et al., 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 S150 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). In the mass spectrometer, 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). Lastly, the activities of two ligninolytic enzymes (peroxidase and phenol oxidase) were assayed in fresh leaf litter within 24 h of sampling to evaluate the effects of N addition on enzymatic lignin-decay. These methods are described in more detail in section 2.5.

To characterize soil chemistry before incubation, a subset of soil samples were separated into their respective O and A horizons and each horizon was individually sieved (<2 mm) to remove coarse woody debris, roots and stones. 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 h and mineral soils at 105 °C for 24 h. Exchangeable soil acidity was evaluated by soil extraction with 1 M KCl and subsequent titration with dilute NaOH. Cation exchange capacity (CEC) was calculated thereafter using the equation, CEC = exchangeable acidity (meq) + exchangeable base cations (meq; Ca, Mg, K, Na).

2.3. Incubation assembly

Intact soil cores, which contained approximately 2/3 organic horizon material (Oe/Oa), and 1/3 mineral soil, were incubated in 10 oz. plastic cups set inside one-gallon glass 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 × 2 cm pieces), representing the dominant litter type at CNAS (~85% of trees in the plots are oak). Intact soil cores were used in the incubation to maintain the natural biophysical and chemical properties of the soil, and fresh litter was used to simulate the litter layer at autumn leaf fall. The mesh, placed between the litter and organic horizon (Oe/Oa), was used to facilitate the completion 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 equal 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 (sensu Kirdittge, 1955 and Naeth et al., 1991) and 60% field capacity (sensu Veihmeyer and Hendrickson, 1949) for the organic horizon (Oe/Oa) and mineral soil components of each core, respectively. Once soil cores were assembled for incubation, Mn amendment treatments were applied to leaf litter at the top of each core. Mn amendments were added using liquid solutions of manganese sulfate tetrahydrate (MnSO₄·4H₂O; 223.05 g mol⁻¹). One of three Mn treatments was applied: ambient (no additional Mn), low Mn, or high Mn. The low Mn amendment rate mirrored Mn concentrations in control N samples and was applied once monthly for six months (in total, ~10 mg g⁻¹ litter). The high Mn amendment rate was 2x that of the low Mn rate (in total, ~20 mg g⁻¹ litter). 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. 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).

2.4. Incubation harvest

Following nearly six months of incubation, remaining litter was removed and weighed to determine mass loss. Litter was then homogenized and subsampled for analysis. A subsample (~1 g) 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 at harvest, as described in section 2.2. A separate ~0.5 g subsample of fresh litter was collected for extracellular enzyme analysis, and, 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.

2.5. Extracellular enzyme activity

Within 24 h of harvest, leaf litter was assayed to determine the activities of the lignin-decay enzymes peroxidase and phenol oxidase, following methods outlined in 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-ethylbenzthiazoline-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 s 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 min (ABTS) or 20 min (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). Final enzyme activity values were calculated following methods outlined by DeForest (2009) and are reported as µmole substrate per hour per gram dry litter (µmol h⁻¹ g⁻¹), in which enzyme activity is standardized by litter moisture content.
2.6. Fungal community characterization

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 ITS5 (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 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 ITS5 (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 sequence.

2.7. Bioinformatics

Sequencing adapters 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 were removed (with specific ‘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 (sequences retained reported in Table S1). Filtered sequences were dereplicated using the usearch10 ‘derep_fulllength’ command and input into ITSSx (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). Singleton and chimeras were removed at this step. Fungal taxonomy was assigned in QIIME (Caporaso et al., 2010) by comparing the representative sequence of each OTU to the UNITE database (Koljalg 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 sequence 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 ≥ 98% (E-value based on minimum accepted E-value in QIIME; Caporaso et al., 2010; see also Pearson, 2013). Non-fungal OTUs were manually removed and samples were rarefied to a depth of 13,022 sequences using the ‘rarefy’ function in R (‘vegan’ package; Oksanen et al., 2017). This left 805 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. Thereafter, OTUs with known taxonomies were parsed into functional groups using database curation by Tedersoo et al. (2014). We used the Tedersoo et al. (2014) database in order to directly compare our results to Morrison et al. (2018), a functional analysis of litter fungal communities across N treatments at CNAS, and because this approach has been shown (e.g., by Anthony et al., 2017) to produce results that are consistent with the newer functional guild assignment tool, FUNGuild (Nguyen et al., 2015). We parsed our community data into the following functional groups: white-rot fungi, filamentous saprotrophs (not including white-rot fungi), yeasts and pathotrophs. Ectomycorrhizal fungi comprised 0.9% of OTUs and < 0.01% of sequences and were not analyzed due to the exclusion of plant hosts in the laboratory incubation.

2.8. Statistical analyses

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” (sum total relative abundance of lignin, phenol, 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 incubation experiment, the effects of Mn and N treatments on mass loss, percent change in aromatics and ligninolytic enzyme activities were assessed using two-way ANOVA. Enzyme activity values were square-root transformed to meet assumptions of normality and homoscedasticity of residuals.

We assessed the effects of Mn and N treatments on fungal community composition with a permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001). The analysis was conducted on Bray-Curtis distances calculated from the log(x+1)-transformed OTU relative abundance values (‘vegan’ package; Oksanen et al., 2017). To visualize these effects, we performed non-metric multidimensional scaling (NMDS) ordination on the same matrix of Bray-Curtis distance values. To assess the influence of Mn and N treatments on individual OTUs or fungal functional groups, we conducted two-way ANOVA on OTU or functional group relative abundance. When necessary, data were arcsine-transformed to meet homogeneity of variance assumptions. If the analysis resulted in a significant interaction between Mn and N (P < 0.05), 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.

Lastly, we used partial least squares regression (PLSR) to (1) confirm that Mn is an important element influencing ligninolytic enzyme activity in soils at our study site, and (2) to identify the primary factors (i.e., nutrients) influencing the activity of these enzymes in our Mn addition experiment. PLSR is a multivariate analysis well-suited to datasets with large numbers of predictor variables that are auto-correlated (Carrascal et al., 2009). We performed PLSR using the NI-PALS algorithm in JMP Pro v. 13 (SAS Institute, Cary, NC) following methods outlined by Smith et al. (2018).

2.9. Accession numbers

Sequence data have been deposited in the NCBI Sequence Read Archive at accession number SRP150852.

3. Results

3.1. Background litter and soil chemistry

Long-term (28 years) N enrichment increased litter N concentrations by 25%, with concomitant reductions in 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 additions. The relative abundance of aromatic compounds, representing the sum total relative abundance of lignin, phenols and other aromatics, also declined, from 29% (N0) to 16% (N150; Fig. S1). We assayed fresh leaf litter from the field N treatments and found a suppression of phenol oxidase (POX) activity with N addition (P < 0.001), but no difference in peroxidase activity
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; 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.

### Table 1

Initial litter chemistry, representing the starting quality of litter inputs to the incubation experiment. Mean concentrations (n = 6) of total aromatics (sum of the relative abundance of 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).

<table>
<thead>
<tr>
<th>Component</th>
<th>Litter origin</th>
<th>Percent change from N0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N0</td>
<td>N50</td>
</tr>
<tr>
<td>Aromatics (%)</td>
<td>28.6 (1.51)⁴</td>
<td>26.5 (2.96)⁴</td>
</tr>
<tr>
<td>N (%)</td>
<td>1.50 (0.07)⁴</td>
<td>1.73 (0.05)ab</td>
</tr>
<tr>
<td>C (%)</td>
<td>57.3 (1.33)⁴</td>
<td>59.6 (0.19)⁣</td>
</tr>
<tr>
<td>C:N</td>
<td>33.0 (2.19)⁴</td>
<td>29.6 (0.85)⁣</td>
</tr>
<tr>
<td>Mn (mg/g)</td>
<td>2.96 (0.06)⁴</td>
<td>1.28 (0.08)⁣</td>
</tr>
<tr>
<td>Ca (mg/g)</td>
<td>5.74 (0.11)⁴</td>
<td>4.16 (0.12)⁣</td>
</tr>
<tr>
<td>K (mg/g)</td>
<td>1.32 (0.09)⁴</td>
<td>1.19 (0.05)⁣</td>
</tr>
<tr>
<td>Mg (mg/g)</td>
<td>1.04 (0.04)⁴</td>
<td>0.93 (0.03)⁣</td>
</tr>
<tr>
<td>P (mg/g)</td>
<td>1.06 (0.04)⁴</td>
<td>1.13 (0.03)⁣</td>
</tr>
<tr>
<td>Al (mg/kg)</td>
<td>515 (180)⁴</td>
<td>197 (19.6)⁣</td>
</tr>
<tr>
<td>B (mg/kg)</td>
<td>15.3 (0.58)⁴</td>
<td>12.1 (0.42)⁣</td>
</tr>
<tr>
<td>Cu (mg/kg)</td>
<td>6.29 (0.34)⁴</td>
<td>6.13 (0.60)⁣</td>
</tr>
<tr>
<td>Fe (mg/kg)</td>
<td>379 (223)⁣</td>
<td>222 (24.7)⁣</td>
</tr>
<tr>
<td>Mn (mg/kg)</td>
<td>35.7 (2.11)⁣</td>
<td>47.2 (3.10)⁣</td>
</tr>
<tr>
<td>PER</td>
<td>1.22 (0.17)⁣</td>
<td>3.66 (0.77)⁣</td>
</tr>
<tr>
<td>POX</td>
<td>284 (40.2)⁴</td>
<td>112 (10.1)⁣</td>
</tr>
</tbody>
</table>

(Table 1). For this reason, when we conducted our PLSR analysis of field enzyme data, we only included POX as a response variable. PLSR analysis revealed that, of the 11 litter nutrients measured, variation in N, Mn, B, Ca and Mg explained most of the variation in field POX activity, and the direction of the relationship between N and POX was opposite that of the other nutrients (Fig. 5a).

Soil pH of the organic horizon declined from approximately 4.0 in the N0 treatment to 3.4 in N150 (P = 0.009; Table 2). Mineral soil pH followed a similar trend, decreasing from 4.7 (N0) to 3.9 (N150; P < 0.001). Correspondingly, exchangeable soil acidity increased with N addition up to 29% in the organic horizon (P = 0.005) and 65% in mineral soil (P = 0.001). 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 total Mn concentrations in leaf litter at harvest (i.e., after ~6 months of decomposition). All litter accumulated Mn, and Mn-amended samples accumulated the most (P < 0.0001; Fig. S3). Partial least squares regression of litter lignin abundance, mass loss and ligninolytic enzyme activities (Fig. 1a and 1b) as the sum of peroxidase (PER) and phenol oxidase (POX) activities, because PER and POX responded similarly to Mn amendments. Partial least squares regression of lignin turnover in the highest N treatment (P < 0.001; Fig. S1).

3.2.2. Litter mass loss and ligninolytic enzyme activities

Long-term N enrichment reduced total litter mass loss by ~16% (P = 0.003; Fig. S4). 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, where mass loss was 6–19% greater in high Mn compared to ambient Mn samples (Fig. S4); but, there was no apparent change in mass loss with Mn in the N150 treatment. The percent change in litter lignin throughout decomposition varied by N treatment, but not by Mn treatment (N: P = 0.001; Mn: P = 0.60; N × Mn: P = 0.74; two-way ANOVA). Chronic N enrichment increased lignin retention in the highest N treatment (P = 0.001; Fig. S1).

Long-term N enrichment repressed ligninolytic enzyme activity (P = 0.004; two-way ANOVA; Fig. 1a), particularly in the highest N (N150) treatment. Manganese amendments significantly ameliorated this response, elevating the activity of these enzymes (P < 0.0001; two-way ANOVA). 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—670% above ambient Mn levels compared with 205% and 390% in the N0 and N50 treatments, respectively (Fig. 1a). There was a significant correlation between enzyme activity and total Mn (P < 0.0001; R² = 0.481; Fig. 1b), where total Mn represents the initial litter Mn concentration plus the cumulative amount of Mn added over the course of the incubation.

### Table 2

Soil characteristics showing the effect of chronic N additions on pH, exchangeable acidity and cation exchange capacity (CEC) for 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; one-way ANOVA).

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<td></td>
<td>N0</td>
<td>N50</td>
</tr>
<tr>
<td>pH (O)</td>
<td>4.03 (0.10)⁴</td>
<td>3.82 (0.18)ab</td>
</tr>
<tr>
<td>pH (A)</td>
<td>4.66 (0.07)⁴</td>
<td>4.45 (0.06)⁣</td>
</tr>
<tr>
<td>Acidity (O)</td>
<td>10.5 (0.67)⁣</td>
<td>13.5 (0.73)⁣</td>
</tr>
<tr>
<td>Acidity (A)</td>
<td>5.70 (0.51)⁣</td>
<td>6.24 (0.58)⁣</td>
</tr>
<tr>
<td>CEC (O)</td>
<td>15.2 (1.15)⁣</td>
<td>18.0 (0.97)⁣</td>
</tr>
<tr>
<td>CEC (A)</td>
<td>6.22 (5.45)⁣</td>
<td>6.79 (0.61)⁣</td>
</tr>
</tbody>
</table>
PER + POX confirmed that lignin-decay enzyme activity increased primarily as a result of Mn amendment, rather than via hidden or joint effects of other co-varying nutrients (Fig. S2b). Specifically, total Mn was the variable with the strongest loading on the most explanatory PLSR latent factor (accounting for 44% of variation in lignin-decay enzyme activity). Other PLSR factors accounted for no more than 27% of the variation.

### 3.2.3. Fungal community composition

Both long-term N enrichment and Mn amendments altered the composition of the litter fungal community (Fig. 2; N: \( P = 0.001 \); Mn: \( P = 0.001 \); N × Mn: \( P = 0.007 \); PerMANOVA); however, the specific effects of these treatments varied by treatment and fungal functional group. For example, chronic N enrichment reduced the relative abundance of filamentous saprotrophs, the dominant fungal functional group in our study, from 80% to 43% (ambient Mn samples). Manganese amendments compounded this effect, further decreasing filamentous saprotroph relative abundance (N: \( P < 0.0001 \); Mn: \( P = 0.032 \); N × Mn: \( P = 0.07 \); two-way ANOVA; Fig. 3a).

These 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 greatest abundance (∼60% of the total community) in the highest N, highest Mn treatment. Notably, one OTU (Coccinonectria rusci; OTU2) comprised the majority of this response in the highest N, highest Mn treatment. The relative abundance of C. rusci increased from ∼5% in highest N, ambient Mn samples to 50% in highest N, highest Mn samples (P = 0.003; Tukey HSD test; Fig. 4).

Chronic N addition increased the relative abundance of pathotrophs from ∼3% (N0) to ∼11% (N150) of the fungal community (ambient Mn samples; P = 0.009; two-way ANOVA; Fig. 3c). Manganese amendments did not significantly influence pathotroph relative abundance (P = 0.105; two-way ANOVA); however, mean pathotroph relative abundance was lower in the highest N, highest Mn treatment (4.9%) compared with the highest N, ambient Mn treatment (11%). A post-hoc least-squares means comparison of these two treatments suggests that pathotroph relative abundance declined with Mn amendment in the highest N, highest Mn samples (P = 0.034), returning pathotroph relative abundance to levels comparable to those of N0 and N50 treatments. There was no difference in pathotroph abundance between N0 and N50 treatments (P = 0.83). 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 (P = 0.002; Tukey HSD test; Fig. 3d). Yeast relative abundance was 0.2% in N0 samples (ambient Mn), compared to 0.9% in N150 samples (ambient Mn). Adding Mn counteracted this effect, reducing the average relative abundance of yeasts back to ∼0.2% in the highest N, highest Mn treatment (P = 0.041; Tukey HSD test). Manganese amendments did not significantly influence yeast relative abundance in the N0 or N50 treatments. White-rot fungi comprised the smallest proportion of the fungal community (<1% relative abundance). The relative abundance of white-rot fungi was elevated in the highest N treatment (P = 0.006; two-way ANOVA), but was unaffected by Mn amendment (Fig. S5).

4. Discussion

We show that long-term (28 years) 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 documented a shift in fungal community composition with Mn addition that helps explain the observed functional response.
served in the N-amended plots at CNAS, Mn is highly vulnerable to horizon and mineral soils. At very low pH levels (3–4), like those observed in amended plots may be driven by reduced soil pH in both the organic and foliar and litter Mn concentrations. At CNAS, Mn leaching in N-amended plots is the primary source of plant-available Mn; Perez and Jeffries, 1992; Hatakka, 1994), while providing the necessary conditions. Manganese enhances litter decomposition by stimulating lignin decay (Berg et al., 2007); however, microbes tend to decompose lignin only after other more labile compounds have been depleted (Berg and Matzner, 1997; Berg and McClougherty, 2014). Thus, Mn amendments were associated with increased mass loss (6–19% higher) in control and intermediate N treatments (P = 0.075; Fig. S4), but not Mn amendments associated with increased mass loss regardless of N treatment, especially given the high rate of ligninase production under N amendment at six months.

Manganese and lignin-decay enzyme activity

Following six months of Mn amendment, the activities of ligninolytic enzymes (PER and POX) increased dramatically, confirming that this increase in enzyme activity was primarily driven by the addition of Mn, rather than by a hidden or joint effect of nutrients that co-vary with Mn (e.g., calcium). We found that after Mn amendment, Mn was the single main variable influencing the activity of ligninolytic enzymes, which represented a large shift from field conditions at CNAS, where N was the primary variable controlling enzyme activity (PLSR; Fig. S2a). These findings suggest that Mn amendments reversed N-induced limitations on enzyme activity and indicate that one important mechanism whereby N reduces ligninase activity is through indirect changes to soil and litter Mn status. Interestingly, these results are consistent with recent findings that N deposition reduces the relative abundance of Mn peroxidase transcripts associated with highly-ligninolytic litter decomposing fungi (Entwistle et al., 2018a).

4.3. Manganese and litter mass loss

Although Mn amendments significantly increased lignin-decay enzyme activity, this response did not translate to differences in total litter mass loss. This disconnect may be explained by the specific role of Mn in lignin decay. Manganese enhances litter decomposition by stimulating lignin decay (Berg et al., 2007); however, microbes tend to decompose lignin only after other more labile compounds have been depleted (Berg and Matzner, 1997; Berg and McClougherty, 2014). Thus, Mn amendments were expected to increase lignin and litter mass loss during late-stage decomposition, but not to influence mass loss during earlier stages of decay. For example, Berg et al. (2007) evaluated the relationship between litter Mn concentration and rates of litter mass loss at different stages of litter decay, finding that the relationship strengthened as litter decay proceeded.

In our incubation experiment, high N (N150) samples retained more lignin (+133%) than control (+37%) and intermediate (+57%) N treatments, suggesting that N150 samples were in an earlier stage of decay. This disparity in litter decay stage may explain why Mn amendments were associated with increased mass loss (6–19% higher) in control and intermediate N treatments (P = 0.075; Fig. S4), but not Mn amendments associated with increased mass loss regardless of N treatment, especially given the high rates

4.4. Fungal community response

In addition to the clear increase in ligninolytic enzyme activities, we observed a significant shift in fungal community composition with Mn amendment, which may be related to 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., 2018) and pathogenic fungi in soils (Morrison et al., 2016). We show a reversal of these N-induced 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. By definition, as the relative abundance of one taxon or functional group increases, that of another must decrease. Further, fungi vary in their ITS gene copy numbers (e.g., ascomycetes vs. basidiomycetes), and thus, relative sequence abundance of the functional groups evaluated in this study may not translate to differences in biomass of these groups (Baldrian et al., 2013). Therefore, to generate hypotheses about the link between altered fungal functional group relative abundance and community function, we limit our interpretation of these data to comparisons with relative abundance data from published N addition studies and evaluate relative changes in functional groups independent of one another.

In our study, the relative abundance of yeasts increased from 0.2% to 0.9% relative abundance with chronic N enrichment (under ambient Mn conditions). Morrison et al. (2018) demonstrated a ~200% increase in yeast relative abundance under long-term N enrichment in the field at the same study site, from 2.0% (N0) to 5.8% (N150). Although yeasts represented a smaller proportion of the fungal community in our
incubations, we show a 350% 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 C compounds (e.g., sugar monomers; Botha, 2011). For this reason, an increase in the dominance of yeasts has been suggested as a mechanism for reduced decomposition and increased soil C storage (Treseder and Lennon, 2015), which may explain soil C accumulation under long-term N enrichment (Morrison et al., 2018). We observed 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 documented 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 appeared to reduce pathotroph relative abundance (post-hoc least-squares means test), 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 obvious in this context. It is likely that many taxa in this category were acting as saprotrophs because facultative saprotrophy is a well-established alternate function of ‘pathotrophs’ (Parfitt et al., 2010; 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 tradeoffs associated with the dual-ecology lifestyle. These tradeoffs include possessing genes encoding only one, rather than the entire suite of class II peroxidases (e.g., MnP in the case of H. irregulare; Yakovlev 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 agaricomycetes (phylum 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. S6a). 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 appeared to reduce the relative 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. (2018), 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. This response was driven by a decrease in the relative abundance of ascomyceteous and zygomyceteous saprotrophs. The relative abundance of basidiomyceteous saprotrophs was unaffected by Mn amendment. Ascomyceteous and zygomyceteous 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 ascomyceteous and zygomyceteous 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 test this idea, we created a synthetic ‘weak’ decomposer category in which we summed the average relative abundance of yeasts, pathotrophs and zygomyceteous filamentous saprotrophs and regressed this group against ligninase activity. This model explained slightly more variation in enzyme activity than the pathotroph-only model (Fig. S6b). We excluded ascomyceteous filamentous saprotrophs from the model because the relative abundance of this group was not correlated with ligninase activity.

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, the relative abundance of OTUs with no annotation was greatest, increasing to >50% of the fungal community, where both N and Mn levels were highest. 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 be expected to have a low capacity for ligninase production (Floudas et al., 2012). Further, the relative abundance of this OTU was only weakly positively correlated with ligninolytic enzyme activities ($P = 0.049; R^2 = 0.08$). 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. (2018) 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. S5). 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 (Hofmockel et al., 2007; Treseder, 2008; Waldrop and Zak, 2006). While a decline in white-rot taxa with N enrichment has been demonstrated in wood collected from the forest floor of a simulated N deposition study in northern Michigan (Entwistle et al., 2018b), this trend is not ubiquitous across all sites and soil materials. This study showed an increase in the relative abundance of white-rot taxa in soils, and other studies have shown a similarly inconsistent response of lignin-decomposing taxa in leaf litter (Morrison et al., 2018) and organic horizon material (Entwistle et al., 2013). Therefore, the increase in dominance of ‘weak’ decomposers under N deposition may play a greater role in reduced lignin and litter decomposition than previously thought. In fact, Entwistle et al. (2018b) showed a significant increase in the relative abundance of soft-rot, cellulolytic and hemicellulolytic fungi with N enrichment, and this response was more consistent across soil materials. A complementary phenomenon, reduced expression of lignin-decay enzymes, has also been linked to increased dominance of weaker decomposers (Hesse et al., 2015). While overall lignocellulosic gene transcription was reduced in response to N enrichment, ascomycete dominance increased and their relative role in the transcription of
lignocellulosic genes was enhanced relative to basidiomycetes, leading the authors to conclude that ascomycetes may play an “expanded role” in plant litter decomposition under simulated N deposition (Hesse et al., 2015). 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 one potentially important mechanism reducing ligninolytic enzyme activity and altering fungal community composition under long-term atmospheric N deposition. We applied Mn amendments to chronically N-fertilized soils to evaluate 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 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 primarily 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 addition 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 may represent a mechanism that helps to explain reduced decomposition and soil C accumulation under this global change factor.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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