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Warming alters fungal communities and litter chemistry with implications for soil carbon stocks

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ABSTRACT

Rates of leaf litter decay are generally expected to accelerate with increasing temperature. However, chronic temperature elevation may cause changes in the quality of litter residues or in the composition of decomposer communities that result in unexpected decay responses. We performed a 23-month litter decomposition study at a long-term soil warming experiment to determine how soil warming affects litter decay rate, litter chemistry, extracellular enzyme activities, and fungal community composition. Experimental soil warming did not affect litter mass loss, but did result in a 23% increase in the relative abundance of lignin; a concomitant shift in enzyme activity wherein lignin-decomposing enzymes had higher activity in the heated treatment while cellulose-decomposing enzymes were suppressed; and a shift in the composition of the active fungal community. In particular, ectomycorrhizal fungi were three times more abundant in the heated treatment than the control, and shifts in fungal community composition were significantly correlated with the observed changes in extracellular enzyme activities. Our results suggest that warming altered the trajectory of decay resulting in litter residues enriched in lignin compounds and that were populated by a higher relative abundance of ectomycorrhizal fungi. These shifts may have altered the temperature sensitivity of litter decay by reducing the quality of litter residues and changing fungal community function.

1. Introduction

Rates of leaf litter decay increase with temperature across latitudinal gradients and in laboratory incubations (e.g., Hobbie, 1996; Conant et al., 2008, 2011) as expected given basic thermodynamic assumptions. However, litter decay is a dynamic process typified by sequential decay of different pools of compounds with varying lability (Melillo et al., 1982; Šnajdr et al., 2011) and a rapid succession of the microbial taxa that perform decomposition as different compounds are targeted (Frankland, 1966; Stursova et al., 2012; Voříšková and Baldrian, 2013). Long-term exposure to elevated temperatures alters the composition and physiology of soil microbial communities (Deslippe et al., 2012; Frey et al., 2013; DeAngelis et al., 2015; Pold et al., 2015) and may cause changes in soil organic matter decay rates and/or the kinds of compounds targeted by microbes (Pisani et al., 2015; Pold et al., 2017; Romero-Olivares et al., 2017). Because long-term warming may also affect the composition or activity of microbes most prevalent in litter decay, litter decomposition in soils exposed to decadal warming may not be reflective of short-term temperature responses, ultimately resulting in different kinds or amounts of organic matter entering soil carbon (C) pools. Studies of litter decomposition under long-term *in situ* temperature manipulations are rare (Conant et al., 2011) and there are few studies that describe the effects of long-term chronic warming on the diversity and composition of the litter microbial community (but see Christiansen et al., 2016; Treseder et al., 2016).

Soil fungi are the primary producers of extracellular enzymes that break down cellulose and lignin (Schneider et al., 2010, 2012), the most abundant components of plant material, and they dominate litter decomposition in temperate forests (Baldrian et al., 2012; Rousk and Frey, 2015). However, different types of fungi have varying capacities to break down cellulose and lignin (Fernandez-Fueyo et al., 2012; Floudas

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et al., 2012; Morin et al., 2012; Kohler et al., 2015). The primary decomposers of lignin are saprotrophs that break apart lignin in order to access cellulose and other labile C compounds, but some ectomycorrhizal fungi (ECMF) also have the potential to produce lignin-decomposing enzymes (Bödeker et al., 2009, 2014; Kohler et al., 2015). Because these different groups have varying functional capacity, extracellular enzyme production (both types and amounts) may be altered by long-term soil warming as fungal community composition changes.

Total fungal abundance in soil often declines in response to long-term experimental warming (Rinnan et al., 2007; Frey et al., 2008; DeAngelis et al., 2015; Liang et al., 2015) and fungal species composition can be affected as well (Deslippe et al., 2012; Pold et al., 2015). It is unknown, however, whether certain functional groups (i.e., saprotrophs versus ECMF) are differentially sensitive to temperature shifts. Observations of increased litter decay rates with higher temperatures (Conant et al., 2008, 2011) suggest greater abundance/activity of fungal saprotrophs, and increased relative abundance of saprotrophs with filamentous growth forms (i.e., excluding yeasts) has been observed in Arctic soils exposed to experimental warming (Treseder et al., 2016). Reponses of ECMF to warming vary and depend in part on the effects of warming on plant growth and plant photosynthetic rates (Clemmensen et al., 2006; Fernandez et al., 2017). Positive responses of aboveground plant biomass to warming are associated with increased ECMF biomass (Clemmensen et al., 2006) and favor ECMF species that form extensive foraging mycelia (Deslippe et al., 2011). Conversely, when warming reduces plant photosynthetic rates, ECMF species that form low-biomass ectomycorrhizae are favored (Fernandez et al., 2017).

To the extent that warming changes the relative abundance or activity of fungal functional groups and thus the extracellular enzymes produced, it also has the potential to affect the chemistry of litter residues that remain after decomposition, because different decomposer communities produce residues with distinct chemistries (Wickings et al., 2011, 2012). Long-term C dynamics are affected by the chemistry of litter residues, in part because of the greater activation energies required to decompose recalcitrant versus labile compounds (Davidson and Janssens, 2006), which make decay of recalcitrant organic matter more sensitive to temperature increases (Fierer et al., 2005; Conant et al., 2011). Litter chemistry may also affect the efficiency with which the fungal community incorporates C into biomass (Frey et al., 2013), as well as influence the potential for organic matter association with mineral surfaces (Jilling et al., 2018), both of which are important controls on long-term soil C storage (Wieder et al., 2013).

Our objectives in this study were to determine the effects of experimental soil warming on litter decay dynamics – including decay rates and the chemistry of litter residues remaining after decomposition – and to explore the underlying mechanisms of litter decay by examining fungal community composition and associated enzyme activities. We conducted a two-year litter decomposition study within a long-term (~10 yr) soil warming experiment, measuring litter mass loss, litter chemistry, fungal diversity and community composition, and extracellular enzyme activities in control and heated plots continuously warmed to 5 °C above ambient.

2. Materials and methods

2.1. Site description, experimental design, and sample collection

This research was performed in an even-aged mixed deciduous forest at the Harvard Forest Long-term Ecological Research (LTER) site in Petersham, Massachusetts, USA (42° 28′ N, 72° 10' W). Vegetation at the site is dominated by red (*Quercus rubra*) and black (*Q. velutina*) oak, which associate with ectomycorrhizal fungal symbionts, with lesser contributions of red maple (*Acer rubrum*) and American beech (*Fagus grandifolia*). Soils are coarse sandy loam Typic Dystrudepts of the Canton series, and mean weekly air temperature ranges from −6 °C in January to 20 °C in July (Melillo et al., 2011). Two adjacent 30 × 30 m megaplots were established in 2003, with one plot serving as a control and the other heated continuously to 5 °C above ambient soil temperature throughout the year by buried heating cables (Melillo et al., 2011). Each megaplot is divided into thirty-six 5 × 5 m subplots, with outer subplots excluded from sampling to reduce edge effects. The two adjacent plots share the same site history, and pre-treatment measurements indicated the plots were very similar in terms of standing woody biomass, woody increment, and soil respiration rates prior to the inception of the experiment (Melillo et al., 2011).

Leaf litter was collected at senescence in fall 2009 from the control plot by use of mesh baskets suspended above the forest floor. We excluded litter from the heated treatment to control for changes in litter chemistry caused by warming – at the time of litter collection senescent litter of oak, which dominates the site, had a 12% higher N concentration and a 10% lower C:N ratio in the heated treatment relative to the control. Collected litter was air-dried, separated by species, weighed, and cut into pieces ~3 × 3 cm. Litterbags (0.3 mm mesh) were prepared using 10 g of mixed litter representing the relative proportions of each tree species at the site (i.e., 70% oak, 20% maple, 10% beech). Litterbags were separated into two compartments with approximately two thirds of the litter placed in one compartment designated for measures of litter mass loss, enzyme activities and litter chemistry, and the remaining third in a separate compartment for determination of fungal community structure (diversity and composition). This separation facilitated immediate preservation at the time of harvest of the litter designated for fungal community analyses. Ten replicate litterbags were placed on the forest floor in each experimental treatment (control or heated) in November 2010 in 10 subplots in each treatment megaplot. Litter bags were placed within the freshly fallen litter layer by first gently removing the layer of freshly fallen leaves, pinning the bags in place, and then replacing the leaves to cover the litterbags. Litterbags were harvested in October 2012 after 23 months of decomposition (soil temperatures during this period shown in Supplementary Material Fig. S1). This harvest point is in the middle of predicted phase I decomposition (*sensu* Aber et al., 1990) for oak litter at this site (Aber et al., 1990). At harvest, litterbags were weighed immediately in the field and then the litter from the smaller compartment was removed using sanitized forceps and weighed, homogenized, subsampled and flash frozen in the field with liquid N. Flash frozen samples were transported in liquid N and stored immediately at −80 °C until RNA/DNA extractions were performed. The remaining fresh litter was transported on ice and stored at 4 °C for analysis of mass loss, extracellular enzyme activity, and litter chemistry.

2.2. Litter mass loss, extracellular enzyme activities, and molecular chemistry

Fresh litter samples were processed within three days of harvest. Sampled litter was cut into ~1 × 1 cm pieces and homogenized for subsampling. Moisture content was measured by drying a 1 g subsample at 60 °C for 48 h. Mass loss was determined as the difference between initial dry weight of leaf litter and dry weight at harvest. While the litterbag method may underestimate absolute litter mass loss by preventing litter fragmentation by soil fauna, it does provide a reasonable means to assess relative differences resulting from global change treatment effects on microbial community activity (Cotrufo et al., 2010).

We evaluated potential activities of a suite of extracellular enzymes involved in the decomposition of organic C, N, and P sources. The methods used were described in full by van Diepen et al. (2015) and followed the protocols as outlined by Saiya-Cork et al. (2002) and DeForest (2009). Briefly, enzyme activities were determined using a 0.5 g subsample of field-moist litter which was homogenized in 125 ml sodium acetate buffer at pH 4.7, representing the average pH of the

sampled litter. Activities of the hydrolytic enzymes leucine aminopeptidase (LAP), *N*-acetyl-glucosaminidase (NAG), acid phosphatase (PHOS), β-glucosidase (BG) and cellobiohydrolase (CBH) were measured fluorometrically using MUB-linked substrates, or AMC-linked L-leucine in the case of LAP. Activities of the oxidative enzymes phenol oxidase (PPO, ABTS) and peroxidase (PER, TMB) were measured colorimetrically using two substrates for each enzyme. Phenol oxidase activity was measured using L-3,4-dihydroxyphenylalanine (L-DOPA) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), gross oxidase-peroxidase was measured using L-DOPA + H_2O_2 (PER) and peroxidase-specific activity was measured using 3,3′,5,5′-Tetramethylbenzidine + H_2O_2 (TMB; Johnsen and Jacobsen, 2008).

The remaining litter was frozen at −80 °C, freeze-dried, and ground for analysis of total organic C and N content and molecular litter chemistry. The latter was measured on a subset of five randomly chosen samples from each treatment by determining the relative percentages and ratios of chemical classes determined using pyrolysis-gas chromatography/mass spectrometry (py-GC/MS) as described previously (Grandy et al., 2009; Wickings et al., 2011). Briefly, samples were pyrolyzed at 600 °C and products transferred to a GC where compounds were separated on a 60 m capillary column with a starting temperature of 40 °C followed by a temperature ramp of 5 °C min^{-1} to 270 °C followed by a final ramp (30 °C min^{-1}) to 300 °C. Compounds were transferred to an ion trap mass spectrometer where they were ionized, detected via electron multiplier, and identified using a compound library from the National Institute of Standards and Technology (NIST).

2.3. Fungal diversity and community composition

We assessed fungal community structure (diversity and composition) of both the “total” and “active” fungal community (*sensu* Baldrian et al., 2012) by sequencing ribosomal RNA genes (rDNA) or transcripts (rRNA), respectively. We co-extracted DNA and RNA from 0.5 to 0.75 g of flash-frozen litter from the same subset of five replicate litterbag samples used for the litter chemistry analysis (described above). DNA and RNA were extracted using a MoBio Powersoil Total RNA Extraction kit and DNA elution accessory kit and purified using a Zymo One-Step PCR Inhibitor Removal column. RNA samples were subjected to a DNAse I treatment following the standard factory protocol in order to remove contaminating DNA. RNA was reverse transcribed using a Promega GoScript Reverse Transcription System kit following the factory protocol. The ITS2 region was amplified from DNA and cDNA by PCR with the primers fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) which were modified to include 10 bp molecular identification indices and the Roche 454 Lib-L adaptor A (ITS4) or B (fITS7).

PCR was performed in triplicate following the protocol of Morrison et al. (2016) and triplicate PCR products were then pooled and purified using AMPure paramagnetic beads to remove fragments less than 200 bp. The concentration of purified PCR products was quantified using a Qubit fluorometer and PCR products were then combined into an equimolar amplicon library. Sequencing was performed on a ¼ plate Roche 454 Titanium FLX sequencing run at the Roy J. Carver Biotechnology Center (University of Illinois).

Sequence data were demultiplexed, denoised, and quality filtered using AmpliconNoise (Quince et al., 2011) as implemented in QIIME 1.7.0 (Caporaso et al., 2010). Sequences that had lengths less than 200 bp were removed and remaining sequences were reverse-complemented and the ITS2 region was extracted using the program FungalITSextractor (Nilsson et al., 2010). ITS2 sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using usearch v. 4.2.66 (Edgar, 2010) with the most abundant sequence in each OTU taken as a representative sequence. Chimeras were identified using the uchime *de novo* method implemented in usearch v. 4.2.66 (Edgar, 2010). Representative sequences were identified taxonomically in QIIME 1.7.0 using BLAST (Caporaso et al., 2010) with the default minimum e-value of 0.001 and using the dynamically clustered UNITE fungal ITS database (Kõljalg et al., 2013) as a reference database. Representative sequences that did not have a satisfactory match in the UNITE database were compared to the NCBI non-redundant nucleotide database using blastn. Blast results from NCBI were processed using the LCA algorithm in MEGAN v4.70.4 (Huson et al., 2011) with a low complexity filter of 0, minimum support of 3, top 5% of matches, and other parameters set to default values. OTUs with a single sequence (singletons) and OTUs that were assigned to taxa other than fungi were manually removed. Chimeric, singleton, and non-fungal OTUs were excluded from all further analyses. The number of total and retained sequences at each processing step are reported in Supplementary Material Table S1. After quality filtering, ITS extraction, sequence clustering at 97% sequence similarity, and removal of chimeras, sequences of non-fungal origin, and singletons, the dataset consisted of 41,329 sequences, constituting 709 OTUs. Of the total OTUs, 80% had matches in either the UNITE or NCBI database. The full dataset was subsampled to 800 sequences per sample, a sequencing depth consistent with recent fungal metabarcoding studies (e.g., Clemmensen et al., 2015; Glassman et al., 2017).

Number of observed OTUs, Shannon diversity, and Simpson's diversity were calculated for each sample in QIIME. Trophic status was assigned to OTUs by comparing genus names to the trophic status assigned to fungal genera by Tedersoo et al. (2014). Though we recognize that these binnings are likely to be overly simplistic due to different autecologies within guilds, they do provide a means to summarize highly diverse communities in an ecologically meaningful way (Nguyen et al., 2016). We assigned trophic status to OTUs at the family level when genus names could not be identified. This was achieved by determining the proportion of genera within a family that shared a trophic status according to Tedersoo et al. (2014). If the proportion of genera was over 95% we assigned that trophic status to the family as a whole; if over 75%, we assigned a “putative” trophic status. Assignments were checked manually for accuracy. If OTUs could not be identified to the family level they were assigned a function of unknown. Our approach is similar to that used by FUNGuild (Nguyen et al., 2016) in that it is based on the same primary database (derived from Tedersoo et al., 2014), but allows classification at the family level whereas FUNGuild primarily considers genus-level assignments. To compare the two approaches, we performed assignments using FUNGuild and found that similar numbers of annotations were made using either approach. Two hundred seventy-nine OTUs (51.0% of OTUs after subsampling to equal sequencing depth per sample) were classified by our approach excluding “putative” assignments versus 274 OTUs (50.0%) using FUNGuild.

2.4. Statistical analyses

Treatment level mean comparisons of litter mass loss, extracellular enzyme activities, and source material of litter chemical compounds determined by py-GC/MS were performed in R v3.1.3 (R Core Team, 2015) using one-way ANOVA. Model residuals were checked for normality and homogeneous variance. When the assumption of normality was violated, data were log-transformed, and when the assumption of homoscedasticity was violated, mean comparisons were performed using a generalized least squares model allowing for heterogeneous variance structure in R package nlme (Pinheiro et al., 2015). To compare means of fungal taxonomic or functional groups, OTU richness, Shannon diversity, and Simpson diversity, we used linear mixed effects models with temperature treatment (control versus heated), nucleic acid pool (DNA versus RNA) and their interaction as fixed effects. We also included litterbag sample as a random effect to account for lack of independence arising from sampling RNA and DNA pools from the same samples. Assumptions of normality and homogeneity of variance were checked as described above.

We used multiple approaches to test for effects of soil warming on fungal taxon relative abundance. To provide a qualitative description of

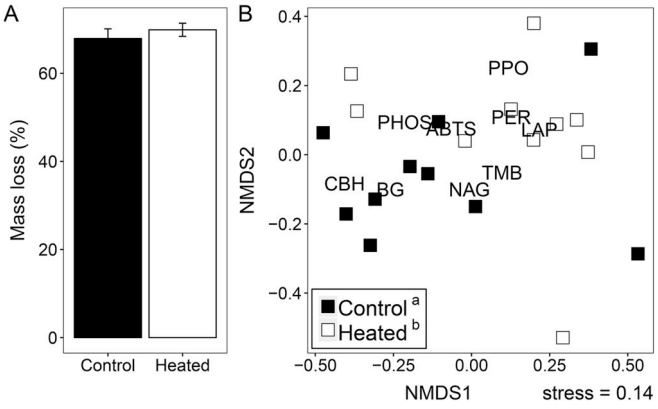

Fig. 1. Mass loss (A) and NMDS ordination of enzyme activities (B). Enzyme activities were relativized by the maximum value for each enzyme before ordination. Superscript letters in panel B figure legend indicate significant differences between treatments. Abbreviations in panel B: BG = beta-glucosidase; CBH = cellobiohydrolase; PPO = polyphenol oxidase (L-DOPA); PER = gross oxidase-peroxidase (L-DOPA + H_2O_2); ABTS = polyphenol oxidase; TMB = peroxidase; LAP = leucine aminopeptidase; NAG = *N*-acetyl glucosaminidase; PHOS = acid phosphatase.

the effects of increased temperature on OTU relative abundance and relate those to taxonomic identity, we calculated Cohen's *d* effect size (Cohen, 1992) for each OTU, calculated Euclidean distance between observations, and performed hierarchical agglomerative clustering of the Euclidean distance matrix with Ward's d clustering method. We then created tanglegrams (Galili, 2015) of the dendrograms resulting from hierarchical clustering of effect sizes and taxonomic distance (Pienkowski et al., 1998; Clarke and Warwick, 2001) for all OTUs that occurred in at least 25% of samples. We excluded OTUs with less than 25% frequency to restrict the analysis to relatively common groups, because we expect the more frequent community members to be more relevant to functional processes (Magurran and Henderson, 2003; Unterseher et al., 2011). We also used Indicator Species Analysis (ISA; De Cáceres and Legendre, 2009; De Cáceres et al., 2010) to test for fungal OTUs with ≥25% frequency that had large positive or negative responses to warming in frequency and relative abundance. ISA was also used to determine litter chemical compounds identified by py-GC/MS that were indicators of control or heated treatments.

To examine the multivariate treatment effects of warming on enzyme activities, litter chemistry, and fungal community composition, we calculated Bray-Curtis distance after relativization by maximum value (enzyme activities) or log + 1 transformation (litter chemistry and fungal community data) and performed NMDS, PERMANOVA (*adonis*) tests, and PERMDISP tests (Oksanen et al., 2015). PERMANOVA was performed with temperature treatment (control versus heated) and nucleic acid pool (DNA versus RNA) as independent variables and litterbag as sample strata, with 99 data permutations (Anderson, 2005). To examine the relationship between enzyme activities and fungal community composition we performed Mantel tests on Bray-Curtis distance matrices and performed linear regressions of enzyme activities against fungal functional group relative abundance. We compared enzyme activities and litter chemistry by fitting (*envfit*) enzyme activities and the relative abundance of chemical source groups (e.g., lignin, polysaccharides, proteins), against the NMDS ordination calculated from relative abundance of individual chemical compounds (Oksanen et al., 2015). We used the Benjamini and Hochberg FDR procedure (Verhoeven et al., 2005) to control for Type I error arising from multiple comparisons in ISA and linear models. A p-value < 0.05 was taken as significant in all statistical tests.

2.5. Accession numbers

Sequence data have been deposited in the NCBI Sequence Read Archive at accession number SRP090501.

Table 1
Biochemical responses to experimental warming after two years of litter decomposition. Standard errors are presented in parentheses. Different letters denote significant differences between treatments ($P < 0.05$); significant differences are also highlighted in bold.

Biochemical parameter	Warming treatment	
	Control	Heated
Litter C (%)	51.35 (0.54)[a]	52.05 (0.34)[a]
Litter N (%)	**2.16 (0.03) [b]**	**2.32 (0.03) [a]**
Litter C:N	**23.8 (0.4) [a]**	**22.5 (0.4) [b]**
Moisture (%)	**67.92 (1.29) [a]**	**63.20 (1.01) [b]**
Net N mineralization (% mineralized yr^{-1})	9.67 (2.78)[a]	9.37 (2.02)[a]
Enzyme activity (μmol hr^{-1} g $litter^{-1}$)		
Leucine aminopeptidase[1]	26.24 (10.77)[a]	23.42 (15.77)[a]
N-acetyl glucosaminidase	1.02 (0.09)[a]	0.99 (0.15)[a]
Acid phosphatase	4.68 (1.96)[a]	7.55 (2.17)[a]
β-glucosidase	1.81 (0.29)[a]	0.98 (0.31)[a]
Cellobiohydrolase	**0.42 (0.10) [a]**	**0.15 (0.08) [b]**
Polyphenol oxidase (L-DOPA)	**7.94 (1.96) [b]**	**15.89 (2.02) [a]**
Peroxidase (L-DOPA)	16.16 (3.96)[a]	14.56 (2.10)[a]
Polyphenol oxidase (ABTS)	79.15 (9.91)[a]	86.42 (14.08)[a]
Peroxidase (TMB)	**67.44 (6.16) [b]**	**99.44 (8.02) [a]**
Litter chemistry (% relative abundance)		
Aromatics	5.35 (0.94)[a]	5.37 (0.54)[a]
Lignin	**33.76 (0.81) [b]**	**41.50 (1.69) [a]**
Lipids	20.90 (1.65)[a]	19.67 (1.93)[a]
Polysaccharides	9.85 (1.26)[a]	7.82 (1.08)[a]
Nitrogen bearing compounds	3.68 (0.47)[a]	3.60 (0.44)[a]
Proteins	2.51 (0.13)[a]	2.26 (0.10)[a]
Phenols	0.79 (0.12)[a]	0.60 (0.05)[a]
Compounds of unknown origin	**23.17 (0.63)[a]**	**19.18 (1.89)[b]**

[1]Units for this enzyme are nmol hr^{-1}g $litter^{-1}$.

3. Results

3.1. Litter mass loss, enzyme activities, and chemistry

Soil warming had no effect on litter mass loss, which averaged 68.9% across all samples; however, it did significantly alter enzyme activities (Fig. 1). Experimental warming increased the activities of polyphenol oxidase and peroxidase and decreased the activity of cellobiohydrolase (Table 1). Warming also significantly altered litter chemistry (Fig. 2), with the relative abundance of lignin-derived compounds being 23% higher in the long-term warming treatment compared to the control (Table 1 and solid vectors Fig. 2). We note that because the litter used for this experiment was collected from the control treatment, any differences in chemistry must be a result of

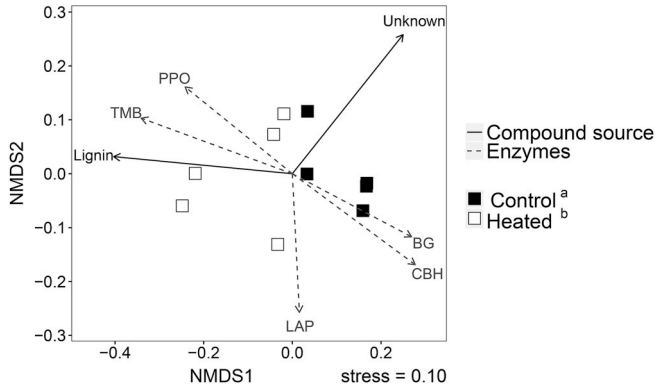

Fig. 2. NMDS ordination of chemical compound relative abundance generated by py-GC/MS analysis of plant litter decomposed for two years under experimental warming. Data were log + 1 transformed prior to analysis. Points represent the chemical composition of different samples. Vectors represent the correlations of relative abundance of compound source classes (solid lines; unknown compound class refers to compounds that were detected by py-GC/MS but which are of unknown origin) or enzyme activities (dashed lines) with the underlying chemical compound matrix. Only significant correlations are plotted. Enzyme abbreviations: BG = beta-glucosidase; CBH = cellobiohydrolase; PPO = polyphenol oxidase (L-DOPA); TMB = peroxidase; LAP = leucine aminopeptidase.

changes in decay dynamics and not due to differences in the initial chemistry of senescent litter. The differences in litter chemistry driven by warming were clearly associated with variation in enzyme activities (enzyme activities with significant correlation with litter chemistry are represented by dashed vectors in Fig. 2). Cellulose-decomposing enzymes and compounds of unknown origin had higher abundance in the control treatment, while lignin-decomposing enzymes (TMB, PPO) and lignin compounds were more abundant in litter in the heated treatment. Though it may be somewhat counterintuitive that lignin-decomposing enzymes had higher activity in the heated treatment where lignin was also at a higher concentration, we assume this is a response to the litter chemistry at the time of sampling (Sinsabaugh and Folstad Shah, 2011) and is not reflective of enzyme activities over the course of decomposition. Indeed, higher lignin relative abundance in the heated treatment suggests that lignin-decomposing enzymes were less active over the course of decay. Indicator species analysis identified six litter compounds as indicators of either the control or heated treatment (Table 2). These included compounds derived from proteins, lipids and polysaccharides in the control treatment versus proteins and lignin in the heated treatment. The most abundant control treatment indicator was *n*-triacontane (C30), a lipid likely derived from plant material (Rielley et al., 1991; Marseille et al., 1999), while the most abundant heated treatment indicator was vanillin, a lignin monomer.

Table 2
Results of indicator species analysis listing chemical compounds with high frequency and/or relative abundance in control (gray rows) or heated (white rows) litter, performed on chemical compounds observed with at least 25% frequency as determined by py-GC/MS. Only significant results are reported. The r_{pb} column gives the value of the point biserial regression coefficient which indicates the strength of the association ranging from 0 to 1, analogous to a Pearson correlation coefficient (De Cáceres et al., 2010). Compound source material is indicated, as well as the average relative abundance for each treatment (with standard errors given in parentheses) and the rank order of compound abundance (the most abundant compound in the dataset would have a rank order of 1).

Compound name	r_{pb}	*P* value	Compound source	Relative abundance (%)		Rank order
				Control	Heated	
Control treatment						
Benzenepropanenitrile	0.889	0.02	Protein	0.13 (0.02)	0.00 (0.00)	106
n-Triacontane (C30)	0.74	0.03	Lipid	7.19 (0.40)	2.99 (1.29)	3
5-methyl-2(5H)-Furanone	0.697	0.03	Polysaccharide	0.12 (0.03)	0.03 (0.02)	100
Biphenyl	0.675	0.02	Aromatic	0.06 (0.02)	0.02 (0.01)	128
3-hydroxy-2-methyl-4H-Pyran-4-one	0.664	<0.05	Polysaccharide	0.90 (0.17)	0.40 (0.12)	34
3,4-dimethyl-Phenol	0.611	0.04	Aromatic	2.10 (0.23)	1.31 (0.28)	18
Heated treatment						
1,2-diethyl-Benzene	0.863	0.02	Aromatic	0.00 (0.00)	0.04 (0.01)	154
Benzonitrile	0.793	0.02	Protein	0.01 (0.002)	0.02 (0.002)	178
4-hydroxy-3-methoxy-Benzaldehyde (vanillin)	0.75	0.02	Lignin	0.67 (0.09)	1.16 (0.12)	28
Monobenzone	0.744	0.04	Unknown origin	0.00 (0.00)	0.06 (0.01)	139
1,2,3,4-tetramethyl-Benzene	0.732	0.04	Aromatic	0.002 (0.002)	0.04 (0.01)	160
1,3,5-Cycloheptatriene	0.495	0.04	Unknown origin	0.00 (0.00)	0.02 (0.01)	176

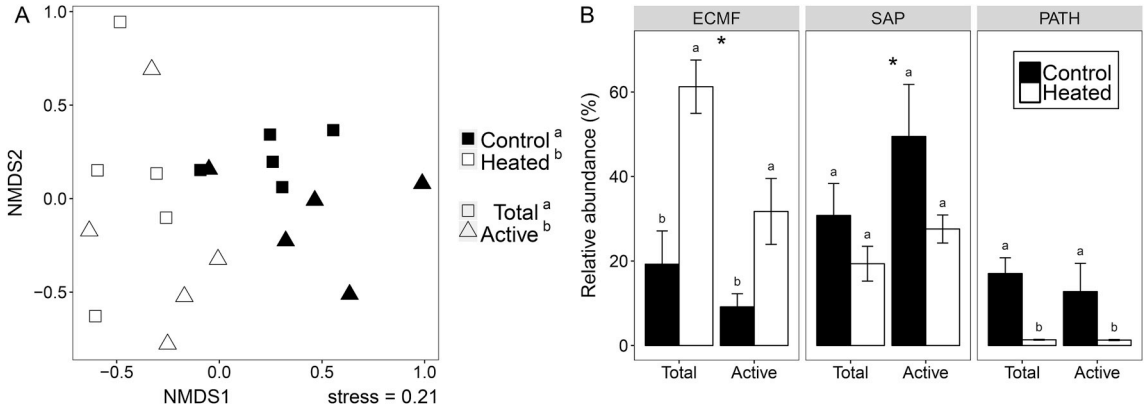

Fig. 3. NMDS ordinations of fungal community composition (A) and mean relative abundance of fungal functional groups (B) in leaf litter after two years of decomposition. Different letters in the panel A figure legend indicate significant differences between treatments or the total (DNA) and active (RNA) community. Error bars in panel B indicate standard error, different letters above bars indicate significant differences between treatments, and asterisks indicate a significant difference in mean relative abundance of functional groups in the total or active community. Abbreviations in panel B: ECMF = ectomycorrhizal fungi; SAP = saprotrophic fungi; PATH = plant pathogenic fungi. Functional group assignments to individual OTUs are shown in Fig. 4 and Fig. S2 column "Trophic status."

3.2. Fungal diversity and community composition

Soil warming did not alter OTU richness in the total fungal community (DNA), but increased OTU richness in the active community (RNA) by 33%, with 73 OTUs per sample found on average in the control treatment compared to 97 in the heated treatment. Warming had no effect on Simpson diversity or Shannon diversity, averaging 0.84 and 3.99, respectively, across all samples. However, there was a warming-induced shift in fungal community composition in both the total (DNA) and active (RNA) communities (Fig. 3A). Community composition was also different in the total (DNA) versus active (RNA) communities, though this difference was small relative to the warming effect. Basidiomycetes made up a larger proportion of sequences than ascomycetes when averaged across total and active communities, and increased from 55% of the community in the control treatment to 62% in the heated treatment ($P = 0.02$). Warming decreased the relative abundance of ascomycetes from 35% to 26% ($P = 0.02$).

Ectomycorrhizal fungi were favored by warming, while warming decreased the relative abundances of saprotrophs and plant pathogens, the latter significantly (Fig. 3B). Plant pathogens made up a large proportion of the community in control litter, representing 17% of the total community, versus 1.4% in the heated treatment. Despite their assignment to the category plant pathogen (Tedersoo et al., 2014), the majority of species in this group likely function as saprotrophs in a litter decay context. For example, the genus *Venturia* made up 85% of the plant pathogen group in terms of relative sequence abundance, and *Venturia* species often persist as saprotrophs on senescent litter after initially parasitizing living plants (Frankland, 1998). There were also differences in the relative levels of activity of different functional groups, as determined by comparing relative abundance in the total (DNA) and active (RNA) communities (Fig. 3B). Saprotrophs made up 25% of the total pool across all samples on average, compared to 39% of the active pool, while ECMF made up 40% of the total pool and 20% of the active pool, suggesting that saprotrophs as a group were more active than ECMF. However, there was no effect of warming treatment on this trend (i.e., no interaction between treatment and total versus active community abundance), suggesting that warming did not differentially affect relative activity levels of the groups and that the effects of warming on functional group relative abundance were consistent in both the total (DNA) and active (RNA) communities.

Some fungal taxa (OTUs) increased significantly in relative abundance with warming and some decreased, and though there was no clear relationship between taxonomic identity and warming response overall, several taxonomic or functional groups did show consistent responses to warming (Fig. 4 and Supplementary Fig. S2). For example, saprotrophs with high potential for lignin decomposition (Osono and Takeda, 2002; Jordaan and Leukes, 2003; Martínez Ferrer et al., 2005; Osono, 2007; Šnajdr et al., 2010), such as *Mycena* (most abundant OTU at 7%), *Rhodocollybia* (3rd most abundant OTU at 6%) and *Crepidotus* all had negative responses to warming. Similarly, the genus *Venturia* responded negatively to warming. This genus included the second most abundant OTU (7% global relative abundance). In contrast, the majority of ECMF taxa responded positively to warming. These included OTUs of the genera *Russula*, *Cortinarius*, *Laccaria*, and *Cenoccocum*. The genus *Russula* contained the fourth (5% relative abundance) and seventh (3% relative abundance) most abundant OTUs.

Community composition of the total fungal community (DNA) did not correlate well with enzyme activities (Mantel r = 0.18, P = 0.15); however, the active fungal community (RNA) showed a significant correlation with the suite of enzyme activities we measured (Mantel r = 0.41, $P = 0.002$). In the active community, only ECMF and saprotrophs had significant correlations with individual enzymes after correcting for multiple comparisons by linear regression (Fig. 5). Active ECMF relative abundance (RNA) was positively correlated with leucine aminopeptidase (LAP) activity. Relative abundance of active saprotrophs (RNA) was positively correlated with β-glucosidase (BG) activity and negatively correlated with peroxidase (TMB) activity. In addition, in the total community (DNA) ECMF relative abundance was positively correlated with peroxidase activity (TMB; $r^2 = 0.57$, $P = 0.01$) and negatively correlated with β-glucosidase activity (BG; $r^2 = 0.44$, $P = 0.04$).

4. Discussion

We examined the biochemical responses (mass loss and extracellular enzyme activities) and fungal communities associated with decaying litter in soils exposed to 5 °C elevated temperature for nine years at the time of our sampling. We show that warming increased the relative abundance of lignin in leaf litter residues despite no significant change in litter mass loss after two years of decomposition. This shift in litter chemistry was associated with changes in fungal community composition and activity – wherein warming favored ECMF and lignin-decomposing enzyme activity and suppressed litter saprotrophs and cellulose-decomposing enzyme activity.

Litter mass loss did not increase with warming, a result that contradicts results from laboratory incubations of soil or litter, where decay and respiration rates generally increase with temperature (Conant et al., 2008, 2011). Similar to our observations, litter mass loss in boreal

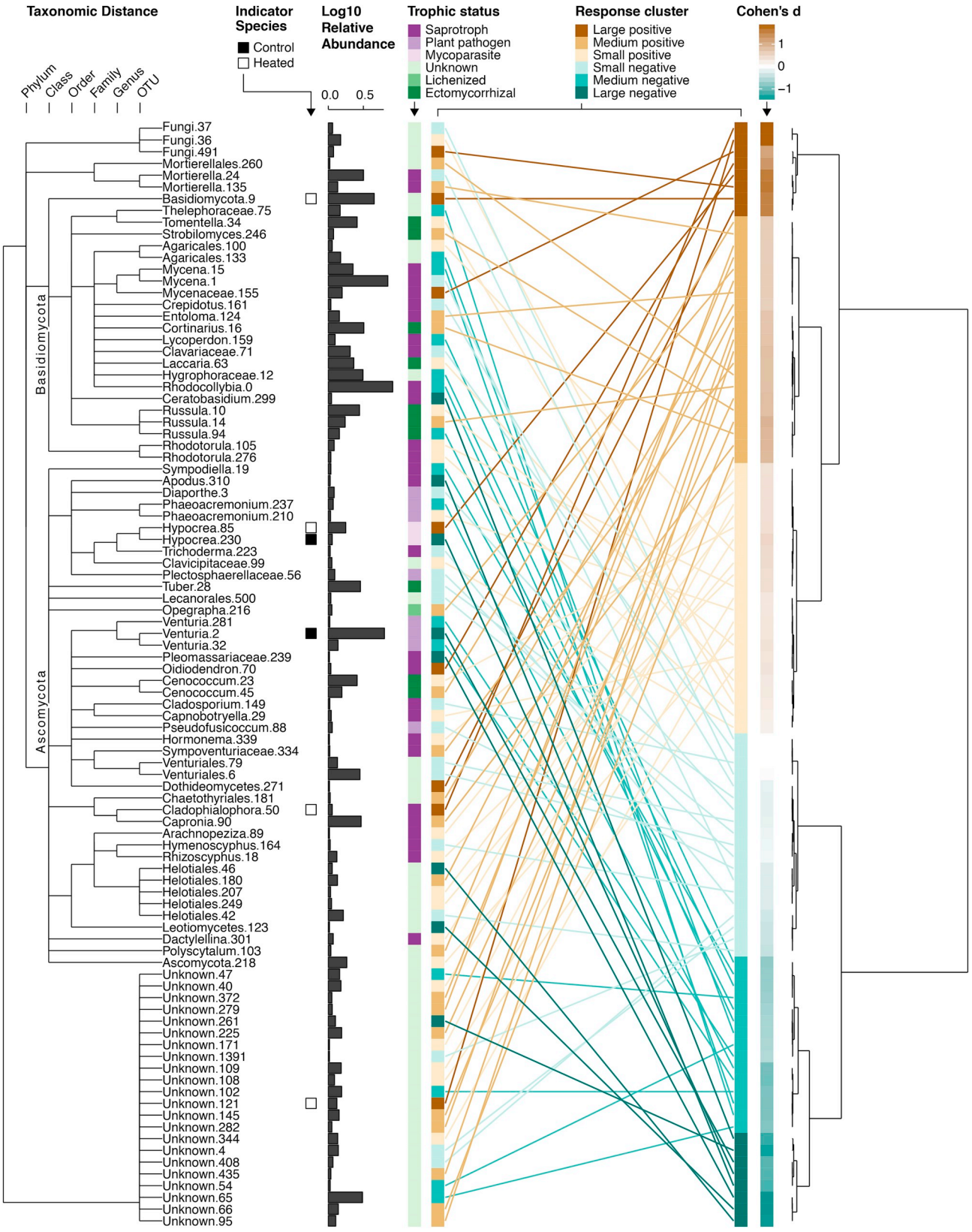

Fig. 4. Relationships among individual fungal taxa, their respective functional groupings, and warming treatment response for the active (RNA) community. Those OTUs identified as indicator species of a warming treatment are shown in column "indicator species". Global OTU relative abundance was log10 transformed for readability. Trophic status classifications were assigned according to Tedersoo et al. (2014). "Response Cluster" shows the direction (positive or negative) of OTU relative abundance in response to warming treatment based on agglomerative hierarchical clustering of the warming response effect size (Cohen's d). The same analysis for the total (DNA) fungal community is shown in Supplemental Fig. S2.

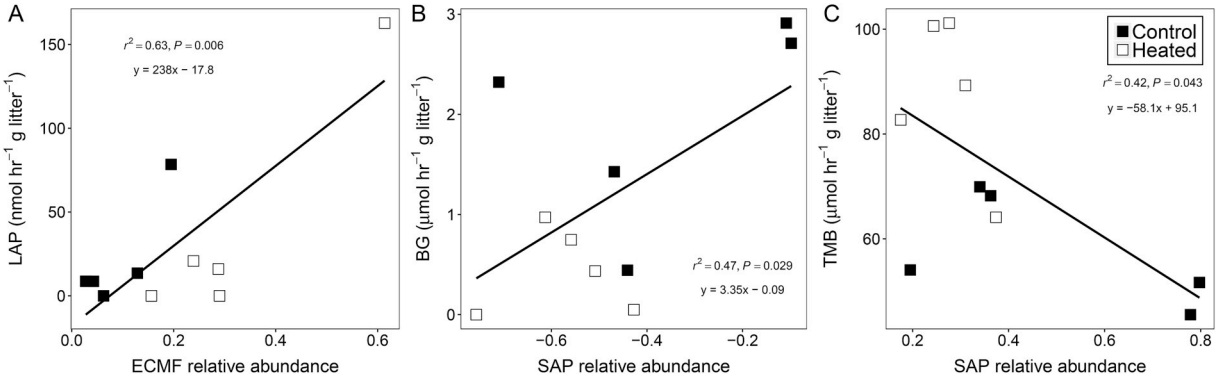

Fig. 5. Relationships between active (RNA) ectomycorrhizal fungi relative abundance and leucine aminopeptidase activity (A), and active saprotroph relative abundance and beta-glucosidase activity (B) and peroxidase activity as measured by the TMB substrate (C). These three regressions are presented because they were the only significant correlations of active fungal functional groups with enzyme activities after correction for multiple comparisons. Overall, the active community had a higher correlation with enzyme activity than the total community (Mantel $r_{active} = 0.41$, $P_{active} = 0.002$; $r_{total} = 0.18$, $P_{total} = 0.15$) Abbreviations: ECMF = ectomycorrhizal fungi; SAP = saprotrophic fungi.

regions exposed to *in situ* warming manipulations was either unchanged (Christiansen et al., 2016) or decreased (Romero-Olivares et al., 2017). However, in the boreal ecosystems, these results were attributed at least partly to decreased litter moisture content with warming. Although we observed a significant decrease (−6.9%) in litter moisture content in the heated treatment at our site (Table 1), samples in both treatments were well within an optimal range for microbial activity (Ilstedt et al., 2000; Moyano et al., 2013; Strickland et al., 2015) and litter mass loss was not correlated with moisture content ($r^2 = 0.08$, $P = 0.2$), suggesting that moisture limitation was not a prominent control on mass loss rates in our system. This interpretation is corroborated by long-term measurements at the site – organic horizon soils tend to be drier in the heated treatment, but this difference is most pronounced at high moisture content and converges during drier conditions (below ~50% moisture during the time period of our experiment). While lower moisture may have played some role in limiting mass loss rates in the heated treatment, it seems unlikely to be the primary driver of decay dynamics in this experiment.

Instead, we believe it is likely that observed changes in fungal community composition, enzyme activity, and litter chemistry may explain the lack of a litter decay response. Nine years of soil warming appears to have altered the composition of the fungal species pool available to colonize and decompose litter, such that different compounds were preferentially decomposed in the heated treatment and decay rates were not accelerated as predicted by first order thermodynamics. Specifically, ligninolytic saprotrophs decreased in relative abundance in both the total (DNA) and active (RNA) communities, concomitant with an enrichment of lignin in litter residues. An entirely different result was observed in a boreal system where warming favored ligninolytic filamentous fungi (Treseder et al., 2016) and increased rates of lignin decomposition (Romero-Olivares et al., 2017). It is unclear what controls these system-specific dynamics, but the relative importance of moisture limitation or background nutrient availability may play a role in determining community responses (Romero-Olivares et al., 2017). For example, and as discussed above, boreal systems often experience stronger suppression of soil moisture in warming treatments than we observed at our site (Allison and Treseder, 2008; Christiansen et al., 2016).

In addition to decreased relative abundance of ligninolytic saprotrophs, we observed an increase in ECMF relative abundance in both the total and active community. Warming also increased tree productivity at this site and increased the N concentration of tree foliage and litter (Melillo et al., 2011), which may favor higher relative abundance of ECMF (Clemmensen et al., 2006; Deslippe et al., 2011). Litter fungal communities that are dominated by ECMF relative to saprotrophs are associated with lower litter decay rates (Bödeker et al., 2016), and ECMF are thought to compete with saprotrophs for nutrients (Averill et al., 2014; Fernandez and Kennedy, 2016), potentially limiting saprotrophic contributions to decay. Competition between these groups may therefore play a role in the overall community response.

Saprotrophic fungi and ECMF were also associated with different kinds of enzyme activity wherein saprotrophic fungi were positively correlated with cellulose decomposition and ECMF were positively correlated with lignin and organic N decomposition (Fig. 5), confirming previous results that show correlations between ECMF abundance and ligninolytic enzyme activity at the ecosystem scale (Talbot et al., 2013, 2015; Bödeker et al., 2014). Genetic evidence shows that some of the more abundant ectomycorrhizal species in our study (e.g., *Russula* and *Cortinarius* species; Fig. 4 and Fig. S2) have the potential to produce lignin-decomposing enzymes like peroxidases (Bödeker et al., 2009; Kohler et al., 2015). However, while ECMF may produce some kinds of lignin decomposing enzymes, they generally lack the full complement of genetic machinery required to perform complete lignin breakdown (Kohler et al., 2015; Talbot et al., 2015), suggesting that their increased dominance relative to saprotrophs contributed to the enrichment of lignin we observed. Consistent with this idea, the abundance of eukaryotic genes encoding carbohydrate-degrading enzymes decreased in the organic soil horizon at this site, with a concomitant increase in abundance of carbohydrate-degrading genes associated with *Actinobacteria* (Pold et al., 2016) which, similar to ECMF, have only a limited ability to break down lignin (Větrovský et al., 2014). Together these results point to a replacement of fungal saprotrophs that are strong lignin decomposers with a suite of ECMF and bacteria that perform incomplete lignin degradation.

4.1. Synthesis

To put our results into context, we synthesize our own and previously published data, summarizing changes in ecosystem C and N cycling, along with the growth or abundance of different groups of organisms (plants, ECMF, saprotrophic fungi) in response to prolonged warming of a decade or more (Fig. 6A). Within the first few years of warming, soil respiration and N mineralization are stimulated (Melillo et al., 2002, 2011; Contosta et al., 2011) leading to a decline in the labile soil C pool. Increased N mineralization stimulates tree growth (Melillo et al., 2011; Dawes et al., 2017), which in turn favors ECMF (Clemmensen et al., 2006; Deslippe et al., 2011). We note that our data represent the relative abundance of ECMF within the whole fungal community, which does not necessarily indicate an increase in absolute abundance. Indeed, total fungal abundance was reduced by warming at

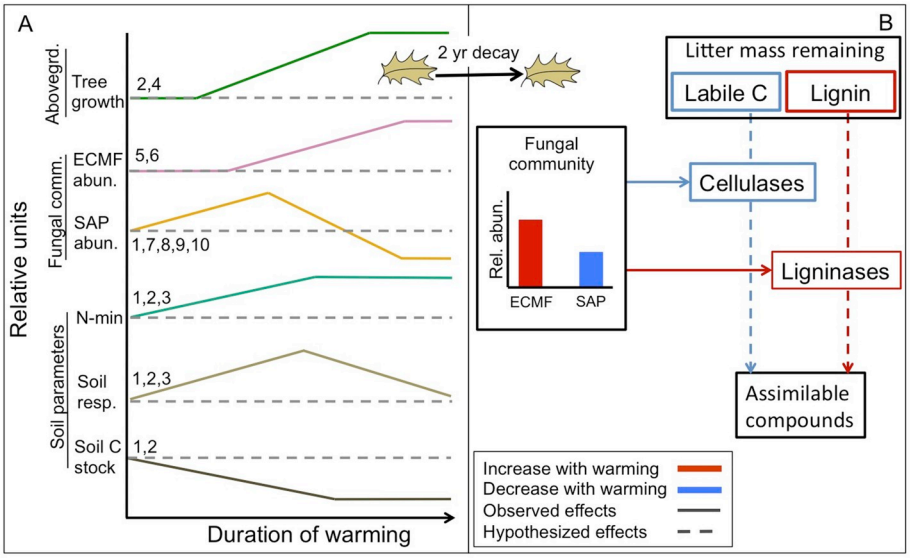

Fig. 6. Conceptual diagram representing generalized temperate forest ecosystem responses to experimental soil warming over time based on published literature (A) and the effects of experimental soil warming on fungal-mediated litter decomposition as observed in our study (B). In panel A, the x-axis represents the duration of warming from initiation through a decade, approximately, but this diagram is meant to represent general ecosystem responses over the course of warming and not necessarily an exact time scale. The y-axis represents relative units of different parameters. Number annotations in panel A are references to citations listed below. Dashed horizontal lines represent control conditions, such that an increase above this line indicates a relative increase with warming. The leaf symbol indicates the approximate time period of our litter decomposition experiment. In panel B, arrows represent fluxes of C or enzymes, while boxes represent C pools after two years of decomposition. Abbreviations: Above grd. = aboveground response; Fungal comm. = fungal community responses; ECMF = ectomycorrhizal fungi; SAP = saprotrophic fungi; N-min = N mineralization rate; Soil resp. = soil respiration rate. Citations listed in panel A: 1. Melillo et al. (2002); 2. Melillo et al. (2011); 3. Contosta et al. (2011); 4. Dawes et al. (2017); 5. Clemmensen et al. (2006); 6. Deslippe et al. (2011); 7. Frey et al. (2008); 8. DeAngelis et al. (2015); 9. Averill et al. (2014); 10. Bödeker et al. (2016).

a separate experiment at our research site (Frey et al., 2008; DeAngelis et al., 2015). The concomitant decline in the relative abundance of saprotrophic fungal species (this study) and lower total fungal abundance in warmed soils generally (Frey et al., 2008; DeAngelis et al., 2015) suggest that as warming continues, the combined pressures of competition with ECMF (Averill et al., 2014; Bödeker et al., 2016) and lower labile C availability (Melillo et al., 2002, 2017; Bradford et al., 2008) reduce the abundance of saprotrophic fungi, corresponding with the often observed acclimation (i.e., reduced respiratory response) of soil C mineralization following 6–8 years of warming (Melillo et al., 2002). Our litter decay study, occurring at 7–9 years of warming, appears to fall within this phase of ecosystem response.

The results of our litter decay experiment and hypothesized outcomes for ecosystem C stocks and fluxes are summarized in Fig. 6B. After two years of litter decomposition, the chemistry of leaf litter decaying in the heated treatment had greater relative abundance of lignin and lower relative abundance of more labile compounds like plant lipids and polysaccharides, despite no significant change in overall litter mass loss (Fig. 6B box “Litter mass remaining”). We also observed greater activity of lignin-decomposing enzymes and lower activity of cellulose-decomposing enzymes (Fig. 6B solid arrows and “Cellulases” and “Ligninases” boxes), responses that were associated with shifts in the relative abundance of ECMF and saprotrophs (Fig. 6B “Fungal community” box). It is important to note that the changes in extracellular enzyme activity we observed at the end of the decay series were likely a response of the decomposer community to the chemistry of litter present at that time point (Sinsabaugh and Shah, 2011) and were not necessarily reflective of enzyme activities throughout the course of decomposition. Indeed, increased lignin relative abundance in the remaining litter suggests that the fungal community was more active in decomposition of labile substrates than lignin over the course of decay.

The shift in litter chemistry we observed is presumably reflected in the pool of assimilable compounds available for microbial uptake (dashed lines and “Assimilable compounds” box in Fig. 6B), having potential effects on future ecosystem C fluxes and storage. For example, the quality of C substrates available for uptake can affect microbial carbon use efficiency (CUE), both directly (Frey et al., 2013) and by selecting for communities with different intrinsic efficiencies (Kallenbach et al., 2016). In addition, the temperature sensitivity of decomposition varies based on resource quality (Fierer et al., 2005; Conant et al., 2011), such that changes in the chemistry of the resource pool available to microbes may affect the temperature response of decomposition rates and C storage long-term (Davidson and Janssens, 2006). The consequences for soil C stocks from a shift in litter chemistry are unclear, but longer-term studies suggest that microbial communities will continue to change in response contributing to uncertainty in soil C-climate feedbacks (Melillo et al., 2017).

5. Conclusions

Our results are consistent with recent research showing that historical climate shapes the response of litter decay rates to contemporary conditions by altering microbial community structure (Strickland et al., 2015; Hawkes et al., 2017) and that this climate legacy can be relatively resistant to contemporary processes like dispersal (Hawkes et al., 2017). The fungal community present in the heated treatment appeared to be less active in lignin decomposition despite higher activity of lignin decomposing enzymes at the time of sampling. This may be due to replacement of the saprotrophs that decompose lignin in ambient temperature conditions with ectomycorrhizal fungi that have incomplete lignin decomposition pathways (Talbot et al., 2015). The changes in community composition we observed here, presumably in combination with direct effects of warming on decomposition processes, have resulted in a change in the chemistry of remaining litter residues despite having no effect on decay rate overall. Similarly, microbial communities associated with different agricultural cropping regimes have been shown to impart a distinct chemical signature to litter residues after decomposing a common litter to the same stage of decay (Wickings et al., 2011, 2012). Together, the changes we observed in fungal community composition and litter chemistry in the heated treatment are likely to impact soil C stocks, but the direction of this response is unclear due to remaining uncertainties including the effects of litter chemistry on microbial CUE (Frey et al., 2013; Kallenbach et al., 2016), potential systematic differences in CUE of ectomycorrhizal fungi and saprotrophs which are currently unknown, influence of chemistry on the potential for organic matter association with mineral surfaces (Jilling et al., 2018), and differences in the temperature sensitivity of labile C and complex C (lignin) decomposition (Davidson and Janssens, 2006).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.02.005.

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