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ORIGINAL ARTICLE

Ectomycorrhizal fungi contribute to soil organic matter cycling in sub-boreal forests

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Soils of northern temperate and boreal forests represent a large terrestrial carbon (C) sink. The fate of this C under elevated atmospheric CO_2 and climate change is still uncertain. A fundamental knowledge gap is the extent to which ectomycorrhizal fungi (EMF) and saprotrophic fungi contribute to C cycling in the systems by soil organic matter (SOM) decomposition. In this study, we used a novel approach to generate and compare enzymatically active EMF hyphae-dominated and saprotrophic hyphae-enriched communities under field conditions. Fermentation-humus (FH)-filled mesh bags, surrounded by a sand barrier, effectively trapped EMF hyphae with a community structure comparable to that found in the surrounding FH layer, at both trophic and taxonomic levels. In contrast, over half the sequences from mesh bags with no sand barrier were identified as belonging to saprotrophic fungi. The EMF hyphae-dominated systems exhibited levels of hydrolytic and oxidative enzyme activities that were comparable to or higher than saprotroph-enriched systems. The enzymes assayed included those associated with both labile and recalcitrant SOM degradation. Our study shows that EMF hyphae are likely important contributors to current SOM turnover in sub-boreal systems. Our results also suggest that any increased EMF biomass that might result from higher below-ground C allocation by trees would not suppress C fluxes from sub-boreal soils.
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Introduction

Northern temperate and boreal forests represent a large carbon (C) sink, with an estimated half a gigatonne of C sequestered in above ground biomass every year (Myneni *et al.*, 2001). Much of this C is eventually transferred below ground, either after the death or annual senescence of vegetation or directly via photosynthetic transfer to root systems and associated ectomycorrhizal fungi (EMF). These soils store up to three times the amount of C as is stored in the above ground vegetation, primarily in the form of soil organic matter (SOM) (Schmidt *et al.*, 2011). These C sinks have the potential to become C sources under the projected global climate change (IPCC, 2007). Although some climate models assume increased C storage in temperate forest soils because of the increased plant allocation of photosynthetic C to roots and symbiotic fungi (Drigo *et al.*, 2010; Orwin *et al.*, 2011; Clemmensen *et al.*, 2013), there is a high degree of uncertainly in this assumption (Higgins and Harte, 2012). Increased C allocation with elevated CO_2 may not only be short-lived (Norby *et al.*, 2010), but concomitant increases in mycorrhizal and mycorrhizosphere activity may stimulate decomposition of previously recalcitrant SOM (Cheng *et al.*, 2012; Phillips *et al.*, 2012b). Fundamental gaps in our understanding of terrestrial C cycling, including the relative contributions of EMF and other fungi to the release of SOM-C (Bargett, 2011), limit the predictive capability of current models (Von Lützow and Kögel-Knabner, 2009; Hayes *et al.*, 2012).

Fungi, especially saprotrophic fungi, are thought to dominate organic matter turnover in temperate forest soils (Baldrian, 2008). Certain groups of saprotrophic fungi, specifically the white rot fungi, have unique abilities to degrade wood because they possess numerous copies of class II peroxidases (Floudas *et al.*, 2012). Both saprotrophic and EMF species, however, produce a range of hydrolytic and oxidative enzymes that have the potential to break down C-containing compounds and mobilize nutrients from SOM (Courty *et al.*, 2010; Floudas *et al.*, 2012). Although photosynthates are likely the primary source of C used by EMF under ideal conditions (Treseder *et al.*, 2006; Wolfe *et al.*, 2012), current research suggests that these fungi may also

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directly (Vaario *et al.*, 2012) or indirectly (Rineau *et al.*, 2012) access SOM-C pools.

Recalcitrant SOM, including lignin and humic complexes, is predominantly found in soil horizons dominated by EMF mycelial networks (Lindahl *et al.*, 2007). These mycelia are present at up to 600 kg of hyphae per hectare (Wallander *et al.*, 2001; Hendricks *et al.*, 2006) and account for up to one-third of the total microbial biomass in coniferous forests (Högberg and Högberg, 2002; Cairney, 2012). Although EMF mats are known to increase total soil CO_2 respiration (Phillips *et al.*, 2012a), the full contribution of these fungi, and their extensive mycelia, to decomposition processes remains unknown (van der Wal *et al.*, 2012). Given the current interest in the C sequestration potential of boreal and temperate forests (De Luca and Boisvenue, 2012), as well as the influence of any increased C allocation below ground by plants, the role of EMF mycelia in SOM-C turnover warrants increased investigation (Talbot *et al.*, 2008; Ekblad *et al.*, 2013).

Current research to estimate the extent and activity of EMF mycelia rely on methods to exclude roots and most saprotrophic fungi from a given zone in the soil profile. Hyphal in-growth cores or bags are commonly used (Wallander *et al.*, 2001). These hyphae traps are usually filled with an inert substrate, such as sand, that minimizes colonization by saprotrophic fungi (Korkama *et al.*, 2007; Lindahl *et al.*, 2007; Wallander *et al.*, 2010). In some cases, additional nutrient sources are added to ensure sufficient hyphal colonization or to evaluate the impact of limiting nutrients on EMF biomass production (Hagerberg and Wallander, 2002; Hagerberg *et al.*, 2003; Hedh *et al.*, 2008; Potila *et al.*, 2009; Berner *et al.*, 2012). However, the captured extramatrical hyphae may not accurately represent the composition or abundance of EMF communities in the surrounding soil (Hendricks *et al.*, 2006; Kjøller, 2006). Further, enzyme expression and activity is downregulated when no nutrients are present (Wright *et al.*, 2005). In order to estimate the contribution of EMF to the terrestrial C cycle, the potential SOM-degrading enzyme activity in locally abundant EMF hyphae needs to be determined.

To investigate the relative contribution of EMF mycelia to enzyme activities in a sub-boreal spruce forest, we buried mesh bags filled with sterilized organic material with or without a surrounding sand barrier. Bags with a sand barrier were served to trap enzymatically active EMF hyphae while excluding obligately saprotrophic hyphae. Filling the mesh bags with fermentation-humic (FH) substrate taken from the same soil in which the traps were to be buried enabled a direct comparison between the EMF hyphae in the mesh bags and the surrounding FH layer. Based on our previous research, which showed that EMF root tips in these ecosystems had high hydrolytic and oxidative enzyme activities (Jones *et al.*, 2010, 2012), we hypothesized that EMF-dominated communities would have a SOM-degradation potential comparable to that of saprotroph-dominated communities. High-throughput sequencing, which combined with comprehensive measures of enzymes involved in SOM turnover, allowed us to evaluate the relative abundance and structure of fungal communities and to elucidate the potential role of EMF fungi in the soil C cycle.

Materials and methods

Field site

The field site was part of a British Columbia Ministry of Forests study that investigated the impact of fertilization on forest productivity and below-ground ecosystem processes (Brockley and Simpson, 2004). The current study occurred at the Crow Creek site, established in 1994 near Houston, British Columbia, Australia (54° 20′ 126° 17′) in the sub-boreal spruce biogeoclimatic zone, on Eluviated Dystric Brunisol to Orthic Humo-Ferric Podzol soils. Each treatment within the site consisted 0.164 hectare plots replicated three times. The current study utilized the non-fertilized control and two fertilized (annually and every 6 years) treatments; for further site information refer to Brockley and Simpson (2004). During the season of this study, the FH layer averaged 33% total C, 1.06% total N, 64% organic matter, NH_4-N, NO_3-N and available P of 46, 2.9 and 346 mg kg^{-1}, respectively, and $pH_{(CaCl2)}$ 4.35 (British Columbia Ministry of Forests Research Laboratory, Victoria, British Columbia, Australia).

Mesh bag construction for EMF extramatrical hyphal capture

FH layer substrate was collected from each treatment plot in the fall of 2008. Ten random subsamples per plot were composited, sieved (4.5 mm) and stored at 4 °C until use. Eleven hyphal trap bags (4.5 × 3.5 cm) were constructed for each treatment plot (99 bags in total) using 50 μm mesh (NITEX 03-50/31; Sefar Inc., Depew, NY, USA). Each bag was filled with 15 g FH substrate, heat-sealed and then sterilized by gamma irradiation with a total dose of 6 Mrad (2 × 3 Mrad with a 1 week resting interval). Post irradiation, bags were stored at 4 °C for an 8-week stabilization period to minimize residual enzyme activity (Lensi *et al.*, 1991). Substrate from one bag from each plot was serially diluted in monopotassium phosphate buffer, plated in triplicate on modified Melin–Norkrans or tryptic soy agar plates (Phillips *et al.*, 2006) and incubated for 1 month to assess for fungal or bacterial contamination, respectively. No bacterial or fungal growth was observed on culture plates after 1 month of incubation, indicating that two consecutive doses of irradiation effectively eliminated residual spores.

Irradiation did not significantly change the pH of the substrates. Another sub-sample of these controls was assessed for residual enzyme activity and pH. Bags intended to capture EMF but exclude saprotrophic hyphae ($n = 5$ per plot) were placed within sand-filled plastic containers (NetPak, Coquitlam, British Columbia, Australia). These containers were punched with circular holes (6 mm diameter; 28 holes per horizontal face and 10 holes per vertical face) to allow hyphal access, lined with a 50-μm NITEX mesh and filled to a depth of 1 cm with sterile silica sand (HCl acid washed and autoclaved). Substrate-containing mesh bags were placed on the sand, covered with 1 cm additional sand and then a final top layer of mesh was sealed to the initial liner using silicone sealant to form a continuous mesh envelope (Supplementary Figure 1). Bags intended to capture both EMF and saprotrophic hyphae ($n = 5$ each plot) were also encapsulated within another layer of mesh, but had no intervening sand barrier.

Hyphal trap bags were buried at the Crow Creek site on 5 June 2009. Each set of bags (with or without sand barriers, +SB and –SB, respectively) were placed in the FH layer of the treatment plots from which the substrates were originally taken. One pair of +SB and –SB bags was buried at five random spots within each plot, 60 cm from the base of a tree and 60 cm from each other. All bags and an FH sample (10 × 10 cm) from between each set were harvested on 1 October 2009. Upon return to the lab, the five +SB, −SB or FH sub-samples from individual plots were composited, so that a single representative +SB, −SB and FH sample was obtained per plot. Sand from the +S mesh bags was also composited on a plot basis. Half of each composited sample was frozen at −20 °C for subsequent molecular analysis and half was stored at 4 °C overnight for use in enzyme assays.

Enzyme assays

All +SB, −SB, FH, sand and residual-activity control samples, as well as EMF tips selected from the FH samples, were assessed for their organic matter breakdown potential. The assessed enzymes included β-1,4-glucosidase (EC 3.2.1.21), cellobiohydrolase (EC 3.2.1.91), β-1,4-xylosidase (EC 3.2.1.37), peroxidase (EC 1.11.1.7), laccase (EC 1.10.3.2), phenol oxidase (EC 1.14.18.1 and EC 1.10.3.2), β-1,4-N-acetylglucosaminidase (EC 3.2.1.52), acid phosphatase (EC 3.1.3.2), leucine aminopeptidase (EC 3.4.11.1) and sulfatase (EC 3.1.6.1). Note that although laccases are phenol oxidases, the term laccase is used in the study to differentiate a 2,2′-azino-bis(3-ethylbenzothiazoline-6 sulfonic acid) diammonium salt (ABTS) assay from a L-3, 4-dihydroxyphenylalanine (L-DOPA) assay. Full details of assay conditions are provided in Supplementary Table S1. All assays except laccase, peroxidase and phenol oxidase were fluorometric.

For +SB, −SB, FH and residual-activity control assays, 5 g of sample was homogenized with a sterile mortar and pestle, 0.1 g of sub-sample was transferred to a sterile Nalgene bottle containing 100 ml of 50 mM sodium acetate buffer (pH 5.0), and then suspensions were shaken for 1 h at 500 r.p.m. Sand samples were similarly processed using 10 g of sample and 1 g of sub-sample. Fluorometric and colorimetric assays were then performed according to previously published methods (Saiya-Cork *et al.*, 2002 and Sinsabaugh *et al.*, 2003), fully described in the Supplementary Information.

For EMF tip assays, roots from the FH sample were gently washed, separated under a dissecting microscope, and ~7 turgid EMF tips from each of the four most common observed morphotypes were randomly selected from each sample. Tips were cleaned of debris and placed in individual microsieves in 96-well microplates containing 75 mM Tris-maleic acid buffer (pH 4.5) to equilibrate for at least 5 min before beginning the sequential enzyme assays (Supplementary Table S1). Fluorometric and colorimetric assays were then performed according to previously published methods (Courty *et al.*, 2005 and Pritsch *et al.*, 2004), fully described in the Supplementary Information. After the final assay, tips were removed from the sieves and scanned (Scanmaker 8700; Microtek Lab, Sante Fe Springs, CA, USA), and their surface areas were determined (WinRHIZO, Regent Instruments, Québec, QC, Canada). Tips were then frozen at −80 °C for subsequent molecular analysis. The total enzymatic activity of EMF tips per plot was determined by averaging the activities of all tips. EMF taxon-specific activities were determined by averaging the activity of the (up to) seven EMF tips per plot, whose identities were confirmed by morphological and molecular identification.

Identification of fungal communities

Sequencing of ectomycorrhizal tips. DNA was extracted from EMF tips using the Sigma Extract-N-Amp Plant Kit (Sigma-Aldrich, St Louis, MO, USA). Extraction solution (25 μl) was added to each EMF tip that was incubated at 95 °C for 10 min, after which 25 μl of dilution solution was added. The ITS1f and ITS4 primers (White *et al.*, 1990; Gardes and Bruns, 1993) were used to amplify the fungal ITS1, 5.8S and ITS2 region. Each 50 μl reaction contained 1.5 μl of template DNA, 2.5 μl of each 10 μM primer and 25 μl GoTaq master mix (Promega, Madison, WI, USA). Following an initial denaturation step of 95 °C for 3 min, amplification proceeded for 35 cycles of 94, 50 and 72 °C per min, with a final extension step of 10 min at 72 °C. PCR products were visualized on 1.4% agarose gels (SYBR Safe; Invitrogen, Carlsbad, CA, USA). PCR products producing single bands were cleaned using the Mag-Bind E-Z pure (Omega Bio-tek Inc., Norcross, GA, USA) system and

sequenced (Applied Biosystems; 3130xl Genetic Analyzer, Foster City, CA, USA). If more than one band was produced (that is, from some tips morphologically identified as *Cenococcum spp.*), bands were extracted from the gel using an E.Z.N.A gel extraction kit (Omega Bio-tek Inc.) before cleanup and sequencing. Sequences were submitted for comparison with the GenBank databases using the BLAST algorithm (Altschul *et al.*, 1997).

Sequencing of fungi in hyphal trap bag and FH substrates. Total community DNA was extracted from 0.5 g of all +SB, −SB and FH samples using the UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). DNA was extracted from 5 g sand samples using a previously published bead-beating method (Phillips *et al.*, 2006) suitable for large samples. Duplicate DNA extractions were performed for each sample and pooled.

The ITS1 and partial 5.8S regions were amplified using the ITS1f (Gardes and Bruns, 1993) and ITS2r (White *et al.*, 1990) primer pair (Integrated DNA Technologies, Coralville, IA, USA). Amplicons were generated for 454-pyrosequencing in a single-step reaction using the following fusion primers: 5′-TitaniumA-Multiplex Identifier-ITS1f-3′ and 5′-TitaniumB-ITS2r-3′ (Supplementary Table S2). Triplicate amplifications were performed for each sample. Each 50 μl amplification reaction contained 0.2 μM each primer, 200 μM dNTP mix, 2 mM $MgSO_4$, 1× PCR reaction buffer, 1 U Platinum Taq DNA Polymerase (Invitrogen) and 5, 10 or 20 ng of sample DNA. Following an initial denaturation step of 94 °C for 2 min, amplification proceeded for 35 cycles of 94 and 55 °C per 30 s, and 68 °C per min, with a final extension of 68 °C for 10 min. PCR products were visualized on 1.4% agarose gels (SYBR Safe; Invitrogen), replicate reactions were pooled and PCR amplicons were cleaned using the Mag-Bind E–Z pure (Omega Bio-tek Inc.) purification system.

Amplicon concentration was determined spectrophotometrically (NanoDrop ND-1000 Spectrophotometer) and both concentration and purity (that is, complete removal of primer-dimers) were verified on a 1.4% agarose gel (SYBR Safe; Invitrogen). Equimolar PCR amplicons from each sample (equivalent to 200 ng each) were pooled to create two Amplicon Libraries that which were sequenced on half a plate each using the 454 GS FLX Titanium platform (Roche Diagnostics, Basel, Switzerland) at the Génome Québec Innovation Center, Montreal, Quebec, Canada. Results were delivered as tag-sorted sequences, quality controlled for the presence of the complete primer-tag.

Bioinformatic analysis

Primer sequences were removed and additional quality filtering and trimming was performed using MOTHUR (Schloss *et al.*, 2009). Sequences with any ambiguous (N) bases, more than eight homopolymers, shorter than 180 or longer than 400 bp, or with greater than 1 bp difference in the primer sequence were removed. Subsets of the excluded sequences were manually assessed for fungal group-specific biases. The quality filtered and trimmed sequences were then processed using the FungalITS extractor software (Nilsson *et al.*, 2010), which extracts the variable ITS1 of the nuclear ITS1 region from the sequence, thus eliminating false alignments and clusters being generated based on the conserved 18S and 5.8S regions. The extracted ITS1 sequences were clustered at 98% (Ryberg *et al.*, 2008) similarity (global alignment) using CD-Hit-Est (Huang *et al.*, 2010). Representative sequences from all clusters (operational taxonomic unit, OTU) were used for BLAST searches against fully identified entries in INSD (Benson *et al.*, 2011) and UNITE (Abarenkov *et al.*, 2010a), using the PlutoF online server (Abarenkov *et al.*, 2010b). A minimum of 97% similarity across 97% of the sequence length was required for positive taxonomic identification at any level. Singleton OTUs with 100% similarity to reference sequences not otherwise identified in the data set were retained, and all other singletons were discarded before statistical analysis. All sequences, fastq formatted on the Galaxy online server (Blankenberg *et al.*, 2010), were deposited in the NCBI Short Read Archive under the accession number SRP022584.

All OTUs greater than four reads within the data set were placed into designated ecological function groups, determined by cross referencing Lawry and Diederich (2003), Rinaldi *et al.* (2008), Hibbet *et al.* (2000) and Moore *et al.* (2011). The groups were EMF, other mycorrhizal (ericoid, arbutoid, monotropoid and arbuscular) fungi, saprotrophs, pathogens, endophytes, yeasts and lichen fungi. Fungi known to belong to more than one of these groups were designated as mixed functional groups, whereas those whose ecological function is unverified were designated as functional group Unknown.

Statistical analysis

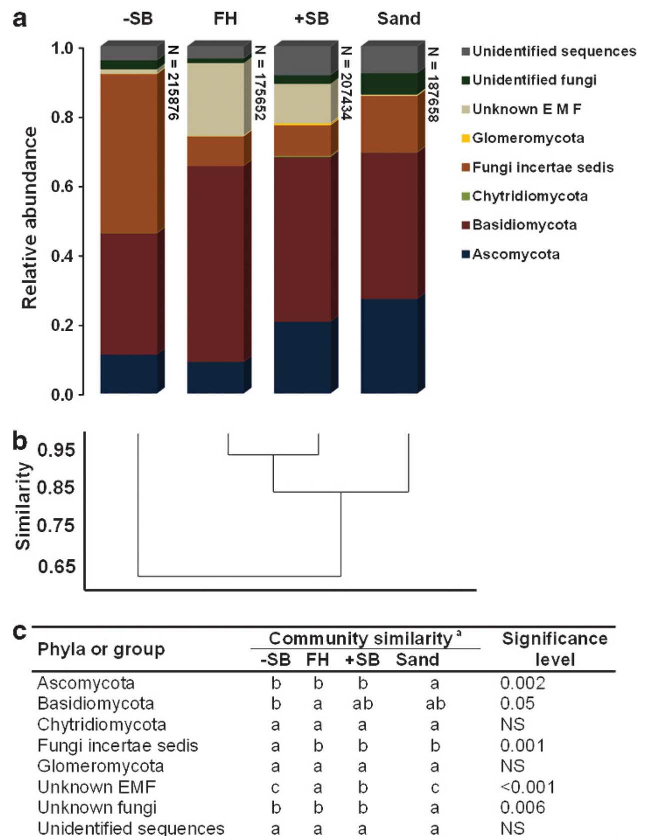
Before statistical analysis, 454 taxonomic data were normalized by dividing the absolute abundance of individual OTUs by total OTU's within a sample (Gihring *et al.*, 2012). Data were log transformed wherever required to meet the assumptions of different statistical tests (Kenkel, 2006). Relationships between the +SB, −SB, FH and sand substrates were assessed using the statistical software package PAST (v 2.17; Hammer *et al.*, 2001). Differences in taxonomy and enzyme activity were examined by analysis of variance (Welch's analysis of variance, if variances are unequal, as assessed using the Levene statistic), followed by a Tukey or Mann–Whitney test to determine where significant differences occurred. Relationships between enzyme activities were assessed using Spearman's r correlation test.

Cluster analysis of functional and taxonomic fungal groups was performed using a correlation matrix based on Pearson's r correlation (1-r; PAST 2.17; Bolhuis and Stal, 2011). Species richness (S), evenness (H/ln(S)) and diversity (Fisher's Alpha (α), Shannon (H) and Simpson's indices) of the fungal communities were calculated using procedures available in PAST (v 2.17; Hammer *et al.*, 2001). Patterns of enzyme activity were assessed by principle component analysis (PCA; Kenkel, 2006) in PC-ORD (McCune and Mefford, 2011). Relationships between enzyme activity and community structure were assessed by regressing enzyme PCA-loading factors with fungal community abundance (log transformed). Fungal communities significantly ($P < 0.05$) associated with patterns of enzyme activity were illustrated as joint plot vectors overlaid on the enzyme ordinations (Ramette, 2007).

Results

General sequencing statistics
A total of 1 151 401 sequences were generated by pyrosequencing the ITS1 region of DNA extracted from hyphal trap substrates, barrier sand and forest floor samples. Quality filtering removed 362 946 sequences, primarily due to length constraints or lack of a complete primer sequence, leaving a total of 788 455 ITS1 region sequences for analysis. The 36 individually assessed samples generated an average of 32 000 sequences and an average of 22 000 quality filtered sequences (Supplementary Table S3). Clustering at 98% similarity resulted in an average of 546 OTUs per sample (singletons included), with rarefaction curves for most samples beginning to plateau (Supplementary Figure S2). The use of a stringent 98% clustering level meant that some similar OTUs were split into different clusters. These clusters were combined if they were identified through BLAST analysis, representing the same fungal OTU. A total of 5541 singletons clusters (clusters containing 1–2 sequences) that did not match any database sequences were removed from the data set (Supplementary Table S3).

Fungal taxonomic diversity
Approximately 94% of the sequences were matched to fungal sequences in the INSD and UNITE databases (Figure 1a). A total of 353 fungal OTUs were identified, including 243 fungi identified to the genus level (Supplementary Table S4). A large percentage of the identified fungi, found in all systems, were members of *Basiodiomycota* and *Ascomycota* (Figure 1a). In hyphal traps without a sand barrier (−SB) however, 44% of the sequences belonged to Fungi *Incertae sedis*. Hyphal traps with a sand barrier (+SB) and the surrounding FH layer contained numerous sequences that matched those of unidentified EMF amplified from root tips (21% and 11%, respectively). At this level of taxonomy,



Phyla or group	Community similarity[a]				Significance level
	-SB	FH	+SB	Sand	
Ascomycota	b	b	b	a	0.002
Basidiomycota	b	a	ab	ab	0.05
Chytridiomycota	a	a	a	a	NS
Fungi incertae sedis	a	b	b	b	0.001
Glomeromycota	a	a	a	a	NS
Unknown EMF	c	a	b	c	<0.001
Unknown fungi	b	b	b	a	0.006
Unidentified sequences	a	a	a	a	NS

Figure 1 Fungal taxonomic diversity in the different hyphal trap bags, sand barriers and FH layers of the forest floor. −SB, hyphal trap bags with no sand barrier; FH, *in situ* fermentation-humic layer; +SB, hyphal trap bags with sand barrier; Sand, sand barrier; $n = 9$ each. (**a**) The relative abundance of different phyla or group in each compartment. The total number of contributing sequence reads is indicated next to each histogram. (**b**) Cluster analysis of taxonomic diversity indicating the percent similarity (based on Pearson's r correlation) of communities in the different compartments. (**c**) Differences in the abundance of each taxonomic group. Communities in the same row with different letters are different at the indicated significance level; NS, not significant. Unknown EMF indicates that the best matching sequences belonged to unidentified ectomycorrhizal tips. Unknown fungi indicates that the best matching sequence belonged to the kingdom Fungi.

FH and +SB fungal communities clustered at a high level of similarity, whereas −SB communities formed a distinct cluster (Figure 1b). This separation was primarily due to significantly higher numbers of Fungi *I. sedis* in the absence of a sand barrier (Figure 1c). The presence of these fungi separated these −SB communities into distinct clusters at all levels of taxonomy (Supplementary Figure S3).

The lowest richness and diversity occurred in −SB communities, which had significantly fewer fungal taxa than the FH samples (Table 1). The FH communities had the highest level of diversity; however, all communities were populated by a few relatively abundant taxa (Table 2). Notably, the Fungi *I. sedis* saprotroph *Mortierella* was the single-most abundant taxon (44% of sequences) found in −SB communities. By contrast, *Mortierella spp.* accounted for only 8% of sequences in FH and

Table 1 Genus level fungal richness and diversity estimates

	Richness (S)	*Evenness (H/ln(S))*	*Shannon (H)*	*Simpson (D)*	*Fisher's Alpha (α)*
−SB	92.7 (15.2) b	0.436 (0.104)	1.99 (0.52)	0.707 (0.184)	12.2 (2.1) c
FH	110.3 (12.8) a	0.494 (0.038)	2.32 (0.21)	0.817 (0.031)	15.4 (1.7) a
+SB	98.4 (7.0) ab	0.467 (0.112)	2.14 (0.52)	0.761 (0.112)	13.3 (1.1) bc
Sand	102.7 (9.9) ab	0.501 (0.060)	2.32 (0.29)	0.794 (0.056)	14.2 (1.4) ab

Abbreviations: FH, *in situ* fermentation-humic layer; −SB, hyphal trap bags with no sand barrier; +SB, hyphal trap bags with sand barrier; Sand, sand barrier.
Data are provided as means (n = 9) with standard deviation in brackets. Data in the same column with different letters are significantly different at P < 0.05.

Table 2 The percent relative abundance of the most common fungal[a] sequences in the hyphal trap bags with and without a sand barrier, sand barriers and the adjacent fermentation-humic layer of the forest floor

−SB		*FH*		*+SB*		*Sand*	
Mortierella	43.5 (22.9)	*Piloderma*	32.3 (4.9)	*Amphinema*	33.6 (19.9)	*Amphinema*	28.4 (17.6)
Amphinema	16.6 (11.6)	EMF tips	21.3 (7.3)	EMF tips	11.5 (7.3)	*Mortierella*	15.7 (14.6)
Wilcoxina	5.8 (4.9)	*Mortierella*	8.3 (5.6)	*Wilcoxina*	11.1 (17.3)	*Wilcoxina*	8.0 (11.9)
Piloderma	4.8 (4.7)	*Cortinarius*	6.8 (6.2)	*Mortierella*	8.3 (5.0)	*Thelephoraceae*	5.7 (11.1)
Tylospora	3.8 (4.5)	*Tylospora*	6.5 (4.9)	*Thelephoraceae*	5.2 (7.9)	*Cenococcum*	2.9 (3.7)
Thelephoraceae	2.2 (2.4)	*Amphinema*	2.7 (1.6)	*Piloderma*	2.2 (2.5)	*Verticillium*	2.9 (2.3)
Tuber	1.5 (3.3)	*Wilcoxina*	1.7 (2.0)	*Ceratobasidiaceae*	1.5 (2.2)	*Ceratobasidiaceae*	2.8 (3.9)
EMF tips	1.4 (1.2)	*Hygrophorus*	1.6 (2.2)	*Cenococcum*	1.5 (2.8)	*Calycina*	2.0 (5.4)
Lactarius	1.0 (2.6)	*Thelephoraceae*	1.2 (1.8)	*Inocybe*	1.3 (2.0)	*Helotiales*	1.8 (2.0)
Cortinarius	0.9 (2.5)	*Cenococcum*	1.1 (0.8)	*Hygrocybe*	0.7 (2.1)	*Cryptococcus*	1.5 (0.9)
Sebacina	0.7 (1.7)	*Helotiales*	0.8 (0.3)	*Cryptococcus*	0.6 (0.4)	*Lecanicillium*	1.0 (0.8)
Verticillium	0.7 (0.7)	*Cadophora*	0.6 (0.6)	*Glomus*	0.6 (1.2)	*Cladosporium*	0.8 (0.7)
Russula	0.6 (1.1)					*Chalara*	0.7 (0.7)

Abbreviations: −SB, hyphal trap bags with no sand barrier; FH, *in situ* fermentation-humic layer; +SB, hyphal trap bags with sand barrier; sand, sand barrier.
A higher taxonomic level is given if the sequence could not be assigned to a genus. Data are provided as means ($n = 9$) with s.d. in brackets. EMF tips, matching sequences belonged to unidentified ectomycorrhizal tips.
[a]The most common (>0.5% relative abundance) fungal groups are given at the genus level.

+SB communities and 16% of sequences in sand communities. Instead, these communities were largely dominated by EMF, including *Piloderma spp.* (FH, 32% of sequences) and *Amphinema spp.* (+SB, 34% of sequences; and sand, 28% of sequences). Although *Piloderma spp.* were the dominant EMF found in the *in situ* FH layer, *Amphinema spp.* and *Wilcoxina spp.* were better able to colonize both types of hyphal traps.

Fungal functional diversity

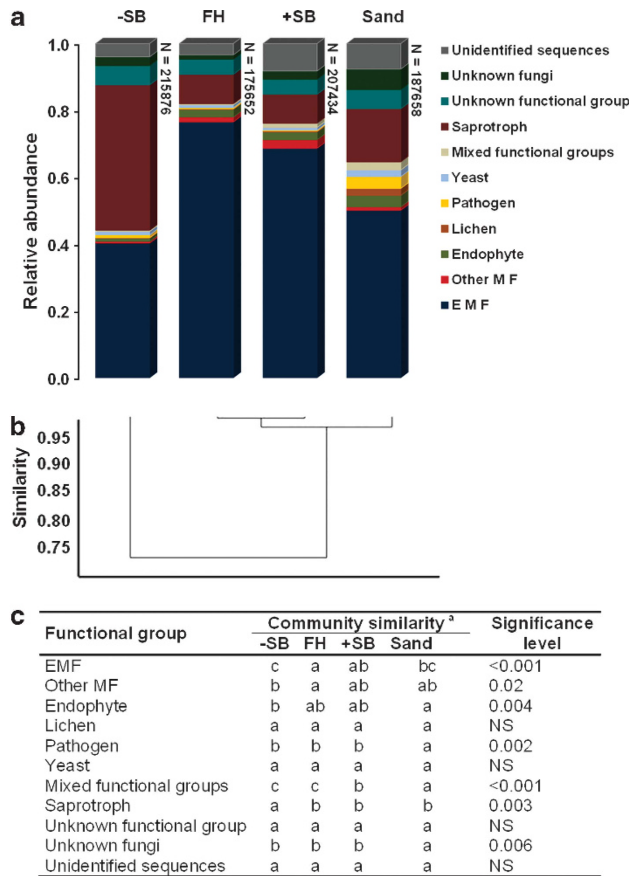
The presence of a sand barrier had a significant impact on fungal groups colonizing the hyphal traps. Hyphal traps with a sand barrier were primarily colonized by EMF, whereas hyphal traps without a sand barrier were equally colonized by saprotrophs and EMF (Figure 2a). As previously noted, −SB traps were heavily colonized by *Mortierella spp.,* which comprised the majority of the identified saprotrophs. The trophic groups that colonized the +SB traps were highly comparable to those in the surrounding FH layer (Figure 2b), with the two groups clustering at ∼98% similarity. Both of these communities contained significantly lower populations of known saprotrophs than the −SB community (Figure 2c).

Ectomycorrhizal tip diversity

A total of 226 EMF tips were selected, based on abundant morphotypes, from the FH layer adjacent to the hyphal trap bags. Sequencing of the complete ITS region confirmed EMF tip identity at the following relative abundances: *Cenococcum* (24%), *Piloderma* (24%), *Amphinema* (17%), *Tylospora* (12%), *Cortinarius* (12%), *Hygrophorus* (5%) and *Hysterangium* (3%) *spp.* Approximately half of the *Cenococcum* tips exhibited multiple banding patterns after PCR amplification of the ITS region. Band-specific sequencing confirmed that these tips contained *Cenococcum* sequences in association with *Phialocephala*, *Piloderma*, *Mycena* or *Cadophora spp.* sequences (Supplementary Figure S4). For the purposes of enzyme activity assays (below), these tips were considered as *Cenococcum* based on the distinctive mantle formed by this fungus.

Activity of enzymes associated with SOM breakdown

The hydrolytic and oxidative enzyme activity potentials were assessed for all components of the hyphal trap systems, the adjacent FH layer and residual-activity controls (Table 3), and for EMF tips sampled from the FH layer (Table 4). Residual



Functional group	Community similarity [a]				Significance level
	-SB	FH	+SB	Sand	
EMF	c	a	ab	bc	<0.001
Other MF	b	a	ab	ab	0.02
Endophyte	b	ab	ab	a	0.004
Lichen	a	a	a	a	NS
Pathogen	b	b	b	a	0.002
Yeast	a	a	a	a	NS
Mixed functional groups	c	c	b	a	<0.001
Saprotroph	a	b	b	b	0.003
Unknown functional group	a	a	a	a	NS
Unknown fungi	b	b	b	a	0.006
Unidentified sequences	a	a	a	a	NS

Figure 2 Fungal functional group diversity in the different hyphal trap bags, sand barriers and FH layers of the forest floor. −SB, hyphal trap bags with no sand barrier; FH, *in situ* fermentation-humic layer; +SB, hyphal trap bags with sand barrier; Sand, sand barrier; $n=9$ each. (**a**) The relative abundance of the different ecological functional groups in each compartment. The total number of contributing sequence reads is indicated next to each histogram. (**b**) Cluster analysis of functional group diversity, indicating the percent similarity (based on Pearson's r correlation) of communities in the different compartments. (**c**) Differences in the abundance of each functional group. Communities in the same row with different letters are different at the indicated significance level; NS, not significant. Other mycorrhizae: ericoid, arbutoid, monitropoid and arbuscular mycorrhizae; mixed functional groups: belong to more than one functional group. Unknown fungi indicates that the best matching sequence belonged to the kingdom Fungi.

enzyme activity was detected for most enzymes in the gamma-irradiated control soils, which were kept under dry and sterile conditions in the lab. This residual activity was generally lower than that in the field samples, however, β-1,4-xylosidase and cellobiohydrolase activities were not statistically different from the hyphal traps (Table 3). Exploratory PCA analysis showed that activity patterns in the control samples were distinct from those in the field samples (Supplementary Figure S5), indicating that active fungi had a dominant influence on enzyme activity under the study conditions. These differences in magnitude and pattern, combined with the likelihood that residual enzymes would have been degraded under non-sterile field conditions (Allison, 2006), informed the decision not to subtract the control from field enzyme activities.

Enzyme activity in the sand barrier surrounding the +SB traps was either at or below the limit of detection, even though all sand contained visible hypha and diverse fungal communities (see Table 1). The +SB community (predominantly mycorrhizal) and the −SB community (saprotrophic and mycorrhizal) enzyme activity levels were in the same range as those of the FH community (Table 3). EMF-dominated +SB communities had significantly ($P<0.01$) higher levels of phenol oxidase (L-DOPA assay) and aminopeptidase activity than −SB or FH communities, and higher peroxidase activity and β-1,4-glucosidase than −SB communities. Activities of all other enzymes did not differ among fungal communities.

Enzyme activities associated with EMF tips were highly variable within a given genus, resulting in few significant differences between genera (Table 4). Nevertheless, *Amphinema* tips had significantly higher ($P<0.05$) enzyme activities associated with cellulose and hemicellulose degradation than did *Piloderma* or *Cenococcum* tips. The hydrolytic enzyme activity of these EMF tips was highly correlated with that of the FH layer from which they were sampled (Table 5). Both the average tip activity and the individually assessed activities of *Amphinema* and *Piloderma spp.* were positively associated with cellobiohydrolase, glucosidase and xylosidase activities within the surrounding FH layer.

Impact of fungal community structure on enzyme activity

The relationships between fungal community structure and the activity of enzymes associated with organic matter breakdown were explored by PCA. Sand samples were not included as they exhibited little enzyme activity. Differences in enzyme activity patterns between the *in situ* FH layer and hyphal traps were best described by the first three PCA axes (Figure 3). Location along PCA axis 1 (PC1) was driven primarily by the activities of cellulose- and hemicellulose-degrading enzymes, which strongly overlapped in the FH layer and +SB traps. Activities in these communities were also strongly associated along PC3, reflecting similar patterns of lignin degradation (peroxidase) potential. The combined effects of the PC1- and PC3-loading factors resulted in a distinct separation of the −SB traps. Both FH and −SB differed from +SB along PC2, however, reflecting variation in enzymes associated with phosphorus release, lignin degradation (laccase versus phenol oxidase) and nitrogen release.

The relative abundance of fungi in a given community was significantly associated ($P<0.05$) with patterns of enzyme activity. Joint plot vectors in Figure 4 illustrate the strength and direction of significant correlations between fungal communities

Table 3 Potential organic matter breakdown activity in the hyphal trap bags, associated sand, adjacent forest floor and residual-activity controls

Enzyme	Enzyme activity (μmol h^{-1} g^{-1})					
	General role	−SB	FH	+SB	Sand	Control
Acid phosphatase	Phosphate mobilization	3.06 (0.51) b	5.11 (1.06) a	2.53 (0.80) b	<0.01 (0.00) d	1.63 (0.36) c
Sulfatase	Sulphate-ester hydrolysis	0.03 (0.01) b	0.06 (0.02) a	0.04 (0.01) b	<0.01 (0.00) c	ND
Leucine aminopeptidase	Nitrogen mobilization	0.01 (0.02) b	0.01 (0.02) b	0.04 (0.03) a	ND	ND
β-1,4-N-Acetylglucosaminidase	Chitin degradation	1.54 (0.42) a	2.13 (0.98) a	1.79 (0.47) a	<0.01 (0.00) b	0.21 (0.40) b
β-1,4-Glucosidase	Cellulose degradation	2.23 (0.69) b	3.05 (0.91) ab	3.44 (1.12) a	0.01 (0.00) c	0.70 (0.56) c
Cellobiohydrolase	Cellulose degradation	0.77 (0.58) ab	0.87 (0.56) ab	1.07 (0.61) a	<0.01 (0.00) c	0.36 (0.20) bc
β-1,4-Xylosidase	Hemicellulose degradation	0.12 (0.06) ab	0.22 (0.18) a	0.19 (0.10) a	<0.01 (0.00) b	0.10 (0.05) ab
Laccase[a]	Lignin degradation	13.3 (12.5) ab	19.0 (11.3) a	5.4 (8.3) b	ND	4.13 (4.6) b
Peroxidase[a]	Lignin degradation	19.2 (15.4) c	39.2 (18.4) ab	48.9 (16.5) a	ND	27.5 (12.6) bc
Phenol oxidase[a]	Lignin degradation	0.5 (1.3) b	0.7 (1.1) b	3.5 (2.6) a	ND	1.54 (1.00) ab

Abbreviations: control, residual enzyme activity in irradiated controls (irradiated control bags not placed in the field); FH, *in situ* fermentation-humic layer; ND, no activity detected; −SB, hyphal trap bags with no sand barrier; +SB, hyphal trap bags with sand barrier; Sand, sand barrier. Data are provided as means ($n=9$) with s.d. in brackets. Data in the same row with different letters are significantly different at $P<0.01$.
[a]Activities given as mmol h^{-1} g^{-1}.

Table 4 Potential organic matter breakdown activity of ectomycorrhizal tips

Enzyme	Enzyme activity (pmol min^{-1} mm^{-2})					
	Piloderma	*Amphinema*	*Cenococcum*	*Cortinarius*	*Tylospora*	EMF[a]
Acid phosphatase	12.71 (7.75)	11.48 (7.65)	8.93 (5.47)	13.56 (11.36)	9.77 (7.63)	10.15 (3.59)
Sulfatase	1.08 (1.13)	0.82 (0.53)	0.77 (0.74)	2.17 (3.26)	1.00 (0.80)	1.16 (0.72)
Leucine aminopeptidase	0.46 (0.19)	0.41 (0.11)	0.50 (0.23)	2.94 (4.78)	0.42 (0.32)	0.54 (0.24)
β-1,4-N-acetylglucosaminidase	3.87 (1.46)	3.57 (1.14)	2.87 (1.48)	6.05 (6.37)	2.79 (1.77)	3.68 (0.89)
β-1,4-Glucosidase	3.16 (1.19) ab	5.78 (2.48) a	1.75 (0.98) b	2.76 (4.12) ab	3.45 (2.23) ab	3.47 (1.37)
Cellobiohydrolase	0.63 (0.28) b	1.09 (0.37) a	0.42 (0.27) b	0.65 (1.18) ab	0.76 (0.39) ab	0.72 (0.34)
Xylosidase	0.22 (0.18) b	0.64 (0.38) a	0.20 (0.10) b	0.13 (0.13) b	0.49 (0.37) ab	0.34 (0.20)
Laccase[b]	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)	0.02 (0.02)	0.03 (0.01)	0.02 (0.01)
Number of plots (n)	8	7	8	5	5	9

Data are provided as means (n = number of plots the tip genera were isolated from. Note that not all genera were found in all plots) with s.d. in brackets. Data in the same row with different letters are significantly different at $P<0.01$. Refer to Table 3 for the general role of each enzyme in organic matter breakdown.
[a]EMF, average activity of all ectomycorrhizal tips, not included in statistical analysis.
[b]Laccase activity given in U min^{-1} mm^{-2}.

Table 5 Direct correlation (Spearmans r) between enzyme activities of the ectomycorrhizal tips selected from the FH layer of the forest floor and the activities of the FH layer

	Enzyme	EMF tips[a]	*Amphinema*	*Piloderma*
FH layer	Xylosidase	0.850**	0.729*	NS
FH layer	Cellobiohydrolase	0.883**	0.712*	0.750*
FH layer	β-Glucosidase	0.833**	0.797*	NS

Abbreviation: FH, *in situ* fermentation-humic layer.
Correlations are significant at *$P<0.05$, **$P<0.01$. Note that only enzymes and EMF tips that were significantly related to FH are shown.
[a]EMF tips, correlation with the average activity of all ectomycorrhizal tips.

and enzyme-loading factors at three different levels of community structure. At the ecological function level, the abundance of saprotrophic fungi was strongly related to the patterns of enzyme activity that separated −SB communities from the other two communities along PC1 and PC3 (Figure 4a). This association continued at the higher (Figure 4b, that is, Fungi *I. sedis*) and lower taxonomic levels (Figure 4c, that is, *Mortierella spp.*). By contrast, the enzyme-loading factors along axes PC1 and PC3 that influenced +SB and FH community clustering were generally correlated with the relative abundance of EMF and other mycorrhizal fungi. The abundance of specific EMF genera also strongly correlated with the enzyme-loading factors of PC2 (Figure 4c), which as mentioned above, largely reflects variations in enzymes associated with phosphorus and nitrogen release and lignin degradation. These fungi included *Amphinema*, which was strongly associated with +SB enzyme patterns, and *Piloderma*, *Cortinarius* and *Tylospora*, which were strongly associated with FH enzyme patterns.

Discussion

A large fraction of terrestrial ecosystem C pools, with estimates as high as 1700 PgC (De Luca and Boisvenue, 2012), are sequestered as SOM in the

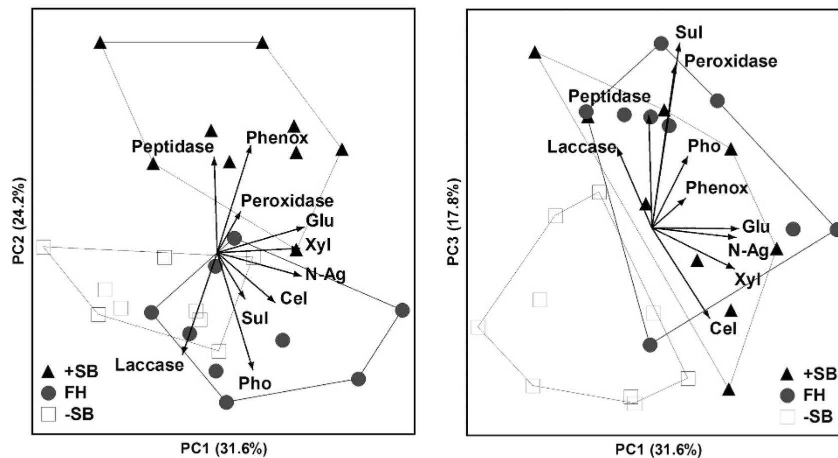


Figure 3 PCA ordination of potential enzyme activity in the hyphal trap bags and the FH layers of the forest floor. The proportion of variance explained by each axis is shown, and the contribution of each enzyme in the ordination is indicated by the plot vectors. −SB, hyphal trap bags with no sand barrier; FH, *in situ* fermentation-humic layer; +SB, hyphal trap bags with sand barrier. Cel, cellobiohydrolase; Glu, β-1,4-glucosidase; N-Ag, β-1,4-N-acetylglucosaminidase; Pho, acid phosphatase; Sul, sulfatase; peptidase, leucine aminopeptidase; Lac, laccase; perox, peroxidase; phenox, phenol oxidase PC1 is associated with Glu (r = 0.895), Cel (r = 0.599), Xyl (r = 0.851) and N-Ag (r = 0.865); PC2 is associated with Pho (r = -0.799), peptidase (r = 0.650), Lac (r = -0.695) and Phenox (r = 0.729); PC3 is associated with Sul (r = 0.798), peptidase (r = 0.484) and Perox (r = 0.702). All associations are significant at $P<0.05$.

soils of temperate and boreal forests (Schmidt *et al.*, 2011). Increased temperature and atmospheric CO_2 levels associated with climate change are predicted to increase the decomposition of this SOM-C pool (Fierer *et al.*, 2005; Conant *et al.*, 2008; Craine *et al.*, 2010). The majority of this SOM is found in the FH horizons dominated by EMF and not saprotrophic fungi (Lindahl *et al.*, 2007). Quantifying the contribution of EMF mycelia to SOM turnover is, therefore, of critical importance to Cmodels (Cairney, 2012).

Potential contribution of EMF mycelia to soil organic C turnover

It has generally been believed that saprotrophic fungi bear primary responsibility for recalcitrant SOM-C decomposition in forest soils (Baldrian, 2008). Recently, Talbot *et al.* (2013) concluded that EMF and saprotrophic communities have complementary roles in SOM turnover. Specifically, in pine forests in a Mediterranean climate, they found that EMF species richness explained SOM-nitrogen (N) decomposition patterns, whereas saprotrophic community structure explained SOM-C decomposition patterns. In the current study, we directly measured the potential enzyme activity in FH-filled mesh bags colonized by the two different trophic groups (colonization by obligate saprotrophs controlled by the presence or absence of a sand barrier) in a sub-boreal spruce system, and compared those activities with that of the surrounding FH horizon. We found that communities dominated by EMF hyphae exhibited significant potential to degrade both SOM-C and SOM-N. The oxidative and hydrolytic enzyme activities exhibited by these EMF communities were comparable to or higher than those measured in communities enriched with saprotrophic fungi. None of the measured enzymes were specifically associated with saprotrophs, suggesting that it is premature to generalize about the role of these two trophic groups across ecosystems.

The EMF-dominated mesh bag communities and FH horizon had significantly similar patterns of cellulolytic and hemicellulolytic enzyme activity. Although these C-targeting hydrolase activities are generally attributed to saprotrophic fungi (Talbot *et al.*, 2013), this was not the case in our spruce soils where communities with abundant saprotrophs exhibited lower hydrolytic enzyme activity. The single-most abundant taxonomic group that correlated with these activities matched unidentified EMF from root tips. Some EMF, such as *Laccaria bicolor*, have retained low levels of cellulolytic and hemicellulolytic hydrolases in their genomes (Martin *et al.*, 2008; Nagendran *et al.*, 2009; Martin *et al.*, 2010). Although gene presence does not automatically indicate functionality, several EMF, including *L. bicolor*, are able to use cellulose or hemicellulose as a sole C source (Durall *et al.*, 1994; Vaario *et al.*, 2012). The abundant unidentified EMF in our systems may have similar hydrolytic activity, an occurrence that may be favoured by a number of site-specific factors.

Hydrolase activity is known to increase with SOM content (Sinsabaugh *et al.*, 2008). The FH horizon in our spruce soils contained 64% SOM, almost half of which was cellulose and hemicellulose (data not shown). However, the most abundant saprotroph in our systems, *Mortierella spp.*, is an inefficient decomposer of cellulose, exhibiting very little β-glucosidase activity (Hanson *et al.*, 2008; Allison *et al.*, 2009). The significantly lower β-glucosidase

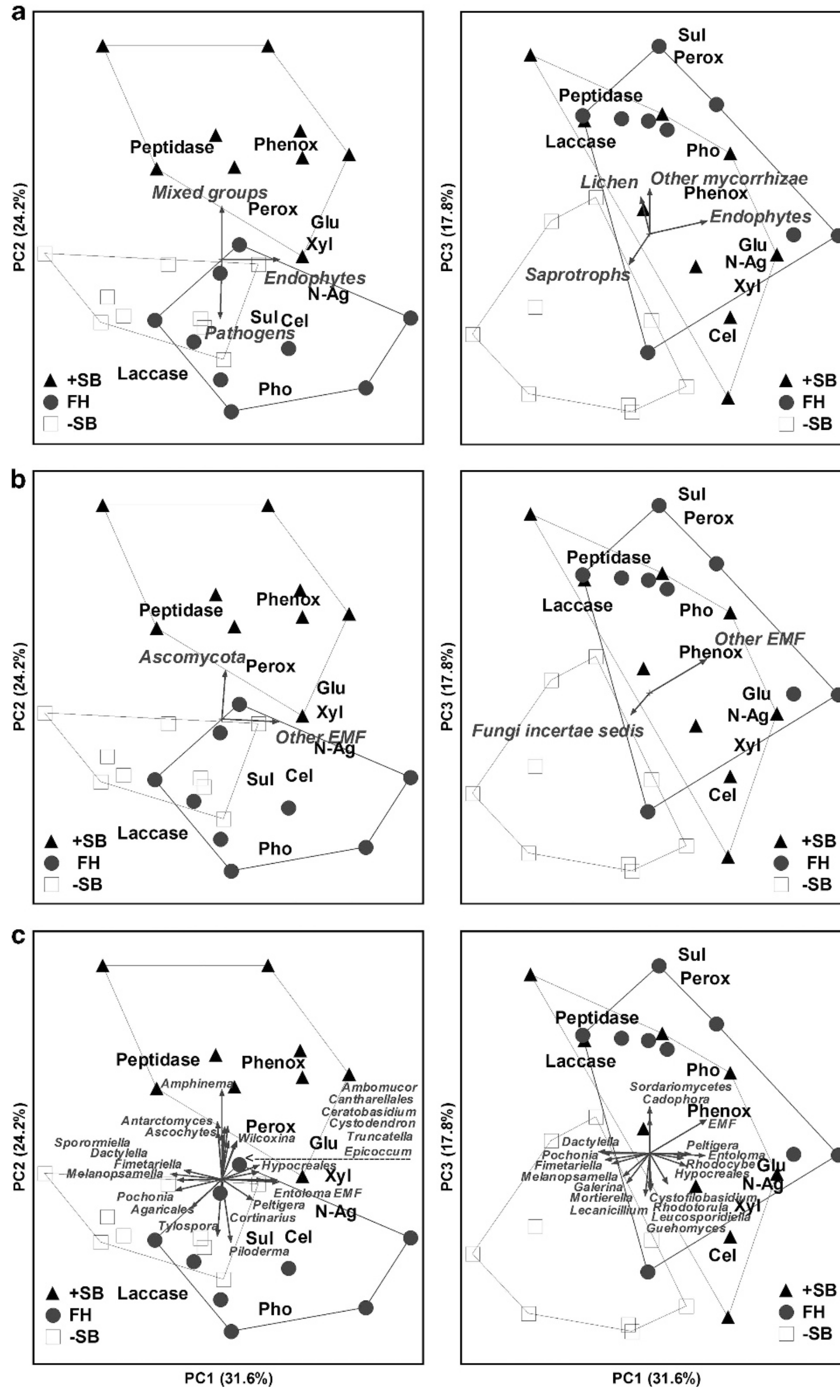


Figure 4 Relationship between fungal communities and the potential enzyme activities in hyphal trap bags and the FH layers of the forest floor. −SB, hyphal trap bags with no sand barrier; FH, *in situ* fermentation-humic layer; +SB, hyphal trap bags with sand barrier. Joint plots vectors indicate the strength and direction of significant ($P<0.05$) correlations between enzyme PC-loading factors and fungal communities at the level of (**a**) ecological function, (**b**) phyla and (**c**) genus or closest identity. The direction and strength of enzyme correlations contributing to each PC axis (as in Figure 3) are indicated with the abbreviations Cel, cellobiohydrolase; Glu, β-1,4-glucosidase; N-Ag, β-1,4-N-acetylglucosaminidase; Pho, acid phosphatase; Sul, sulfatase; peptidase, leucine aminopeptidase; Lac, laccase; Perox, peroxidase.

activity in our saprotroph-dominated hyphal traps is likely related to the abundant (50% of the total sequences) *Mortierella spp.* colonizing these traps. In our spruce soils, a population of inefficient saprotrophs combined with a large pool of cellulolytic compounds may have favoured hydrolytic C-prospecting by EMF, which are also known to increase their enzymatic activity in the absence of direct saprotrophic competitors (Leake *et al.*, 2001; Pereira *et al.*, 2012). Although we cannot exclude

potential bacterial activity, a metaproteomic study of *Fagus sylvatica* L. litter (Schneider *et al.*, 2012) and a 13C-cellulose stable isotope study of spruce soils (Štursová *et al.*, 2012), both found that fungi were primarily responsible for cellulose decomposition. In the latter study, *Amphinema spp.* were identified as having incorporated 13C from cellulose into their DNA. In our study, the cellulose and hemicellulose-degrading activities of EMF tips, and in particular *Amphinema* tips, correlated highly with those activities in the FH layer. Although requiring further research, the strength of the correlations ($r > 0.7$) and the interrelatedness of the enzymes involved make it unlikely that this is a spurious phenomenon.

Our results also suggest that EMF in spruce soils are directly involved in the breakdown of lignin and humic complexes, supporting research that suggests that increased mycorrhizal activity associated with climate change will stimulate decomposition of previously highly recalcitrant SOM-C (Cheng *et al.*, 2012; Phillips *et al.*, 2012b).The oxidative enzymes involved in the decomposition of these compounds are broadly classified as phenol oxidases (including laccase and tyrosinase) and peroxidases (including lignin and broad spectrum peroxidases) (Sinsabaugh, 2010; Theuerl and Buscot, 2010). Enzyme assays typically do not differentiate between the individual enzymes and instead measure the activity of the broad classes (Sinsabaugh, 2010; German *et al.*, 2011; Eichlerová *et al.*, 2012). In the current study, the potential phenol oxidase activity in EMF-dominated mesh bags was significantly higher than that of the FH or saprotroph-dominated bags when measured using a L-DOPA assay, but lower when measured using an ABTS assay. Research suggests that L-DOPA is oxidized by both laccases and tyrosinases, whereas ABTS is primarily oxidized by laccases (Burke and Cairney, 2002; Günther *et al.*, 1998; Eichlerová *et al.*, 2012). The EMF in our mesh bags did not have the relevant laccases to effectively oxidize ABTS, a fundamental difference that separated these communities from the otherwise trophically similar communities in the surrounding FH layer. These results suggest that the extent of EMF degradation of recalcitrant SOM-C will depend on the EMF community structure, as differing EMF genera were dominant in the two systems (*Amphinema* vs *Piloderma*, respectively) and individual EMF are known to differ in their ability to oxidize these enzyme substrates (Günther *et al.*, 1998; Burke and Cairney, 2002; Tedersoo *et al.*, 2012). *Amphinema* were also the most abundant EMF in the saprotroph-dominated bags, but they were outnumbered by lignin-degrading saprotrophic *Mortierella spp.* (Hanson *et al.*, 2008; Allison *et al.*, 2009). Although Allison *et al.* (2009) found no link between lignin degradation by *Mortierella spp.* and L-DOPA-phenol oxidase activity, our *Mortierella*-dominated communities exhibited substantial ABTS-phenol oxidase activity. Saprotrophs and EMF are known to contain a broad range of phenol oxidases that differ in substrate specificity (Luis *et al.*, 2004; Kellner *et al.*, 2010). Until this complex group of enzymes are better understood, researchers investigating the relative contributions of EMF and saprotrophic fungi to SOM-C turnover should include numerous phenol oxidase assays, as reviewed by Sinsabaugh (2010) in parallel.

Release of SOM-degrading enzymes by EMF does not necessarily imply the uptake of any released C. For example, phenol oxidase activity by EMF hyphae is associated with the exploration and the concomitant mining of polyphenolic-rich humic compounds for other nutrients (Talbot *et al.*, 2008, 2013; Rineau *et al.*, 2012). The EMF mycelia colonizing the mesh substrates may have been similarly prospecting for nitrogen, as there was a positive relationship between L-DOPA-phenol oxidase activity and protease activity ($r = 0.731$). However, the significantly higher levels of peroxidase activity in the EMF-dominated systems were not directly associated with protease activity, suggesting that additional polyphenolic degradation may be occurring independently of nitrogen acquisition. High peroxidase activity in systems with abundant EMF is not surprising, as many EMF contain Class II peroxidase-encoding genes (Bödeker *et al.*, 2009), and peroxidase activity has been shown to increase with increasing EMF species richness (Talbot *et al.*, 2013). The combined and complex oxidase enzyme activity in our EMF-dominated systems suggests that EMF mycelia are actively breaking down recalcitrant SOM.

Effectiveness of modified hyphal traps to isolate EMF mycelia

Hyphal trap bags are a crucial tool for assessing the relative contributions of EMF and saprotrophic mycelium to SOM cycling (Wallander *et al.*, 2001). Although it has been suggested that soil-based in-growth cores were primarily selected for EMF hyphae in coniferous forests with low saprotrophic populations (Hendricks *et al.*, 2006), comparable approaches in our study (that is, bags without a sand barrier) were equally colonized by both trophic groups. Surrounding these mesh bags with a sand barrier, however, trapped a fungal community that was highly similar to that of the adjacent soil, with $\sim$70% EMF sequences and fewer than 9% saprotrophic sequences. Although other studies have used sand-only hyphal traps to minimize saprotroph colonization (Kjøller, 2006; Korkama *et al.*, 2007; Wallander *et al.*, 2010; Walker *et al.*, 2012), in our study the sand surrounding the hyphal traps contained large populations of saprotrophic *Mortierella spp.* (16% of the total sequences). *Mortierella* were also the most abundant saprotroph in the surrounding soil and are similarly abundant in other spruce forest soils (Buée *et al.*, 2009). These fungi are

known to colonize the surface of spruce roots (Salt, 1977) and may have incidentally explored the sand in association with EMF hyphae. Our results suggest that sand-only hyphal traps may not effectively isolate EMF hyphae in soils where *Mortierella* are abundant.

The fungal communities in our saprotroph-excluding mesh bags were also taxonomically similar to those found in the surrounding soil, although differences did emerge at the genus level. The most abundant EMF in the hyphal traps were *Amphinema* and *Wilcoxina*, but in the surrounding soil were *Piloderma*, Cortinarius and Tylospora. These different colonization patterns could be because of explorer type, as EMF colonizing the +SB hyphal traps would need to explore at least the width of the sand barrier away from a colonized root tip. Although *Amphinema* and *Piloderma* are both considered medium distance explorers, *Piloderma* may also form mats with a limited range of exploration (Agerer, 2001; Kluber *et al*., 2011). Berner *et al.* (2012) also hypothesized that short-term mesh bag studies may preferentially select for early EMF colonizers. The most abundant EMF in our mesh bags, *Amphinema* and *Wilcoxina*, are highly competitive early colonizers of seedlings, both in greenhouses (Jones *et al*., 1997; Menkis *et al*., 2005) and under field conditions (Gagné *et al*., 2006; Barker *et al*., 2012; Walker *et al*., 2012). Early colonizing *Amphinema* and *Wilcoxina* may have out-competed other species, including less competitive *Piloderma spp.* (Erland and Söderström, 1991; Erland and Finlay, 1992; Wu *et al*., 1999). Differences in fungal nutritional requirements might also influence colonization. For example, *Cortinarius spp.* avoid nutrient-limited sand hyphal traps (Kjøller, 2006; Berner *et al*., 2012). These EMF may have similarly avoided the sand barrier in the current study, as *Cortinarius* were more abundant in hyphal traps without a sand barrier.

Conclusions

Using novel microcosms of sterile, FH substrate surrounded by a barrier of sand, we determined that both oxidative and hydrolytic enzyme activities in EMF-dominated substrates are comparable to or greater than that exhibited by microcosms enriched in saprotrophic hyphae. Collectively, our data supports a substantial role for EMF hyphae in the turnover of both recalcitrant and labile SOM in sub-boreal spruce stands. Furthermore, it expands our ability to predict feedbacks between elevated atmospheric CO_2 and soil C fluxes.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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