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LETTER

Ectomycorrhizal fungi slow soil carbon cycling

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Abstract

Respiration of soil organic carbon is one of the largest fluxes of CO_2 on earth. Understanding the processes that regulate soil respiration is critical for predicting future climate. Recent work has suggested that soil carbon respiration may be reduced by competition for nitrogen between symbiotic ectomycorrhizal fungi that associate with plant roots and free-living microbial decomposers, which is consistent with increased soil carbon storage in ectomycorrhizal ecosystems globally. However, experimental tests of the mycorrhizal competition hypothesis are lacking. Here we show that ectomycorrhizal roots and hyphae decrease soil carbon respiration rates by up to 67% under field conditions in two separate field exclusion experiments, and this likely occurs via competition for soil nitrogen, an effect larger than 2 °C soil warming. These findings support mycorrhizal competition for nitrogen as an independent driver of soil carbon balance and demonstrate the need to understand microbial community interactions to predict ecosystem feedbacks to global climate.

Keywords

Biogeochemistry, ecosystem ecology, mycorrhizal fungi, soil carbon, soil ecology, soil nitrogen.

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INTRODUCTION

Carbon (C) storage in terrestrial ecosystems is regulated by C inputs from net primary production and C outputs due to decomposition and respiration by microbial decomposers (Schlesinger & Bernhardt 2012). Nitrogen (N) limitation is pervasive in terrestrial ecosystems, and can limit fluxes of C through both primary producers and free-living microbial decomposers (Schimel & Weintraub 2003; LeBauer & Treseder 2008). Plants with root-associated ectomycorrhizal (EM) fungal symbionts dominate boreal, temperate, montane and some tropical ecosystems (Read 1991; Torti *et al*. 2001). These fungi produce enzymes that degrade organic N, which can unlock N trapped in soil organic matter to fuel plant primary production (Rineau *et al*. 2012; Shah *et al*. 2015). However, by doing so EM fungi may induce or exacerbate N limitation of free-living microbial decomposers. Theoretical models predict that competition between EM fungi and free-living decomposers for N will slow soil C cycling and increase soil C storage (Orwin *et al*. 2011), which is supported by a global pattern of increased soil C storage in EM ecosystems (Averill *et al*. 2014). Yet, despite the potential importance for predicting soil and ecosystem C storage, there is still no direct test of the mechanism of action (Fernandez & Kennedy 2015).

Previous work on this topic has focused on laboratory studies which have shown evidence that EM fungi selectively mine organic N (Abuzinadah *et al*. 1986; Bending & Read 1995; Rineau *et al*. 2012) and slow decomposition of plant litter (Gadgil & Gadgil 1975). Although these laboratory studies are compelling mechanistically, they cannot evaluate the relevance of ectomycorrhizal-decomposer competition for biogeochemical cycling in real-world conditions. There are notable correlations between fungal community composition and soil C sequestration, however these remain to be experimentally and mechanistically vetted (Clemmensen *et al*. 2013, 2015). Furthermore, while some field experiments report changes in decomposer activity and soil C turnover when roots and EM fungi are excluded (Gadgil & Gadgil 1971; Lindahl *et al*. 2010; Ekblad *et al*. 2013; Phillips *et al*. 2013; Brzostek *et al*. 2015), they cannot account for disturbance effects of treatments that may generate a temporary increase in labile C inputs to soil, increasing free-living microbial activity. Forest girdling experiments have shown positive effects of EM fungi and plant roots on soil C cycling, however again community effects are confounded with disturbance of the treatments (Högberg *et al*. 2001; Subke *et al*. 2004, 2011). Finally, it has been suggested that inhibition of soil C cycling by the presence of EM roots may be driven by a soil drying effect rather than competition between EM fungi and soil saprotrophs *per se* (Bending 2003; Koide & Wu 2003). Because of these shortcomings, there is still no clear biogeochemical test of EM-decomposer competition altering soil C and N cycling.

We conducted two complementary field experiments to test for and quantify EM-root inhibitory effects on soil C and N cycling. Both explicitly included controls for the disturbance involved in experimentally excluding EM fungi. First, we tested the effect of EM exclusion on soil C and N cycling over the course of one growing season in an old growth temperate forest using mesh exclusion bags ('EM-exclusion experiment'). Second, we determined if the effect of EM fungal inhibition was quantitative by leveraging a gradient of EM fungal abundance over the course of an entire year, which included EM exclusions across the gradient created by trenching ('EM-gradient experiment'). The gradient study allowed us to control for the role of soil drying, as drying effects should be present in both the low- and high-EM forest sites. We measured changes in soil respiration per gram microbial biomass (hereafter, 'biomass-specific respiration') and per gram soil, as well as indicators of microbial N availability. Biomass-specific respiration rates have been widely used to better understand soil

respiration responses to temperature (Bradford *et al*. 2008; Hartley *et al*. 2008; Karhu *et al*. 2014) and soil moisture (Waring & Hawkes 2014), and allow us to detect shifts in microbial activity due to release from N limitation.

METHODS

Site description and experimental design

The experiments were conducted at the Harvard Forest in Petersham, MA, USA (42°32′ N, 72°11′ W). Experimental plots were established within three forest types, chosen to represent low-, medium- and high-EM abundance. Low-EM sites were girdled, ~ 130-year-old *Tsuga canadensis* stands, originally designed to simulate hemlock woolly adelgid infestation. Since girdling in 2005, the forest has been re-growing as EM black birch. Medium-EM abundance sites were ~ 132-year-old second growth *Tsuga canadensis* stands used as experimental controls from the girdling treatments. High-EM abundance sites were 200+-year-old, *Tsuga canadensis* stands. Sites have previously been described in Finzi *et al*. (2014). The old growth sites were used for the EM-exclusion experiment, whereas all sites were used for the EM-gradient experiment. Figure S1 in Supporting Information shows the spatial distribution of sites.

Within each experiment we used a different method to exclude roots and mycorrhizal fungi. The EM-exclusion experiment used a fine mesh, while the EM-gradient experiment used a trenching technique. In addition to our goal of reducing EM abundance, mycorrhizal exclusion has an obvious disturbance effect. By severing roots and fungal hyphal networks we create a pulse of fresh substrates that may increase microbial activity, which would be confounded with the predicted release from EM inhibition. We controlled for this disturbance by explicitly incorporating disturbance controls into our design with both methods.

In the EM-exclusion experiment we constructed two types of mesh bags: (1) mycorrhizal exclusion bags made of 1-μm nylon mesh to exclude both roots and mycorrhizal fungi and (2) disturbance controls constructed of 2-mm fibreglass window screen to allow entry of roots and mycorrhizal fungi. Both types of mesh bags (12×12 cm^2) were filled with ~ 100 g of sieved, field moist organic horizon material. Three replicates of each treatment were installed in each of six plots within the high-EM site ($n = 36$). Bags were placed approximately in the centre of the soil organic horizon. Treatments were installed during the first week of June 2013 and harvested during the third week of August 2013. Harvested bags were homogenised by hand, roots were removed and subsampled for chemical analyses.

In the EM-gradient experiment, four 30×30 m^2 plots were established in each of the low-, medium- and high-EM sites as described in Finzi *et al*. (2014). In 2011, trenching was used to experimentally reduce EM abundance using two 60×60 cm^2 trenches per plot. The trenches were dug through the soil organic horizon to 30- cm depth from the top of the mineral soil horizon, lined with 2-mm thick plastic and then back filled. In-growth bags made of 2-mm window screen as above were filled with sieved organic horizon material and placed both inside and outside trenches at an equal distance from the trench perimeter. Bags were incubated for up to 13 months in the field, in the approximate centre of the soil organic horizon. Two in-growth bags were placed inside and outside of each trench, resulting in four disturbance control and four exclusion observations per plot. Bags were harvested from the field in June and August 2012 and transported on ice to Boston University where they were processed within 24 h.

Quantification of ECM abundance using sand in-growth technique

We quantified EM abundance across the forest-age gradient, using the sand in-growth technique (Wallander *et al*. 2013). Mesh bags of 50-μm ($8 \times 8 \times 1$ cm^3) were filled with ~ 100 g of acid-washed sand. The 50-μm mesh allows for the in-growth of fungal hyphae, but not roots. Furthermore, because the sand is depleted of C, mycorrhizal fungi that receive C from plants dominate. The sand in-growth bags therefore act as a hyphal 'trap', in which EM fungi survive longer than free-living fungi. This approach has been validated using trenching and ^{13}C analysis of in-growth hyphae (Wallander *et al*. 2001), and ITS sequencing showing that EM fungi dominate fungal community composition within the in-growth bags (Parrent & Vilgalys 2007; Wallander *et al*. 2010, 2013). Four sand bags were installed in each plot, outside of trenches, in July 2011 and harvested in August 2012. We quantified fungal abundance in sand in-growth bags by extracting and quantifying the fungal biomarker ergosterol.

Ergosterol was extracted and quantified from sand using the methods of Hobbie *et al*. (2009), extraction details are presented in Appendix S1. Two samples returned values an order of magnitude greater than the rest, and were determined to most likely be a result of contamination. These samples were excluded from the final analysis.

Fungal community characterisation

DNA was extracted from in-growth cores (0.2 g) using standard methods. Purified DNA extracts were amplified by PCR in triplicate using the ITS1F/ITS2 primer pair (White *et al*. 1990; Gardes & Bruns 1993) and sequenced (2× 250 bp) on an Illumina MiSeq V2 (for details, see Appendix S1). Paired end sequences were assembled and quality filtered, and downstream OTU clustering and taxonomy assignment were performed using the QIIME pipeline (v. 1.7) (Caporaso *et al*. 2010), the uclust algorithm and the UNITE fungal database (Edgar 2010; Kõljalg *et al*. 2013). Further details on sequence analysis are present in Appendix S1.

Fungal to bacterial ratios

We used quantitative PCR (qPCR) to determine relative abundances of fungi and bacteria. We used the 5.8S/ITS1f primer pair for fungi, and the Eub338/Eub518 primer pair for bacteria as described in Fierer *et al*. (2005). qPCR reaction conditions are described in Appendix S1. Fungal to bacterial (F : B) ratios were calculated based on the number of ITS and 16S copies detected in each sample.

Soil and microbial biomass C and N pools

Inorganic N concentrations were determined from 10 g soil extracted with 2 M KCl (Shepherd *et al*. 2001). Ammonium and nitrate concentrations were determined colorimetrically (Sims *et al*. 1995; Doane & Horwath 2003). Extractable organic N in KCl extracts was measured in microplates using the OPAME method (Jones 2002). Soils were dried at 100 °C to constant mass, ground with mortar and pestle, weighed, wrapped in tin capsules and run on an NC2500 Element Analyzer for total C and N (CE Elantec, Lakewood, NJ, USA). Gravimetric soil moisture was measured on 5 g soil subsamples dried for at least 24 h at 100 °C.

Microbial biomass was quantified using chloroform fumigation and extraction with 0.5 M K_2SO_4 (Vance *et al*. 1987). Controls were immediately extracted in 50 mL centrifuge tubes. Fumigation was performed by placing a cotton ball within the centrifuge tube, pipetting 3 mL of chloroform onto the cotton ball, capping the tube and then incubating the samples in the dark for 7 days (Wallenstein *et al*. 2006). After incubation, samples were vented and extracted. Organic C and N content of extracts was determined with an Apollo 9000 TOC/TN Analyzer (Teledyne Tekmar, Mason, OH, USA) using an arginine standard. We applied correction factors to scale from extractable microbial C and N to total microbial C and N (Vance *et al*. 1987).

Soil microbial C and N fluxes

Soil respiration was measured by placing 20 g of field moist soil into a 488-mL glass mason jar fitted with a septum for headspace sampling. Soils equilibrated for 24 h after being weighed into jars. Three gas samples were taken from each jar over 3 h. CO_2 was measured using an infra-red gas analyser (EGM-4; PP Systems, Amesbury, MA, USA). We measured gross N depolymerisation rates using the assay described in Watanabe & Hayano (1995), modified by Lipson *et al*. (1999).

We quantified potential net N mineralisation by incubating soils for 7 days at laboratory temperature before extraction with 2 M KCl as described earlier. Daily net N mineralisation was calculated as the difference in inorganic N between incubated and non-incubated soil subsamples divided by the number of days incubated.

Soil enzyme activities

We measured the activities of four hydrolytic enzymes involved in the decomposition of C and N: beta-glucosidase (BG), cellobiohydrolase (CBH), n-acetyl glucosaminidase (NAG) and leucine aminopetidase (LAP). These were chosen because they are considered important indicator enzymes for understanding microbial function (Sinsabaugh *et al*. 2008). Soil enzyme activities were measured fluorometrically (German *et al*. 2011) on frozen (−20 °C) soil subsamples at saturating substrate concentrations.

Biomass-specific activities

Because EM fungi can represent one-third or more of the total soil microbial biomass (Högberg & Högberg 2002), exclusion of EM fungi may lead to a net decline in total microbial biomass and associated fluxes, even if free-living decomposer biomass has increased, as well as the turnover of soil C. This is because the source of respiration measured with EM fungi present is a mixture of recent photosynthate transferred to EM fungi and soil organic C processed by free-living decomposers, while the source of respiration when EM fungi are excluded is solely soil organic matter. For this reason, we report both respiration mg^{-1} microbial biomass (herein biomass-specific respiration) as well as respiration g^{-1} soil C. This allows us to capture changes in the rate of soil C turnover due to a comparatively more active microbial biomass.

Potential enzyme activities as well as the gross N depolymerisation rate were also analysed per unit microbial biomass. Biomass-specific enzyme rates and potential activities can reflect microbial investment or allocation in these resource acquisition strategies. These activities and rates are herein referred to biomass-specific activities and rates.

Statistical analysis

Ergosterol concentrations of sand in-growth bags were averaged within plot, and then analysed as a function of site using ANOVA. We analysed all other response variables in a mixed-effects framework using the lme function in the nlme package for R-statistical software (Pinheiro *et al*. 2014; R Core Team 2014). Plot was coded as a random effect so that treatments and disturbance controls from within the same plot were paired (Gelman & Hill 2007). In the EM-gradient experiment, we first tested for an effect of trenching on soil moisture, or an interaction between trenching and site, given past reports that trenching can increase soil moisture (Koide & Wu 2003). We found no effect of treatment or treatment × site and therefore proceeded with analysis without modelling soil moisture effects. We tested for main effects of stand age and mycorrhizal exclusion (trenching), as well as an interaction between the two predictors. Season (June or August harvesting) was initially included as a random effect, but was removed from models in the EM-gradient experiment because it was not significant. When interactive effects were not significant predictors for a response variable in the full model, we only included main effects. If interactive effects were significant we tested for differences between trench and control treatments within each site using the glht function within the multcomp package in R (Hothorn *et al*. 2008). In the EM-exclusion experiment, we tested for main effects of mycorrhizal exclusion. Furthermore, the 1-μm mesh used to implement mycorrhizal exclusions increased gravimetric soil moisture by ~ 5% relative to controls ($P = 0.07$). To account for this we fitted all models with soil moisture as a covariate. We calculated variance inflation factors (Zuur *et al*. 2010), modified for a mixed-effects framework, to ensure that treatment and soil moisture were not collinear. The highest model variance inflation factor was 1.13, implying that collinearity was not an issue and effects of soil moisture and treatment could be reliably separated with statistical models. Response variables were natural log transformed if the model residuals did not meet the assumptions of the linear regression as indicated by heteroscedasticity of errors. In very few cases this

resulted in excluding 1–2 observations from an analysis if the value of the response variable was zero. We present the response of total enzyme activity as well as subsets of enzymes that target on C-containing compounds (BG + CBH) and enzymes that target compounds that contain both C and N (NAG + LAP). We did not perform any multiple comparison corrections, as this reduces Type I errors at the expense of Type II errors. In either case, we are more concerned about errors of magnitude and direction, both of which are better addressed by fitting multilevel models, as done in this analysis. This topic is discussed at length in Gelman *et al.* (2012).

Fungal community composition was analysed as a function of site, trenching treatment, plot and their interaction with PERMANOVA using the adonis function in the vegan package (Oksanen *et al.* 2015) for R statistical software (R Core Team 2014) based on Bray–Curtis dissimilarities calculated from an abundance matrix of fungal OTUs. Results were visualised with non-metric multidimensional scaling in PC-Ord (McCune & Mefford 2011). All mixed-effects regression output can be found in Appendix S2.

RESULTS

In the EM-gradient experiment, EM abundance based on ergosterol concentrations within sand in-growth bags increased significantly across the stand age gradient ($P < 0.05$, Fig. 1a). Molecular metrics of EM sequence relative abundance and fungal : bacterial gene copy ratio trended to increase across the gradient, although differences were not significant (Fig. 1b), consistent with the findings of the ergosterol analysis. Both EM sequence relative abundance and fungal : bacterial gene copy ratio were reduced within the trench treatments ($P < 0.01$, Fig. 1c), resulting in conditions similar to the low abundance EM black birch stands (Fig. 1b).

Fungal communities differed across the EM-gradient sites ($F = 5.26$; partial $r^2 = 0.099$, $P < 0.001$) and between the EM-exclusion treatments ($F = 4.62$; partial $r^2 = 0.043$, $P < 0.001$) based on PERMANOVA (Fig. 2). Trenching had a unique effect in each site ($F = 2.05$; partial $r^2 = 0.038$, $P < 0.001$), resulting in subsets of the local fungal community. The first two axes captured 31% of the variation. Axis 1 was correlated with % soil C ($r^2 = 0.41$; $P < 0.001$). Based on the 20 most abundant OTUs by sequence abundance, we saw that control treatments were dominated by taxa in the genera *Amanita*, *Lactarius* and *Russula*, as well as 14 fungal OTUs unresolved to genus level, representing 70% of all control sequences. Exclusion treatments were dominated by *Amanita*, *Lactuarius*, *Tylopulus*, *Russula*, *Boletus*, *Scleroderma*, *Paxillus*, *Clavulina* and *Phylloporus*, and nine fungal OTUs that could

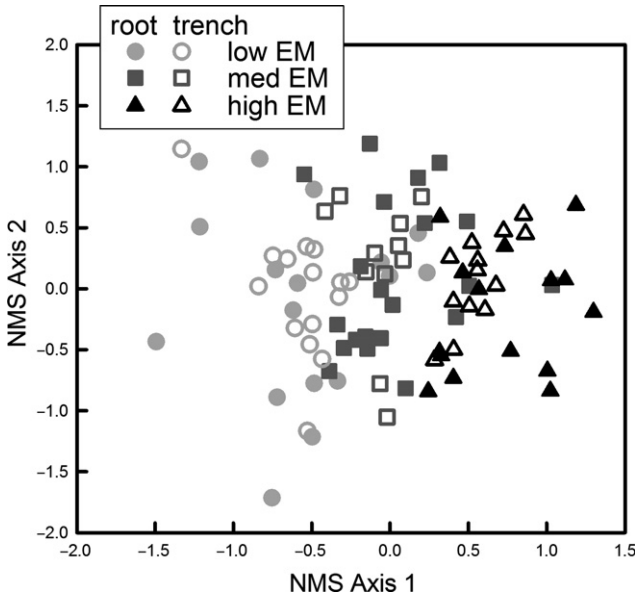

Figure 2 Non-metric multidimensional scaling ordination of fungal communities based on ITS sequences from sites across the ectomycorrhizal gradient, including both control and trenched samples. Final stress was 18.73 for a three-dimensional solution after 114 iterations.

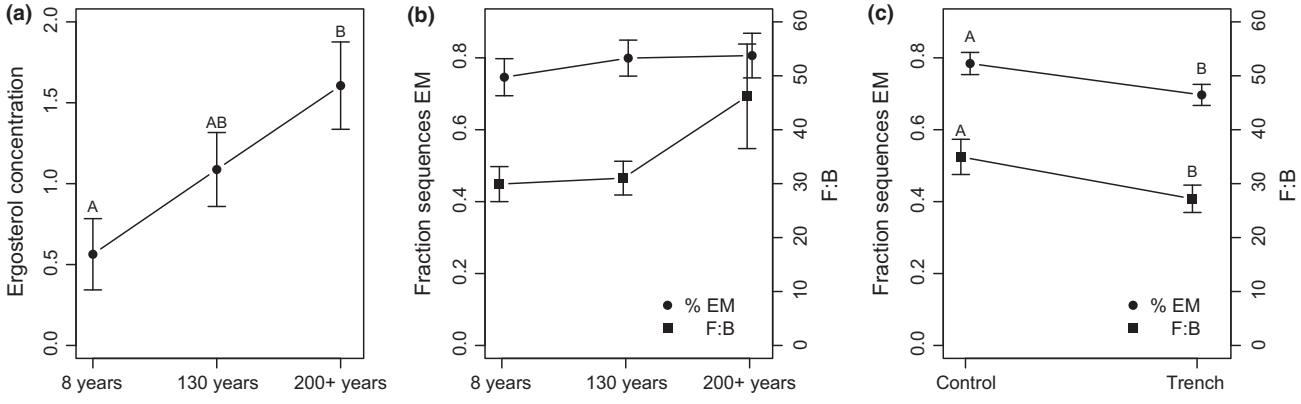

Figure 1 Microbial community patterns across sites and trenches in the ectomycorrhizal (EM)-gradient experiment. (a) Ergosterol concentrations from sand in-growth bags are plotted by site in units of μg ergosterol g^{-1} sand. Letters denote significant differences at $P < 0.05$. (b) Relative abundance of EM sequences and fungal to bacterial ratios (F : B) by site. Fraction of sequences EM is calculated as the number of fungal sequences designated EM divided by the total number of fungal sequences from each sample. F : B is based on the number of copies of fungi and bacteria from qPCR assessment. (c) Responses of EM sequence relative abundance and F : B to EM exclusion by trenching. Asterisks denote differences are significant at $P < 0.05$. Centre values are means and error bars represent one standard error of the mean.

not be assigned to known genera, representing 66% of all exclusion sequences. We caution against over interpreting the most abundant OTUs, as it has been well demonstrated that sequence abundance of individual OTUs does not necessarily reflect the abundance of fungal taxa within or across sample (Nguyen *et al*. 2015).

Total microbial biomass varied across treatments and experiments. Total microbial biomass declined in response to EM exclusion within the EM-exclusion experiment (Fig. 3a). Within the EM-gradient experiment, total microbial biomass declined in low-, but not medium- or high-EM sites (Fig. 3b).

In the EM-exclusion experiment, we found that the exclusion of EM fungi increased soil C respiration by 39% (Fig. 4a) and biomass-specific soil respiration by 64% (Fig. 4b). EM exclusion was associated with multiple signals of increased microbial N availability, including a 181% increase in the N mineralisation rate (Fig. 4c), a 441% increase in biomass-specific N mineralisation (Fig. 4d), 41% decline in the gross N depolymerisation rate (Fig. 4e) and a 38% decline in biomass-specific N depolymerisation (Fig. 4f), an indicator of microbial allocation to N acquisition. Despite the decline in microbial allocation to N acquisition, there was no change in total enzyme activity g^{-1} soil (Fig. 4g), but overall microbial allocation to decomposition increased when EM fungi were excluded, resulting in an 87% increase in biomass-specific enzyme activity (Fig. 4h).

Across the EM gradient, microbial biomass C : N was highest at the high-EM sites ($P < 0.01$, Fig. 5a). Trenching did not alter biomass C : N. Biomass-specific N depolymerisation rates increased across the gradient and were highest in the high-EM sites ($P < 0.01$, Fig. 5b). Microbial biomass per unit soil C was lowest in high-EM sites ($P < 0.001$, Fig. 5c). There was a significant interaction between site and treatment such that biomass per gram soil C declined in trenches within the low-EM sites ($P < 0.001$), but not medium- or high-EM sites. N depolymerisation rates declined in trench treatments ($P < 0.01$, Fig. 5d). Biomass-specific C and N degrading activities (BG + CBH and NAG + LAP) followed the same pattern across sites, with high-EM sites having the highest biomass-specific activities ($P < 0.05$). Biomass-specific enzyme activity increased in trenches ($P < 0.05$, Fig. 5e). Trenching did not change biomass-specific N-degrading enzyme activity.

Biomass-specific respiration was highest in the high-EM sites ($P < 0.05$). There was an interaction between site and trenching treatment such that biomass-specific respiration increased within the trenches only at the high-EM site, but not medium- or low-EM sites ($P < 0.05$, Fig. 6a). N mineralisation also increased within trenches and the effect was driven by the high-EM site (site by treatment interaction $P = 0.06$, Fig. 6b). Total respiration per gram soil C declined in low-, but not medium- or high-EM sites (Fig. 6c).

DISCUSSION

Multiple lines of evidence have led to the emerging idea that EM fungi significantly contribute to the decomposition and selective mining of organic N from soils (Lindahl *et al*. 2010; Rineau *et al*. 2012; Bödeker *et al*. 2014; Lindahl & Tunlid 2015; Shah *et al*. 2015). This leads to the theoretical prediction that selective decomposition and uptake of organic N by EM fungi will drive N limitation of free-living decomposers, thereby slowing soil C respiration and increasing soil and ecosystem C storage (Orwin *et al*. 2011). Here we demonstrate that EM saprotroph competition is occurring under field conditions and resulting in reduced rates of both soil C and N cycling when EM abundance is sufficiently high. Exclusion of EM fungi led to increases in soil C respiration and N mineralisation compared to disturbance controls, shifts in microbial allocation to decomposition and a shift to a more bacteria-dominated microbial community. Furthermore, EM inhibition of soil C and N cycling was limited to old-growth temperate forests where EM fungi were greatest in abundance. This field experiment validates a novel mechanism of soil C

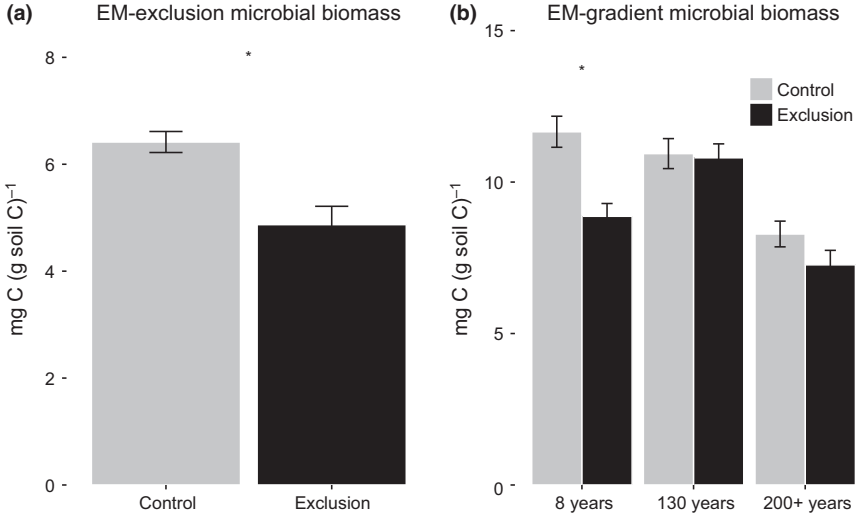

Figure 3 Changes in microbial biomass in each experiment (mg C g C^{-1}). Asterisks denote significant differences at $P < 0.05$. Note that ages in panel b are associated with low, medium and high abundance of ectomycorrhizal fungi within the total soil microbial community.

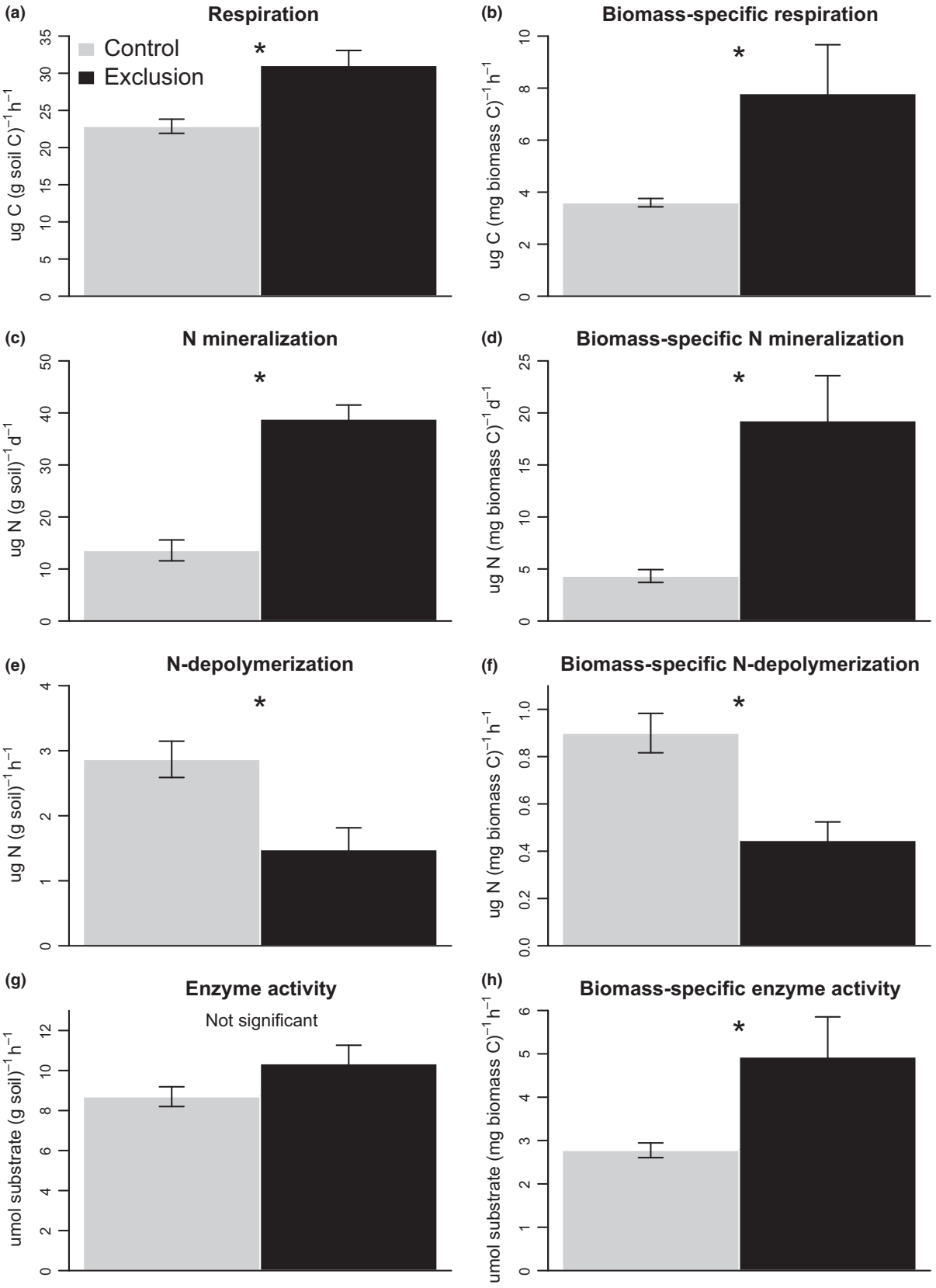

Figure 4 Biogeochemical responses in ectomycorrhizal exclusion experiment. We report both total and biomass-specific responses. Biomass-specific responses are normalised by the microbial biomass C pool. Here we report (a) soil respiration, (b) biomass-specific soil respiration, (c) net N mineralisation, (d) microbial biomass-specific N depolymerisation rate and (e) mass-specific total enzyme potential activity (sum of beta-glucosidase, cellobiohydrolase, n-acetyl-glucosaminidase and leucine amino peptidase). Asterisks denote differences that are significant at $P < 0.05$. Errors bars represent $\pm$ 1 SE.

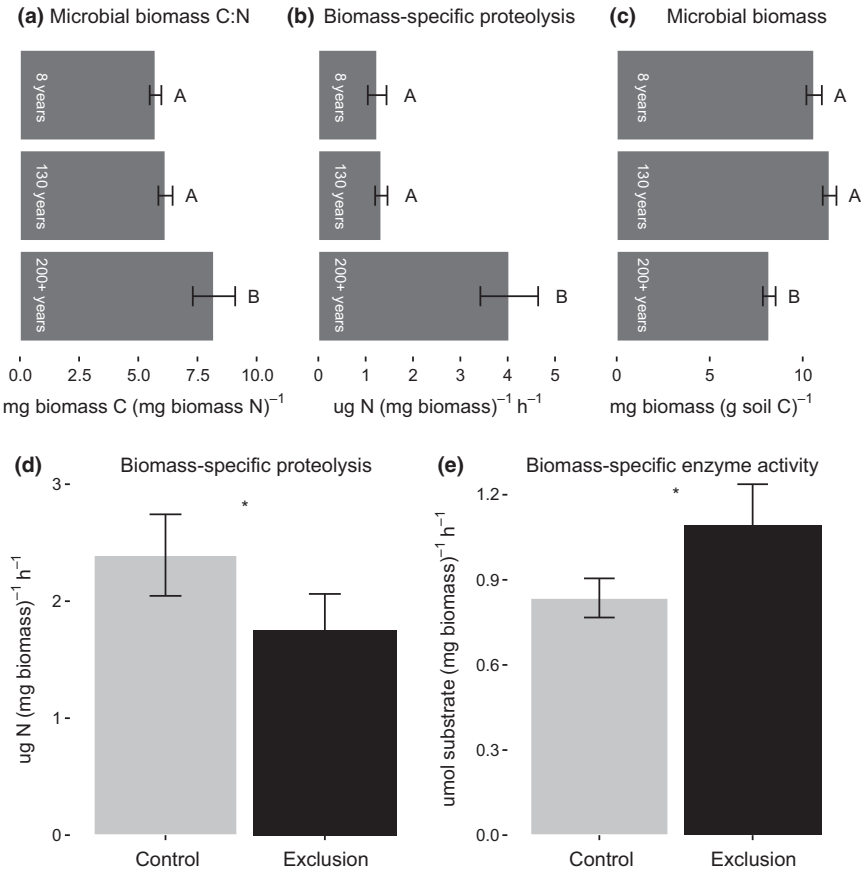

Figure 5 Allocation patterns and responses in the ectomycorrhizal (EM)-gradient experiment: (a) microbial biomass C : N across sites (g C g N^{-1}), (b) biomass-specific gross N depolymerisation rate (μg N mg C^{-1} h^{-1}), (c) microbial biomass C per g soil (mg C g C^{-1}), (d) biomass-specific gross N depolymerisation rates (μg N mg C^{-1} h^{-1}) and (e) biomass-specific C degrading enzyme concentrations (μmol mg C^{-1} h^{-1}). Note that the site ages correspond to low, medium and high abundance of EM fungi within the soil microbial community; control and exclusion refer to in-growth bags placed outside and inside trenches respectively. Letters and asterisks indicate significant differences ($P < 0.05$).

stabilisation, and implies that future changes in EM abundance in response to N deposition, warming, elevated CO_2 and aggrading forests will alter the strength of the EM-competitive interaction, and the magnitude of the C sink maintained by EM-competitive interactions.

Past experiments to exclude EM fungi have been poorly controlled with respect to disturbance, making it difficult to compare the current study with previous findings. Results from past studies are equivocal, with both increases and decreases in microbial activity in response to experimental EM or root plus EM exclusion (Gadgil & Gadgil 1971, 1975; Lindahl *et al.* 2010; Brzostek *et al.* 2015). However, none of these experiments controlled for disturbance caused by the experimental treatments, preventing separation of EM and disturbance effects. Hence, prior results showing increases in microbial activity in response to EM exclusion may be an artefact of experimental treatments.

Factors other than EM fungal exclusion were unlikely to explain our results. For example, there was a small, 5% increase ($P = 0.07$) in gravimetric soil moisture when roots and EM fungi were excluded in the EM-exclusion experiment, but changes in soil moisture are insufficient to explain the observed responses for several reasons. First, all models were fit with soil moisture as a covariate when testing for exclusion effects in the mycorrhizal exclusion experiment. Variance inflation factors justified inclusion of soil moisture, as soil moisture and treatment were not so confounded as to preclude separating their effects in any multiple regression model (Zuur *et al.* 2010). All reported exclusion effects were significant despite inclusion of soil moisture in every statistical model. Second, roots were excluded in both the high- and low-EM stands in the EM-gradient experiment. If soil drying were driving the increase in decomposition observed in exclusion treatments, then we would expect to see increases in decomposition in both the low- and high-EM sites. This was not the case, as increases in C cycling were limited to exclusion treatments within high-EM sites. Finally, no differences in soil moisture could be detected in the EM-gradient experiment exclusion treatments ($P > 0.40$).

Similar to our argument against soil moisture driving the observed responses to EM exclusion, our experimental design eliminates the possibility that biogeochemical differences were solely artefacts of our exclusion treatments. Disturbance alone cannot explain observed responses, because both control and exclusion treatments were disturbed in a similar manner. If trenching were responsible for the observed responses, then

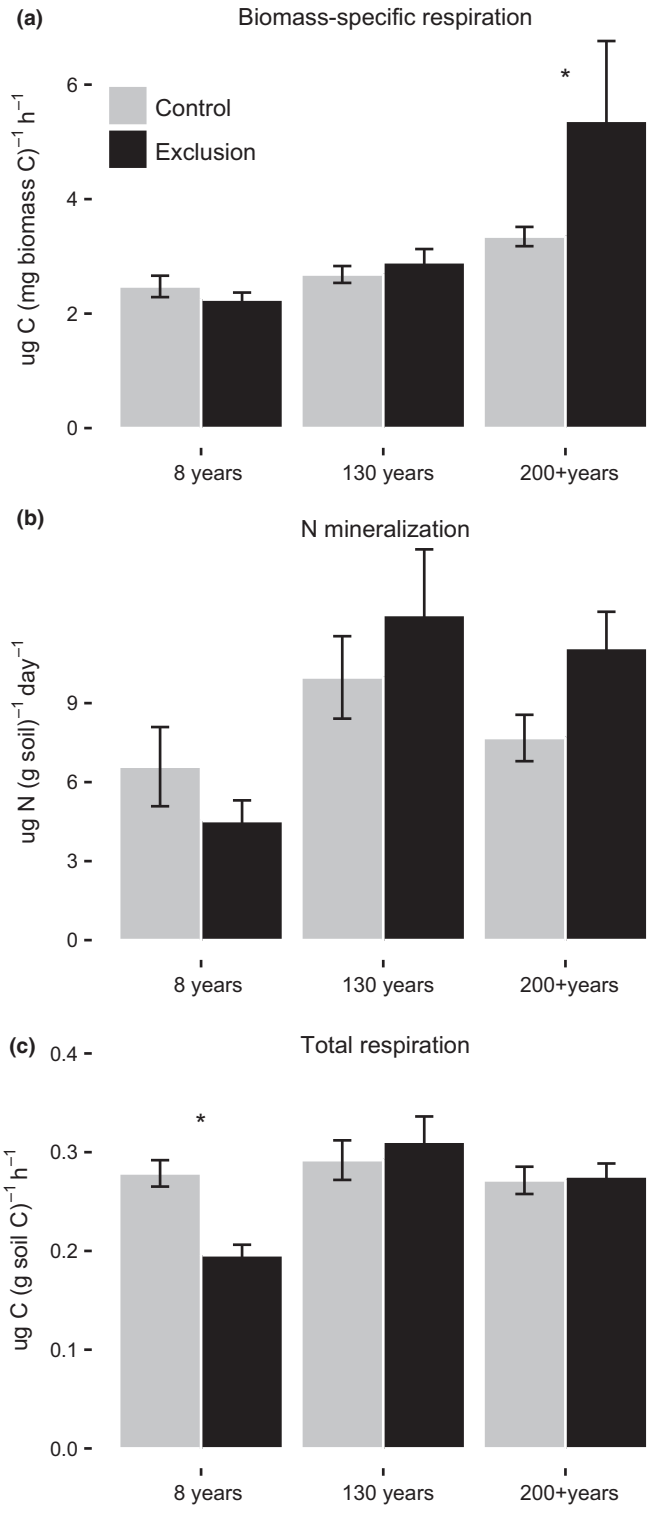

Figure 6 The *x*-axis age gradient reflects an ectomycorrhizal (EM) abundance gradient, from lower to higher across younger to older sites. (a) There was a significant interaction between biomass-specific respiration (μg C mg C^{-1} h^{-1}) and site in the EM gradient experiment, such that increases in biomass-specific respiration in response to EM exclusion by trenching were limited to the oldest, highest abundance EM site ($P < 0.05$). (b) N mineralisation followed a similar pattern, with a near significant increase in N mineralisation limited to the oldest, high-EM sites ($P = 0.07$). (c) Total respiration per gram soil C declined in young, but not medium or high EM sites ($P < 0.05$). Asteriks denote significant differences at $P < 0.05$.

we would expect both low- and high-EM sites to exhibit similar responses to EM exclusion. Different exclusion treatments (mesh vs. trenching) also produced C and N cycling responses of a similar magnitude in high-EM sites. It is possible that differences in fine root N uptake may explain differences across the EM gradient; however, fine root biomass did not differ significantly ($P = 0.19$) within the 2-mm in-growth bags used as disturbance controls and the trend was towards increased fine root density in the low-EM sites. Hence, if fine roots controlled the observed effects, we would expect to see the strongest inhibition of soil C cycling at the low-EM sites, which is directly opposite to the measured rates. It is also possible the 1-μm mesh in the EM-exclusion experiment excluded soil predators, confounding microbial predation and the presence of EM fungi. However, predation generally increases rather than decreases decomposer activity (Ingham *et al.* 1985; Moore *et al.* 2003), and therefore cannot explain our findings. Finally, inhibition of decomposer activity by EM fungi may have been driven by competition for some soil nutrient other than N. We cannot rule out this possibility, but do note the extensive literature on the role of EM fungi on N acquisition within the soil horizons observed here (Read 1991; Lindahl & Tunlid 2015).

Despite the increases in soil C and N cycling, we observed a decline in microbial biomass when EM fungi were excluded in the first experiment (Fig. 3a) and either a decline or no change in microbial biomass at the highest EM sites in the gradient experiment (Fig. 3b). This is unsurprising, however, considering that EM fungi can represent one-third or more of the total soil microbial biomass (Högberg & Högberg 2002). Therefore, exclusion of EM fungi may lead to a net decline in total microbial biomass, even if free-living decomposer biomass has increased, as well as the turnover of soil C. This also supports our use of biomass-specific respiration, as it allows us to capture changes in the rate of soil C turnover due to a comparatively more active microbial biomass in the absence of EM fungi.

We also observed an inconsistency between the response of potential N-targeting enzyme activities and the gross rate of N depolymerisation in response to EM exclusion. Gross N depolymerisation declined in both experiments, while N-degrading enzyme potentials were constant or increased. We believe this may be due to the fact that N-degrading enzymes also return organic C to microbes, and increasing investment in N-degrading enzymes has been shown to be an optimal strategy to maximise microbial C acquisition when N limitation is relieved (Averill 2014). Hence, these contrasting findings are not necessarily inconsistent with a shift from microbial N to C limitation, especially in conjunction with observed increases in N mineralisation and C respiration in EM-exclusion treatments.

Our design is not perfectly controlled, as there are C inputs from primary productivity present in the root and EM in-growth disturbance controls that are absent in the EM-exclusion treatments. EM hyphal turnover itself can also be an important component of ecosystem C input (Wilkinson *et al.* 2011; Clemmensen *et al.* 2013). Ideally we would create treatments where roots could grow in without EM fungi, and also add EM fungal necromass equal to any EM fungal

turnover in the disturbance controls. Because we cannot include these inputs in the EM-exclusion treatments, we have likely underestimated the inhibitory effect of EM fungi on soil C cycling, as these organic matter inputs would likely further increase soil microbial activity in the EM-exclusion treatments compared to disturbance controls.

Recently, Clemmensen *et al.* (2015) showed a shift from EM to ericoid mycorrhizal dominated fungal communities across a boreal forest gradient in C storage. It may be that specific members of EM and other mycorrhizal fungi play an important role in stabilizing soil C. It is possible that certain species of EM and ericoid mycorrhizal fungi engage more in N-competitive mechanisms than other species, and this is reflected in the shift in community composition by Clemmensen *et al.* (2015). We observed a different set of species dominating our soils by sequence abundance in the present study, however we caution against over interpreting community composition data based on sequence abundance.

Based on these field experiments, we support a novel mechanism of soil C storage predicted from ecological theory (Orwin *et al.* 2011), with the potential to explain the global pattern of elevated soil C storage observed in EM ecosystems (Averill *et al.* 2014). The effect of EM fungi on soil respiration is larger than the 21% increase found for 2 °C forest soil warming (Wang *et al.* 2014). Changes in plant allocation to EM fungi due to warming, forest age, elevated CO_2 and other global change factors will likely alter the strength of the competitive interaction between EM fungi and free-living decomposers (Garcia *et al.* 2008; Clemmensen *et al.* 2013). Positive or negative feedbacks to climate change are possible, depending on whether or not this interaction is common, and whether global change results in a net decrease or increase in the abundance of EM fungi relative to other microbial functional groups in soil. Acknowledging and modelling ecological interactions within soil microbial communities, and in particular with EM fungi, has the potential to transform our understanding of how C is distributed across the Earth.

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AUTHORSHIP

CA performed all laboratory and fieldwork. CA and CVH designed the study, performed data analysis and wrote the manuscript.

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