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Differences in microbial community response to nitrogen fertilization result in unique enzyme shifts between arbuscular and ectomycorrhizal dominated soils

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Abstract:

While the effect of nitrogen (N) deposition on belowground carbon (C) cycling varies, emerging evidence shows that forest soils dominated by trees that associate with ectomycorrhizal fungi (ECM) store more C than soils dominated by trees that associate with arbuscular mycorrhizae (AM) with increasing N deposition. We hypothesized that this is due to unique nutrient cycling responses to N between AM and ECM dominated soils. ECM trees primarily obtain N through fungal mining of soil organic matter subsidized by root-C. As such we expected the largest N induced responses of C and N cycling to occur in ECM rhizospheres and be driven by fungi. Conversely, as AM trees rely on bacterial scavengers in bulk soils to cycle N, we predicted the largest AM responses to be driven by shifts in bacteria and occur in bulk soils. To test this hypothesis, we measured microbial community composition, metatranscriptome profiles, and extracellular enzyme activity in bulk, rhizosphere, and organic horizon (OH) soils in AM and ECM dominated soils at Bear Brook Watershed in Maine, USA. After 27 years of N fertilization, fungal community composition shifted across ECM soils, but bacterial communities shifted across AM soils. These shifts were mirrored by enhanced C relative to N mining enzyme activities in both mycorrhizal types, but this occurred in different soil fractions. In ECM stands these shifts occurred in rhizosphere soils, but in AM stands they occurred in bulk soils. Additionally, ECM OH soils exhibited the opposite response with declines in C relative to N mining. As rhizosphere soils account for only a small portion of total soil volume relative to bulk soils, coupled with declines in C to N enzyme activity in ECM OH soils, we posit that this may partly explain why ECM soils store more C than AM soils as N inputs increase.

Introduction:

Atmospheric deposition of N has fueled increases in tree growth across temperate forests (Quinn Thomas *et al.*, 2010; Averill & Waring, 2018). However, increased detrital inputs cannot fully account for the rate at which soil carbon (C) stocks increase in response to N fertilization (Pregitzer *et al.*, 2008; Zak *et al.*, 2008). Rather, most evidence points to reductions in soil C decomposition to explain this phenomenon (Fog, 1988; Zak *et al.*, 2008; Janssens & al., 2010; Edwards *et al.*, 2011; Frey *et al.*, 2014; Morrison *et al.*, 2016). While N-induced reductions in decomposition, particularly soil respiration are well-documented, the mechanism by which N reduces or alters soil microbial activity is less clear. Most research connects N induced shifts in fungal community structure and gene

expression to declines in soil C decomposition (Frey *et al.*, 2004; Waldrop *et al.*, 2004; Freedman *et al.*, 2015; Entwistle *et al.*, 2018; Zak *et al.*, 2019; Entwistle *et al*., 2020). In contrast, other studies highlight shifts in bacterial community composition and function as drivers of these declines (Freedman & Zak, 2014; Freedman *et al.*, 2016a; Carrara *et al.*, 2018). While policy has driven reductions in atmospheric N deposition in some regions including the northeast US, global N deposition is expected to double over the next century (Galloway *et al.*, 2004; Reay *et al.*, 2008). As such, uncovering mechanisms that explain how N induced microbial shifts vary across forest types and horizons is paramount to informing models that predict the fate of the terrestrial C sink.

Differences between biogeochemical cycling in soils dominated by arbuscular mycorrhizal fungi (AM) or ectomycorrhizal fungi (ECM) may explain why soil C responses to N fertilization are linked to shifts in fungal community structure and function in some experiments and bacteria in others (Phillips *et al.*, 2013). Two key factors in the AM vs ECM dichotomy that may drive these differences are (1) dependence on rhizosphere stimulation of decomposition to access nutrients, and (2) fungal vs bacterial control of decomposition. Below, we will outline how we expected these differences to influence divergent biogeochemical responses to N fertilization between mycorrhizal types.

To meet their N demand, trees that associate with ECM fungi (herein ECM trees) invest a substantial amount of C belowground to prime microbial decomposition of organic matter in the rhizosphere (Hobbie, 2006; Yin *et al.*, 2013; Brzostek *et al.*, 2014, 2015). Microbes use this C to produce extracellular enzymes that mine N from soil organic matter thus, increasing tree N supply (Phillips *et al.*, 2013; Cheeke *et al.*, 2017; Lin *et al.*, 2017). When N limitation is alleviated through fertilization, we expected ECM trees to allocate less C to microbes belowground to obtain it. We hypothesized that reduced belowground C allocation to microbes, coupled with enhanced N supply would shift microbial nutrient demand in the rhizosphere towards C limitation. In order to maintain biomass C:N, we further hypothesized that microbes would enhance production of C mining relative to N mining extracellular enzymes. As decomposition in soils dominated by ECM trees (herein ECM soils) is driven by mycorrhizal and free-living fungi that mine nutrients from soil organic matter, we hypothesized that shifts in C and N availability would drive changes in fungal community composition as competition for resources changes (Cheeke *et al.*, 2017).

Trees that associate with AM fungi (herein AM trees) invest less C belowground to rhizosphere microbes to obtain N than ECM trees and rely mainly on bacterial dominated inorganic N cycling in bulk soils to meet N demand (Averill *et al.*, 2014; Midgley & Phillips, 2014; Cheeke *et al.*, 2017). Here, bacteria scavenge and mineralize organic N leading to enhanced tree N supply (Phillips *et al.*, 2013). We hypothesized that as N fertilization enhanced microbial N availability, microbes in AM bulk soils would move toward C limitation. To maintain biomass C:N, we hypothesized that microbes would enhance the production of C relative to N mining enzyme in bulk soils. As bacteria are the major drivers of N cycling and decomposition in soils dominated by AM trees (herein AM soils), we hypothesized that these shifts in C and N availability would result in changes in free-living bacterial community composition.

To test these hypotheses, we established a network of AM and ECM dominated plots at the long-term (27 years) watershed scale N fertilization experiment at the Bear Brook Watershed in Maine, USA. We used an analysis of bacterial 16S ribosomal RNA and fungal 28S genes to examine N induced changes in both bacterial and fungal community structure in bulk, rhizosphere, and organic horizon soils separately. We further examined microbial function by analyzing the soil metatranscriptome and examining the presence of a suite of gene transcripts involved in carbon decomposition. In order to link genes to biogeochemistry, all of these measurements were done in tandem with measurements of extracellular enzyme activities in bulk, rhizosphere, and organic horizon soils that mineralize simple and complex C as well as N and phosphorus (P).

Methods:

Study site:

The Bear Brook Watershed in Maine (hereafter Bear Brook) is a unique watershed-scale fertilization experiment that has been used to examine the impact of N fertilization on biogeochemistry for 27 years. Here we advance previous research that has sought to understand the impact of N deposition on C and nutrient cycling and retention in forests by applying a mycorrhizal lens to this system. Prior research at Bear Brook has shown that N fertilization leads to increased stream water N exports, increased accumulation of N in hardwood biomass, increased foliar N concentration, and base cation depletion (Fernandez *et al.*, 2003; Elvir *et al.*, 2006; Patel *et al.*, 2019). The site is located in eastern Maine near the town of Beddington (44°52′15″N, 68°06′25″W) on the

southwest slope of Lead Mountain (Wang & Fernandez 1999). The experiment consists of two adjacent watersheds West Bear (10.3 ha), and East Bear (11.0 ha). West Bear was aerially fertilized every two months between 1989 and 2016 at a rate of 25.2 kg N ha^{-1} yr and 28.8 kg S ha^{-1} yr^{-1} in the form of solid pellet $(NH_4)_2SO_4$ compared to ambient deposition in 2016 of 1.5 kg N ha^{-1} yr and 2.1 kg S ha^{-1} yr^{-1} as wet deposition (National Atmospheric Deposition Program, Greenville, ME; Jefts *et al.*, 2004; Fatemi *et al.*, 2016). As 2016 was the last year of fertilization, these measurements are among the last taken during 27 years of watershed fertilization at this site. Average precipitation is ~1400 mm and mean annual temperature is 5.6°C (Patel *et al.*, 2018). Soils are acidic, have low cation exchange capacity and base saturation, and are characterized as coarse-loamy, isotic, frigid Typic Haplorthods with an average depth of 0.9m (Norton *et al.*, 1999, Jefts *et al.*, 2004; Fernandez *et al.*, 2007).

We acknowledge that whole watershed fertilization experiments are limited to pseudo-replication as each watershed represents a treatment with only one sample (Hulbert 1984), however prior to treatment these watersheds had similar tree species composition, soil type, and element fluxes (Norton *et al.*, 1999, Wang & Fernandez, 1999). It is noteworthy that pH is an important driver of microbial community diversity, biomass, and enzyme activity (Lauber *et al.*, 2009; Sinsabaugh, 2010; Rousk & Bååth, 2011; Fierer *et al.*, 2012; Kaiser *et al.*, 2016); however, there is little pH difference between the fertilized and reference watersheds (3.97 vs 4.01 in OH and 4.18 vs 4.28 at 5 cm depth; Jefts *et al*., 2004) and no difference in microbial biomass (Wallenstein *et al.*, 2006).

Experimental design:

In order to detect possible differences in N fertilization response between mycorrhizal types, we established a plot network of 6 AM and 6 ECM dominated (>65% diameter at breast height; Table S1) 10 x 10 m plots in the lower elevation hardwood zone of both the reference and fertilized watershed in May 2016 (N=24 plots). Tree species were similar between watersheds with AM trees represented by red maple (*Acer rubrum*) and sugar maple (*Acer saccharum*) and ECM trees represented by American beech (*Fagus grandifolia*), grey birch (*Betula populifolia*), and yellow birch (*Betula alleghaniensis*).

To capture variability across the growing season, we sampled soils in each plot in May, July, and September of 2016. In each plot we extracted three 10 x 10 cm OH layers and homogenized them

into a single sample defining this as the OH soil fraction. Next, we sampled four 5 cm diameter mineral soil cores to a depth of 15 cm beneath the OH layer and homogenized these by plot. All samples were kept on ice and transported to West Virginia University for further processing within 48-72 h. Upon return to the lab, we separated rhizosphere soil from mineral soil samples via the soil-adhesion method wherein the rhizosphere soil fraction was operationally defined as soil that remained clung to roots after modest shaking (Phillips & Fahey, 2005). While this method assumes that rhizosphere effects are uniform along the length of fine roots rather than being concentrated at root tips, this method has proven useful in delineating differences in rhizosphere and bulk mineral biogeochemistry in previous studies (Phillips & Fahey, 2006, 2008; Brzostek *et al.*, 2013, 2015; Fahey *et al.*, 2013; Yin *et al.*, 2014; Carrara *et al.*, 2018). Remaining mineral soil was defined as the bulk soil fraction. After removal of roots, all soils were passed through a 2 mm sieve and stored at -80˚C until further analysis. To preserve soil RNA for transcriptomic analysis during the July sampling date, we sampled OH soil and separated mineral soil into rhizosphere and bulk fractions in the field and immediately preserved in them in Lifeguard Soil Preservation Solution (MoBio, Carlsbad, CA).

Extracellular enzyme activity:

To determine the extent to which N fertilization impacts microbial allocation to extracellular enzymes, we assayed the potential activity of hydrolytic enzymes that release N (N-acetylglucosaminidase; NAG), phosphorus (acid phosphatase; AP), and simple carbon (ß-glucosidase; BG). In addition, we measured microbial allocation to complex C degrading oxidative enzymes phenol oxidase and peroxidase. Briefly, 1g of thawed soil was homogenized in 50mM sodium acetate buffer (pH 5.0). Next, hydrolytic activities were determined using a fluorometric microplate assay with methylumbelliferone-linked substrates and oxidative enzymes using a colorimetric microplate assay with L-3,4-dihydroxyphenylalanine linked substrates (Saiya-Cork *et al.*, 2002). It is important to note that we measured potential enzyme activity under substrate saturated conditions. Under these conditions, the potential enzyme activities reported here can be used as a proxy of microbial enzyme pool size or microbial investment in each decomposition pathway. In order to determine the extent to which N fertilization alters microbial allocation to carbon or nutrient acquisition, we opted to express enzyme activity as ratios between C, N, and P acquiring enzymes (Midgley & Phillips, 2016). We used t-tests to determine significant differences between enzyme ratios, in order to compare fertilized

and reference enzyme activities within each horizon and mycorrhizal type. T-tests were used rather than 2-way ANOVA as bulk, rhizosphere, and OH soils are ecologically distinct and considered to be unique sample groups.

To compare enzyme profiles between fertilized and reference soils at a broader resolution, we examined differences through similarity matrices. For each soil fraction within both mycorrhizal types, we calculated Bray-Curtis similarity of the five enzymes assayed using the vegdist function within the vegan package for R v 1.2.5033 (Oksanen et al., 2015; R Core Team, 2020). Next, profile similarity was compared by permutational multivariate analysis of variance (PERMANOVA) using the adonis function to determine if the centroids of the enzyme profiles varied significantly. For visualization of these data, we used non-metric dimensional scaling (NMDS) to generate NMDS scores of the enzymes for each soil fraction and mycorrhizal type and present the data to highlight N induced shifts in enzyme profiles.

Bacterial and fungal community composition and metatranscriptomics:

Due to logistical and time constraints, all bacterial 16S, fungal 28S and metatranscriptomic data were collected during only the July sampling date. This date was chosen to capture microbial community structure and function at the peak of the growing season. To determine the bacterial and fungal community composition and gene expression in response to long-term N fertilization, DNA and RNA were simultaneously extracted for each soil fraction from ECM and AM dominated plots using the MoBio RNA Powersoil Kit supplemented with the MoBio DNA Elution Accessory Kit, following the manufacturers protocol. Samples were quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Next-Generation (NGS) amplicon-sequencing libraries were prepared using a two-step protocol, where step 1 amplifies the region of interest and step 2 adds a unique index and Illumina adapters. In step 1, each reaction contained: 5 ng of template DNA, 5 uM of F primer, 5 uM of R primer, 1X of KAPA HiFi Buffer (KAPA Biosystems, Roche Cape Town, South Africa), 0.3 mM of KAPA dNTPs, and 0.5 units KAPA HiFi HotStart DNA polymerase. Bacterial primers were S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21which target the V3-V4 16S region (Klindworth *et al.*, 2013) and fungal primers were LR22R and LR3 which target the D2 hypervariable region of the fungal large ribosomal sub unit (Mueller *et al.*, 2016) (primer sequences in Table S2). Thermocycler conditions were as follows: an initial

denaturation at 95˚C for 3 min, followed by 15 cycles of 98˚C for 20 s, 63˚C for 30 s, and 72˚C for 30 s, and a final extension of 72˚C for 5 min. PCR samples were purified using the AxyPrep Mag PCR Clean-up protocol (Axygen Biosciences, Union City, CA, USA) and quantified on the Qubit 2.0 Fluorometer (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA). In step 2, unique index combinations were assigned to each sample (Table S2). Each reaction contained: 1 ng of the AxyPrep cleaned product from step 1, 5 uM of F primer, 5 uM of R primer, 1X of KAPA HiFi Buffer, 0.3 mM of KAPA dNTPs, and 0.5 units KAPA HiFi HotStart DNA polymerase. Thermocycler conditions were as follows: an initial denaturation at 95˚C for 3 min, followed by 22 cycles of 95˚C for 30 s, 56˚C for 30 s, and 72˚C for 30 s, and a final extension of 72˚C for 4 min. PCR samples were purified using the AxyPrep Mag PCR Clean-up protocol (Axygen Biosciences, Union City, CA, USA) and quantified on the Qubit 2.0 Fluorometer with Qubit dsDNA HS reagents (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA). Samples were pooled in equal molar concentrations and sequenced on the Illumina MiSeq (250 bp paired-end reads) at the West Virginia University Genomics and Bioinformatics Core Facility (West Virginia University, Morgantown, WV, USA).

Sequence processing, diversity analysis, and classification was performed in Qiime2-2019.4 (Bolyen *et al.*, 2019). Low quality nucleotides, adapters, and primer sequences were trimmed and quality trimmed reads were processed using the DADA2 function to output representative sequences. DADA2 further processes the reads by removing of phiX reads, chimeric reads, and identical reads, and correcting polymerase-induced errors, and merging the forward and reverse reads, to produce a minimized representative data set (Callahan *et al.*, 2016). The representative data set from DADA2 was aligned for diversity analysis and phylogenetic tree reconstruction using mafft. Bacterial 16S sequences were classified using the silva-132-99-nb-classifier.qza provided by Silva (Quast *et al.*, 2013; Yilmaz *et al.*, 2014). Fungal 28S sequences were classified against the large subunit database from UNITE (97% threshold) (Nilsson *et al.*, 2019). To determine the impact on N fertilization on broad-level species composition, all fungal and bacterial phyla representing more than 1% of abundance across plots were examined for significant shifts in relative abundance. In addition, fungal families representing more than 3% of abundance across all plots were compared between N fertilized and references soils. Finally, as *Actinobacteria* and *Proteobacteria* have the ability to create ligninolytic enzymes and are involved in organic matter decomposition (Freedman & Zak, 2014), we

examined the impact of N fertilization on the relative abundance of *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*.

To further analyze fungal and bacterial community response to N fertilization, all ASVs were normalized by dividing the number of sequences of each ASV by total sequences in each sample. Next, the ASVs were used to calculate Bray-Curtis similarity using the vegdist function in the vegan package in R (Oksanen *et al.*, 2015). Using the adonis function, communities were analyzed by PERMANOVA with treatment and soil fraction as main effects for both AM and ECM dominated soils.

RNAs, extracted simultaneously with DNA from the same soil samples, were quantified using the Nanodrop 2000 spectrophotometer and quality was assessed via Agilent bioanalyzer (Agilent, Santa Clara, CA). RNAs were DNase treated with the Turbo DNA-free Kit and preserved with SUPERaseIN (Thermo Fisher Scientific), following manufacturers protocols. DNase treated RNAs were quantified on the Qubit 2.0, using the RNA Broad Range Assay. RNAs from each mycorrhizal type and each soil fraction were pooled in equal molar amounts to produce 12 total RNA pools (2 mycorrhizal types x 3 soil fractions x 2 treatments). RNA pools were submitted to the WVU Genomics and Bioinformatics core facility for Illumina library construction. Illumina sequencing libraries were generated using the ScriptSeq Complete Gold Kit (Epicentre Biotechnologies, Madison, WI), which first performs a ribosomal depletion followed by random hexamer cDNA synthesis and indexing. The adaptor-tagged RNA-Seq libraries were sequenced at the Marshall University Genomics Core Facility (Huntington, WV) on the Illumina HiSeq 2500.

To determine the impact of N fertilization on microbial transcription of genes associated with C turnover, metatranscriptome sequences were queried against a suite of genes involved in the breakdown of lignin, simple carbon, and chitin using the default 'blastx' function in DIAMOND with an evalue cutoff of 1×10^{-5} (v. 2.0.2 *sensu* Buchfink *et al.*, 2014; Freedman *et al.*, 2016b; Table S3). Reference gene databases were downloaded from FunGene and sequences were manually curated as described in Freedman *et al.*, 2016b. Gene abundance per sample ranged from 0-2797 reads and were normalized by dividing by total number of reads per sample and multiplying by 100 (de Vries *et al.*, 2015; Weigold *et al.*, 2016; Table S4).While community analysis was completed at the plot level, RNA was pooled within mycorrhizal type, soil fraction, and treatment for gene transcript abundance

analysis. With limited statistical power, OH, bulk, and rhizosphere fractions were used as replicates to test for significant differences in transcript abundance by using t-tests between treatments within mycorrhizal type (Table S3). To determine the effect N had on total ligninolytic capacity, the relative abundance values of each of the lignin degrading proteins within each treatment and mycorrhizal type was analyzed.

Results:

Extracellular enzyme activity:

The N response of simple C (BG) to nutrient acquiring (NAG & AP) enzyme activity ratios were similar across AM and ECM bulk and rhizosphere soils, but complex C (phenol oxidase & peroxidase) to N ratios varied by soil fraction between AM and ECM plots. For example, in N fertilized AM bulk and rhizosphere soils, the ratio of BG to NAG was 221 and 194% higher respectively, as compared to reference soils (Fig. 1 a,b; $p<0.05$). Similarly, BG to NAG ratios were 106 and 175% higher in N fertilized ECM bulk and rhizosphere soils as compared to reference soils (Fig. 1 a,b; $p<0.05$). BG to AP acquiring enzymes were higher in N fertilized AM bulk and rhizosphere soils by 148 and 142% respectively and also in ECM bulk and rhizosphere soils by 167 and 258% (Fig. 1 c,d; $p<0.05$).

N fertilization induced higher complex C to N enzyme activity ratios in only AM bulk soils and ECM rhizosphere soils. In N fertilized AM bulk soils, phenol oxidase to NAG activity ratios were 185% higher and peroxidase to NAG ratios trended 75% higher (Fig. 1 e,g). N fertilization had no effect on complex C to N enzyme activity ratios in ECM bulk soils. However, in N fertilized ECM rhizosphere soils, peroxidase to NAG activity ratios were 119% higher and phenol oxidase to NAG activity trended 56% higher (Fig. 1 f,h). N fertilization had no effect on complex C to N acquiring enzyme activity ratios in AM rhizospheres.

OH soil enzyme ratios varied by mycorrhizal type. In AM OH soils, N fertilization increased the ratio of BG to AP activity by 141%, but had no significant effect on other enzyme ratios (Table S5). In ECM OH soils N fertilization increased the ratio of BG to AP activity by 206% (Table S5). Conversely, in ECM OH soils N fertilization significantly decreased the phenol oxidase to NAG activity ratio by 76% and the peroxidase to NAG ratio by 69% (Table S5).

Distinctive shifts in enzyme activity of AM bulk soils and ECM rhizosphere soils were further supported by PERMANOVA of Bray-Curtis similarity matrices of enzyme profiles with horizon and mycorrhizal type as factors. PERMANOVA on soil enzyme profile NMDS scores highlighted a marginal N effect in AM bulk soils ($p=0.109$), but no effect in ECM bulk soils (Fig. 2 c,d). Conversely, enzyme profiles were significantly different in ECM rhizospheres ($p=0.021$), but not AM rhizospheres (Fig. 2 e.f). In OH soils, enzyme profiles significantly shifted in both mycorrhizal types (Fig. 2 a,b).

Bacterial and fungal community composition and metatranscriptomics:

There were only minor shifts in dominant fungal phyla across mycorrhizal types and soil fractions. When fungal taxonomy was considered at the phylum level, the three most dominant phyla across all plots were Ascomycota (% relative abundance), Basidiomycota, and Chytridomycota (Table S6). The relative abundance of many fungal taxa were unaffected by N across the plots, however in AM bulk soils, N fertilized soils harbored ~10% fewer basidiomycetes and ~61% more ascomycetes (Table S6). With all soil fractions pooled, there was a marginally significant 14% decline in basidiomycetes in fertilized AM plots compared to control plots (Fig. 3a, $p=0.08$). Fungal families representing >3% of the relative abundance across samples were Amanitaceae, Atheliaceae, Clavariaceae, Cortinariaceae, Hygrophoraceae, Russulaceae, and Tricholomataceae. Across ECM soils, N fertilization enhanced the relative abundance of Russulaceae and reduced the abundance of Atheliaceae (Figure S1). N induced fungal family shifts in AM soils varied across soil fractions (OH, bulk, and rhizosphere) with a notable increase in the relative abundance of Cortinariaceae occurring in both AM rhizosphere and bulk soil fractions (Figure S1).

Major phylum level bacterial shifts were less straightforward with limited N induced shifts occurring across mycorrhizal types and soil fractions (Table S7). N induced shifts in the relative abundance of ligninolytic bacterial classes varied across AM soil fractions with a decline in *Betaproteobacteria* in OH soils (-52%), an increase in *Actinobacteria* (78%) and *Gammaproteobacteria* (47%) in bulk soils, and no significant shifts in rhizosphere soils (Figure S2 a,c,e). In ECM soils, N fertilization reduced the relative abundance of *Betaproteobacteria* across OH (-81%), bulk (-74%), and rhizosphere (-67%) soils (Figure S2 b,d,f).

When considered as total community composition at the ASV level, N fertilization shifted bacterial communities across AM soils, whereas N fertilization shifted fungal community composition in ECM soils. Within AM bulk and rhizosphere soils, bacterial community composition differed with N fertilization (Fig. 4 a,c). Conversely, in ECM soils, N fertilization altered fungal community composition in bulk and rhizosphere soils (Fig. 4 b,d). Additionally, ECM bacterial communities differed between fertilized and control bulk soils ($p<0.05$), but no difference was detected in ECM rhizosphere communities.

We detected limited N induced shifts in transcription of proteins associated with lignin decomposition under N fertilization across both mycorrhizal types (Table S3); however, there was significantly less transcription of genes encoding total lignin degrading enzymes in N fertilized AM soils compared to control when the relative abundance of all transcripts encoding lignin degrading enzymes were combined (Fig. 3b). Abundance of lignin degrading transcripts were pooled to better estimate total ligninolytic potential of soils rather than examining each gene individually.

Discussion:

Understanding mechanisms that drive variability in soil C response to N fertilization across forest types is critical in predicting the fate of the terrestrial C sink. Here, we provide evidence of a mechanism that explains how ECM soils tend to store more C than AM soils under higher N deposition (Averill *et al.*, 2018). We show that while N induced shifts in microbial enzyme allocation toward C mining (relative to N mining) were a common response across mycorrhizal types, this occurs at a much smaller magnitude in ECM soils. In ECM stands, N fertilization enhanced complex C relative to N mining only in rhizosphere soils which occurred concomitantly with shifts in fungal community composition (Fig. 1 e-h, Fig. 4 b,d). These changes reflect a disruption of the rhizosphere-centric, fungal driven, organic nutrient economy of ECM trees. In AM stands, shifts toward complex C mining and away from N mining enzyme activity occurred in bulk soils (Fig. 1 e-h) which account for the majority of forest soil volume. Unlike ECM soils, these shifts were mirrored by shifts in bacterial community composition highlighting N induced changes to the bacterially driven, inorganic nutrient economy of AM trees (Fig. 4 a,c). N induced C losses are likely small in ECM stands, because C relative to N mining enzyme enhancements were isolated to rhizosphere soils, which account for only a small fraction of forest soil volume (Finzi *et al.*, 2015). Additionally, N induced

declines in C mining relative to N mining in ECM OH soils may enhance C gains under long-term N fertilization. Based on these shifts in decomposition, ECM soils likely store more C under N fertilization than AM soils which exhibit enhanced C relative to N mining across bulk soils.

N fertilization likely reduced belowground C allocation in ECM trees to a greater extent than AM trees because they rely more heavily on organic N released by rhizosphere priming to meet their N demand (Phillips *et al.*, 2013; Brzostek *et al.*, 2015). As N supply was enhanced by fertilization, it is probable that ECM trees shifted C allocation from belowground growth towards above-ground tissues (Litton *et al.*, 2007). This reduction in rhizosphere C availability, coupled with enhanced N supply, likely put a C constraint on soil fungi which are the dominant decomposers of high C:N litter (Phillips *et al.*, 2013; Cheeke *et al.*, 2017). As such, there was a shift in fungal community composition and microbes moved toward C limitation which would restrict microbial growth. To maintain growth and biomass C:N, fungi shifted production of extracellular enzymes to match resource constraints which led to an increase in complex C relative to N mining activity by rhizosphere microbes in ECM soils (Fig. 1 f,h). These shifts in fungal community composition were highlighted by an increase in the relative abundance of the family Russulaceae in N fertilized ECM soils (Fig. S1). Russulaceae are considered 'contact explorers' and as such, they preferentially use N in the soluble inorganic form (Defrenne *et al.*, 2019). Therefore, enhanced inorganic N availability as the result of N fertilization may have influenced this increase in Russulaceae. However, other research has shown that genera within Russulaceae vary in their response to N fertilization with some nitrophobic genera exhibiting declines in abundance (Lilleskov *et al.*, 2011). Regardless, the family Russulaceae contain a range of ectomycorrhizal and saprotrophic organic matter decomposers (Looney *et al.*, 2018) and therefore, it is possible that the increase in their abundance is linked to the observed increase in C relative to N mining enzyme activity in N fertilized ECM rhizospheres.

N fertilization responses in AM soils were the result of a more direct effect on bacterial shifts in C and N demand and were likely less influenced by declines in plant-C allocation to the rhizosphere (Brzostek *et al.*, 2015). AM litter has much lower C:N than ECM litter (Phillips *et al.*, 2013). This allows them to rely more heavily on rapid bacterial N cycling in bulk soils to meet N demand rather than sending C to prime organic N release in the rhizosphere (Midgley & Phillips, 2014). As N fertilization increased N supply in AM bulk soils, bacteria became constrained by C

availability. This resulted in a shift in bacterial community composition and resource demand which led to an enhancement of C relative to N mining enzymes in bulk soils (Fig. 1 e,g). This shift appears to be partially driven by an increase in the relative abundance of taxa that are involved in organic matter decay (*Actinobacteria* and *Gammaproteobacteria*; Freedman & Zak, 2014), which may partially account for the observed increase in C relative to N mining enzymes in bulk soils (Fig S2).

Differences between AM and ECM trees in how they couple C and N cycles belowground has proven to be a powerful framework for explaining variability in biogeochemical cycles across forest ecosystems (Phillips *et al.*, 2013; Averill *et al.*, 2014, 2018; Brzostek *et al.*, 2015; Midgley *et al.*, 2015; Midgley & Phillips, 2016; Terrer *et al.*, 2016; Cheeke *et al.*, 2017). Past research has shown that (1) ECM trees prime soil decomposition to a greater extent than AM trees (Brzostek *et al.*, 2015), (2) low C:N, AM litter drives rapid decomposition and high inorganic N availability in AM soils, and (3) decomposition is driven more by fungi in ECM soils and bacteria in AM soils (Phillips *et al.*, 2013; Cheeke *et al.*, 2017). Here we expand on this framework by showing that these differences in belowground traits appear to drive variability in the response of soil decomposition to N fertilization and may explain why ECM soils appear to gain more soil C than AM soils under N fertilization (Midgley & Phillips, 2016; Averill *et al.*, 2018). N fertilization drove changes in ECM soils primarily through localized shifts in rhizosphere enzyme profiles that occurred in tandem with shifts in fungal communities. The increases in C mining in ECM rhizospheres were also counterbalanced by a reduction in C mining in ECM organic horizons. In AM soils, however, N fertilization led to more widespread bulk soil enzyme shifts which were mirrored by bacterial community changes. Thus, we hypothesize that differences between mycorrhizal associations in how they couple C and N cycles belowground drive who responds to enhanced N availability (i.e., fungi vs. bacteria), where this response occurs (i.e., rhizosphere vs. bulk soil), and potentially whether the system gains or loses soil C. Future research is needed to examine whether this mechanism holds across forest ecosystems, under other forms of N deposition (i.e., ammonium vs nitrate) and whether these responses operate under ambient N deposition loads. Also, future research that examines this mechanism below 15 cm is needed to determine if mycorrhizal type drives N responses in deeper soil C pools where decomposition may be constrained by oxygen, moisture, or mineral protection.

In addition to providing a plausible mechanism to explain ECM soil C gains under elevated N deposition, these results may also shed light on why microbial community responses to N deposition vary across studies. While much research highlights shifts in fungal community composition to explain N induced decomposition shifts (Frey *et al.*, 2004; Waldrop *et al.*, 2004; Freedman *et al.*, 2015; Entwistle *et al.*, 2018; Zak *et al.*, 2019; Entwistle *et al*., 2020), others link these shifts to changes in bacterial community composition and metabolism (Freedman & Zak, 2014; Freedman *et al.*, 2016a; Carrara *et al.*, 2018). We show that fungal communities shifted in ECM soils and bacterial communities shifted in AM soils. As such, variation in microbial responses across studies may be explained by relative mycorrhizal dominance. As these divergent microbial shifts between mycorrhizal types were apparent at such a small scale (10 x 10 m plots within a 10.3 ha watershed) it is possible that small differences in the relative abundance of AM and ECM trees can have sizable impacts on dominant microbial responses (i.e. bacterial vs fungal) to N fertilization.

Regardless of soil fraction, these shifts in enzyme activity appear to be driven by changes in microbial resource stoichiometry wherein microbes alter enzyme production as N limitation is alleviated and C limitation becomes more pronounced. This work builds on previous research that highlights the ability of microbes to alter allocation of resources to extracellular enzymes based on the relative demand of C and N to maintain growth (Schimel & Weintraub, 2003; Allison & Vitousek, 2005; Allison *et al.*, 2010; Sinsabaugh & Follstad Shah, 2012). This effect was observed in an increase in the ratio of BG to NAG activity across bulk and rhizosphere soil fractions in both mycorrhizal types which was driven primarily by a reduction in NAG activity (Fig. 1a,b, Table S8). However, the complex C to NAG activity response varied between mycorrhizal types with increases occurring in in ECM rhizospheres and in AM bulk soils (Fig. 1 e-h). While most studies report declines or no change in oxidative enzyme activity in response to N fertilization (Fog, 1988; Saiya-Cork *et al.*, 2002; DeForest *et al.*, 2004; Frey *et al.*, 2004, 2014; Zak *et al.*, 2008; Sinsabaugh, 2010), these distinct ECM and AM-associated increases in complex C to NAG activity ratios were partially driven by increased peroxidase activity in ECM rhizospheres and by increased phenol oxidase activity in AM bulk soils (Table S8). One explanation for this could be that microbes in ECM rhizospheres were pushed further toward C limitation than bulk soils due not only to N limitation alleviation, but strong declines in root-C inputs. In AM bulk soils, where saprotrophic communities are adapted to

scavenging inorganic N, high inputs of N may result in C restriction of microbial growth which may be dampened in AM rhizospheres due to some access to root-C. In either case, these mechanisms may act on soil microbes to enhance production of complex C mining enzymes in order to maintain biomass C:N. While it appears that shifts in C and N availability are driving these changes in microbial enzyme investment, we acknowledge that these enzymes operate on individual bonds present in complex soil organic matter pools and therefore the efficiency of specific enzymes to enhance microbial access to either nutrients or C may vary.

We found little evidence that the abundance of gene transcripts involved in decomposition were correlated with microbial community composition or enzyme activity. In ECM soils, shifts in community composition of fungi were not coupled with significant shifts in the relative abundance of ligninolytic enzyme transcripts (Fig. 3b). In AM soils, N fertilization reduced total ligninolytic transcript abundance which occurred in tandem with a marginally significant 14.3% ($p=0.08$) decline in basidiomycete relative abundance (Fig. 3 a,b). Here, it is possible that long term-N fertilization suppressed fungal activity in AM soils without altering fungal community composition on a broader scale, however reductions in gene transcription were not mirrored by declines in ligninolytic enzyme activities (Deforest *et al*., 2004). In ECM soils, it is possible that strong reductions in belowground C allocation to mycorrhizal symbionts resulted in an enhancement of free-living fungi relative to symbiotic fungi. This change could be responsible for the observed fungal community shifts in ECM soils as well as the changes in rhizosphere enzyme profiles without necessarily altering the rate of ligninolytic gene transcription across soil fractions. Regardless, we found little evidence that the relative abundance of genes involved in decomposition was a direct metric of the enzymatic potential of these soils. However, as transcriptomic data were pooled to one sample per mycorrhizal type, soil fraction, and treatment we lack the statistical power to examine changes in fungal transcription at finer scales.

Documented soil C cycling responses to N fertilization range from being driven primarily through shifts in fungi (Frey *et al.*, 2004; Freedman *et al.*, 2015; Entwistle *et al.*, 2018; Zak *et al.*, 2019; Entwistle *et al.* , 2020), to declines in belowground C allocation by trees and shifts in bacteria (Freedman & Zak, 2014; Freedman *et al.*, 2016a; Carrara *et al.*, 2018). Here we provide evidence that differences in C and nutrient cycling strategies between AM and ECM trees result in distinct fungal

shifts in ECM soils and bacterial shifts in AM soils. Further, in ECM stands we find that N induced fungal shifts occur in tandem with rhizosphere-isolated enhancements in C mining relative to N mining enzyme activity, whereas the same enzyme shifts occur across AM bulk soils. These results coupled with declines in C relative to N mining in ECM OH soils provide a mechanism that may explain variability in documented microbial responses to N fertilization, and ultimately why ECM soils tend to store more C than AM soils under enhanced N. As N deposition regimes continue to change across much of the developing world (Kanakidou *et al.*, 2016), including this mechanism in our conceptual framework may prove useful in enhancing the predictive capabilities of models that estimate the fate of the land C sink.

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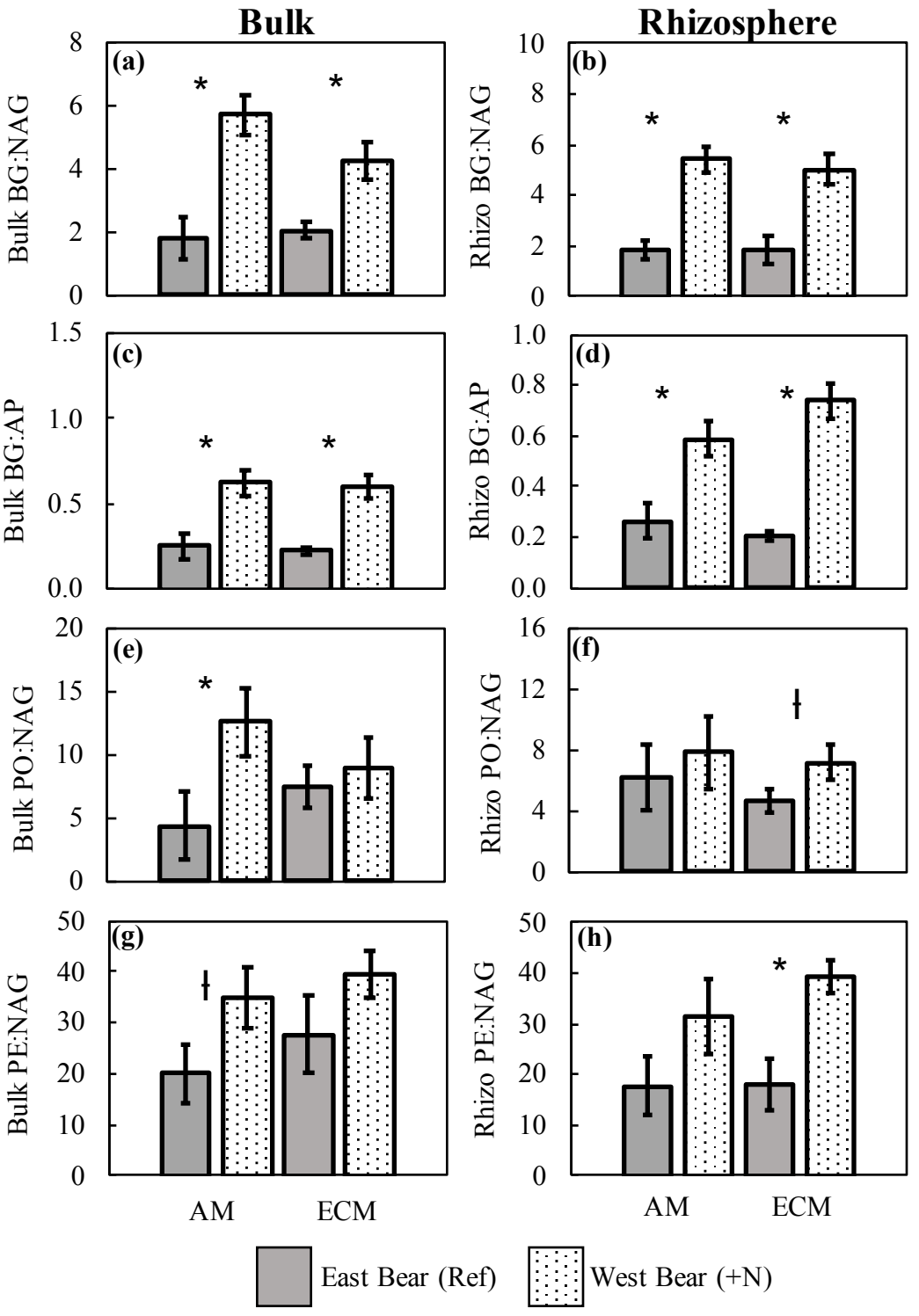
Figure Captions:

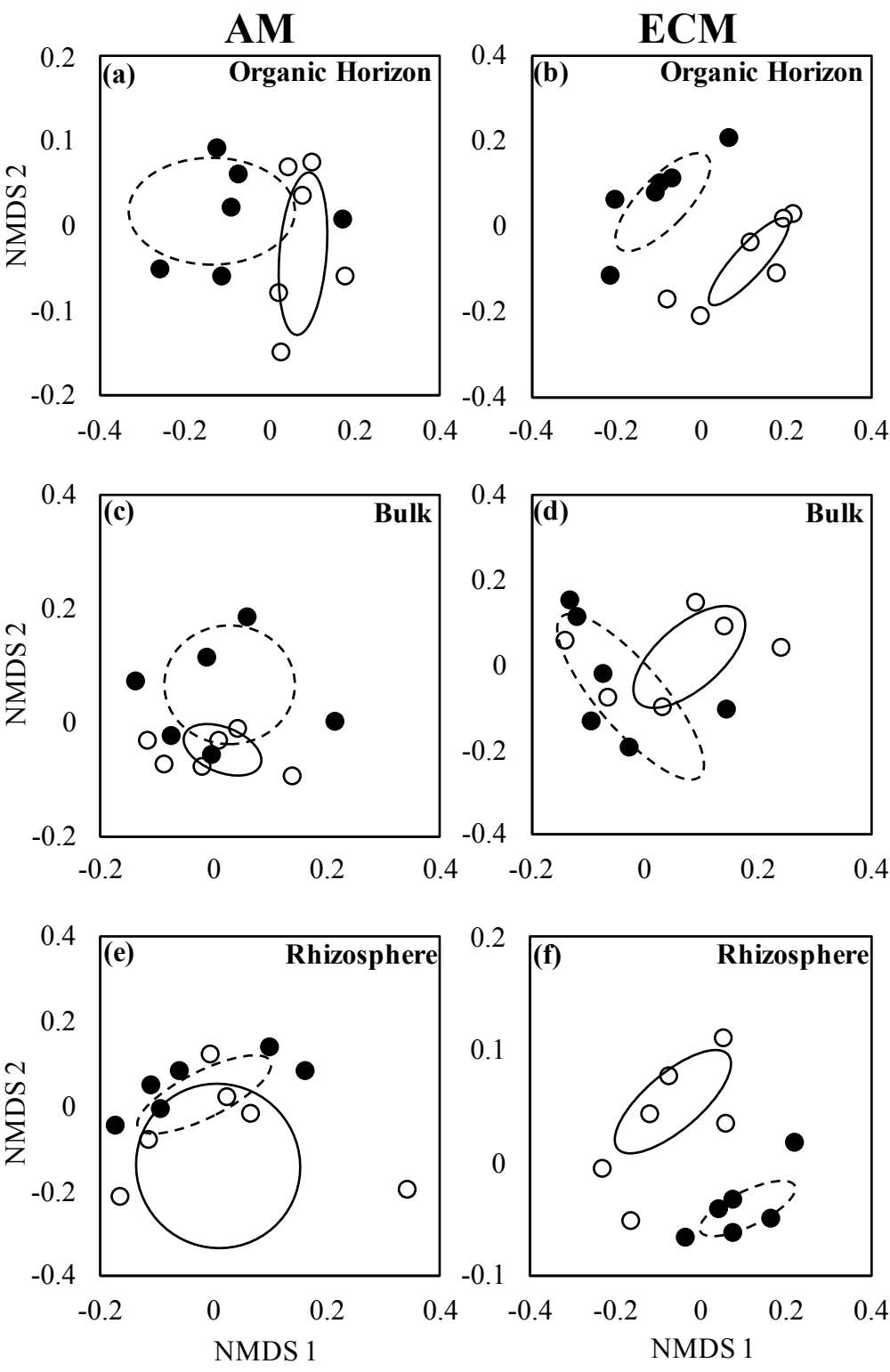
Figure 1. N fertilization enhanced the ratio of simple C (ß-glucosidase) to nutrient enzyme activities across AM and ECM bulk and rhizosphere soils, but enhanced complex C (phenol oxidase & peroxidase) to N only in AM bulk soils and ECM rhizosphere soils. Values are overall seasonal mean ratios (mean +SE) of (a&b) ß-glucosidase: N-Acetylglucosaminidase, (c&d) ß-glucosidase: acid phosphatase, (e&f) phenol oxidase: N-Acetylglucosaminidase, (g&h) peroxidase: N-Acetylglucosaminidase. Values were measured in May, July, and September across all plots (n=6 plots). Asterisks indicate $p<0.05$ for t-tests between N fertilized and reference plots within mycorrhizal type. Crosses indicate $p<0.10$.

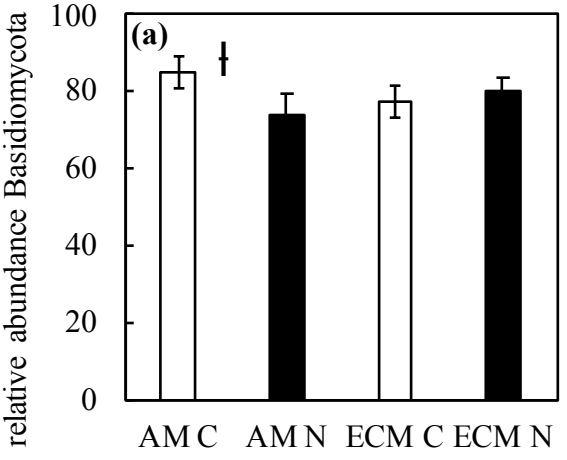
Figure 2. N fertilization altered enzyme profiles in AM bulk soils and ECM rhizosphere soils. Scatterplots are NMDS1 vs NMDS2 of the enzyme profiles for (a) AM OH soils $p=0.023$ (b) ECM OH soils $p=0.002$ (c) AM bulk soils $p=0.095$, (d) ECM bulk soils $p=0.172$, (e) AM rhizosphere soils $p=0.577$, (f) ECM rhizosphere soils $p=0.021$. Closed circles are fertilized plots, open circles are reference plots.

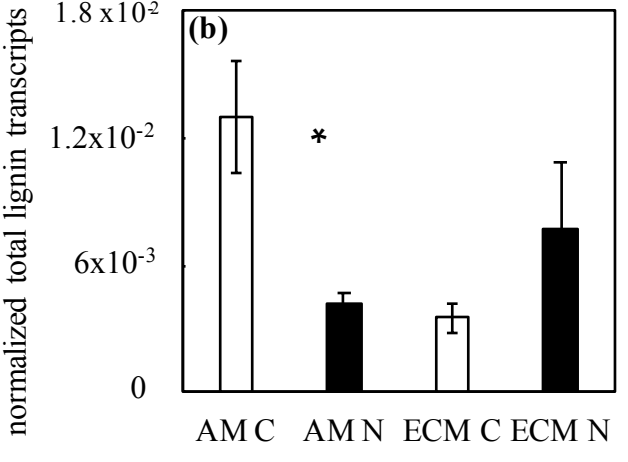
Figure 3. N fertilization reduced Basidiomycota abundance and ligninolytic gene transcription in AM soils, but not ECM soils. Symbols represent significant differences between reference and N fertilized values within mycorrhizal type. For (a) n=6 plots per mycorrhizal type per treatment, (b) n= 3 samples per mycorrhizal type per treatment. Asterisk indicates $p<0.05$, cross indicates $p<0.10$.

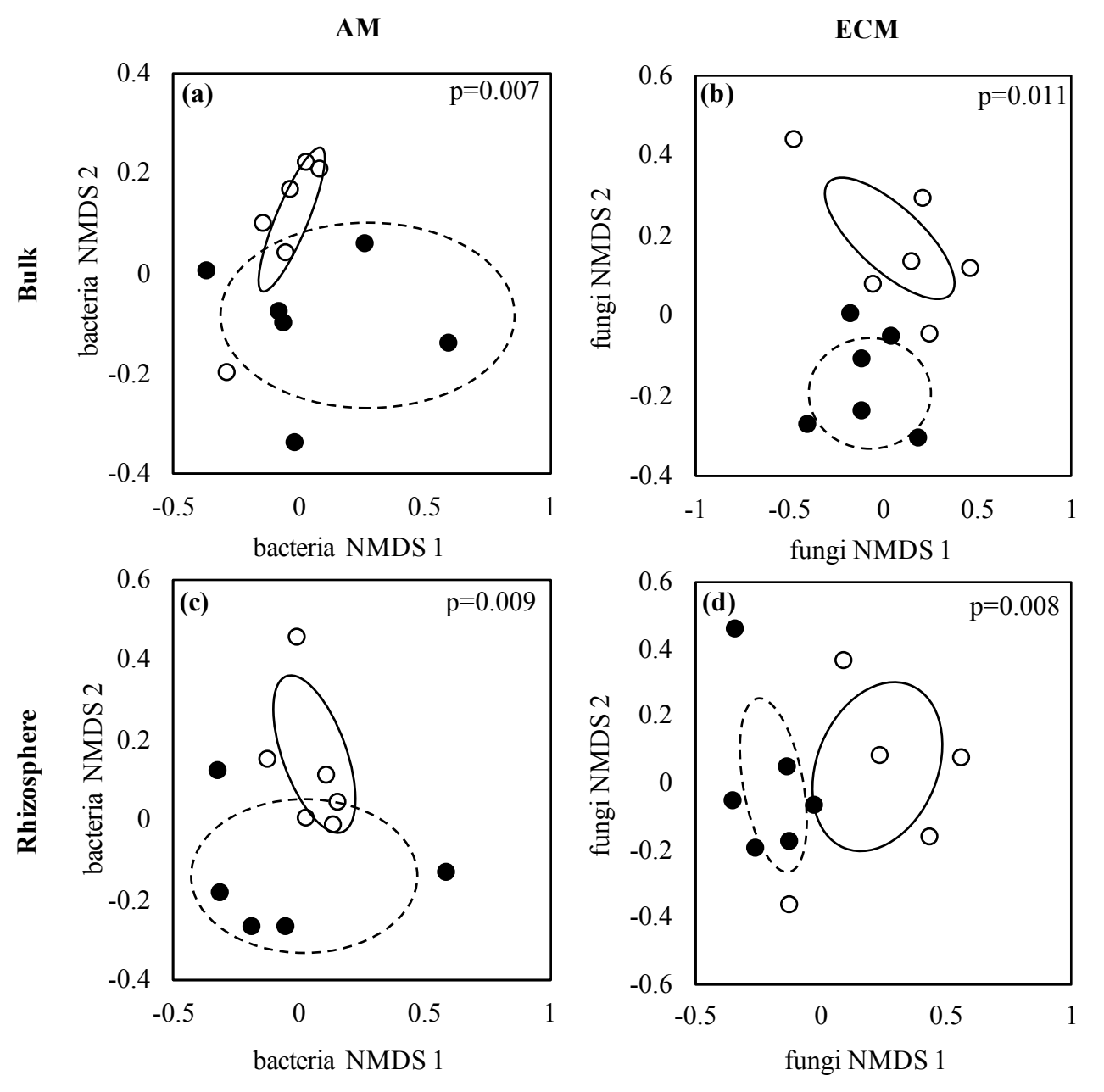
Figure 4. N Fertilization altered bacterial communities in AM soils and fungal communities in ECM soils. Scatterplots are NMDS1 vs NMDS2 of the community structure for (a) AM bulk soils bacteria, (b) ECM bulk soils fungi, (c) AM rhizosphere soils bacteria, (d) ECM rhizosphere soils fungi. Closed circles are fertilized plots, open circles are reference plots. P values indicate significant differences between community composition.


Bulk
Rhizosphere
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
Bulk BG:NAG
Rhizo BG:NAG
Bulk BG:AP
Rhizo BG:AP
Bulk PO:NAG
Rhizo PO:NAG
Bulk PE:NAG
Rhizo PE:NAG
AM
ECM
East Bear (Ref)
West Bear (+N)


AM
ECM
(a) Organic Horizon
(b) Organic Horizon
(c) Bulk
(d) Bulk
(e) Rhizosphere
(f) Rhizosphere
NMDS 1
NMDS 2


(a)
relative abundance Basidiomycota
100
80
60
40
20
0
AM C
AM N
ECM C
ECM N


(b)
normalized total lignin transcripts
1.8 x10-2
1.2x10-2
6x10-3
0
*
AM C
AM N
ECM C
ECM N


AM
ECM
Bulk
Rhizosphere
(a)
p=0.007
bacteria NMDS 2
bacteria NMDS 1
(b)
p=0.011
fungi NMDS 2
fungi NMDS 1
(c)
p=0.009
bacteria NMDS 2
bacteria NMDS 1
(d)
p=0.008
fungi NMDS 2
fungi NMDS 1