RESEARCH ARTICLE

Mycorrhizal fungal communities respond to experimental elevation of soil pH and P availability in temperate hardwood forests

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One sentence summary: Though decades of acidic deposition have contributed to reduce soil pH in forests worldwide, experimentally raising pH changes mycorrhizal fungi colonizing tree roots and alters phosphorus availability.

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ABSTRACT

Many forests are affected by chronic acid deposition, which can lower soil pH and limit the availability of nutrients such as phosphorus (P), but the response of mycorrhizal fungi to changes in soil pH and P availability and how this affects tree acquisition of nutrients is not well understood. Here, we describe an ecosystem-level manipulation in 72 plots, which increased pH and/or P availability across six forests in Ohio, USA. Two years after treatment initiation, mycorrhizal fungi on roots were examined with molecular techniques, including 454-pyrosequencing. Elevating pH significantly increased arbuscular mycorrhizal (AM) fungal colonization and total fungal biomass, and affected community structure of AM and ectomycorrhizal (EcM) fungi, suggesting that raising soil pH altered both mycorrhizal fungal communities and fungal growth. AM fungal taxa were generally negatively correlated with recalcitrant P pools and soil enzyme activity, whereas EcM fungal taxa displayed variable responses, suggesting that these groups respond differently to P availability. Additionally, the production of extracellular phosphatase enzymes in soil decreased under elevated pH, suggesting a shift in functional activity of soil microbes with pH alteration. Thus, our findings suggest that elevating pH increased soil P availability, which may partly underlie the mycorrhizal fungal responses we observed.

Keywords: acid deposition; arbuscular mycorrhizal fungi; ectomycorrhizal fungi; phosphorus; 454-sequencing; phosphatase

INTRODUCTION

Human activities have increased the atmospheric inputs of both nitrogen (N) and acidic compounds to most forest ecosystems in eastern North America, Europe and eastern Asia. Many of these forests have undergone significant soil acidification since the middle of last century and are likely approaching N saturation due to past and current atmospheric N deposition levels (Aber,

McDowell and Nadelhoffer 1998; Lovett and Goodale 2011). Such changes in soil chemistry also impact the availability of other essential nutrients in forest ecosystems. In particular, phosphorus (P) becomes less biologically available with decreasing soil pH, whereby aluminum (Al) is mobilized below pH 5 and geochemically binds inorganic P (P_i) (Thomas and Hargrove 1984; Goldberg, Davis and Hem 1996). Thus, despite the prevailing paradigm that temperate forests are N limited, increased N inputs, combined with soil acidification, leads to the biogeochemical expectation of P limitation over time (Cross and Schlesinger 1995). However, few studies in acidic temperate forests provide evidence for P limitation of trees (e.g. Casson, Eimers and Watmough 2012), and several conclude that no such P limitation exists for trees (Wood, Bormann and Voigt 1984; Finzi 2009). If P is indeed less biologically available at low pH, it suggests the existence of a mechanism that allows trees to overcome the P limitation (DeForest *et al.* 2012; Kluber *et al.* 2012).

One such mechanism may involve changes in the belowground activities of soil organisms, particularly mycorrhizal fungi, which live in symbiosis with plant roots and, in exchange for carbon, provide the plant with nutrients, especially N and P (Read and Perez-Moreno 2003; Smith and Read 2008). Mycorrhizal fungi may be important for maintaining the nutrition of host plants in acidic, P-limited soils since the extraradical hyphae of mycorrhizal fungi may provide the majority of plant P through soil foraging activity, with estimates of 80% to as much as 100% of plant P provided by mycorrhizal fungi (Marschner and Dell 1994; Smith, Smith and Jakobsen 2003). In addition, mycorrhizal fungi can increase P availability by secreting phosphatases into soil to degrade organic P (P_o; Courty *et al.* 2010; Burke, Smemo and Hewins 2014), excreting organic acids that mobilize P from mineral complexes (e.g. leaching of polyphenolics or calcium oxalate; Jongmans *et al.* 1997; Antunes *et al.* 2007) or immobilizing soluble Al through sequestration in fungal tissue (Aguilera *et al.* 2011). While links between P availability and mycorrhizal phosphatase production have not been observed in culture (Olsson *et al.* 2006; Nygren and Rosling 2009), non-mycorrhizal fungi are known to increase phosphatase activity under P starvation (Kaffman *et al.* 1994; van Aarle, Olsson and Söderström 2001). It has also been suggested that EcM fungal diversity may be more important than the level of mycorrhizal colonization for plant P uptake (Baxter and Dighton 2001). Furthermore, evidence from manipulative field studies have shown that P amendment influences phosphatase production in soil (DeForest *et al.* 2012; Kluber *et al.* 2012), and soil P availability is correlated with mycorrhizal fungal community structure (Burke *et al.* 2009, 2011; Coince *et al.* 2013). Thus, changes belowground communities may be responsible for shifts in soil enzyme activity that potentially increase plant P acquisition.

However, because P availability is linked to soil pH, the response of mycorrhizal fungi to changes in soil P may be inherently linked to changes in soil acidity. An increasing number of studies have suggested that soil pH may be the primary factor affecting soil fungal and bacterial community structure, diversity and biomass in a variety of ecosystems (Bååth and Anderson 2003; Fierer and Jackson 2006; Lauber *et al.* 2009; DeForest and Scott 2010; Rousk *et al.* 2010), and changes in soil pH have been shown to influence mycorrhizal fungal species richness (reviewed in Lauber *et al.* 2009), root colonization (Cairney and Meharg 1999; Coughlan *et al.* 2000; van Aarle, Olsson and Söderström 2002), biomass (van Aarle, Olsson and Söderström 2002) and community structure (Lehto 1994; Wallander *et al.* 1997; Erland and Taylor 2002; Kjøller and Clemmensen 2009; Rineau and Garbaye 2009; Dumbrell *et al.* 2010). It is plausi-

ble that soil acidification alters mycorrhizal fungal community composition both directly as well as indirectly, such as through its effect on P availability. For example, soils with low pH may harbor different mycorrhizal fungal species as compared to high-pH soils simply because those species capable of maintaining growth and cellular function in acidic environments would survive over those adapted to more basic conditions (reviewed in Lauber *et al.* 2009). However, because P_o is likely the dominant form of available P in acidic temperate forests (Yanai 1992), we might also expect those soil fungi most capable of efficiently liberating and capturing P_o to be favored in low-pH soils where P_i is limiting; however, this topic remains largely unexplored.

In addition, temperate hardwood forests are dominated by tree species that form mycorrhizal relationships with either ectomycorrhizal (EcM) or arbuscular mycorrhizal (AM) fungi. Because of their different functional attributes, EcM and AM fungal communities may be expected to respond differently to changes in soil chemistry. For example, EcM fungi are expected to increase plant access to both soluble and insoluble inorganic as well as organic nutrients, including recalcitrant organic nutrients, since they have the capacity to produce phosphatases that degrade P_o, and organic acids that mobilize P from mineral complexes (Jongmans *et al.* 1997; Smith and Read 2008; Courty *et al.* 2010; Burke, Smemo and Hewins 2014). Conversely, while AM fungi increase plant access to soluble P_i, they have not been shown to produce organic acids (Antunes *et al.* 2007) and are generally believed to not produce extracellular phosphatase enzymes (Smith and Read 2008). However, there is evidence that AM fungi can immobilize soluble Al (Aguilera *et al.* 2011), and it has been suggested that increases in rhizosphere pH due to AM fungal presence can increase P mobilization. Despite these characteristics of AM fungi, in acidic forest soils where organic pools of nutrients dominate, plants with roots colonized by EcM fungi may have greater access to soil nutrients than trees colonized by AM fungal species due to the ability of EcM fungi to produce phosphatases and organic acids. Consequently, AM and EcM fungi may constitute different functional groups from the perspective of nutrient acquisition as determined by their differential ability to access organic forms of nutrients. However, there is increasing evidence that substantial variation exists in the response of EcM fungi to soil nutrient availability, with some EcM fungi displaying an ability to degrade and access organic forms of nutrients while others appear to lack this ability (Smith and Read 2008; Courty *et al.* 2010; Pena and Polle 2014). Thus, different nutrient acquisition functional groups (e.g. groups specialized for P acquisition) may exist within both AM and EcM fungi. If this is the case, the structure of mycorrhzial communities within forests is likely to impart a large influence on the ability of plants to overcome acid-induced P limitation.

In order to understand the interacting effects of soil pH and P availability on mycorrhizal fungi and soil microorganisms, large-scale manipulative experiments are needed. In this study, we present an examination of both EcM and AM fungi, as well as soil microbial activity and biomass, in an ecosystem-level manipulation in which we increased soil pH and P availability across six temperate hardwood forests. The purpose of the treatments was to provide two mechanisms to increase the availability of P in soil, indirectly via pH manipulation and directly by adding phosphate, where the combined treatment of pH and P should maximize the availability of P. Previous work from our experimental plots suggests that mycorrhizal fungal community structure and soil microbial function respond rapidly (less than one year) to increases in P availability (DeForest *et al.* 2012; Kluber *et al.* 2012), but responses by AM fungi to pH were less evident than for EcM

fungi. However, evidence from the literature suggests that strong responses by mycorrhizal fungi to increases in soil pH may take longer to develop (Bakker, Garbaye and Nys 2000). There is also a need for further studies evaluating the response of potential mycorrhizal functional groups (e.g. groups specialized for P acquisition) to changes in soil chemistry, and especially P availability given the widespread occurrence of acidic deposition in northern forest ecosystems.

We hypothesized that our soil pH and P treatments would alter the P acquisition strategy of soil fungal and microbial communities, thus, driving a community structure change for AM and EcM fungi. Our expectation was that soil acidification has led mycorrhizal fungal communities to shift and include a greater percentage of taxa reliant on and efficient at liberating P_o from soil organic matter. Due to their different functional attributes, AM and EcM fungi were expected to respond differently to our experimental manipulations of soil pH and P availability. For example, we hypothesized that AM fungal communities would be largely influenced by the availability of labile forms of P, while EcM fungal community structure would likely to be positively correlated with the availability of recalcitrant forms of P and the production of soil enzymes, including phosphatases. We also expected EcM fungal taxa to exhibit divergent responses to P pools and enzyme activity, indicating the presence of P functional groups within the EcM fungi.

MATERIALS AND METHODS

Site description and experimental design

We established an ecosystem-level pH and P manipulation experiment in the fall of 2009 in 72 experimental plots within six forest blocks and split between two distinct physiographic regions of Ohio. The northern plots (36 plots, three forests) were established within mixed hardwood forests of the glaciated Allegheny plateau, while the southern plots (36 plots, three forests) were established within similar forests on the unglaciated Allegheny plateau. Forest regions are ~270 km apart. Plots in the northern forests (hereafter glaciated forests) are located on loamy to silty loam soils with an average pH of 4.3, while the southern forest plots (hereafter unglaciated forests) are located on similar soils with an average pH of 4.7. Average temperature and precipitation for the glaciated forests is 8.1°C and 1200 mm, respectively, while the unglaciated forests have mean temperature and precipitation of 10.7°C and 1000 mm. We chose these regions because they differ in soil age and developmental processes, and, therefore, could respond differently to changes in soil pH and P availability.

Elevated pH and P treatments were administered to the 72 plots (measuring 30 × 40 m; 800 m^2) by the application of limestone (Hi-Ca lime; high calcium to magnesium ratio) and triple super phosphate, respectively. On average, the glaciated and unglaciated sites received 11.4 and 7.3 Mg (or tonnes) ha^{-1} of Hi-Ca lime in 2009 and an additional 2.0 Mg ha^{-1} of Hi-Ca lime in late October 2010 to maintain soil pH levels. Limestone was added to elevated pH and elevated pH + P plots to attain a target pH between 5.8 and 6.2, which previous work had determined would be sufficient to immobilize reactive Al in these soils (DeForest and Scott 2010). We also applied ~42 kg P ha^{-1} as triple super phosphate to both elevated P and elevated pH + P plots, and this application occurred in the spring of 2010 and 2011. Both Hi-Ca lime and triple super phosphate was supplied by the Andersons, Inc. (Maumee, OH, USA). Although we established all of our plots in mixed hardwood forests, tree community composition varied among the six forest blocks, and the glaciated sites

contained a higher percentage of AM trees (*Acer* spp. 30%–68% of basal area) compared to the unglaciated forests (*Acer* spp. 10%–16% of basal area). For a full description of the field sites and experimental set-up, please refer to DeForest *et al.* (2012) and Kluber *et al.* (2012).

Soil and root sampling

Since unglaciated forests are often 2–4 weeks ahead of glaciated forests phenologically, we sampled unglaciated plots on August 25 and 26 2011, and glaciated plots were sampled on September 12 and 13 2011 following methods described in Kluber *et al.* (2012). In each plot, the litter layer was removed and 10 soil cores (4 cm in diameter and 5 cm deep, as this is the zone of highest fine root growth and activity in our forests) were collected 1 m from 10 randomly selected trees measuring more than 6 cm diameter at breast height. Samples were pooled by plot and kept at 4°C until processed in the laboratory. Fine roots were separated from soil by sieving (2 mm), washed with deionized water and sorted into three categories: AM roots, EcM roots, and woody roots (no AM or EcM roots, all diameter sizes). Categories were confirmed with a dissecting microscope between 20–40× and all senescent root material was discarded. For each plot, live EcM root tips were counted (as a measure of abundance) and pooled into a single composite sample—rather than being separated into morphotypes—and stored at −70°C prior to DNA extraction and pyrosequencing. Similarly, AM roots were combined into one sample per plot, lyophilized, and used for DNA extraction and quantification through qPCR. We considered roots not colonized by EcM fungi as potentially AM roots and treated them as such for analysis. Since most of the trees in the plots potentially colonized by AM fungi consist of *Acer* spp. (*Acer rubrum* and *A. saccharum*) and *Liriodendron tulipifera*, and roots of both species are easy to identify with a dissecting scope, incorrect assignment of AM roots is unlikely. Soil cores were not collected near areas containing herbaceous understory plants in order not to include herbaceous roots or their potential influence on AM communities in our samples.

Analysis of soil chemistry and extracellular enzymes

Soil pH was measured by adding deionized water to sieved field fresh soil (1:2 H$_2$O) and recorded after 30-m incubation with shaking. Gravimetric water content was determined by drying a soil subsample for 48 h at 105°C, while total C and N was determined through dry combustion using an ECS 4010 CHNSO elemental analyzer (Costech Analytical, Valencia, CA). Biologically available soil P was quantified using sequential fractionation into most labile P (resin P) and more recalcitrant forms (bicarbonate extractable P and hydroxide extractable P). We also measured the occluded P fractions of hydroxide SOC P, strong acid P (HCl P) and residual P (Johnson, Frizano and Vann 2003; DeForest *et al.* 2012), as well as total P, and all P fractions were measured by inductively coupled plasma spectrometry. Potential extracellular enzyme activity was measured on fresh soils within 24 h of collection using (MUF)-linked substrates as described previously (DeForest *et al.* 2012). We measured enzymes involved in C cycling (β-1,4-glucosidase and cellobiohydrolase), N cycling (β-N-acetylglucosaminidase and leucine aminopeptidase) and P cycling (phosphomonoesterase and phosphodiesterase) after homogenizing 5 g of soil in 500 ml of 50 mM acetate buffer (pH = 5). All samples were measured in black 96-well plates and activities are expressed per gram of soil dry weight; calculations were as described previously (DeForest 2009).

PLFA analysis of microbial biomass

We used phospholipid fatty acid (PLFA) analysis to estimate changes in microbial biomass (total bacterial, total fungal, and AM fungal) and fungal/bacterial ratios across treatments (Tunlid *et al.* 1989; Vestal and White 1989; Zelles 1999; Olsson and Wilhelmsson 2000). Analytical recovery was determined by adding phospholipid 19:0 standard (Avanti Polar Lipids, Inc., Alabaster, AL, USA), and total lipids were extracted from freeze-dried soil. Polar and non-polar lipids were separated and quantified using a HP GC-FID (HP6890 series, Agilent Technologies, Inc. Santa Clara, CA, USA) as described elsewhere (DeForest *et al.* 2004; DeForest and Scott 2010), and biomarkers were identified using the Sherlock System (v. 6.1, MIDI, Inc., Newark, DE, USA).

Molecular analysis of arbuscular mycorrhizal fungal communities

Lyophilizing AM roots allowed for the quantification of dry root biomass prior to DNA extraction. The amount of freeze dried AM roots was highly variable, ranging from 3 to 1054 mg root dry weight per plot, with a mean of 237 mg. All roots removed from the soil cores were then homogenized prior to DNA extraction following the procedure described in Kluber *et al.* (2012), using the bead beating and phenol/chloroform protocol described previously (Burke *et al.* 2009). AM fungal community structure was determined with TRFLP, using the primers AM1 (Helgason *et al.* 1998) and NS31 (Simon, Lalonde and Bruns 1992) and the endonuclease HinfI (Promega Corporation, Madison, Wisconsin, USA), as described previously (Kluber *et al.* 2012). TRFLP profiles were generated at the Biotechnology Resource Center (Cornell University) and analyzed using Peak Scanner Software (version 1.0, Applied Biosystems™, Foster City, California, USA, 2006) following procedures in Burke, Dunham and Kretzer (2008). Our previous study found that using this primer set resulted in recovery of only sequences with high similarity to taxa in the Glomeraceae (Kluber *et al.* 2012). However, to further assess the specificity of our reactions, we cloned and sequenced PCR products, screened 96 clones, and grouped sequences into Operational Taxonomic Units (OTUs) as previously described (Kluber *et al.* 2012). As noted in the results section (and see Table S1, Supporting Information), 93 clones had high similarity to AM fungi, while three clones did not yield sequence data of sufficient quality to permit assignment to taxa. This effort further confirmed the specificity of our primer set for AM fungi. We used the copy number of AM fungal 18S rRNA genes as an estimate of AM fungal root colonization, which has been shown to be a reliable method for estimating AM fungal root colonization in previous studies (Isayenkov, Fester and Hause 2004; Alkan *et al.* 2006). However, it is important to note that qPCR measures gene copy number, which can be used as an indicator of total AM fungal DNA in roots, but it may not relate directly to microscopic root colonization estimates in some cases (Gamper *et al.* 2008). We followed qPCR procedures previously outlined in Kluber *et al.* (2012) and quality control guidelines established by Bustin *et al.* (2009). Briefly, three replicate qPCR reactions were run for each sample using primers AM1 and NS31 and SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, California, USA) on a MiniOpticon realtime PCR detection system (Bio-Rad Laboratories, Inc., Hercules, California, USA). The gene copy number was determined by comparing the quantification cycle (C_q) to a standard curve using the CFX Manager™ software, version 2.0 (Bio-Rad Laboratories, Inc., Hercules, California, USA). The C_q was determined manually for each run, to optimize the reaction efficiency (97.4%–100.0%) and standard curve r^2 (0.990–0.998). Reaction specificity was determined by melt curves and visual estimation on 2% agarose gels, and all no template controls were below detection. The number of 18S rRNA gene copies per μl of extracted sample DNA was averaged across the three qPCR method replicates and reported in two ways: an estimate of AM fungal root colonization, expressed as gene copies per gram of dry root tissue used for DNA extraction; and an estimate of AM fungal abundance from the mass of roots within the volume of soil sampled, expressed as gene copies per cm^3 of soil.

Molecular analysis of ectomycorrhizal fungal communities

DNA was extracted from the pooled EcM root tips using bead beating and phenol/chloroform purification as previously described (Burke *et al.* 2009). All EcM root tips collected from a plot were pooled and used for extraction (the number of root tips per plot ranged from 4 to 1176, with an average 267 of root tips per core). DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Inc., Waltham, Massachusetts, USA), and the three replicate treatment plots per forest were pooled, which resulted in 24 total samples (three forests × four treatments × two regions). The volume of each DNA sample used for pooling was dependent on the number of root tips present in the sample, where the ratio of DNA molar concentration (ng DNA/μl extractant) between the pooled samples was the same as the ratio of root tips between the samples. Final template concentration was standardized to 25 ng μl^{-1}. The fungal ITS region was amplified using a 25 μl reaction containing 22.5 μl Platinum PCR Super Mix High Fidelity (Invitrogen™, Carlsbad, California, USA), 1 μl of template, 0.4 μg μl^{-1} bovine serum albumin and 0.1 μM of each primer. PCR conditions were 3 min at 94°C, followed by 33 cycles of 94°C for 45 s, 50°C for 30 s and 72°C for 60 s, with a final extension of 72°C for 10 min. We used primers ITS1-F (Gardes and Bruns 1993) and ITS2 (White *et al.* 1990) to amplify the ITS1 region of the rRNA gene, which have proven effective in previous pyrosequencing studies of fungal diversity in forest soils (Buée *et al.* 2009). To facilitate library multiplexing, we used a set of 24 Multiplex Identifier (MID) sequences attached to the ITS1-F primer which permitted separation of sequence reads into treatment × forest × region combinations. Sequencing was completed on the Genome Sequencer FLX 454 System, and fungal ITS sequence data were processed using the QIIME pipeline to group OTUs at a 97% similarity, remove chimeras and screen against the UNITE database (version 5.0) to assign taxonomy. Our pyrosequencing effort yielded over 101 643 sequences. We eliminated raw sequence reads that were <200 bp in length or that contained more than 2 primer mismatches. Sequences with homopolymer runs of >6, with more than six ambiguous bases or that had an average quality score <25 were also deleted from the analysis. In total 72 128 sequences met quality control criteria with an average sequence length of 344 bp. The UCLUST algorithm (Edgar 2010) was used to group OTUs at a 97% similarity cutoff, and the BLAST algorithm was used to screen OTUs against the UNITE database to assign taxonomy. A total of 3174 OTUs were detected among all samples analyzed. The most abundant sequence for each OTU was selected as a representative and used for assigning taxonomy (maximum E-value of 1e^{-3}; minimum percent identity of 90%). The taxonomy assignment for each OTU was resolved at the genus level if possible. If the OTU taxonomy assignment could not be resolved at the genus level, it was resolved to the lowest taxonomic group

possible with a 90% similarity cut off. Though some OTUs matched with non-EcM fungal taxa (<1.4% of the total sequence number matched with non-EcM fungal genera or with groups that are exclusively not EcM fungi, such as the Glomeromycota and Chytridiomycota), they were retained in an attempt to assess the fungal OTUs that were associated with live EcM root tips. Overall, 67% of sequences matched with known or putative EcM fungal taxa (as per Rinaldi, Comandini and Kuyper 2008), while the remaining 31.6% could not be successfully resolved to the genus level, but many of these sequences could be assigned to families or orders that contain known EcM fungal genera. (Sequences that were identified to the orders Thelephorales and Sebacinales or families Sebacinaceae or Russulaceae were included in this estimate of EcM fungal OTUs because these groups are known to be largely, or exclusively in the case of the Thelephorales, ectomycorrhizal, and our samples were live EcM root tips).

Statistical analyses

Treatment effects on measured variables were tested using a factorial design, which allowed us to assess both the main effect of pH or P manipulation independent of each other as well as their interaction. Significant interactions indicate non-additive (i.e. synergistic or antagonistic) effects of the elevated pH and P treatment (Quinn and Keough 2002). When significant interactions were found, interaction plots and pairwise comparisons between each combination of factor levels [ambient pH + ambient P (i.e. control), elevated pH + ambient P (i.e. elevated pH treatment), ambient pH + elevated P (i.e. elevated P treatment), elevated pH + elevated P] were used to determine the nature of the interaction.

We analyzed treatment effects with linear mixed-effects (LME) models conducted in R v2.15 (R Development Core Team 2013) using the *nlme* (v3.1) package (Pinheiro *et al.* 2013). Region (glaciated versus unglaciated sites) and the treatments were fixed effects, while forest block was a random effect. Akaike information criteria were calculated for each model to determine whether to include forest stand as a random effect and/or to model heterogeneity within region (Zuur *et al.* 2009). If necessary to improve assumptions of normality and equality of variances, data were natural log or square root transformed. Post-hoc pairwise comparisons were made with the *multicomp* (v1.2) package (Hothorn, Bretz and Westfall 2008; Bretz *et al.* 2011). Significance with the LME models was defined at $\alpha = 0.05$.

We used non-metric multidimensional scaling (NMS) procedures available through PC-ORD 4 (MjM Software, OR, USA) to compare AM and EcM fungal communities between regions and among treatments. Prior to NMS, singletons were removed from both the AM and EcM fungal data sets, resulting in the exclusion of 12 AM fungal OTUs and 79 EcM fungal OTUs, and retention of 20 AM fungal OTUs and 104 EcM fungal OTUs for NMS. Analysis of treatment effects on both AM and EcM fungal communities was conducted on proportional data, not raw TRFLP fluorescence or raw sequence read counts, respectively, and, as such, were arcsine squareroot transformed prior to analysis. NMS was run using Sørenson distance and a random starting configuration. Soil variables were related to fungal community patterns using Pearson correlations against NMS axes. Significant r values at $\alpha = 0.05$ were determined by the sample size (72 for the AM fungal data set and 24 for the EcM fungal data set) according to Zar (1998). Non-parametric permutation procedure (PERMANOVA) was used to test the effects of region, pH treatment, and P treatment on fungal communities and was conducted using the *vegan* (v2.0-10) package in R (Oksanen *et al.* 2013). Rar-

efaction curves were generated for the root fungal communities associated with live EcM root tips in Analytic Rarefaction, v1.3 (Holland 2003) by summing the number sequence reads per OTU within each treatment, while indicator species analysis for these communities was conducted in PC-ORD 4. Correlations between EcM and AM fungal taxa and P fractions or phosphatase enzymes were made in SigmaStat (v3.5, Systat Software, San Jose, California, USA). All graphs were produced in SigmaPlot (v10, Systat Software, San Jose, California, USA).

RESULTS

Soil chemistry

Elevated pH and elevated pH + P plots had a soil pH (5.8 average of all 36 plots) that was 1.5 units higher than control and elevated P plots (4.3 average of all 36 plots), which contributed to lower total acidity, and dramatically lower extractable Al in elevated pH compared to ambient pH treatments (Table 1). Soil pH was slightly higher (11%) in the unglaciated region overall, while acidity and extractable Al exhibited regional variation that interacted with pH manipulation (Table 1; Fig. S1, Supporting Information). Although control and elevated P plots in the glaciated region were highest (by 2.5–9 times) in total acidity (pairwise P < 0.01), extractable Al was highest in these same plots in the unglaciated region. Total soil C and N were 46% and 55% greater, respectively, in glaciated sites compared to the unglaciated sites, but there was no treatment effect (Table 1). However, a significant pH × P interaction was found for soil N (Table 1), but this is likely driven by the unglaciated region (Fig. S1, Supporting Information). Soil C:N ratio did not differ between regions or across treatments.

Elevated pH and P treatments had a significant effect on most P fractions despite underlying regional differences, (Table 1). Resin P—the most readily available P fraction—exhibited the strongest response to P addition and the clearest pattern of P and pH treatment interaction (Table 1). P addition elevated Resin P to 5.9 times that of ambient P plots, but pH treatment had the opposite effect, reducing Resin P in elevated pH plots by about half that in ambient pH plots (Table 1; Fig. S2, Supporting Information). Resin P was two times greater in the unglaciated region, while other P pools displayed the opposite pattern and were higher in the glaciated region. Among the other available, but more recalcitrant P fractions, hydroxide P and bicarbonate P exhibited a similar treatment response as resin P, with significant increases with P addition (at least for hydroxide P) and significant decreases with pH manipulation. Responses to treatments were less clear among the more occluded P fractions and were confounded by significant interactions, but the significant P × pH interactions for many of measured P pools suggests that elevating P and pH may have acted synergistically in the elevated pH + P plots (Table 1; Fig. S2, Supporting Information).

Enzyme activity and PLFA analysis

Only the activities of P-acquiring enzymes were affected by treatments. Activity of phosphomonoesterase declined in the elevated pH and elevated pH + P plots compared to those at ambient pH (Fig. 1; Table 2), while phosphodiesterase activity was decreased in the elevated pH, elevated P and elevated pH + P plots compared to controls (Fig. 1; Table 2). Overall, soil enzyme activity was significantly greater (by 2–3 times) in glaciated plots than unglaciated plots for all measured extracellular enzymes, except for β-N-acetylglucosaminidase (NAGase) (Table 2).

Table 1. Mean ± standard error for soil chemistry along with P-values showing the effect of region (R), pH treatment and P treatment from the LME models with forest blocks as the random effect. Units are cmol(+) (kg soil)⁻¹ for total acidity and exchangeable aluminum, while soil C and N are expressed as mg (kg soil)⁻¹, C:N is a molar ratio and units for all P fractions are mg P (kg soil)⁻¹.

Treatment	Soil pH	Total acidity	Ex Al[a]	Soil C	Soil N	C:N	Resin P	Bicarb P[b]	Hydroxide P	Hydroxide SOC P	HCl P	Residual P	Total P
Glaciated													
Control	3.9 ± 0.1	9.3 ± 0.9	0.7 ± 0.1	61.2 ± 8.2	3.7 ± 0.4	16.5 ± 0.7	1.1 ± 0.2	32.8 ± 2.2	99.4 ± 9.8	132.4 ± 14.1	9.3 ± 3.1	119.6 ± 9.8	394.6 ± 32.1
Elevated pH	5.6 ± 0.1	0.8 ± 0.2	0.1 ± 0.1	54.5 ± 2.8	3.3 ± 0.2	16.4 ± 0.6	0.7 ± 0.2	19.3 ± 1.4	84.3 ± 7.5	96.1 ± 8.0	12.1 ± 1.5	129.1 ± 9.7	341.7 ± 23.4
Elevated P	4.1 ± 0.1	9.0 ± 1.3	1.1 ± 0.4	58.8 ± 5.2	3.9 ± 0.4	15.4 ± 0.8	8.7 ± 1.2	35.0 ± 2.4	109.9 ± 6.4	162.6 ± 15.9	11.2 ± 2.7	141.0 ± 17.1	476.2 ± 47.0
Elevated pH + P	5.5 ± 0.2	1.2 ± 0.4	0.1 ± 0.1	49.4 ± 4.4	3.2 ± 0.3	15.8 ± 0.7	2.8 ± 0.5	22.7 ± 1.7	94.3 ± 5.8	114.0 ± 9.0	20.1 ± 4.5	134.2 ± 14.6	388.1 ± 31.5
Unglaciated													
Control	4.5 ± 0.2	4.4 ± 0.5	3.4 ± 0.5	33.5 ± 3.5	2.0 ± 0.2	17.0 ± 1.5	2.4 ± 0.2	11.5 ± 1.8	61.9 ± 4.1	49.4 ± 3.5	2.3 ± 0.5	100.3 ± 11.9	227.7 ± 18.2
Elevated pH	6.2 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	40.2 ± 3.7	2.4 ± 0.3	17.9 ± 1.6	1.7 ± 0.3	4.9 ± 1.2	57.4 ± 6.1	64.4 ± 14.1	7.5 ± 1.4	135.5 ± 19.1	271.4 ± 37.1
Elevated P	4.6 ± 0.2	3.0 ± 0.6	2.1 ± 0.5	40.5 ± 4.3	2.5 ± 0.2	16.6 ± 1.8	15.9 ± 1.8	11.3 ± 2.6	94.9 ± 6.8	86.6 ± 6.0	11.5 ± 2.7	135.1 ± 15.9	355.4 ± 26.6
Elevated pH + P	6.0 ± 0.2	1.0 ± 0.3	0.4 ± 0.2	39.2 ± 2.8	2.2 ± 0.2	18.2 ± 1.2	6.9 ± 1.5	7.1 ± 1.3	66.1 ± 4.6	59.4 ± 2.9	12.6 ± 1.8	124.8 ± 11.2	276.2 ± 19.5
P-values													
R	0.057	**0.023**	**0.049**	**<0.001**	0.054	0.393	**0.001**	**0.004**	0.108	**0.012**	0.084	0.811	0.108
pH	**<0.001**	**<0.001**	**<0.001**	0.871	0.478	0.562	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.354	**0.012**
P	0.688	0.643	0.568	0.746	0.395	0.109	**<0.001**	0.089	**<0.001**	**<0.001**	**<0.001**	0.092	**<0.001**
R × pH	0.767	**<0.001**	**<0.001**	0.107	0.098	0.504	0.435	**0.001**	0.844	0.055	0.363	0.453	0.239
R × P	0.605	0.558	0.080	0.317	0.616	0.570	0.454	0.417	0.131	0.334	**0.008**	0.939	0.218
pH × P	0.127	0.119	0.098	0.294	**0.047**	0.559	**0.005**	0.434	0.079	**0.014**	0.056	**0.041**	**0.016**
R × pH × P	0.700	0.462	**0.013**	0.694	0.597	0.935	0.583	0.791	0.090	**0.034**	**0.029**	0.328	0.081

*Significant P-values shown in bold (α = 0.05).

[a] Ex Al—exchangeable aluminum.

[b] Bicarb P—bicarbonate extractable phosphorus.

Unglaciated plots had a significantly higher abundance of PLFA biomarkers used to estimate non-AM fungal biomass (18:2ω6, 18:2ω9c and 18:0) compared to glaciated plots and the biomarker for AM fungal biomass (16:1ω5c) was greater in elevated compared to ambient pH treatments, although this differences was only approaching significance (Fig. 2; Table S2, Supporting Information). Collectively, this contributed to significantly higher amounts of total fungal biomarkers and significantly lower bacterial:fungal ratio in elevated pH and elevated pH + P plots compared to ambient pH plots (control and elevated P), as well as in the unglaciated region compared to the glaciated region (Fig. 2; Table S2, Supporting Information). Bacterial and total microbial biomass did not differ between regions or treatments (Fig. 2; Table S2, Supporting Information).

Arbuscular mycorrhizal fungal communities

AM fungal root biomass was four times greater in the glaciated compared to the unglaciated region, and treatment differences were not observed (Table S3, Supporting Information). However, the colonization rate of roots (18S rRNA gene copies g⁻¹ dry root tissue) in elevated pH plots was nearly double that of ambient pH plots and 1.7 times greater in the unglaciated than in the glaciated region (Fig. 1; Table S3, Supporting Information). Because of the greater mass of AM roots in the samples from glaciated plots, when 18S rRNA gene copy number was expressed as AM fungal abundance (i.e. standardized to the mass of roots per soil core), the pH effect was masked in the unglaciated region and only significant in the glaciated region (pairwise $P < 0.001$). Further, the regional difference in AM fungal abundance was driven by the particularly high abundance levels in elevated pH plots in the glaciated region as compared to unglaciated plots in general (pairwise $P < 0.001$). Finally, no difference in AM fungal abundance or colonization was observed in response to elevating P (Fig. 1; Table S3, Supporting Information).

We successfully recovered 93 clones representing AM fungi from our samples, which separated into 38 OTUs when grouped with 97% sequence similarity (Table S1, Supporting Information). BLAST searches to the DDBJ/EMBL/GenBank databases confirmed that we recovered only AM fungi from our root samples and that all 93 sequences matched with high similarity for Glomeromycota taxa (Table S1, Supporting Information) (Note: we recovered no non-target sequences. Out of a total of 96 clones, three failed to sequence properly and could not be assigned to taxa.) These sequences are publicly available through DDBJ/EMBL/GenBank databases under accession numbers HG426184–HG426276.

The NMS ordination of AM fungal communities showed differences between regions and between elevated and ambient pH plots, with regions separating mostly along axis 2 and pH treatments separating along both axis 1 and 2 (Fig. 3). There was no clear difference between elevated and ambient P plots (Fig. 3). The NMS patterns were confirmed by three-Way PERMANOVA, which found significant effects of region ($F = 3.8$; $P < 0.01$) and pH treatment ($F = 3.3$; $P = 0.01$), but not P treatment ($F = 0.4$; $P = 0.8$). Complete results from the PERMANOVA can be found in Table S4 (Supporting Information). The AM fungal community shifts were correlated with a number of environmental variables, including all measured P fractions, except for the most readily available P fraction, resin P (Fig. 3; Table S5, Supporting Information). Hydroxide available SOC P, HCl extractable P and residual P pools were negatively correlated with axis 1, while bicarbonate P, hydroxide P and soil acidity, were positively correlated with axis 2. Total P and soil pH were correlated with both axes. These
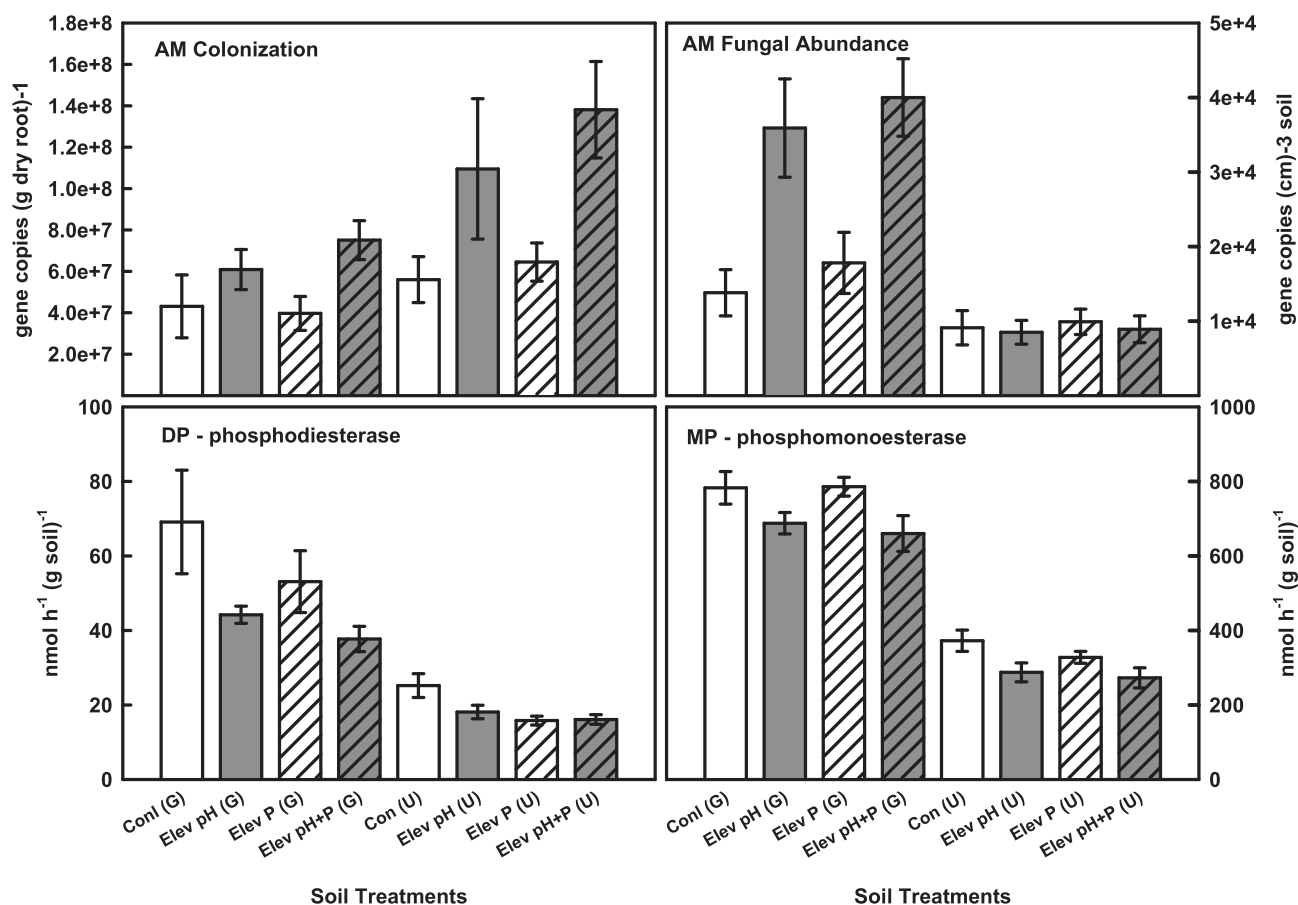
Figure 1. Bar graph showing mean (± standard error) AM fungal colonization (top left), AM fungal abundance (top right), phosphodiesterase activity (bottom left) and phosphomonoesterase activity (bottom right) in each treatment plot separated by region. Conl—control treatment (white bars), Elev pH—elevated pH treatment (gray bars), Elev P—elevated P treatment (white hashed bars), Elev pH + P—elevated pH + P treatment (gray hashed bars). G—glaciated region, U—unglaciated region.

correlations reflect differences in soil chemistry between the study regions in addition to those created by experimental lime addition (Table 1).

Through correlating the abundance of individual AM fungal TRFs with soil chemistry and enzyme activity, we found that some AM fungal TRFs were significantly negatively correlated with phosphatase activity in soil (Fig. 4) and that many were also significantly negatively correlated with bicarbonate extractable P and hydroxide extractable P, two bioavailable, but more recalcitrant pools of soil P (Fig. 5). We found no significant correlations for resin P among AM fungal TRFs.

Ectomycorrhizal fungal communities

The pyrosequencing effort yielded between 1700 and 3697 sequences for each pooled root sample within each forest for 24 treatment × site combinations and a total of over 72 000 sequences that met our quality control criteria. All sequences are publicly available through DDBJ/EMBL/Genbank under Bioproject number PRJNA239366 (Biosample accession number SAMN02727892). The sequencing effort found that the fungal communities associated with live EcM fungal tips were dominated by taxa of the Boletales, Cortinariaceae, Pezizales, Russulaceae, Sebacinaceae and Thelephoraceae (Fig. S3, Supporting Information). A single unidentified Basidiomycota OTU was the most abundant group in the glaciated ambient pH plots, totaling almost 22% of recovered sequences (Fig. S3 and Table S6, Sup-

porting Information) but the abundance of this group was much lower in other treatment plots.

While the rarefaction curves generated with the 454-sequencing OTUs begin to level off, they do not reach a full plateau (Fig. S4, Supporting Information). Compared to rarefaction curves generated with our previous direct sequencing effort in 2010, though, rarefaction curves from the current study suggest that this sequencing effort profiled the richness of EcM root associated fungi to a larger extent than our previous morphotyping and direct sequencing (compare Fig. S4, Supporting Information with fig. 2 from Kluber *et al.* 2012). In addition to the methodological differences between the two years, our EcM fungal morphotyping survey from 2010 eliminated any non-EcM fungal genera from our analyses, while the rarefaction curves shown here include all OTUs that were obtained with 454-sequencing. However, only 1.4% of the sequences and 5.2% of OTUs (166 out of 3174 OTUs) matched with non-EcM fungal taxa (see Materials and Methods section), indicating that the majority of sequences and OTUs included in our rarefaction analyses were EcM fungal taxa. As in Kluber *et al.* (2012), our findings also indicate that the EcM fungal richness was similar among treatments (Fig. S4, Supporting Information). Further, because our rarefaction curves within each treatment did not reach a plateau (despite the fact that this sequencing effort generated over 72 000 sequences that grouped into 3174 OTUs), a larger sequencing effort may yield additional OTUs (Fig. S4, Supporting Information).

Table 2. Mean ± standard error for enzyme activity measurements along with *P*-values showing the effect of region (R), pH treatment, and P treatment from the LME models with forest blocks as the random effect. Enzyme activities are expressed as nmol h^{-1} (g soil)$^{-1}$.

Treatment	BG[a]	Cell[b]	DP[c]	MP[d]	NAG[e]	LAP[f]
Glaciated						
Control	119.2 ± 19.4	25.7 ± 5.0	69.1 ± 13.9	782.7 ± 43.8	145.9 ± 35.9	78.5 ± 6.1
Elevated pH	131.9 ± 12.8	26.5 ± 3.3	44.2 ± 2.3	687.7 ± 28.8	119.4 ± 12.9	84.5 ± 4.9
Elevated P	139.1 ± 18.0	33.5 ± 6.2	53.1 ± 8.3	785.7 ± 25.3	132.8 ± 26.3	79.2 ± 3.5
Elevated pH + P	123.4 ± 12.0	26.4 ± 3.1	37.7 ± 3.4	660.0 ± 48.1	93.5 ± 11.1	78.5 ± 8.0
Unglaciated						
Control	40.3 ± 7.7	6.6 ± 1.6	25.2 ± 3.2	372.3 ± 28.7	61.5 ± 8.2	21.0 ± 5.7
Elevated pH	71.3 ± 20.5	14.2 ± 6.1	18.1 ± 1.8	287.4 ± 25.5	93.4 ± 16.3	24.3 ± 4.8
Elevated P	52.2 ± 15.0	9.8 ± 3.9	15.8 ± 1.2	327.7 ± 16.1	82.3 ± 22.5	22.4 ± 4.0
Elevated pH + P	52.5 ± 14.5	8.5 ± 4.0	16.1 ± 1.3	272.7 ± 27.1	83.3 ± 15.0	20.3 ± 5.6
P-values						
R	**0.015**	**0.019**	**0.001**	**<0.001**	0.151	**0.001**
pH	0.497	0.983	**0.003**	**<0.001**	0.625	0.644
P	0.913	0.660	**0.003**	0.333	0.569	0.574
R × pH	0.409	0.293	0.267	0.352	0.062	0.756
R × P	0.656	0.395	0.691	0.689	0.629	0.857
pH × P	0.157	0.164	0.237	0.993	0.254	0.384
R × pH × P	0.954	0.933	0.343	0.485	0.964	0.949

*Significant *P*-values shown in bold (α = 0.05).
[a]BG—β-1,4-glucosidase.
[b]Cell—cellobiohydrolase.
[c]DP—phosphodiesterase.
[d]MP—phosphomonoesterase.
[e]NAG—β-N-acetylglucosaminidase.
[f]LAP—leucine aminopeptidase.

Overall EcM fungal abundance (tip number cm^{-3}) was not significantly different across regions or treatments (Table S3, Supporting Information). However, NMS ordination of EcM fungal communities (Fig. 6) showed clear differences between regions and between elevated and ambient pH treatments, but a less clear effect of P treatment. A three-way PERMANOVA found significant regional differences ($F = 1.7$, $P = 0.04$) and pH treatment differences ($F = 2.0$, $P = 0.01$) in EcM fungal communities but no significant effects of P treatment ($F = 0.9$, $P = 0.5$), confirming NMS ordination results (Table S4, Supporting Information). We also found significant correlations between soil environmental conditions and axes of the ordination (Fig. 6; Table S5, Supporting Information). Total P content, soil C and the activity of three extracellular enzymes (including both phosphatases) were significantly correlated with axis 1, reflecting the EcM fungal community differences between regions, while pH and acidity were significantly correlated with both axes, reflecting changes in chemistry associated with lime addition (Fig. 6; Table S5, Supporting Information).

Indicator species analysis found that some taxa were significant indicators of either region or elevated pH plots (Table 3). For example, the EcM fungal genera *Genea, Inocybe, Laccaria* and *Scleroderma* were all indicators of glaciated forests, whereas the Cortinariaceae and the genus *Tricholoma* were all significant indicators of our unglaciated forests (Table 3). Additionally, Sebacinaceae and Thelephoraceae were significant indicators of elevated pH plots, Results from the full indicator species analysis, as well as the average percent sequence abundance for each taxa can be found in the Tables S6–8 (Supporting Information).

Analysis of specific EcM fungal taxa and their functional response to soil chemistry and enzyme activity found large variability. Some EcM fungal taxa were significantly negatively correlated with phosphatase activity in soil, whereas others were significantly positively correlated with phosphatase activity (Fig. 7). We found similar results for response to more recalcitrant pools of soil P (Fig. 8) with taxa displaying both positive and negative correlations. EcM fungal genera that were significantly correlated with phosphatase activity included *Genea* and *Inocybe* (short distance; mycelia morphology as per Agerer 2001) and *Laccaria* (medium distance), while the genus *Tricholoma* (medium distance) was significantly negatively correlated. Six genera were significantly correlated with bicarbonate available P (Fig. 8); positively correlated genera were *Laccaria* (medium distance) and *Leotia*, while *Otidea*, Cortinariaceae (medium distance) and *Tricholoma* (medium distance) were negatively correlated. None of the EcM fungal taxa that were significant indicators of lime addition were found to be significantly associated with enzyme activity or P pools. However, all the taxa that were positively correlated with enzyme activity were significant indicators of glaciated forests (*Genea, Inocybe* and *Laccaria*), while the one genus negatively correlated with enzyme activity (*Tricholoma*) was a significant indicator of unglaciated forests (Table 3; Fig. 7). Both Cortinariaceae and *Tricholoma* were significant indicators of unglaciated forests and were significantly negatively correlated with more recalcitrant P pools. Of additional interest was the response of the fungal taxa *Archaeorhizomyces*. Although it is not certain if this taxa forms EcM fungal relationships with tree roots, it is commonly found on plant roots and is not a plant pathogen (Rosling *et al.* 2011). We found that it was a significant indicator of unlimited plots (low-pH soils) and was significantly positively associated with both phosphatase enzymes as well as bicarbonate available P (Table 3; Figs 7 and 8).

DISCUSSION

The elevation of soil pH and P availability in our hardwood forest plots resulted in treatment differences in the belowground
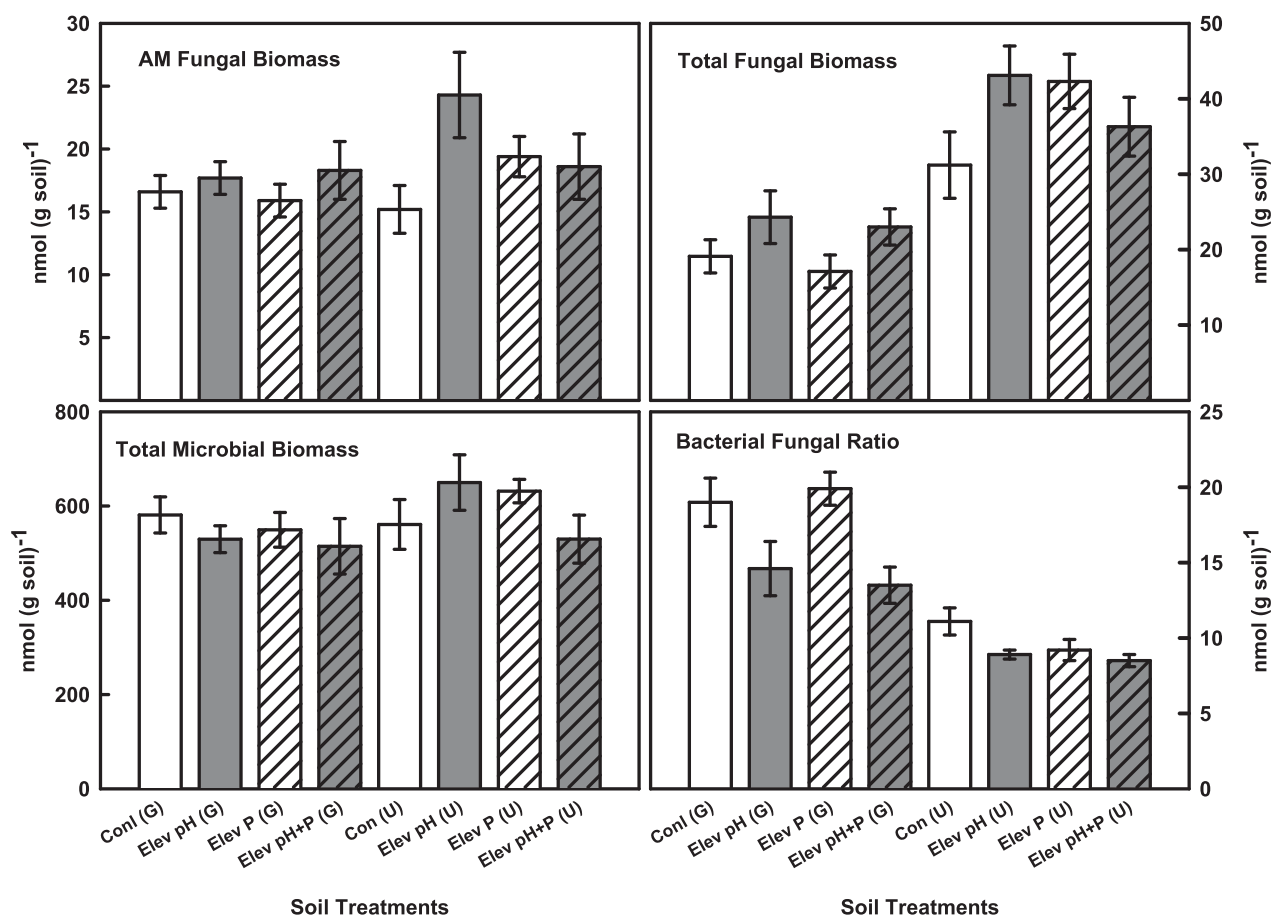
Figure 2. Bar graph showing mean (± standard error) AM fungal biomass (top left), total fungal biomass (top right), total microbial biomass (bottom left) and bacterial to fungal ratio (bottom right) in each treatment plot separated by region. Biomass was estimated using PLFA (details regarding the biomarkers used for each estimate are located in Table S2, Supporting Information). Conl—control treatment (white bars), Elev pH—elevated pH treatment (gray bars), Elev P—elevated P treatment (white hashed bars), Elev pH + P—elevated pH + P treatment (gray hashed bars). G—glaciated region, U—unglaciated region.

environment two years after initiation of the study. We provide evidence that soil pH and P availability influence the community structure of mycorrhizal fungi and the functional activity of soil microbes. Despite the large effect that P amendment had on the measured P pools, both AM and EcM fungi were more responsive to elevated pH than elevated P. Because elevating pH also decreased the activity of phosphatase enzymes, we reason that elevating pH not only influenced the community structure of mycorrhizal fungi, but also affected the overall function of soil microbes in terms of their P acquisition strategies. Decreases in phosphatase enzymes suggest a switch from reliance on P_o to P_i and reflect a possible overall change in biological P demand with pH elevation. Since the changes we observed to P-acquiring enzymes and soil P availability were accompanied by changes to the community structure of AM and EcM fungi, and the abundance of some taxa were significantly associated with P-acquiring enzymes and soil P availability, our findings suggests that P functional groups exist within both AM and EcM fungi. Changes to these groups could affect plant nutrient acquisition in forests undergoing ecosystem acidification.

Effects of pH on soil chemistry and P availability

Elevating soil pH in the current study suppressed the production of both phosphodiesterase and phosphomonoesterase, which has been a consistent trend among previous studies using

this experiment (DeForest *et al.* 2012; Kluber *et al.* 2012). These P-acquiring enzymes are produced by soil microorganisms, as well as plant roots, to access insoluble P_o; thus, a suppression of P-acquiring enzymes is an indicator of reduced biological demand for P (Olander and Vitousek 2000), and may, in effect, be a more biologically relevant estimate of available P than labile P fractions. For example, Shaw and DeForest (2013) found the labile (i.e. resin) P pool to be a small, highly variable pool, that turns over within a week. We suggest that this pH-induced liberated P does not accumulate in the soil solution, but rather is quickly assimilated by soil biota and plants. The uptake of P is known to be facilitated at higher soil pH (Holford 1997; Hinsinger 2001), which can explain why the resin P pool accumulated in the elevated P plots compared to the elevated pH and elevated pH + P treatments (Table 1). Since the elevated P plots were at ambient soil pH, the more acidic conditions could have diminished the capacity for P uptake in microbes and plants. Greater P uptake in higher pH soil may also help explain the greater mycorrhizal colonization and fungal biomass in the elevated pH and elevated pH + P plots.

General effects of pH on mycorrhizal fungi

In addition to changes in P pools and P-acquiring enzymes, we found that elevated pH plots had higher AM fungal colonization (qPCR data) as well as greater overall fungal biomass (PLFA
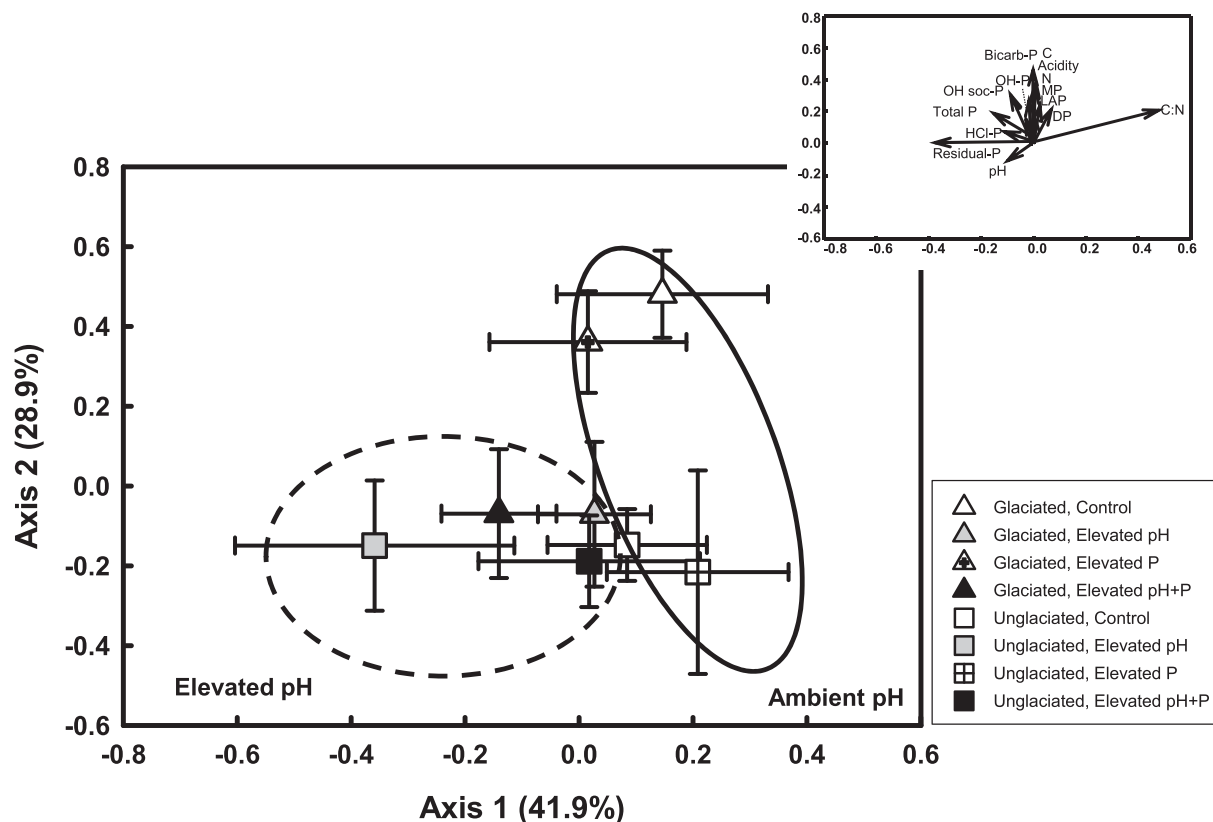
Figure 3. Non-metric multidimensional scaling ordination of AM fungal communities based on terminal restriction fragment length polymorphism analysis of the 18S rRNA gene. The proportion of variance explained by each axis is shown. Cumulative variance was $r^2 = 0.921$ and final stress of the ordination was 11.5%. Centroids and error bars represent the mean and standard errors of axes scores within a given treatment with symbol shapes and colors representing different regions and treatments, as indicated by the legend. Inset is a joint plot of significantly correlated ($P < 0.05$ for $r > 0.232$; $n = 72$) environmental variables. The joint plot vector lengths indicate the strength and direction of the correlations, but for clarity, Pearson correlation coefficients are also reported in Table S5 (Supporting Information).

data), and these changes were accompanied by altered AM and EcM fungal community structure; results not observed in our previous study. We found the Sebacinaceae and Thelephoraceae were significant indicators of pH elevation, and species in these families can often produce substantial extra-radical hyphae [e.g. short, medium and long distance exploration types as per Agerer (2001)]. We reason that the higher AM fungal colonization, and potentially higher fungal biomass, observed in elevated pH and elevated pH + P plots could be attributed to greater fungal growth and hyphae production with the pH manipulation. This is consistent with a number of studies that have found an effect of soil pH on mycorrhizal fungal growth (reviewed in Lauber *et al*. 2009), including van Aarle, Olsson and Söderström (2002), who found an increase in hyphal growth in conditions of higher soil pH. In general, the soil chemistry changes associated with acid deposition has been shown to decrease mycorrhizal colonization of both AM and EcM fungi (reviewed in Rousk, Brookes and Bååth 2010), which is also consistent with our study, where mitigating the effects of acid deposition led to increases in AM fungal colonization. While this conclusion of potentially greater fungal growth, along with the result of a lower ratio of bacterial:fungal biomass in elevated pH plots, seems contradictory to published studies showing that high-pH soils favor bacteria over fungi (e.g. Rousk, Brookes and Bååth 2009), it should be noted that the pH manipulation in the current study raised pH from 4 to 6, on average. Rousk, Brookes and Bååth (2009) found a substantial increase in fungal growth between soil pH 4 and 4.5 and in fungal biomass between soil pH 4 and 6. We sug-

gest that our results may be consistent with an increase in fungal growth and biomass across a narrow pH range. Finally, our PLFA results are marginally suggestive of an increase in AM fungal biomass with elevated pH. We found higher PLFA 16:1ω5c (a biomarker of AM fungi) at higher soil pH; a result consistent with other studies (Frostegård, Bååth and Tunlid 1993; Rousk, Brookes and Bååth 2010). However, PLFA results need to be interpreted with caution as they may not be straightforward indicators of biomass for certain groups, their specificity can be limited to specific cases (Frostegård, Tunlid and Bååth 2011), and this marker, in particular, can confound AM fungi and some groups of Gram-negative bacteria. Nevertheless, AM fungi are known to be less common in acidic soil conditions (van Aarle, Olsson and Söderström 2002), thus supporting the observed increase in AM fungal colonization (and potentially biomass) in our elevated pH and elevated pH + P treatments. This increase in colonization, along with AM and EcM fungal community structure changes with pH alteration are consistent with our hypothesis that mycorrhizal fungi would respond to pH-induced changes in P availability since pH elevation also affected P fractions and phosphatase activity.

An alternate explanation to the mycorrhizal changes with pH alteration is that elevating pH lifted a physiological restraint on fungal growth and activity in the elevated pH and elevated pH + P plots and shifted the community toward taxa better able to grow and survive in higher pH soils. External pH (and, therefore, the external concentration of hydrogen ions) is known to affect cellular function—namely, the proton motive force of the
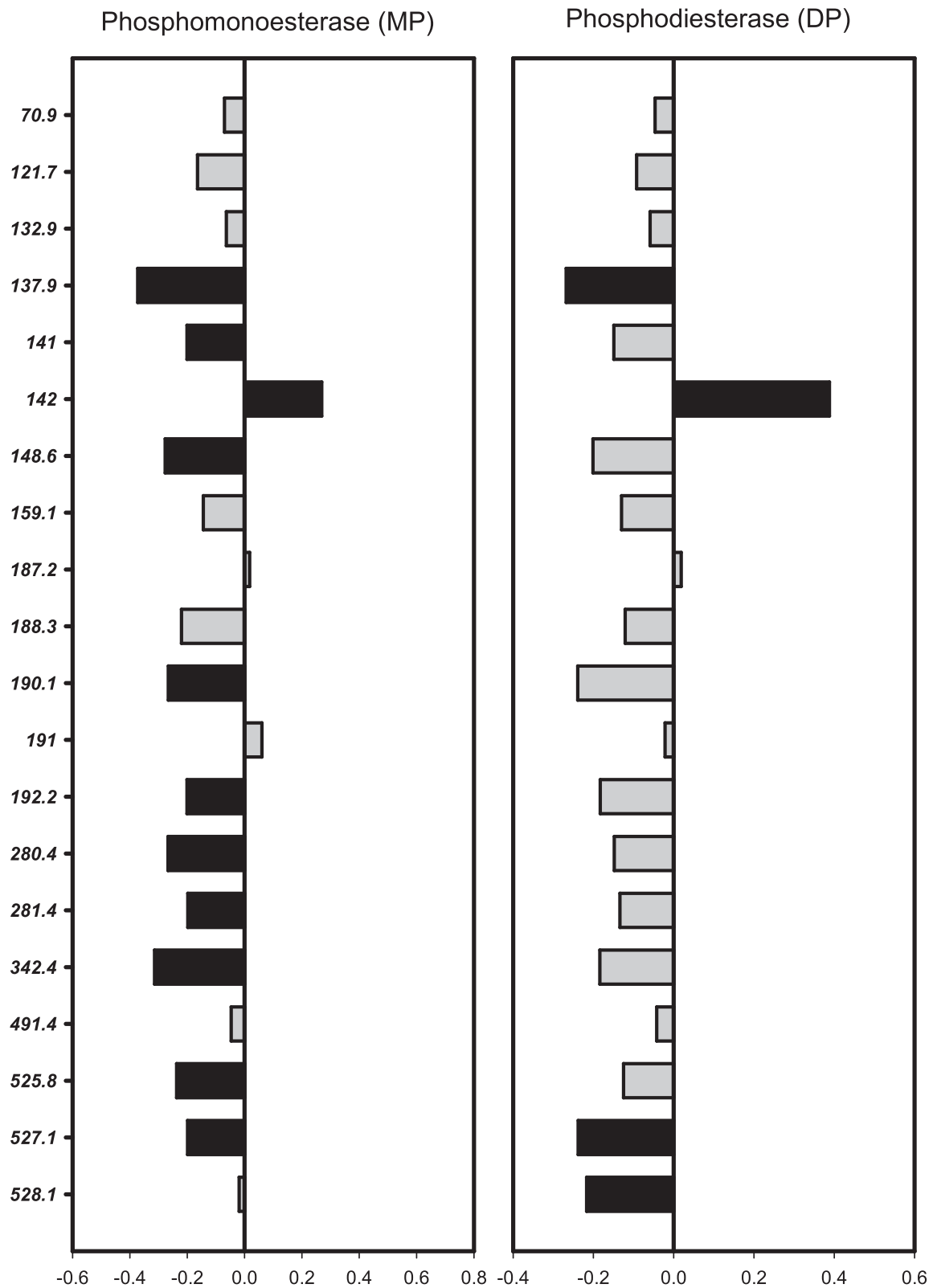
Figure 4. Pearson correlations between AM detected TRFs and soil phosphatase activity. Significant correlations are represented by black bars, whereas gray bars represent nonsignificant correlations ($P < 0.05$ for $r > 0.232$; $n = 72$). The AM TRFs detected on tree roots through molecular methods are listed along the y-axis. Correlations are positive if bars extend to the right side of the figure (>0), and correlations are negative if the bars extend to the left side of the figure (<0). The correlation coefficient (r) is represented along the *x*-axis. Since different sequence OTUs can produce similar TRFs, it is not possible to clearly separate TRFs into AM fungal species with this method.

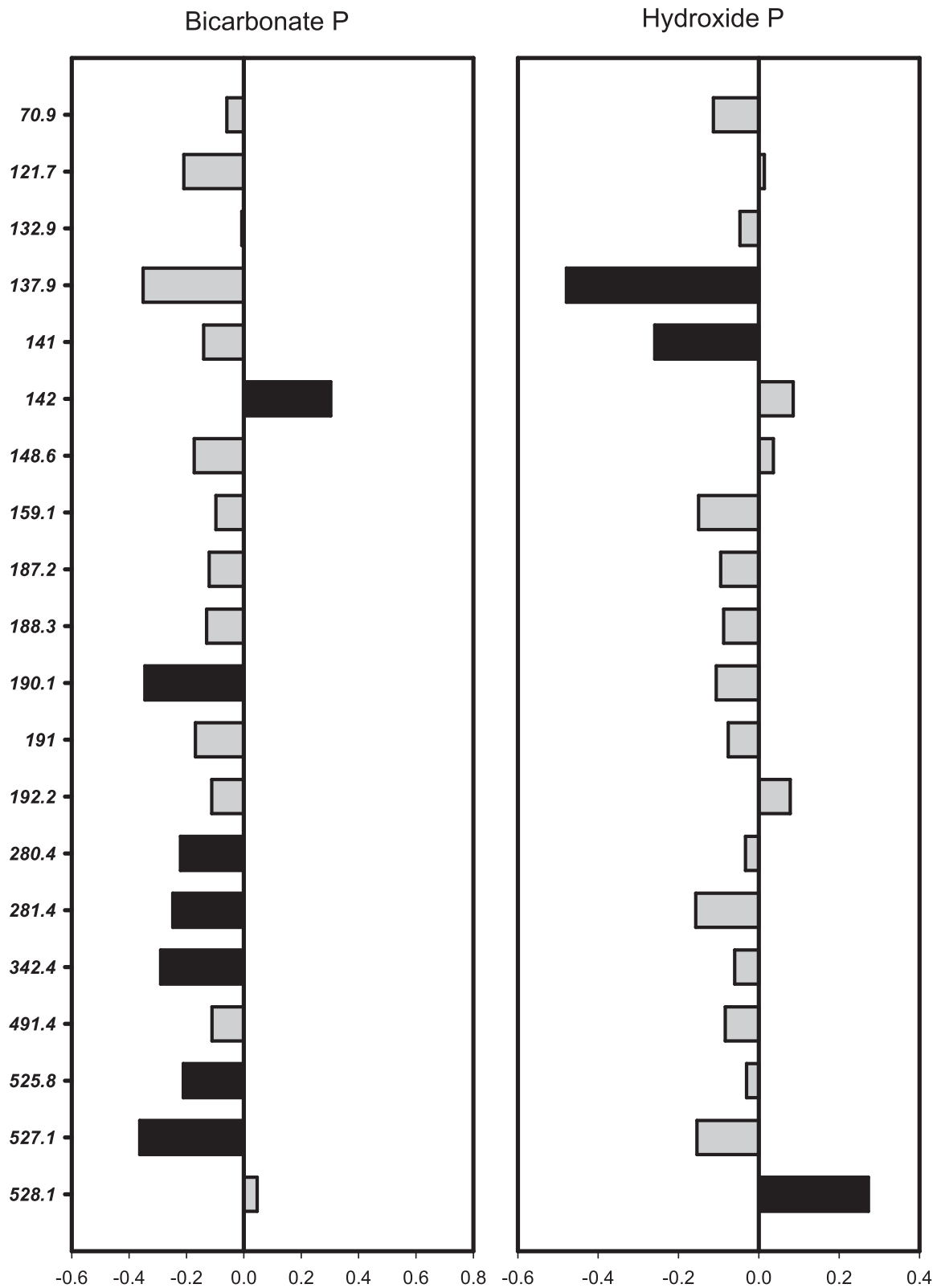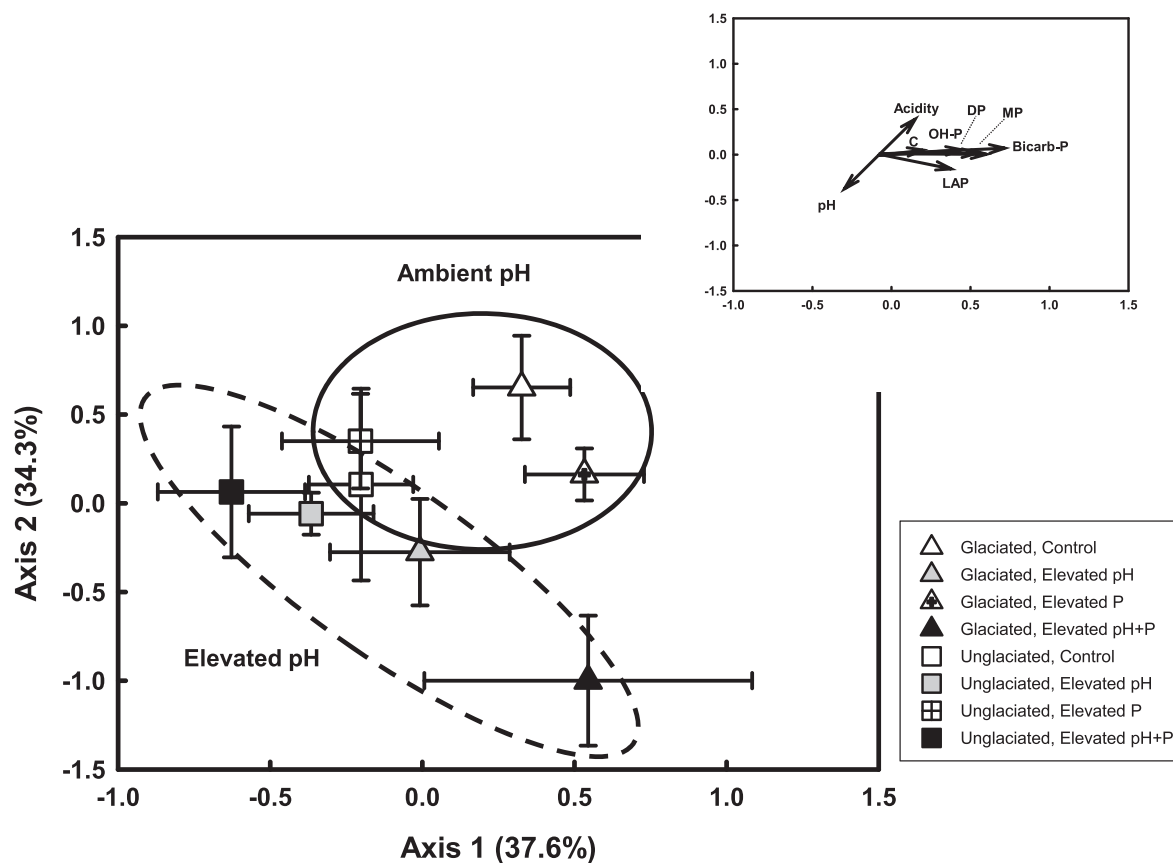**Figure 5.** Pearson correlations between AM detected TRFs and soil bicarbonate extractable P and hydroxide extractable P, two less biologically available P pools. Significant correlations are represented by black bars, whereas grey bars represent nonsignificant correlations ($P < 0.05$ for $r > 0.232$; $n = 72$). The AM TRFs detected on tree roots through molecular methods are listed along the y-axis. Correlations are positive if bars extend to the right side of the figure (>0) and correlations are negative if the bars extend to the left side of the figure (<0). The correlation coefficient (r) is represented along the x-axis. Since different sequence OTUs can produce similar TRFs, it is not possible to clearly separate TRFs into AM fungal species with this method.

Figure 6. Non-metric multidimensional scaling ordination of EcM fungal community based on 454-pyrosequencing. The proportion of variance explained by each axis is shown. Cumulative variance was $r^2 = 0.880$, and final stress of the ordination was 11.5%. Centroids and error bars represent the mean and standard errors of axes scores within a given treatment, as indicated by the legend. Inset is a joint plot of significantly correlated ($P < 0.05$ for $r > 0.404$; $n = 24$) environmental variables. The joint plot vector lengths indicate the strength and direction of the correlations, but, for clarity, Pearson correlation coefficients are also reported in Table S5 (Supporting Information).

cytoplasmic membrane—with some taxa better able to survive in low pH conditions than others (Booth 1985). Our elevated pH treatments may have lifted this cellular restraint, thus increasing mycorrhizal growth and activity and shifting the community structure in the elevated pH treatments. While it is not possible to deduce whether the differences in mycorrhizal colonization or community structure between elevated and ambient pH plots of the current study are a result of the lifting of physiological limitations or of P limitation, our results are consistent with a number of studies that have documented an effect of variation in soil pH on structuring soil microbial communities (Bååth and Anderson 2003; Fierer and Jackson 2006; Lauber *et al.* 2009; Rousk *et al.* 2010), shifting mycorrhizal fungal community structure (Erland and Söderström 1990; Lehto 1994; Wallander *et al.* 1997; Kjøller and Clemmensen 2009; Rineau and Garbaye 2009), altering the presence of fungal hyphae (Lehto 1994; Bakker, Garbaye and Nys 2000; van Aarle, Olsson and Söderström 2002), or changing mycorrhizal fungal root colonization (Ouimet, Camiré and Furlan 1995; Coughlan *et al.* 2000). It cannot be ignored that our elevated pH and elevated pH + P plots likely had a substantial increase in calcium content and, for AM fungi, increased colonization and biomass have been shown to be affected by calcium content of soil (Juice *et al.* 2006) and plant tissue (Ouimet, Camiré and Furlan 1995). It is possible that increases in calcium may be partly responsible for the observed effects on mycorrhizal fungi, but the strong correlations we observed between mycorrhizal

fungal community structure and soil P measurements suggest that P availability played a role.

In addition to the treatment effects on mycorrhizal fungi, regional differences were also apparent, as both EcM and AM fungal community structure, as well as AM fungal colonization, abundance, and AM fungal root biomass, varied between regions. Such regional differences for mycorrhizal communities are not surprising given the high degree of spatial variation for soil fungi (Izzo, Nguyen and Bruns 2006; Dumbrell *et al.* 2010; Shahin *et al.* 2013) and the inability of fungi to disperse readily between the two regions. Although all six forest blocks were located in mixed hardwood forests, glaciated forests had a higher percentage of AM trees, and especially *Acer* spp., compared to unglaciated forests (DeForest *et al.* 2012) and these differences in tree relative dominance could also have contributed to the regional differences we observed. Despite these differences, though, both sites experienced a similar response to pH treatment - the mycorrhizal fungal community structure changes, mycorrhizal fungal colonization, phosphatase suppression, and decreases in biologically available P measurements in the elevated pH and elevated pH + P plots were consistent across regions, which differ in their soil age and soil developmental processes.

In our previous work, which examined the responses of soil microbial and mycorrhizal fungal communities one year following the establishment of the treatment plots, the effects of

Table 3. Indicator species analysis comparing EcM fungal communities between ambient and elevated pH plots and physiographic regions.

Taxa	Max group[a]	Indicator value	Mean	Std. Dev.	*p*
Archaeorhizomyces	Ambient pH	61.8	28.8	8.37	0.0048
Herpotrichiellaceae unid	Elevated pH	59.0	49.9	6.28	0.0894
Helotiales unid	Ambient pH	59.4	53.8	2.86	0.043
Helvella	Elevated pH	33.3	16.5	6.91	0.0894
Clavicipitaceae unid	Ambient pH	33.3	16.1	7.14	0.0902
Sebacinaceae unid	Elevated pH	73.8	55.2	6.75	0.0064
Thelephoraceae unid	Elevated pH	60.0	54.9	3.62	0.0978
Basidiomycota unid	Ambient pH	70.5	58.8	5.15	0.0152

<div align="center">Taxa that are indicators of either glaciated or unglaciated regions during autumn 2011</div>

Taxa	Max group[1]	Indicator value	Mean	Std. Dev.	*p*
Ascomycota unid	Glaciated	33.3	16.4	7.1	0.0944
Genea	Glaciated	47.6	31.1	8.44	0.04
Chaetosphaeria	Glaciated	50.0	22.3	7.86	0.0146
Bionectriaceae unid	Glaciated	33.3	16.5	7.11	0.0976
Cordyceps	Unglaciated	51.9	36.0	8.82	0.0644
Hypocreaceae unid	Unglaciated	61.5	49.3	7.35	0.0778
Inocybe	Glaciated	62.1	43.3	10.68	0.066
Cortinariaceae unid	Unglaciated	44.9	31.4	8.63	0.0894
Laccaria	Glaciated	40.4	22.3	8.35	0.039
Tricholoma	Unglaciated	45.1	28.7	8.35	0.052
Scleroderma	Glaciated	41.7	19.8	7.76	0.0402
Craterellus	Unglaciated	41.7	19.4	7.57	0.0384
Glomeraceae unid	Glaciated	41.7	19.3	6.96	0.037

Only taxa that were significant indicators of treatments are shown. A total of 5000 randomizations were used for the Monte Carlo test. Max Group identifies the group with the highest indicator value for those fungal taxa. Results from the full indicator species analysis can be found in the Tables S7–8 (Supporting Information).

P addition and pH manipulation on belowground communities were not clear (DeForest *et al.* 2012; Kluber *et al.* 2012). For example, we previously found only minor effects of treatment on EcM fungal communities and no treatment effects were observed for AM fungal communities (Kluber *et al.* 2012). One year later, treatment differences between elevated and ambient pH plots were clear. Since the distribution of fungi within a forest stand can be highly site specific, (Izzo, Nguyen and Bruns 2006; Shahin *et al.* 2013), high levels of variability within and across stands is expected thus requiring additional time for a clear response pattern to emerge from the underlying noise. It is also possible that the increasing strength of community responses over time were due to methodological differences, at least for the EcM fungal community, because of the change in profiling technique; in our first year we used direct sequencing of morphotyped root tips (Kluber *et al.* 2012) and 454-sequencing during year two. However, the methods to describe AM fungi between years were consistent, and we observed a similar differentiation of the AM fungal communities between treatments that was observed for the EcM fungal community. Thus, we suggest an overall acclimation of the mycorrhizal fungi to the new environmental conditions that took multiple years to manifest.

Do mycorrhizal functional groups exist within forests?

Although changes in pH affected both AM and EcM fungal community structure, these broad groups of fungi may have responded differently based on their P-acquiring mechanisms, further suggesting that changes to P availability could underlie the response of mycorrhizal fungi to pH treatment. For example, AM fungal taxa were generally negatively correlated with the more recalcitrant available P pools (bicarbonate and hydroxide P) and phosphatase activity, whereas EcM fungal taxa displayed more variable responses, with some taxa positively or negatively correlated with these soil quality metrics. This suggests niche separation between these broad fungal groups and highlights differences in their functional roles within forest systems, with AM fungi not associated with organic P acquisition or cycling whereas some EcM fungi participate in these activities. Overall community structure changes were correlated with the more recalcitrant P pools and phosphatase activity for both EcM and AM fungi, possibly due to shifts toward taxa more reliant on recalcitrant forms of P or more efficient at liberating P_o in acidic conditions. For EcM fungi, these could be taxa capable of producing organic acids or phosphatase enzymes (Jongmans *et al.* 1997; Smith and Read 2008; Courty *et al.* 2010; Burke, Smemo and Hewins 2014), while for AM fungi these could be taxa capable of immobilizing Al or altering rhizosphere pH to mobilize P (Smith and Read 2008; Aguilera *et al.* 2011).

While some EcM fungal taxa were significantly correlated with enzyme activity or P availability, we found no general relationship between EcM fungal taxa that were significant indicators of pH treatment and their functional activity correlations, highlighting the variable responses of the EcM fungi in our study. As we hypothesized, our data do indicate the presence of P functional groups within the EcM fungi with many taxa of short- and medium distance mycelia morphology having significant correlations with soil phosphatase activity. However, our analysis of EcM fungal functional roles is rather conservative since we pooled soil cores within plots for our analysis. We would expect fungi to respond to microsite variability in soil chemistry (Burke *et al.* 2009), and by pooling samples, we may have obscured the signal of EcM fungal response to soil chemistry. For example, the genus *Russula* is frequently associated with phosphatase activity under field conditions (Courty *et al.* 2005) and in pure culture (Burke, Smemo and Hewins 2014), but we found
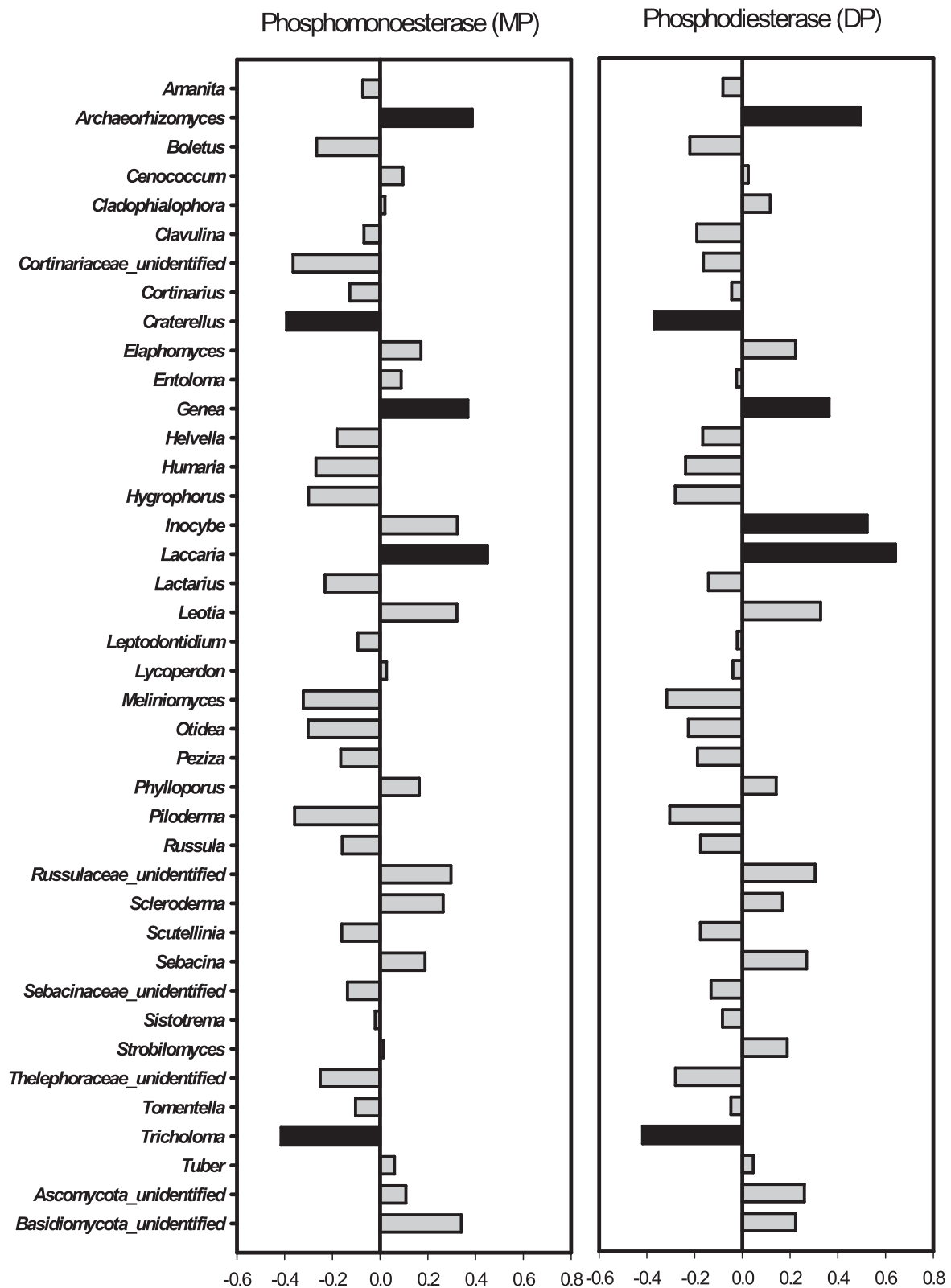
Figure 7. Pearson correlations between EcM fungal taxa and soil phosphatase activity. Significant correlations are represented by black bars, whereas grey bars represent nonsignificant correlations ($P < 0.05$ for $r > 0.404$; $n = 24$). The EcM fungal taxa detected on tree roots through 454-sequencing are listed along the y-axis. Correlations are positive if bars extend to the right side of the figure (>0) and correlations are negative if the bars extend to the left side of the figure (<0). The correlation coefficient (r) is represented along the x-axis.
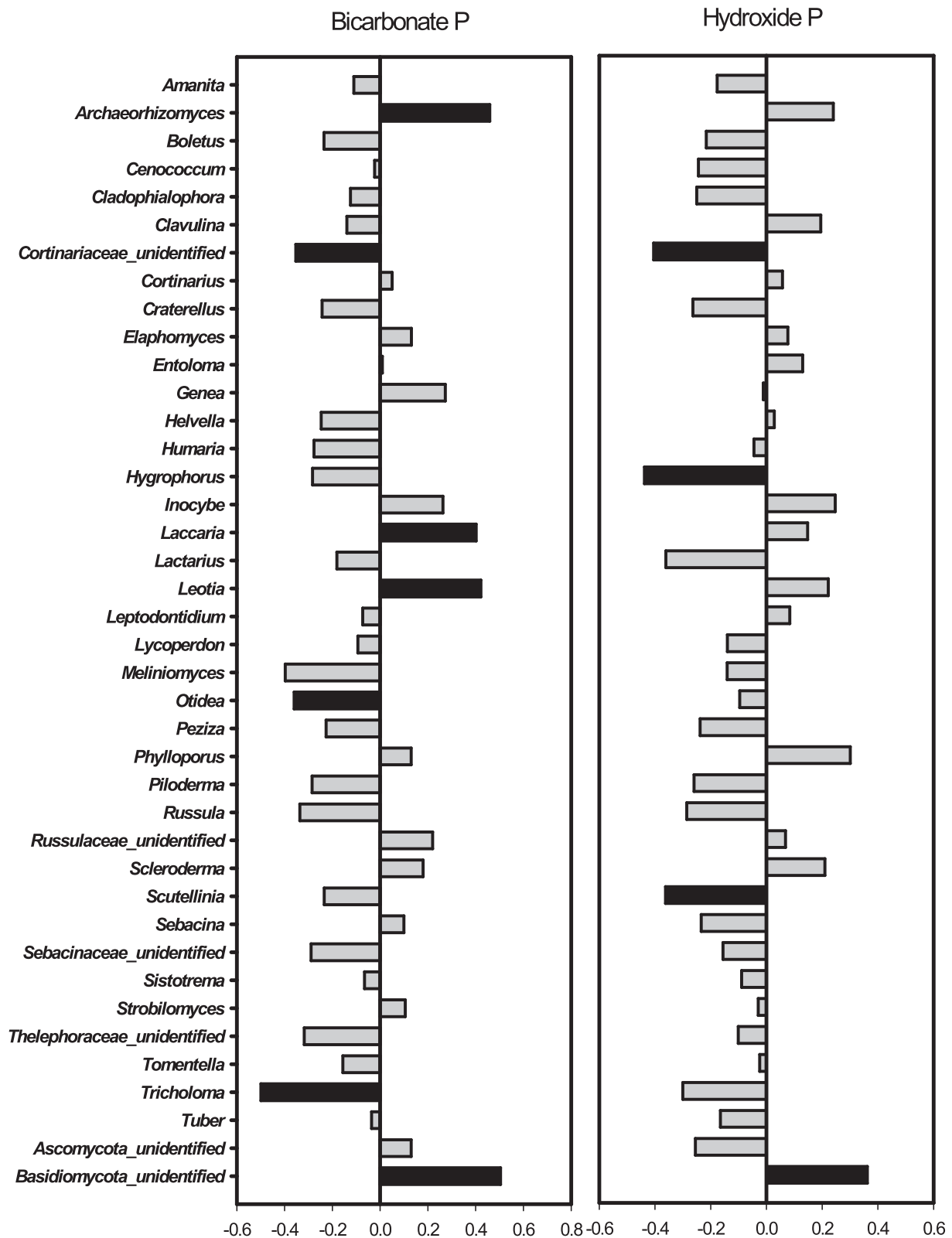
Figure 8. Pearson correlations between EcM fungal taxa and soil bicarbonate available P and hydroxide extractable P, two less biologically available P pools. Significant correlations are represented by black bars, whereas grey bars represent nonsignificant correlations ($P < 0.05$ for $r > 0.404$; $n = 24$). The EcM fungal taxa detected on tree roots through 454-sequencing are listed along the y-axis. Correlations are positive if bars extend to the right side of the figure (>0) and correlations are negative if the bars extend to the left side of the figure (<0). The correlation coefficient (r) is represented along the *x*-axis.

no evidence for a significant relationship between *Russula* taxa and phosphatase activity in the current study. We suggest that to fully describe EcM fungal taxa belonging to the P functional guild, studies examining microsite behavior of fungi and pure culture experiments are needed. Nonetheless, although our results are conservative, it argues for the existence of an EcM fungal P functional group, which could increase plant P acquisition under acid-induced P limitation.

Summary

We found that two years after increasing soil pH and P availability in six temperate hardwood forests, mycorrhizal fungi responded to elevation in soil pH with increases in biomass (AM fungi), increases in root colonization (AM fungi) and shifts in mycorrhizal fungal community structure (AM and EcM fungi). Elevating pH also led to a downregulation in the production of extracellular enzymes used by soil microbes to access organic forms of P and a reduction in bioavailable P pools, suggesting that changes to soil P availability, which are inherently linked to soil pH, may be a factor underlying the changes in mycorrhizal fungal colonization, biomass and fungal community structure seen in the current study. The results presented here provide evidence for our hypothesis that soil pH affects the P acquisition strategy of soil fungal and microbial communities and that under acidic conditions mycorrhizal fungal communities, as well as soil microbial communities, will be dominated by taxa reliant on and efficient at liberating P_o from soil organic matter. We predict that as pH increases and P_i becomes more available in our long-term research plots, these fungal and microbial communities will become increasingly dominated by taxa less efficient at liberating P_o from soil organic matter. We suggest that the shift in the P acquisition strategy of root fungal communities is an important mechanism driving the apparent lack of P limitation above ground in temperate hardwood forests, which have experienced soil acidification and chronic effects of acid and N deposition.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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