Phosphorus cycling in deciduous forest soil differs between stands dominated by ecto- and arbuscular mycorrhizal trees

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Summary

- Although much is known about how trees and their associated microbes influence nitrogen cycling in temperate forest soils, less is known about biotic controls over phosphorus (P) cycling. Given that mycorrhizal fungi are instrumental for P acquisition and that the two dominant associations – arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi – possess different strategies for acquiring P, we hypothesized that P cycling would differ in stands dominated by trees associated with AM vs ECM fungi.
- We quantified soil solution P, microbial biomass P, and sequentially extracted inorganic and organic P pools from May to November in plots dominated by trees forming either AM or ECM associations in south-central Indiana, USA.
- Overall, fungal communities in AM and ECM plots were functionally different and soils exhibited fundamental differences in P cycling. Organic forms of P were more available in ECM plots than in AM plots. Yet inorganic P decreased and organic P accumulated over the growing season in both ECM and AM plots, resulting in increasingly P-limited microbial biomass.
- Collectively, our results suggest that P cycling in hardwood forests is strongly influenced by biotic processes in soil and that these are driven by plant-associated fungal communities.

Introduction

Soil age and climate play a central role in determining the extent to which nitrogen (N) and phosphorus (P) limit biotic processes in forests (Elser et al., 2007; Vitousek et al., 2010; Harpole et al., 2011). In pedogenically ‘young’ soils (e.g. those in temperate and boreal regions), N generally limits forest productivity (Finzi, 2009; Weand et al., 2010; Groffman & Fisk, 2011), whereas P generally limits productivity in highly weathered tropical soils (Walker & Syers, 1976; Turner et al., 2007) due to the formation of recalcitrant inorganic and organic P forms, erosion and leaching losses (Walker & Syers, 1976; Turner et al., 2007). However, in unglaciated temperate forest soils, such as those found in the Midwestern USA, plant and microbial functions may be P-limited. For example, P addition decreased P-releasing enzymatic activity in southern Ohio (DeForest et al., 2012). In addition, this region has high rates of N deposition, which may further exaggerate P-limitation (Jonard et al., 2015). Given the vital role of temperate forests in the land carbon (C) sink (Xiao et al., 2011) and the emerging view that both N and P limit biotic processes in soil and that these are driven by plant-associated fungal communities.

Phosphorus cycling in soil is known to be under both biological and geochemical control, with biological processes generally found to dominate in topsoil where root and microbial density is high (Wood et al., 1984; Achat et al., 2012). Phosphorus is available for uptake by plants and microbes only in its ionic forms (Plassard & Dell, 2010), and the low mobility of P ions in soil results in sharp depletion zones around roots (Lewis & Quirk, 1967). Forest ecosystems can remain productive despite chronically low soil P availability, because microbes can enrich this pool by decomposing P-rich organic matter and weathering P-rich minerals (Johnson et al., 2003; Zhao et al., 2009). Hence, the soil microbial biomass, which can account for up to 78% of the total biomass P in temperate forest soils (Turner et al., 2013), may be the primary driver of P dynamics in these soils (Richardson & Simpson, 2011).

The majority of plants form symbiotic associations with mycorrhizal fungi to acquire P (Plassard & Dell, 2010; Jansa et al., 2011). Increased C allocation to roots and their mycorrhizal partners is a way for plants to alleviate P deficiency (Wallander & Nylund, 1992; van der Heijden, 2001; Kiers et al., 2011). The two types of mycorrhizal fungi that associate with trees, ectomycorrhizal (ECM) fungi and arbuscular mycorrhizal (AM) fungi, both significantly increase P acquisition to plants, primarily by increasing the surface area for P uptake by colonizing soil beyond the root depletion zone (Jones et al., 1998; Smith & Read, 2008; Smith et al., 2011). ECM and AM fungi differ in what substrates from which they can access P. ECM fungi produce enzymes to hydrolyze organic P forms in soil (Häussling & Marschner,
1989; Read & Perez-Moreno, 2003), whereas AM fungi produce these enzymes to a much lesser extent (Joner et al., 2000; Koide & Kabir, 2000). ECM fungi also produce low molecular weight organic acids and chelators (Plassard & Dell, 2010) that have been thought to play a major role in the weathering of minerals (Hoffland et al., 2004). Interestingly, such organic weathering agents are now proposed to play a central role in the destabilization and subsequent decomposition of organic matter in soil (Clarholm et al., 2015). Although there seem to be less substantial differences between AM- and ECM-dominated forests in mineral weathering than previously assumed (Koele et al., 2014), the extent to which soils dominated by ECM- or AM-forming trees vary in their P cycling characteristics is still unknown.

In this study, we compared forest plots dominated either by AM- or ECM-forming trees, but underlain by the same parent materials, to assess the degree to which mycorrhizal association influences P cycling. In a previous investigation, we reported that the upper surface soils of AM-dominated plots have higher pH, lower organic matter content and lower acid phosphatase activity than ECM-dominated plots at this site (Phillips et al., 2013). Hence, we hypothesized that P would be cycled mostly in inorganic forms in soils dominated by AM trees, but in organic forms in soils dominated by ECM trees. Further, because these forests are underlain by ‘unglaciated’ soils that have low P availability (DeForest et al., 2012), we hypothesized that biotic processes would drive seasonal P dynamics in this forest.

Materials and Methods

Site description

This research was conducted at Indiana University’s Moores Creek Research and Teaching Preserve, a c. 80-year-old temperate deciduous forest in south-central Indiana, USA (39°05’N, 86°27’W) with elevation ranging from 165 to 230 m asl. Soils are thin, unglaciated Inceptisols derived from siltstone, shale and, to a lesser extent, limestone. The forest naturally regenerated following the abandonment of farming c. 1930. The climate is humid continental with mean annual precipitation of 1200 mm and mean annual temperature of 11.6°C.

In 2011, 14 plots (20 × 20 m) were identified to represent stands dominated by trees associated with either AM or ECM fungi. AM plots contained a mixture of sugar maple (Acer saccharum), tulip poplar (Liriodendron tulipifera) and sassafras (Sassafras albidum). ECM plots contained pignut hickory (Carya glabra), American beech (Fagus grandifolia), white oak (Quercus alba), red oak (Q. rubra) and black oak (Q. velutina). Plots were assigned to a mycorrhizal association when trees from that mycorrhizal association (AM or ECM) contributed to >80% of the basal area of the plot (n = 7). All plots were located in similar landscape positions and contained more than one tree species from the dominant mycorrhizal association. Slope position and aspect were not correlated with dominant mycorrhizal association (Supporting Information Fig. S1). All sampling was performed in a 15 × 15 m internal plot to avoid edge effects.

Field sampling

In each plot, four litter traps (40 cm diameter, 1-mm mesh) were placed at the corners of an internal 12 × 12 m square. Trap content was collected biweekly from May to November 2011. In the laboratory, litter was air-dried for 4 d at room temperature (21°C) and pooled by plot. For each plot, leaf litter was sorted by species and weighed.

Soil was collected from each plot during the first week of May, July, September and November in 2012. In each plot, four soil cores were collected following a stratified random sampling design by taking one core in each of four quadrants of the internal 15 × 15 m area. We sampled the top 15 cm of the soil (including the organic layer) with a 5-cm-diameter stainless-steel corer. Cores were transported to the lab in their plastic liners and processed within 24 h of sampling by separating cores into the top 0–5 cm and bottom 5–15 cm, and pooling cores from a given plot. Soils were sieved using a 2-mm mesh to homogenize the soil and remove rocks and roots. Five subsamples of each soil sample were immediately stored at 4°C, −20°C, −80°C, freeze- or air-dried until analysis (see later).

Soil solution P concentration data were collected at 13 time points from 1 May until mid-November 2012. The anion exchange membrane (AEM) method developed by Shaw & DeForest (2013) was used to estimate in situ soil solution P concentrations (orthophosphate ion) throughout the growing season, detailed in Method S1. Soil solution P was calculated as average µg P per strip.

Aboveground characteristics

In order to estimate the annual litter P pool, we measured the P content of the three dominant AM and ECM leaf litters (AM: A. saccharum, L. tulipifera and S. albidum; ECM: C. glabra, Q. alba and Q. rubra). For each species, two to three fallen leaves were randomly selected from three plots where those trees were most abundant. Leaves were ground using a mortar and pestle, and 0.2 g was digested in nitric and perchloric acid (Sommers & Nelson, 1972). Because there were no significant differences among tree species within a mycorrhizal association, we used an average leaf litter P for AM-forming tree species (0.49 ± 0.06 mg P g⁻¹) and ECM-forming trees (0.45 ± 0.03 mg P g⁻¹) multiplied by the weight of each litter type in each plot to estimate litter P in mg m⁻². Further, an estimate of annual tree P requirements per plot was derived from the litter P content based on available data from temperate forests (Johnson et al., 2003). Johnson et al. (2003) estimate that P in litter accounts for c. 60% of the annual P requirement of trees, the other parts being wood increment and root growth. Hence, plot average litter P m⁻² was divided by 0.6 to estimate annual tree P requirement m⁻².

Soil characteristics

A field-moist sample of 1–2 g of soil was weighed and dried at 105°C for 1 h for gravimetric soil moisture (SM) determination.
Organic matter (OM) content was determined by ashing dry soils at 450°C for 16 h. Weight loss after ashing was used to calculate the percentage OM. Air-dried soil samples were ground, and total C and N concentrations were determined by dry combustion on a Costech ECS4010 Elemental Analyzer (Costech Analytical, Valencia, CA, USA). Soil pH was determined on 5 g of DW-equivalent soil suspended in 40 ml of 0.01 M CaCl₂ solution. The suspensions were shaken for 1 h and vortexed immediately before analysis. Soil pH was measured using a bench top electrode Ag/AgCl pH meter (VWR, Radnor, PA, USA).

Sequential P extraction

Immediately following sample preparation, 5 g of field-moist soil was subjected to a partial Hedley sequential P fractionation (Hedley et al., 1982a,b). We extracted and quantified inorganic P (Pi) in the following four fractions: resin P, bicarbonate P, hydroxide P and acid P (Cross & Schlesinger, 1995). As bicarbonate P and hydroxide P also contain organic P (Po), we also quantified Po in these fractions. Resin P and bicarbonate P are considered to extract more bioavailable P pools than the more complex P forms extracted by hydroxide P (secondary P minerals) and acid P (calcium bound P minerals) (Cross & Schlesinger, 1995; Yang & Post, 2011). We did not perform complete digests of the soil samples and hence have no estimate of residual P. Thus, the sum of the extracted P fractions — that is, total Pi plus total Po — does not reflect total soil P in these soils. Details on the sequential P extraction procedure are given in Methods S2.

Enzyme activities

The potential extracellular enzyme activities of acid phosphomonoesterase (AP) and phosphodiesterase (PD) were analyzed using previously published methods (Saiya-Cork et al., 2002), detailed in the Methods S3. Measured acid phosphatase activity provides a good estimate of extracellular enzymatic activity in soil because it is known to be unaffected by cell lysis (Blankenship et al., 2014) and intracellular phosphatases mostly have an alkaline pH optima (Plassard et al., 2011). Because freezing is expected to cause the least alteration of soil enzymatic activity during storage (Burns et al., 2013), soils were stored at −80°C before analysis (<1 month).

Soil microbial biomass

Soil microbial biomass was extracted from soils stored at −20°C by fumigating c. 10 g of soil for 4 d in a desiccator with an ethanol-free chloroform atmosphere (Brookes et al., 1982). Organic C was extracted from 10 g of fumigated and unfumigated soils with 50 ml of 0.5 M K₂SO₄ (Vance et al., 1987). Extracts were stored at −20°C until analyzed. Total organic C in extracts was quantified via high temperature oxidation on a Shimadzu TOC analyzer. Microbial biomass C (MBC) was calculated as the difference in extracted C before and after fumigation and corrected assuming an extraction efficiency of 45% (Jenkinson et al., 2004). Total ergosterol in soil was extracted from freeze-dried soils according to Methods S4 and used as an indicator of biomass of Ascomycete and Basidiomycete fungi in soil (Olsson et al., 2003).

Similarly to fresh soils, resin P and bicarbonate P were extracted from 5 g of fumigated soil according to the procedure described for sequential P extraction, detailed in Methods S2. Released microbial biomass P (rMBP) was calculated as the difference in extracted Pi before and after fumigation. We corrected microbial biomass P (MBP) to account for differences in P sorption among plots: MBP = rMBP/(1 − sorption) (Brookes et al., 1982). Phosphorus sorption capacity was determined for soil samples collected in May 2012 and assumed to be constant over time. From each sample, subsamples of 1.2–1.3 g of field-moist soil were equilibrated in 25 ml 1 mM KH₂PO₄ (+P) or 25 ml ddH₂O (noP) by horizontal shaking for 16 h (Giesler et al., 2002). Soil slurries were filtered through pre-rinsed Whatman No. 1 filter papers and extracts were stored at −20°C until analyzed. Sorption was calculated as the percentage of added P remaining in +P solutions minus P released in the noP solutions (Giesler et al., 2002). We calculated the molar ratio of C:P in the microbial biomass (MBC:P) after correcting MBP by assuming an extraction efficiency of 40% (Brookes et al., 1984). However, to avoid overestimating the size of the MBP pool, we did not otherwise correct for extraction efficiency.

Molecular characterization of soil fungal community

Four plots were randomly selected from each mycorrhizal association and total soil DNA was extracted from freeze-dried soil samples (0–5 cm) from May and September. Extractions were performed in duplicate from 0.25 g of soil using the Xpedition™ Soil/Fecal DNA miniprep kit (Zymo Research, Irvine, CA, USA). The fungal barcode region ITS2 rRNA (Schoch et al., 2012) was amplified using gITS7 (Ihrmark et al., 2012) and ITS4 m modified from ITS4 to account for mismatches against the class Archaeorhizomycetes (5′-TCCTC[C/G][G/C]CTTATTGATATGC-3′). Life technology fusion primers were constructed on the forward primer for multiplexing. PCR was performed with 10–20 ng DNA template, 1 x SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and 0.8 nM of each primer on a CFR96 Touch™ Real/Time PCR Detection system (Bio-Rad). Following 10 min of pre-denaturation at 95°C, we ran 27 cycles of 1 min at 95°C, 45 s at 50, 54 and 58°C (performed in parallel reactions), and 50 s at 72°C, followed by 3 min at 72°C. Three annealing temperatures were used to reduce primer bias (Schmidt et al., 2013) and monitoring the runs by real-time PCR ensured that amplification remained linear. All six reactions from each soil sample were combined and purified using the ZR-96 DNA Clean & Concentrator™-5 (Zymo Research). The fungal community sequence analysis is detailed in Methods S5.

Calculations and statistical analyses

Principal components analysis (PCA) of soil parameters was conducted in PC-ORD version 5.33 software (McCune & Mefford, 1999).
Before PCA analysis, we used correlations (Pearson’s r) to evaluate which variables covaried (Minitab 16.0; Minitab Inc., State College, PA, USA) in order to subsequently remove those from the analysis. The enzyme activities AP and PD showed a significant positive correlation, as did Pi content in different fractions (P<0.001). We also performed a number of PCA runs, including selected individual P fractions and enzyme activities in different combinations, to ensure the robustness of the analyses. Here, we present the results based on total Pi, total Po, P sorption, MBC : P, enzyme activity for AP, ergosterol, SM, OM content and pH; all other combinations were very similar in outcome. Ordination results are presented in joint plots. Correlations between soil variables and ordination axes using PC-ORD default settings are reported as r² values and used as indicators for the importance of variables in structuring the ordination.

We used the multi-response permutation procedure (MRPP), a nonparametric procedure in PC-ORD for testing the hypothesis of no difference between two or more groups (McCune & Grace, 2002), in order to test for the effects of mycorrhizal association, soil layer and time. MRPP provides P-values as well as A-values that measure ‘effect sizes,’ representing homogeneity within the group compared with that expected randomly. For instance, perfect homogeneity in the group gives A=1, whereas A values between 0 and 1 indicate that heterogeneity between the groups is greater than that expected by chance.

Mixed linear model analyses were conducted including either one or two factors depending on the tested parameter (see later), followed by pairwise comparisons using Student’s t-test or Tukey’s t-test, respectively. These statistical analyses were performed in JMP 10 (SAS Institute Inc., Cary, NC, USA). Basal area, litter biomass, leaf litter P content and average seasonal in situ soil solution P were analyzed using mycorrhizal association as a fixed effect. Leaf litter P content was analyzed with tree species as a fixed effect. Soil moisture, OM content, C : N ratio, pH, P sorption, total ergosterol, MBC, MBP, MBC : P, and Pi and Po content in all extracted fractions were analyzed using mycorrhizal association, soil layer and their interaction as fixed effects, and plot as a random effect. The same analysis was conducted for P content of all fractions and enzymatic activity normalized by ergosterol as a fixed effect. Leaf litter P content was analyzed with tree species as a fixed effect. Soil moisture, OM content, C : N ratio, pH, P sorption, total ergosterol, MBC, MBP, MBC : P, and Pi

In order to relate aboveground P pool sizes (litter P and estimated annual P requirement of trees; see ‘Aboveground characteristics’) to belowground P fractions, P content in extracted soil fractions was converted to pool sizes for the top 15 cm soil (kg ha⁻¹). Soil weight per surface area was approximated using measurements from 33 plots in three adjacent forest sites: 51.7 ± 15.5 and 133.6 ± 17.2 kg m⁻² for the 0–5 and 5–15 cm soil layers, respectively. No correlation was detected between soil weight and proportion of ECM forming trees in the plots. The P contents of extracted fractions were multiplied by the average soil weight of the soil layer and an estimate of each P pool down to 15 cm depth was produced by addition of values for 0–5 cm and 5–15 cm for each plot. Only plots that had measurements from both soil layers were used in further analysis. Mixed linear models with mycorrhizal association, sampling time and their interaction as fixed effects, and plot as a random effect, were used to analyze the estimated P pool sizes.

**Results**

**Plot level effects of mycorrhizal association**

Based on abiotic soil characteristics, total extracted organic and inorganic P content, MBC : P, ergosterol and acid phosphatase activity, AM plots separated from ECM plots in multivariate space (Fig. 1). The first three PCA axes accounted for 70% of the variation. MRPP analyses revealed significant differences between mycorrhizal associations (A=0.017, P=0.007), soil layers (A=0.066, P<0.001) and over time (A=0.21, P<0.001). The separation was mainly driven by differences in ergosterol (axis 1; r²=-0.83), OM content (axis 1; r²=-0.77) and enzyme activity for AP (axis 1; r²=-0.71), and partly driven by pH (axis 2; r²=-0.51), P sorption (axis 2; r²=0.45) and MBC : P (axis 2; r²=0.44) (Fig. 1). Mycorrhizal association separated mainly along ordination axis 1, whereas soil layers separated mainly along axis 2 (Fig. S2). The layer differences were greater in ECM plots than in AM plots (Fig. S2).

Mycorrhizal association significantly affected most soil characteristics (Table 1) and extracted P fractions (Table S1). Percentage OM (P<0.001), soil C : N ratio (P<0.001) and P sorption (P<0.001) were significantly higher in ECM plots compared with AM plots, whereas pH was significantly lower in ECM compared with AM plots (P<0.001). Microbial biomass C (P=0.008) and total ergosterol (P<0.001) were greater in ECM plots compared with AM plots, but the opposite was true for MBP which was highest in AM plots (P=0.034). Soil moisture was not affected by mycorrhizal association (P=0.543). Many variables also exhibited interactive effects between mycorrhizal association and soil layer (Tables 1, S1). For example, the molar ratio C : P in microbial biomass was significantly higher in ECM plots as a result of high values in the deeper soil layer. Soil moisture, OM content, C : N ratio, MBC and total ergosterol were greater in the 0–5 cm layer than in the 5–15 cm layer (all P<0.001). Soil layer also affected all extracted P fractions with significantly less P in the 5–15 cm soil layer compared with the top 5 cm of soil. Only pH was not significantly affected by soil layer (P=0.256), but there was a significant interaction between mycorrhizal association and soil layer for pH (P=0.001).

In contrast to belowground characteristics, AM and ECM plots were similar in aboveground characteristics, both with respect to basal area (P=0.271) and leaf litter biomass.
correlated with total extracted ergosterol (for AP and 2.3 times higher for DP (Table 1). Total soil phosphatase activities were significantly affected by mycorrhizal association and soil layer with higher activity in ECM plots with the AM plots. No significant difference in MBC content was found between the two categories AM or ECM (P = 0.563). On average, 632 ± 42 mg P m⁻² was delivered to the forest floor by litter. AM plots received marginally more P from litter compared with ECM plots (P = 0.061) (Table 1).

Effects of mycorrhizal association on soil enzymatic activity and microbial communities

Soil phosphatase activities were significantly affected by mycorrhizal association and soil layer with higher activity in ECM plots compared with AM plots (P < 0.001 for both): 1.5 times higher for AP and 2.3 times higher for DP (Table 1). Total soil phosphatase activity (i.e. the sum of AP and PD) was more strongly correlated with total extracted ergosterol (r² = 0.61, Fig. S3a) than with MBC (Fig. S3b). In ECM plots, there was a weak correlation between total phosphatase activity and MBC (r² = 0.43), whereas no correlation was found in AM plots (r² = 0.04) (Fig. S3b). The correlation between total phosphatase and ergosterol was conserved within ECM plots but no correlation between phosphatase activity and ergosterol was found for AM plots (r² = 0.08) (data not shown). There was a strong positive correlation between ergosterol and MBC in ECM plots (r² = 0.86) indicating that fungi in the Dikarya constitute a major fraction of microbial biomass in these plots (Fig. S4). The correlation between ergosterol and MBC was weaker in AM plots (r² = 0.54) (Fig. S4). Despite these patterns, P pool content and enzymatic activity remained significantly different between plots dominated by different mycorrhizal associations after normalization by the amount of fungi in the Dikarya, estimated by extracted soil ergosterol (Table S2). Thus, observed differences in the P content of extracted soil fractions are not driven only by the biomass of fungi in the Dikarya.

Arbuscular and ectomycorrhizal plots harbored distinct soil fungal communities in topsoil. Mycorrhizal association explained 37% of the observed variance in community structure, whereas sampling time explained 10% (Fig. S5). Fungal richness was significantly higher (Adonis, r² = 0.34, P < 0.001) in the AM plots compared with the ECM plots (Fig. 2a). The characterized fungal communities were dominated by taxa belonging to the Dikarya in both AM and ECM plots, and AM fungal communities in these soils thus remain unknown. Ascomycetes were by far the most abundant phyla, constituting > 80% of the rarefied fungal sequence reads, excluding ECM plots sampled in May at which time Ascomycetes contributed to only 50% of the captured fungal community (Fig. 2b). Instead ECM plots sampled in May were dominated by fungal taxa that have a known ECM lifestyle and these constituted 40% of the observed fungal abundance of these samples (Fig. 2c). The relative abundance of ECM fungi was much lower in September than in May but still higher in ECM plots compared with AM plots (Table S3).

Effects of mycorrhizal association on P pool sizes

Above- and belowground P pools were scaled to kg P ha⁻¹ (Table S4) to allow for a comparison of average annual pool sizes (Fig. 3). This comparison indicated that annual P requirements of the trees correspond to approximately one-third of the annual average P in soil microbial biomass. Furthermore, the sum of seasonal average Hedley available pools (resin P, bicarbonate Pi and Po) exceeds the annual P requirement of the trees biomass by two
to five times (Fig. 3). AM plots had significantly more extractable P \((229 \pm 12 \text{ kg ha}^{-1})\) compared with ECM plots \((187 \pm 16 \text{ kg ha}^{-1})\) \((P=0.019)\). The difference was due to significantly more total extractable Po in the AM plots \((P=0.027)\). However, the ECM plots had significantly more available Po compared with AM plots \((P<0.001)\). The opposite was true for the less available complex P fractions, where AM plots had significantly less available P in ECM plots \((P<0.001)\). Overall, Po made up 73 ± 3% of the available P in ECM plots compared with 65 ± 4% in AM plots, whereas Po made up 63 ± 3% of the less-available P in ECM plots compared with 79 ± 1% in AM plots.

Seasonal effects on soil P dynamics

In both AM and ECM plots, there was a significant effect of sampling time on total Pi \((P<0.001)\) (Fig. S6a) and total Po \((P<0.001)\) (Fig. S6b), as well as all individual P fractions, except bicarbonate Po (Table S4). On the one hand, we observed a steady decrease in total extractable Pi over the season from an average of 56 ± 4 mg P kg\(^{-1}\) soil in May to 24 ± 2 mg P kg\(^{-1}\) soil in November (Fig. 4a). Organic P, on the other hand, increased sharply between July and September, from an early season average of 59 ± 5 mg P kg\(^{-1}\) soil to 126 ± 11 mg P kg\(^{-1}\) soil in September (Fig. 4a). Total Po was also significantly affected by mycorrhizal association \((P=0.027)\), but there was no significant interaction effect between mycorrhizal association and sampling time (Fig. S6b).

Over the growing season, there was a significant increase in MBC : P ratios from an average of 25 in May, a ratio c. 50 in July and September, and a final high of 140 recorded in November. MBC : P was also higher in ECM plots compared with AM plots \((P=0.066)\) (Fig. S6c). Changes in MBC : P were driven both by increases in MBC and decreases in MBP (Fig. 4b). Microbial biomass C was significantly affected by mycorrhizal association \((P=0.003)\), season \((P<0.001)\), and their interaction

## Table 1 Average values ± SE, expressed as percentage or per kg of DW soil for arbuscular mycorrhizal (AM)- and ectomycorrhizal (ECM)-dominated plots

<table>
<thead>
<tr>
<th>Aboveground characteristics</th>
<th>AM</th>
<th>ECM</th>
</tr>
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<tbody>
<tr>
<td>Basal area (m(^2) ha(^{-1}))</td>
<td>126 ± 16(^a)</td>
<td>105 ± 9(^a)</td>
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<tr>
<td>Leaf litter (g m(^{-2}))</td>
<td>1509 ± 150(^a)</td>
<td>1224 ± 74(^a)</td>
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<tr>
<td>Total litter P (mg m(^{-2}))</td>
<td>716 ± 70(^a)</td>
<td>560 ± 36(^a)</td>
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<thead>
<tr>
<th>Soil characteristics</th>
<th>0–5 cm</th>
<th>5–15 cm</th>
<th>0–5 cm</th>
<th>5–15 cm</th>
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<tbody>
<tr>
<td>SM percentage M,L,MxL</td>
<td>28 ± 2(^ab)</td>
<td>23 ± 1(^bc)</td>
<td>30 ± 2(^a)</td>
<td>20 ± 1(^a)</td>
</tr>
<tr>
<td>OM percentage M,L,MxL</td>
<td>8.0 ± 0.3(^b)</td>
<td>4.6 ± 0.1(^c)</td>
<td>15.2 ± 1.2(^a)</td>
<td>4.4 ± 0.2(^c)</td>
</tr>
<tr>
<td>C : N M,L,MxL</td>
<td>13 ± 3(^a)</td>
<td>11 ± 0.4(^d)</td>
<td>21 ± 0.9(^a)</td>
<td>17 ± 0.6(^b)</td>
</tr>
<tr>
<td>pH M,L,MxL</td>
<td>5.0 ± 0.1(^b)</td>
<td>4.6 ± 0.1(^b)</td>
<td>4.0 ± 0.1(^c)</td>
<td>4.2 ± 0.1(^c)</td>
</tr>
<tr>
<td>P sorption % M,L,MxL</td>
<td>24 ± 3(^b)</td>
<td>29 ± 4(^bc)</td>
<td>38 ± 1(^ab)</td>
<td>42 ± 3(^a)</td>
</tr>
<tr>
<td>Microbial biomass</td>
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<tr>
<td>Tot ergo mg kg(^{-1}) M,L,MxL</td>
<td>1.5 ± 0.2(^b)</td>
<td>0.5 ± 0.1(^d)</td>
<td>6.3 ± 0.6(^a)</td>
<td>0.9 ± 0.1(^c)</td>
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<td>MBC g kg(^{-1}) soil M,L,MxL</td>
<td>1.3 ± 0.11(^b)</td>
<td>0.5 ± 0.06(^c)</td>
<td>2.0 ± 0.18(^a)</td>
<td>0.6 ± 0.05(^d)</td>
</tr>
<tr>
<td>MBC : P M,L,MxL</td>
<td>22 ± 3(^b)</td>
<td>35 ± 9(^b)</td>
<td>24 ± 4(^b)</td>
<td>63 ± 12(^a)</td>
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<td>Enzyme activities</td>
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<tr>
<td>AP activity M,L,MxL</td>
<td>29 ± 1.7(^bc)</td>
<td>25 ± 1.5(^c)</td>
<td>57 ± 3(^a)</td>
<td>33 ± 2(^b)</td>
</tr>
<tr>
<td>PD activity M,L,MxL</td>
<td>4.6 ± 0.8(^b)</td>
<td>2.1 ± 0.1(^c)</td>
<td>14 ± 1.6(^a)</td>
<td>2.9 ± 0.3(^bc)</td>
</tr>
</tbody>
</table>

Microbial biomass C : P is expressed as a molar ratio (MBC : P). Acid phosphatase activity (AP) and phosphodiesterase (PD) are expressed in arbitrary activity units based on fluorescence in soil enzyme assay. Soil characteristics are further separated by soil depth. SM, soil moisture; OM, organic matter; Tot ergo, total ergosterol. Different letters indicate that values are significantly different based on Tukey t-test. M, Main effect of soil layer. MxL, Interaction effect for mycorrhizal association × soil layer. For each P pool, different letters indicate that values are significantly different based on Student’s t-test (plot characteristics) and Tukey t-test (soil characteristics).

Fig. 2 Top-soil fungal communities in arbuscular mycorrhizal (AM; dark grey) vs ectomycorrhizal (ECM; light grey) plots sampled in May and September. (a) Observed and estimated (Chao 1) fungal richness. (b) Average relative abundance of Ascomycota as percentage of total fungal community. (c) Average relative abundance of ECM forming fungi as percentage of total fungal community. Data are based on rarefied sequence reads and presented as average ± SE.
Fig. 3 Growth seasonal average phosphorus (P) pools scaled to illustrate relative pools sizes in kg P ha\(^{-1}\) down to 15 cm soil depth. Complex and available organic soil P (Po) pools are grey to illustrate that these were significantly different between (a) ectomycorrhizal (ECM)- and (b) arbuscular mycorrhizal (AM)-dominated stands. Biotic pools (annual tree requirement and microbial biomass) are illustrated as circles. Yearly tree P requirements are estimated, derived from the litter P. Uptake to trees is assumed to be predominantly through the microbial biomass P pool. Resin P pool was closely correlated to soil solution P and is thus labeled solute (Solut.). Exchangeable (Exchang.) inorganic soil P (Pi) and available (Avail.) Po represent bicarbonate extracted pools. For less available P forms, (Complex) Po represents the hydroxide extractable fraction and Complex Pi represents the sum of hydroxide and acid extractable Pi. Arrows illustrate the proposed main fluxes in the two stand types over the growth season. Values, except for tree P requirements, are the seasonal averages ± SE.

Fig. 4 Seasonal dynamics of (a) total inorganic phosphorus (P) fractions (Pi; light grey) and total organic P (Po; dark grey) and (b) corrected microbial biomass carbon (MBC) (black) and P (MBP; grey). Values are weighted average across soil layer with ± SEM as error bars. Different letters (above Po and MBP, and below Pi and MBC) indicate significantly different values between sampling times within each pool.
(P = 0.017); MBC was higher in ECM plots compared with AM plots in July and September (Fig. S6d). For both mycorrhizal associations, MBC fluctuated over the season with high values recorded in May and November (Fig. 4b). Microbial biomass P, however, decreased over the season (Fig. 4b) and was not significantly affected by mycorrhizal association (P = 0.973, Fig. S6e).

The summer of 2012 was dry with limited rainfall from mid-May to mid-August. As a result, soil moisture changed significantly over the growing season with the lowest values (14%) recorded in July (Fig. 5a). In situ soil solution P was significantly affected by mycorrhizal association (P < 0.001) and time (P < 0.001), and a significant interaction effect was detected (P = 0.001; Fig. 5a). Seasonal average in situ soil solution P was three times higher in ECM plots at 18 ± 3.2 µg P per strip compared with AM plots at 6.2 ± 0.9 µg P per strip. This difference was the result of an extended peak with significantly higher soil solution P detected in ECM plots compared with AM plots following the first rain, which began to alleviate the drought in early July (c. 16 mm on 7–8 July). The peak in ECM plots was >10 times higher than the peak in AM plots: 89 ± 24.4 compared with 8.3 ± 2.9 mg P m⁻². Among all measured P fractions in the top 5 cm, resin P, MBP and hydroxide Po (Fig. 5b–d) were the only P fractions significantly affected by mycorrhizal association and time; significant differences between AM and ECM plots were observed in all of these in July, the time of lowest soil moisture. Finally, there was no correlation between soil pH and resin P (r² = 0.006) across the season.

**Discussion**

We examined P dynamics in soils in central hardwood forests of southern Indiana where a rich assemblage of tree species forming symbiotic associations with predominantly arbuscular (AM) or ectomycorrhizal (ECM) fungi co-occur on soils developed from the same unglaciated parent material. Accumulating evidence suggests that the mycorrhizal associations of dominant tree

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**Fig. 5** Seasonal dynamics of phosphorus (P) fractions with significant differences between arbuscular mycorrhizal (AM) stands (dark grey) and ectomycorrhizal (ECM) stands (light grey): (a) illustrates in situ soil solution P (µg/strip) collected in 2-wk intervals and percentage soil moisture (dashed line); (b) readily available inorganic soil P (Pi) measured as resin Pi; (c) microbial biomass P (MBP) and (d) less available organic soil P (Po) measured as hydroxide(-extractable) Po. Average values are presented ± SE. In (a), asterisks indicate time points when AM and ECM stands are significantly different; in (b–d) different letters indicate significant differences based on Tukey’s pairwise comparison.
species have distinct effects on ecosystem-level C and N cycling (Phillips et al., 2013). For instance, differences in the enzymatic capacities of AM and ECM fungi have been suggested to be the best predictor of decomposition and C storage in soil at both ecosystem and global scales (Averill et al., 2014). Our study provides evidence that these associations also result in plot-level differences in P dynamics, and that microbial biomass and community compositions drives these dynamics.

In accordance with the recently proposed framework of distinct mycorrhizal-associated nutrient economies (Phillips et al., 2013), we hypothesized that in plots where trees associate with AM fungi, soil P would cycle predominantly in inorganic forms, but that in plots dominated by ECM forming trees, soil P would cycle mostly in organic forms of P. Overall, our findings confirm these expectations. Significantly higher soil phosphatase activity and higher P availability in ECM plots support the hypothesis that these systems cycle organic P substrates to a greater extent than AM plots. In AM plots, organic P was instead found to accumulate in the less-available hydroxide-extractable pool. The sum of all inorganic P pools, however, was not significantly affected by mycorrhizal association.

Our results are in line with the emerging view that P cycling in forest soil systems is controlled by the soil microbial biomass rather than by the annual uptake of trees (Cole et al., 1978; Yang & Post, 2011). As in earlier reports from sites presumed to be P-limited, we found that Hedley available pools exceed tree requirements for P (Johnson et al., 2003; Yang & Post, 2011). This is, in part, because resorption and internal storage of P within woody plants decrease their dependency on de novo P acquisition (Rennenberg & Herschbach, 2013). Furthermore, in our study the seasonal average microbial biomass P (MBP) was about three times bigger than the estimated P requirements of the trees. Similarly, microbial biomass has been shown to constitute 68–78% of the total biomass P in other temperate forests (Turner et al., 2013). Overall, the sum of the two biotic pools – seasonal average MBP and estimated annual P requirements of the canopy trees – exceeded the size of the available P pool, indicating that the microbial biomass drove the observed P dynamics. Seasonal averages provide a conservative estimate of available pool sizes because these pools are expected to have a high turnover rate of days to weeks (Shaw & DeForest, 2013). The trend was stronger for AM plots and whether these are more P-limited than the ECM plots due to the lower ability of their microbial biomass to utilize organic P sources, or whether AM plots have a higher turnover rate of MBP, warrant further study.

Higher concentrations of available P in ECM plots than in AM plots appear to be driven by higher enzymatic activities of fungi in ECM plots due to differences in fungal community composition. High phosphatase activity of ECM fungi (Plaschke & Dell, 2010), the ability of ECM fungi to capture P directly from saprotrophic fungi (Lindahl et al., 1999), and the recently proposed pathway of direct P uptake by ECM fungi (Rennenberg & Herschbach, 2013; Becquer et al., 2014) highlight the importance of Po for ECM fungi and their associated host trees. Across all plots, total phosphatase activity was strongly correlated with total ergosterol, but less so with MBC, supporting the idea that fungi in the Dikarya are predominantly involved in P release from soil organic matter. However, ergosterol alone did not explain soil enzymatic activity or P content in measured P pools because these remained significantly different when normalized by ergosterol. The composition of fungal taxa and taxonomic rank also differed between AM and ECM plots, and the capacities to exude extracellular phosphatases varies significantly among species in the Dikarya, even among ECM fungi (Nygren & Rosling, 2009). Finally, plant roots, bacteria and saprotrophic fungi may all contribute to non-negligible extracellular phosphatase activity and cycling of organic P in AM plots. Thus, community compositional differences may explain observed differences in phosphatase activities and Po availability between AM and ECM plots.

In contrast to our expectations, we found no differences in total Pi in AM and ECM plots and higher in situ soil solution Pi in ECM plots than in AM plots. We had expected that the higher pH of AM soils would result in higher Pi availability in AM plots compared with ECM plots. However, the lack of correlation between soil pH and the available Pi extracted in the Resin fraction across the season suggests that biological processes, rather than chemical processes, control P availability in forest top soils (Wood et al., 1984). Furthermore, higher in situ soil solution Pi in ECM plots was driven by drought-alleviating rains in early July that resulted in a soil solution P peak that was significantly higher in ECM plots compared with AM plots. This peak may be explained by high phosphatase activities in ECM plots that hydrolyzed hydroxide Po and resulted in high resin Pi and bicarbonate Po pools in ECM plot soils. The accumulated resin Pi pool was then detected as a soil solution P peak as soon as drought conditions no longer prevented the mobility of hydrolys products. Lysis of microbial biomass following the rain could also have contributed to the soil solution P peak, as MBP was significantly higher in ECM plots compared with AM plots before the rains. Low average soil solution P concentrations in AM plots, particularity the low peaks following the drought-alleviating rains in July, and undetectable differences in total Pi between AM and ECM plots strongly support the idea that the AM pathway of P acquisition is based primarily on their high-affinity uptake system and fast translocation to plants (Smith et al., 2011). Rapid P uptake may explain the consistently low levels of in situ soil solution P in AM plot soils.

In addition to differences between AM and ECM plots, we observed a seasonal decline in total P and an increase in total P across all plots. The total P decline is in accordance with established knowledge that trees and their mycorrhizal symbionts assimilate inorganic P from the soil solution, which is in equilibrium with absorbed and complexed forms of P (Frossard et al., 2010). In addition, we found that both hydroxide and acid Pi pools varied significantly over the season, suggesting that these pools contribute to seasonal Pi cycling. Although phosphatase enzymes in soil hydrolyze P, de novo formation of Po appeared to exceed the decomposition of organic P substrates during the growing season. Given that the increase in total P happened before litter fall, we conclude that the buildup of Po was likely to be a product of rhizodeposition (e.g. sloughed cells) and turnover...
of the microbial biomass (Richardson & Simpson, 2011). During the winter season, decomposition as well as abiotic processes have the potential to restore inorganic P levels by transforming accumulated organic P. Although seasonal changes in P pools could reflect changes in the extractability of organic material (Chen et al., 2003), significant differences in soil conditions (i.e. pH, OM and P sorption) between mycorrhizal associations suggest that the seasonal decrease in Pi and increase in Po are real changes and are not an artifact of the extraction process. We conclude that over the growing season, inorganic P substrates were assimilated and subsequently transformed to organic P substrates by plants and microbes in both AM and ECM plots.

In addition to changes in total Pi and Po, we found that the microbial biomass became increasingly P-limited over the season, particularly at the end of the growing season. This contradicts the idea that soil microbial biomass has a constant C : P ratio (Cleveland & Liptzin, 2007). This stoichiometric change is likely linked to a seasonal shift from mycorrhizal to saprotrophic life-strategies of the microbial community. During the growing season, when mycorrhizal fungi mediate plant-derived C flux to the soil system, mycorrhizal fungi rapidly assimilate and transfer P to their host trees (Plassard & Dells, 2010). Trees that associate with mycorrhizal fungi experience P-limitation only if they fail to translocate C to their fungal symbionts (Fellbaum et al., 2014). Towards the end of the growing season, as leaves senesce and the belowground C flow to roots decreases, the microbial biomass is expected to shift from a predominantly root symbiotic community to one dominated by free-living C-limited decomposers, as we observed in the fungal community of ECM stands. Taken together, the observed seasonal changes in total Pi, Po and MBP suggest that both AM and ECM stands become more P-limited over the course of the growing season.

Our study provides evidence that tree mycorrhizal associations affect P cycling in hardwood forest soils. Common garden experiments have previously demonstrated that tree species significantly affect soil nutrient cycling and decomposition activity within 30 yr of planting (Hobbie et al., 2007; Vesterdal et al., 2012). In naturally generated forest stands, the causal link between vegetation and soil P cycling cannot be explicitly tested. However, the studied plots have been vegetated for over 80 yr, have the same parental material and experience no systematic climate differences. Hence, it is reasonable to assume that differences in soil P cycling are determined by the current stand dynamics rather than conditions prevailing before establishment. Our results partially support the mycorrhizal-associated nutrient economy framework (Phillips et al., 2013) with ECM plots having more organic P in available forms compared with AM plots where more complex organic P accumulated. However, seasonal buildup of organic P in soil resulted in an increasingly P-limited microbial biomass in both systems. These seasonal differences were larger than those observed between mycorrhizal associations. Soil microbial biomass was the largest biotic P pool, and we propose that changes in seasonal tree derived C alters microbial community composition and, subsequently, the cycling of P in soil. In the future, it will be important to further investigate MBP turnover rates, the importance of C availability for P cycling and the seasonal composition of the microbial community, to better understand how P cycling is controlled in hardwood forest soils and how it may be affected by changing environmental conditions.

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Author contributions

A.R., M.G.M. and R.P.P. planned and designed the study. A.R. and M.G.M. conducted fieldwork and soil analysis. T.C. performed ergosterol analysis and H.U. performed molecular characterization of fungal communities. A.R., M.G.M. and P.F. analyzed the data and A.R., M.G.M. and R.P.P. wrote the manuscript with considerable input from the other authors.

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**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Map of plot locations.

**Fig. S2** PCA showing difference between AM- and ECM-dominated plots, and soil layers.

**Fig. S3** Total soil enzymatic activity vs ergosterol and MBC.

**Fig. S4** Microbial biomass carbon (MBC) and ergosterol.

**Fig. S5** PCA of fungal community structure of topsoil from four AM- and ECM-dominated plots.

**Fig. S6** Seasonal dynamics in total Po and Pi as well as MBC and P.

**Table S1** Seasonal average soil P contents (mg P kg⁻¹ dry soil) by mycorrhizal association and soil depth.

**Table S2** Average soil P contents normalized by soil ergosterol (mg P mg⁻¹ ergosterol).

**Table S3** Average relative abundance of the ten commonest orders of ectomycorrhizal fungi.

**Table S4** Estimated seasonal average size of P pool (kg ha⁻¹).

**Methods S1** *In situ* soil solution P.

**Methods S2** Sequential P extraction.

**Methods S3** Soil enzymatic activity.

**Methods S4** Ergosterol extraction.

**Methods S5** Fungal community sequence analysis.

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