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The cycling of readily available phosphorus in response to elevated phosphate in acidic temperate deciduous forests

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ABSTRACT

The biogeochemical cycling of phosphorus (P) in temperate forest ecosystems is poorly understood relative to nitrogen (N) cycling because of the assumption that N is the main factor that limits plant growth. This dichotomous way of understanding nutrient regimes is likely an oversimplification of these dynamic, interacting cycles within the evermore-dynamic framework of soil development. In order to better understand P cycling in temperate deciduous forests, we sought to quantify labile (resin) P flux and determine if soil shows signs of P stress. Labile P flux was quantified in nine ambient and nine elevated P plots on unglaciated eastern deciduous forests in Eastern Ohio. Anion exchange membranes (AEMs) were deployed *in situ* for two weeks, in the fall and the winter. Flux values were contextualized by resin P pools, N and P mineralization, and phosphatase enzyme activities determined from soil samples collected both at the time of AEM deployment and harvest. Phosphorus flux was 0.64 ± 0.23 μg P d^{-1} (mean ± SD) in ambient plots and 3.47 ± 2.39 μg P d^{-1} in amended P plots. Resin P pools were 4.16 mg kg^{-1} and 10.26 mg kg^{-1} in the control and elevated plots, respectively. Phosphorus turnover rate increased from one week in control plots, to 3 days in elevated P plots. Results indicate mild P stress relative to N, as N was mineralized (0.84 ± 0.18 mg N kg^{-1} d^{-1}) while P was immobilized (-0.22 ± 0.09 mg P kg^{-1} d^{-1}). The elevated P treatment did not increase P mineralization. Phosphatase enzyme activities were significantly suppressed by 34% with P amendment. Results suggest labile P is a shallow pool, but is rapidly cycling.

1. Introduction

Soil nutrient cycling has a commanding influence on plant mineral nutrition and is a vital component of forest ecological dynamics. As such, understanding biogeochemical cycling is important to improve our understanding of ecology. Under the paradigm of "Liebig's Law of the Minimum," temperate forests are considered to be limited largely by nitrogen (N) (Aber et al., 1991; Vitousek and Howarth, 1991). However, this idea is giving way to a co-limitation framework wherein both N and phosphorus (P) limit ecosystem activities (Davidson and Howarth, 2007; Elser et al., 2007). In comparison to N, P cycling is poorly understood in temperate forest ecosystems, as what is known is mostly from tropical ecosystems (Dieter et al., 2010; Porder et al., 2007; Vitousek et al., 2010; Vitousek and Sanford, 1986). This lack of understanding is likely rooted in the relative geographic concentrations of work done on these soil nutrient cycles rather than a clear difference between forests occurring in these differing climate regimes.

The longstanding, longitudinal nutrient-cycling dichotomy between tropical systems (presumably P limited) and temperate systems (presumably N limited) has been challenged to varying degrees (Dieter et al., 2010; Elser et al., 2007; Johnson et al., 2003). The presumed inherent difference between these systems may not be as well-defined as previously thought, as it is primarily based on the degree of soil weathering (barring depositions, soil activity, etc.) and relative soil age and parent material, rather than geographical or community differences. To determine the extent to which P deficit exists in temperate systems, it is essential to understand P dynamics within the context of other soil properties and processes.

Soil N limitation is associated with earlier stages of soil development (Hedin et al., 2003) as N inputs are primarily mediated by biological processes (Lambers et al., 2008) whereas P inputs are derived from geochemical processes and are not biologically renewed (Walker and Syers, 1976). This means that N limitation generally decreases with time while P limitation increases in the absence of major disturbance (Crews et al., 1995; Wardle et al., 2004). Independent of climate, time since catastrophic disturbance (e.g., glaciation) is an important factor in where P becomes more limiting, relative to N, to ecosystem productivity (Wardle et al., 2004). Therefore, without significant disturbance, P will eventually limit forest productivity. However, there is not a clear shift between N and P limitation in forests, as the two biogeochemical cycles are likely coupled (Lambers et al., 2008).

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The mechanisms of soil P limitation (Vitousek et al., 2010) and enrichment (Porder et al., 2007) are fairly well understood, but to a lesser extent are the forms in which P exists in the soil, as typically measured by chemical (e.g., Hedley) fractionation (Cross and Schlesinger, 1995; Dieter et al., 2010; Johnson et al., 2003). Research is being done to better understand the roles of the different phosphorus fractions and their implications on species composition and ecosystem processes (DeForest and Scott, 2010; DeForest et al., 2012; Siddique et al., 2010; Turner, 2008). For example, in acidic hardwood forests, increasing the availability of P can decrease phosphatase activity, but it depends on ambient P pools (DeForest et al., 2012). While phosphatase activity may be a good metric of P demand and/or enzyme pools, it is not necessarily a good metric of P flux, which has not been well-established in temperate systems. Vandecar et al. (2011) reported that tropical labile soil P flux occurs on a scale of hours or days. However, it is unclear if this rate is reasonable for temperate ecosystems.

Our objectives were to determine the flux of readily available P in acidic temperate hardwood forests. We expected the cycling of P to increase with elevated P, but the activity of phosphatase enzymes to decrease. Additionally, we sought to provide some context regarding the position of these sites on the continuum between N and P limitation by measuring N mineralization and enzyme activities in addition to standard soil properties. We expected to find rapid P cycling and indicators of P limitation in these systems.

2. Materials and methods

2.1. Study sites and experimental design

The sites studied were located in three unglaciated eastern deciduous forests in Southeast Ohio. These sites have an average basal area of 31 m^2 ha^{-1} and are dominated by *Quercus* spp. (60%), *Acer* spp. (13%), and *Fagus grandifolia* (8%) (DeForest et al., 2012). Two of the sites were located at the Waterloo Wildlife Management Area (managed by the Division of Wildlife of the Ohio Department of Natural Resources) and the third was located at Zaleski State Forest (managed by the Division of Forestry of the Ohio Department of Natural Resources). Soils are loam and silt loams and classified as Typic Hapludults and Dystrudepts. These forest sites receive an annual average of 100 cm of precipitation and have an average high July temperature of 28 °C and average low January temperature of −6 °C.

Each of the three sites had six constituent 20 m × 40 m (800 m^2) plots, established in August of 2009 (DeForest et al., 2012). Half of the plots in each site were amended with 41.8 kg P ha^{-1} in the form of TSP (triple super phosphate), while the remainder served as controls. There were nine elevated P plots and nine control plots. The experiment was conducted in October (before litter fall) and December of 2010 to account, presumably, for great differences in P availability at the end of the growing season when available P should be low and after litter fall when available P should be high from low plant demand and recent organic inputs.

At each time, anion exchange membranes (AEMs) were deployed in the mineral soil in triplicate in each plot and left *in situ* for fourteen days. Additionally, soil cores were taken near the deployment sites at a depth of 5 cm at the time of AEM deployment (day 0) and at the time of AEM harvest (day 14) to quantify the difference in the labile P pools between the control and elevated P plots, contextualizing the turnover data.

2.2. Preparation of AEMs

The AEMs used in this experiment were obtained from GE Infrastructure: Water & Process Technologies of Watertown, MA, USA. In raw form, the AEMs are in large sheets, making it necessary to cut them into a more workable size. The AEMs were cut into precise 2 cm × 6 cm (total 24 cm^2) strips using a rotary cutter. It is essential that the AEMs not be allowed to dry out, so during the cutting process they were kept moist with dilute HCl solution. We charged the AEMs by shaking them with 0.5 M $NaHCO_3$ for 15 min and then rinsing them with deionized water (DI). This process was repeated three times. Anion exchange membranes were stored moist at 5 °C in plastic bags until use. After use, we cleaned the AEMs by shaking them with 0.5 M HCl with alternating rinses with DI water. AEMs were reused after cleaning, as multiple uses have not been shown to be detrimental to their effectiveness (Cheesman et al., 2010).

2.3. Calibration of AEMs

We calibrated the AEMs to determine if the amount of P extracted from the AEMs represents the actual amount of P in solution. Charged AEMs were shaken in 25 mL P standards (0, 0.5, 1, 2, 5, 10, or 40 ppm) for 4 h with four replicates per standard. After 4 h, AEMs were removed from the standard solutions and gently rinsed with DI water. Phosphorus was extracted from the AEMs by shaking with 0.5 M HCl for 4 h. To determine the maximum capacity of an AEM strip (24 cm^2), we loaded nine strips with 1 M KH_2PO_4 using methods stated above and extracted and analyzed for phosphate.

2.4. Phosphate analysis

The Murphy–Riley ascorbic acid method was employed to analyze the HCl extract for available phosphate (Kuo, 1996). Steps were taken to assure that the method was appropriate for the samples studied, including the establishment of an ideal incubation time and the testing of different ratios of molybdate working solution to extract solution without neutralization. It was determined that an incubation time of between 18 and 24 h and a ratio of 200 μL of sample to 100 μL of working solution yielded linear standards (0–10 ppm; $r^2 > 0.999$), consistent slope, and the most reliable results. The field deployed AEMs were composited by plot for chemical analysis, and the samples were assayed in quadruplicate and read after incubation on a Synergy HT (BioTek, Winooski, VT, USA) microplate spectrophotometer at 880 nm.

2.5. Field deployment and harvesting of AEMs

Anion exchange membranes were deployed *in situ* in the study plots. Using a syringe, a hole was made in each AEM to thread a fishing line to facilitate recovery. The AEMs were stored in plastic bags until deployment to prevent drying. The AEMs were deployed in triplicate in each plot along the diagonal axis, approximately equidistant from one another within the center of the plot. To deploy the AEMs, the O horizon was gently removed and a putty knife was used to guide insertion into the mineral soil at an angle to a depth of 5 cm. Once deployed, the soil was firmly pressed against the AEM to provide good soil contact and the O horizon was replaced. The line attached to each membrane was then tied to a marker flag. After fourteen days *in situ*, the strips were harvested by locating the marker flags, carefully pulling the line attached to each AEM, and cutting the line from its flag. The AEMs from each plot were stored in individual plastic bags for transport and then cleaned in the lab with a gentle stream of DI water and forceps to remove soil. The moist AEMs were temporarily stored at 4 °C before P analysis.

2.6. Soil sampling and resin P analysis

Within 20 cm of each AEM deployment site, three, 2 cm diameter soil cores were collected 5 cm into the mineral soil, for a total

of nine composited soil cores per plot. Soil was sieved, homogenized, and stored at 4 °C for subsequent analysis. To measure the resin P (labile) fraction, 5 g of sieved soil was weighed into a 50 mL Oakridge high density centrifuge tube, to which 20 mL of DI water and one charged AEM was added. The samples were shaken (175 rpm) horizontally for 4 h; the AEMs were then removed and cleaned of soil with DI water. The AEMs were stored overnight in individual plastic bags and extracted the following day.

2.7. Nitrogen analysis and mineralization

To quantify soil net N mineralization, nitrate (NO_3^-) and ammonium (NH_4^+) analysis were performed both on field-fresh and incubated soils, but only for the first sampling date. Soil was incubated at 22 °C, kept moist, in loosely lidded cups for fourteen days. About half of the soil was analyzed for P to determine P mineralization rates. The initial (un-incubated) samples and the incubated samples were both shaken on a rotary shaker with 1:5 dilutions of soil and 1 M KCl for 1 h. Supernate was removed from the sample after centrifuging at 3000 rpm for 10 min. Ammonium was determined colorimetrically using a salicylate-hypochlorite method (Kempers and Zweers, 1986), while nitrate was determined using a VCl_3/Griess method (Miranda et al., 2001) as modified by DeForest and Scott (2010).

2.8. Enzymes

We measured the activities of phosphomonoesterase (PM) and phosphodiesterase (PD), which can depolymerize labile organic P (e.g., sugar phosphates) and less-labile nucleic acids and phospholipids, respectively (Turner, 2008). Soil enzyme activities were measured at AEM deployment (day 0) and harvest (day 14). Fall samples were sieved and analyzed one day after harvest, whereas winter samples were stored at −20 °C for a few weeks prior to analysis. One-gram of soil was mixed with 50 mM of sodium acetate trihydrate buffer (pH 5.0). We prepared a 200 μM of 4-methylumelliferone (MUB) phosphate and *bis*-(4-MUB) phosphate the day before analysis and stored it at 5 °C. Samples were prepared and dispensed as described in DeForest (2009) into black, 96-well 300 μL microplates with blanks (sodium acetate buffer), reference standards (MUB + buffer), and negative controls (substrate + buffer) for each plate and sample controls (soil slurry + buffer), quench (MUB + soil slurry), and assay (soil slurry + substrate). After all substrates were dispensed, the plates were incubated in the dark at 20 °C.

Due to problems with adding NaOH (DeForest, 2009; German et al., 2011), we measured enzyme activity without adding NaOH or anything else (DeForest et al., 2012). Because P availability and other soil environment conditions (i.e., season) can greatly influence enzyme activity (Paz-Ferreiro et al., 2011), we measured fluorescence of a well-plate several times to provide a more robust estimation of enzyme activity, as opposed to the common single measurement method (DeForest, 2009; DeForest et al., 2012; Saiya-Cork et al., 2002). To determine the correct incubation times, fluorescence was measured sequentially at least seven times between 0.5 and 23 h after substrate addition for both PM and PD for the first sampling date. Sampling times were removed from the calculation to achieve linear results with the highest R^2 (e.g., >0.999) among all the samples. This data was used (see results) to find the best incubation times and to provide a comparison with measuring enzymes once per assay. As such, enzyme activities were calculated from four measurement times between 0.5 and 3 h for PM and 3–6.5 h for PD. Enzyme activity was calculated by the slope of product formed by time (V; nM g^{-1} soil h^{-1}).

2.9. Data analysis and statistics

We used a linear mixed-effect (LME) model to determine if elevated P significantly altered the measured variables. Treatment is the fixed effect and forest stand, nested within season, are the random effects. The analysis was conducted using 'lme' function, in addition to the 'anova' function in the R 'nlme' package (v.2.13.0, R-Project). Linear regressions analysis was performed with using the 'lm' within the 'anova' function in R (R-Project, 2010). We used the 'slope.com' function in the 'smatr' package to compare slopes among groups. Shapiro–Wilk normality test was used to determine normally and Bartlett's test to determine homoscedasticity among treatments. Variables were $\log_{10}$ transformed, if necessary, to ensure homogeneity of variance. All differences discussed were significant when P-value was ≤ 0.05.

3. Results

3.1. AEM percent recovery

We observed a difference in the percent recovery of phosphate based on the concentration of solution. Percent recovery of solutions between 0.5 and 2 ppm (low P; 12.5–50 μg P) was around 90% whereas the recovery of solutions between 2 ppm and 40 ppm (high P; 50–1000 μg P) was ~77%. Bartlett-corrected likelihood ratio statistic was 22.8 with a P-value < 0.01 which strongly indicates the slope between initial P and AEM extracted P was different between our categories of low and high P concentrations. We used the results from this experiment to correct AEM values using a second order polynomial (Eq. (1)) because it provided the best fit ($R^2 = 0.9995$) using one equation.

$$\text{Corrected P } (\mu g) = 0.0025\ (\mu g\,P)^2 + 1.0343\ (\mu g\,P) + 1.7092 \quad (1)$$

The highest standard treatment values were not used in Eq. (1) because all samples were well under 125 μg P (5 ppm). We observed that 1 cm^2 can hold approximately 1.03 ± 0.02 (mg P cm^{-2}; SD) or around 24 mg per AEM strip.

3.2. Soil properties and nutrient cycling

The available P pool (i.e., resin P) was significantly ($P < 0.01$) two to three times greater in the elevated P treatments when compared to the control plots. Ambient resin P was 4.3 ± 1.2 μg P g^{-1} in October and 3.9 ± 0.6 μg P g^{-1} in December. Elevated P was 9.8 ± 3.8 μg P g^{-1} in October and 10.8 ± 2.7 μg P g^{-1} in December. We observed no significant ($P = 0.94$) seasonal main effect or an interaction between season and treatment ($P = 0.34$). The flux of available P (μg P/day) was significantly ($P < 0.01$) four times greater in the elevated P treatment when compared to the control (Fig. 1), but was unchanged by season ($P = 0.80$) or by season and treatment interaction ($P = 0.24$). There was a decrease ($P = 0.06$) in net P mineralization in the elevated P treatments when compared to the control (Fig. 2). Extractable pools of NH_4^+ and NO_3^- were similar ($P > 0.13$) between treatments and averaged around 8.6 ± 4.3 μg NH_4^+-N g^{-1} and 0.8 ± 2.1 μg NO_3^--N g^{-1}. Furthermore, net N mineralization was not affected ($P = 0.81$) by the treatment, but was at least 10 times greater than P mineralization (Fig. 2). Nitrification was insensitive to the treatment ($P = 0.19$). Control soil N mineralization was 0.11 ± 0.4 mg N kg^{-1} day^{-1} and elevated P soil was 0.21 ± 0.3 mg N kg^{-1} day^{-1}. There was no significant treatment effect on total C ($P = 0.53$), N ($P = 0.10$), or CN ($P = 0.21$).

3.3. Soil enzymes

Phosphomonoesterase activity showed a significant treatment effect ($P = 0.02$); with elevated P treatment plots having 13% lower

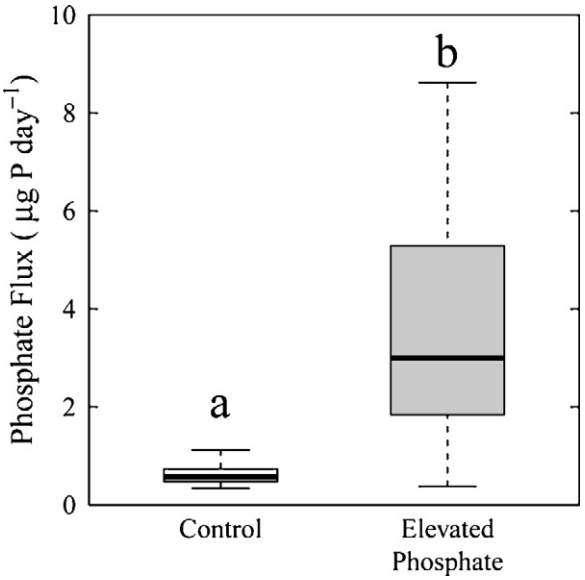

Fig. 1. Phosphate flux in response to elevated soil phosphate in acidic deciduous forest soils. Letters indicate significant differences ($n = 18$).

activity as compared to the controls (Fig. 4). Likewise, PD activity was significantly ($P < 0.01$) lower by 34% in the elevated P treatment when compared to the control. We observed no significant ($P > 0.08$) season or season by treatment interactions with enzyme activities. While not the original purpose for this experiment, we observed that the incubation time for PM and PD were different and PD required an incubation time twice as long as that of PM. Phosphomonoesterase activity was linear (i.e., $R^2 > 0.99$) with time from 30 min to 5 h, however, PD was linear with time from 1 h to 23 h for control plots. However, for elevated P treatments, the first measurement (30 min for PM and 1 h for PD) was often removed to improve linearity. When comparing enzyme activity calculated from individual incubation times with activity based on multiple measurements, we observed strong linearity (i.e., $R^2 > 0.90$) between 2 and 4 h for PM, but it was between 4 and 23 h for PD (Fig. 3). Based on this relationship, optimal single-point incubation time for PM was around 3 h and above 5 h for PD.

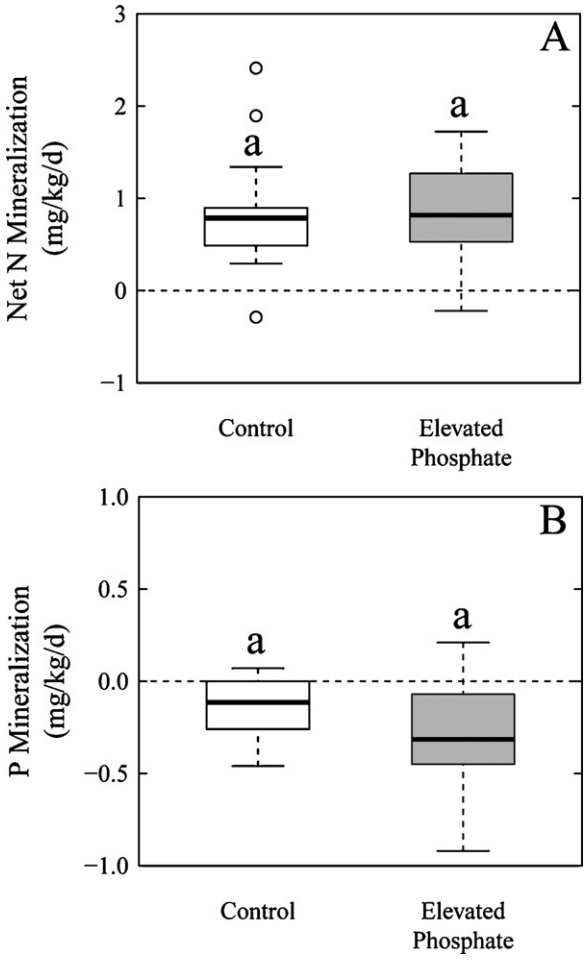

Fig. 2. Net nitrogen mineralization (A) and phosphate mineralization (B) in response to elevated soil phosphate. Letters indicate significant differences ($n = 18$).

4. Discussion

4.1. Nutrient cycling

The primary goal of this study was to quantify labile P flux in acidic eastern deciduous forest soils and to present a practical method for achieving this end. Readily available P turnover in ambient soil conditions was found to be 0.64 μg P a day, which is about 15% of the labile P pool, suggesting it completely turns over within a week. Under the elevated P treatment, P cycled 3.5 μg P a day or 34% of the pool, indicating a turnover of labile P within 3 days. This result is consistent with other experiments where adding fertilizer increased the cycling rate (Magill et al., 1997; Nave et al., 2009; Prescott et al., 1992; Zak et al., 2006). This study did not attempt to capture the nuanced P dynamics of eastern deciduous forest ecosystems, but rather sought to establish a snapshot of the activity of the highly labile resin P pool. Flux data, when coupled with pool data, indicated that the resin P pool in eastern deciduous forests is small but rapidly cycling and fluctuating over time. Because of this fluctuation, resin P flux is likely a more useful metric than resin P pools alone. A tropical dry forest site with similar low labile P status (3.2 ± 0.7 μg P g^{-1} soil) as quantified by Campo et al. (2001) also found indicators of rapid P flux, though focused on transfer of P from litter to mineral soil.

Results also indicated that the relationship between N and P in this acidic temperate forest system is subtle. While general understanding of N vs. P limitation dynamics based on the temperate/tropical dichotomy would indicate N limitation in this temperate region, enzyme activities and N and P mineralization data indicate that there is more P stress than N stress. We found that in both elevated P and ambient soil, N was mineralized while P was immobilized. Elevated P treatments should have caused P mineralization (Prescott et al., 1992), indicating the treatment has yet to alleviate P limitation. It is unclear if the microbial response scales to the plant trophic level. As proposed by DeForest et al. (2012), soil microbes may experience P deficiency, but this may not be observed in vegetation due to better utilization of organic P pools via phosphorus-acquiring enzymes.

While no significant relationships were found between N and P properties, the N mineralization–P immobilization dynamic is indicative of N abundance and a relative lack of P for soil microorganisms, even in the elevated P soil. The P budget described by Yanai (1992) demonstrates positive P mineralization consistent with what one would expect of a temperate forest not limited by P. Additionally, enzyme activities further indicated P stress, with both phosphomonoesterase and phosphodiesterase activities being significantly lower in elevated P plots than in control plots, consistent with the findings of Olander and Vitousek (2000), who found decreased phosphatase activities on P fertilized soils compared to equivalent, unfertilized P limited montane rainforest soils in Hawaii.

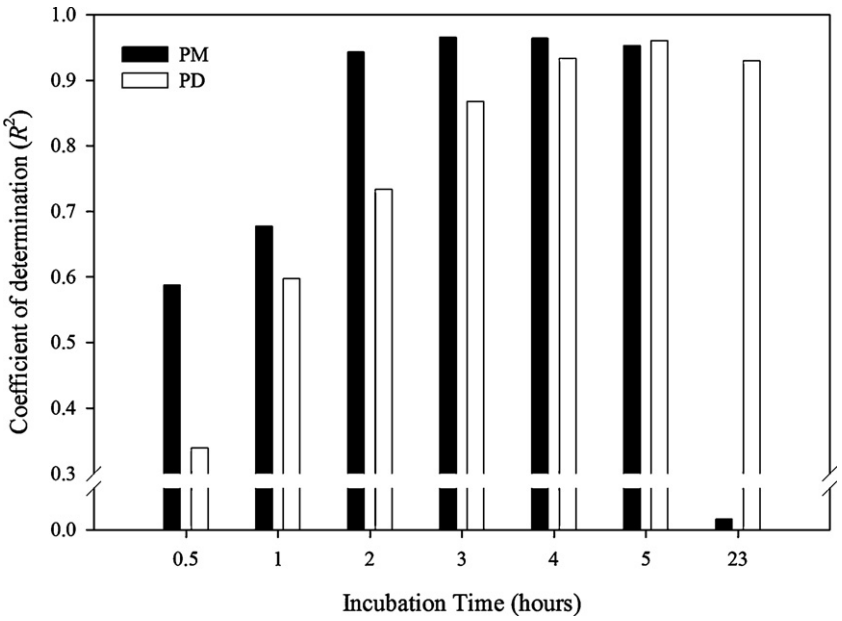

Fig. 3. A correlation of phosphomonoesterase (PM) and phosphodiesterase (PD) enzyme activity based on a single incubation time with enzyme activity calculated from seven measurements. Measuring activity for PM at 2 h or PD at 5 h correlated strongly ($R^2 > 0.95$) to measuring enzyme activity several times.

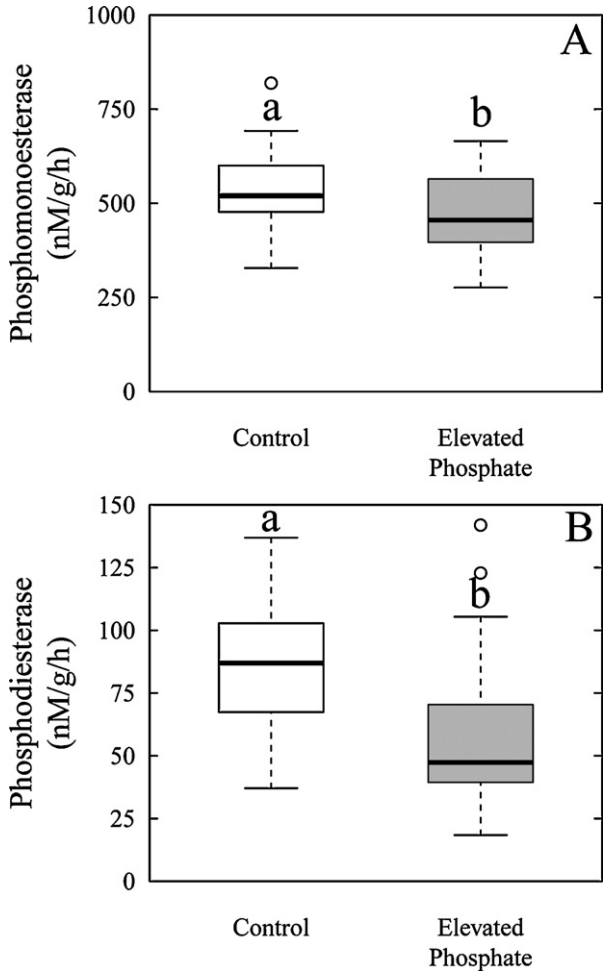

Fig. 4. The enzyme activity of phosphomonoesterase (A) and phosphodiesterase (B) in response to elevated soil phosphate. Letters indicate significant differences ($n = 18$).

The use of field-deployed AEMs provides an opportunity to quantify P flux in a similar manner to the quantification of P pools by the resin P extraction step of the Hedley fractionation method (Johnson et al., 2003). These values provide an important piece of the larger puzzle of P dynamics in forest systems and may be more informative than P pools when comparing different systems. Comparison of flux values, in the context of the other metrics herein, may further support a more nuanced understanding of nutrient dynamics as having less to do with climate (e.g., tropical or temperate) and more to do with the biogeochemical history of the site.

4.2. Enzyme incubations

While enzyme methodology was not an original objective of this experiment, we discovered that the typical incubation times of acid phosphatase might need to be much longer. Thirty minutes is a typical incubation time for acid phosphatase (i.e., a phosphomonoesterase) (DeForest, 2009; Saiya-Cork et al., 2002), but may be underestimating *in situ* activity. For example, if this experiment just used 30 min readings, then reported mean PM enzyme activity would have been 20% lower, but similar if measured once at 3 h. However, there was no clear trend between absolute enzyme activity and 30 min incubation activities (data not shown). Regardless, these results indicated great flexibility in regards to incubation times; from 2 to 5 h for PM and 5 to 23 h for PD. It is unclear if incubation times will alter the story of treatment effects in other investigations, but it may make subtle differences less apparent. In addition to recommendations by German et al. (2011), it is advisable to confirm proper enzyme incubation time(s) to confirm linear activity over time.

5. Conclusions

A tripling of readily available P increased P flux nearly sixfold resulting in a one-week turnover in ambient soils to 3 day turnover in elevated P soils. This quantification, being the primary goal of this investigation, is valuable for the comparative study of P dynamics in forest ecosystems, temperate and tropical. These continued studies are particularly vital with increasing evidence that

the assumptions made about the nutrient status of forests based on the temperate/tropical dichotomy may not always hold true (Elser et al., 2007). This humid temperate site demonstrated indicators of microbial P limitation, with P flux increasing and phosphatase enzyme activities decreasing with P amendment, while convention would indicate it to be N limited. Phosphorus flux results indicate that readily available P pool in unglaciated temperate eastern deciduous forest sites is very shallow but also very dynamic. Understanding this shallow, fast-moving stream of labile P is but a snapshot of the larger P dynamics in eastern deciduous forests but an important step in characterizing the mineral nutrient status of these soils.

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