NUTRIENT LIMITATION IN SOILS EXHIBITING DIFFERING NITROGEN AVAILABILITIES: WHAT LIES BEYOND NITROGEN SATURATION?

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Abstract. The nature of nutrient limitation in large areas of temperate forest may be changing due to human activities. As N availability in these forests increases, other nutrients could increasingly constrain productivity and other ecosystem processes. To determine the nature of nutrient limitation (N, P, and Ca) in forest soils exhibiting differing N availability, we conducted three field studies in the Fernow Experimental Forest, West Virginia, USA. The first used a ubiquitous herbaceous species, *Viola rotundifolia*, to compare indices of N availability to the activity of root-associated phosphomonoesterase (PME) activity at two spatial scales. The second study used fertilized, root in-growth cores to assess the extent of N, P, and Ca limitation. Finally, we measured the root-associated PME activity of *V. rotundifolia* growing in experimental plots that have received various combinations of nutrient additions and harvest treatments.

For entire watersheds, stream water nitrate concentrations were positively related to PME activities ($R^2 = 0.986$). For small plots, PME activities were positively associated with soil nitrate availability ($R^2 = 0.425$), and to a lesser extent with the leaf N concentrations ($R^2 = 0.291$). Root growth into microsites fertilized with P was greater than growth into microsites fertilized with either N or Ca, especially in watersheds with high N availability. Experimental additions of N increased the root-associated PME activity of *V. rotundifolia*, supporting the causality of the relationship between N availability and PME activity. Collectively, our results indicate that, as N availability increases, P becomes increasingly limiting at the sites examined. Understanding how nutrient limitations change during N saturation should improve ecosystem models and better inform our attempts to mitigate any undesired effects.

Key words: central Appalachian (USA) hardwood forest; Fernow Experimental Forest, West Virginia, USA; forest ecosystems; nitrogen saturation; nutrient limitation (N, P, and Ca); phosphatase activity; Viola rotundifolia.

INTRODUCTION

Nutrient limitation is of great economic and ecological importance because it can constrain the productivity of agricultural and natural ecosystems. Billions of dollars are spent every year on the production and use of fertilizers in the United States alone (National Agricultural Statistics Service 2005), and since global food production is highly dependent on nutrient additions, as the human population grows this expenditure is likely to remain significant (Smil 2001). Nutrient limitations not only constrain ecosystem productivity, but also influence its composition, diversity, interspecific interactions, and cycling of other essential materials (Vitousek 2004).

The nature of nutrient limitations in natural ecosystems may be changing because human activities are causing these systems to receive unusually large inputs of essential nutrients (e.g., N, P, and S) from the atmosphere and/or runoff (Smil 2000*a*, *b*, Brimblecombe 2005, Galloway 2005). If sustained, these large nutrient inputs could alter the *current* structure and function of ecosystems, and their susceptibility and responsiveness to *future* changes in the environment (e.g., drought, frost, herbivores, pathogens, and increasing CO_2 levels). Thus, it is fundamentally important to understand how nutrient limitations may change if we hope to predict the future composition and dynamics of ecosystems (Vitousek 2004).

Temperate forests have been previously considered to be nitrogen (N) limited (Vitousek and Howarth 1991). However, this condition may no longer exist in some areas that receive excessive amounts of N deposition from the atmosphere (Aber et al. 1989, 1998, 2003, Fenn et al. 1998). In these regions there is evidence that high amounts of atmospheric N deposition can increase the availability of N to the point that it exceeds (or "saturates") the biological demand for this nutrient. Nitrogen saturation has been variously defined (Ågren and Bosatta 1988, Aber et al. 1989, Stoddard 1994), but in general it is viewed as a continuum of changes that results in a sustained excess of available N relative to an ecosystem's ability to retain it (Aber 1992).

An overabundance of available N may result in several undesired ecosystem effects including greater losses of nitrate to receiving waters, an enhanced loss of

nutrient base cations, increased soil acidity, increased aluminum solubility, imbalances in the nutrient:N ratios of plant tissue, and the elevated production of nitrous oxide—a powerful greenhouse gas (Aber et al. 1989). Ultimately these changes may cause nutritional problems that affect forest productivity (Aber et al. 1995, Magill et al. 2004) and reduce species diversity in a manner similar to that observed in other ecosystems after long-term additions of fertilizer (Bobbink and Willems 1987, Bobbink 1991, Tilman 1996, Roem and Berendse 2002, Stevens et al. 2004).

As the degree of N saturation increases, N becomes less limiting and another nutrient could become increasingly limiting, or at least co-limiting, to plant and microbial productivity. In soils derived from base-poor parent material, two nutrients in particular are likely to limit plant growth as N availability increases—phosphorus and calcium.

Phosphorus (P) limitation may arise because it is an essential nutrient for plants, it is often present as unavailable forms in soils, and because N saturation may decrease the ability of plants to acquire P by causing a decrease in fine-root biomass (Nadelhoffer 2000) and mycorrhizal colonization—although the effect on mycorrhizal colonization seems to be highly variable (Marx et al. 1977, Wallenda and Kottke 1998, Treseder and Allen 2000, Treseder 2004).

In research sites where factorial fertilization trials cannot be conducted, there are several measures that can indicate P limitation, but none are unambiguous. Foliar N:P ratios have been suggested as an indicator of either N or P limitation (Koerselman and Meuleman 1996, Gusewell 2004), but ratios thought to indicate P limitation vary from 12.5 to 26.3 (Tessier and Raynal 2003) and may not reflect known differences in nutrient limitations (Vitousek et al. 1995). Root proliferation into soil microsites enriched in growth-limiting nutrients is well documented (e.g., Drew 1975), and has proven useful in assessing nutrient limitations in forest ecosystems (Cuevas and Medina 1988, Raich et al. 1994, Peterjohn et al. 1999, Stewart 2000, Blair and Perfecto 2001, Gleeson and Good 2003). However, although preferential root growth into nutrient-rich sites is common, it is not universal (Robinson 1994). A third measure of P limitation that has been used in several field studies is the activity of root-associated phosphomonoesterase (PME) activity. The expression of root-associated PME activity is associated with reduced growth (Goldstein et al. 1988a, b) and is regulated by P limitation (Olander and Vitousek 2000, Phoenix et al. 2003, Vance et al. 2003). The induction of phosphatase production is a ubiquitous response of higher plants to P starvation (Duff et al. 1994), and it can enhance P availability by releasing ester-bound phosphate groups from a variety of organic compounds. Molecular studies reveal that the production of acid phosphatase is tightly linked to the P status of plants, quickly induced prior to the onset of severe P starvation, and proportional to the extent of P deprivation (Baldwin et al. 2001, Miller et al. 2001, Raghothama and Karthikeyan 2005). Therefore, the production of extracellular acid phosphatases appears to be a specific response to P deficiency that is capable of significantly increasing P availability. Even so, there remains uncertainty as to the relationship between root phosphatase exudation and P acquisition (Kochian et al. 2004), and one study found that an increase in mycorrhizal colonization suppressed any change in root-associated PME activity when plants were exposed to P limitation (Cumming 1996). Thus, it appears that an increase in root-associated PME is sufficient to indicate P limitation, but such a response is not always necessary to meet the P requirements of plants under P-limiting conditions. Since no method of detecting P limitation (in the absence of fertilization trials) is necessarily conclusive, in this study we measure two indices of P limitation (root growth into fertilized microsites and root-associated PME activity), and examine a limited set of existing data for a third (foliar N:P ratios).

As N availability increases, calcium (Ca) limitation might also arise because this element makes important contributions to the structural integrity of plant tissues, and because it is essential for numerous physiological processes (McLaughlin and Wimmer 1999). Furthermore, Ca availability can be diminished by several factors including elevated losses in stream water that often accompany an increase in nitrate loss, reductions in atmospheric inputs over large regions (Hedin et al. 1994), substantial removals of Ca in harvested plant tissue (Johnson et al. 1988), and reduced plant uptake by greater amounts of soluble aluminum in acidified soils (Shortle and Smith 1988, Marshner 1995). Indeed, concern has been expressed about the potential for a widespread depletion of base cations in forest soils by acid deposition and N saturation (Federer et al. 1989, Likens et al. 1996, Adams 1999, DeHayes et al. 1999, Adams et al. 2000, Tomlinson 2003).

Evidence for Ca limitation under conditions of increased N availability can be difficult to detect (DeHayes et al. 1999). However, mass-balance calculations suggest that it may become more evident in the near future. The leaching of Ca from forest soils in the eastern United States is believed to have increased to 10 times the amount that occurred before the Industrial Age, thereby helping to create the net loss from forests that are typically found today (Federer et al. 1989). The extent to which this discrepancy between inputs and outputs results from rock weathering is difficult to determine (Blum et al. 2002, Bailey et al. 2003), but losses of Ca are certainly compounded by tree harvesting, which can remove 90% of the aboveground pool (and 13–19% of the total [plant + soil] pool) of this nutrient. As a consequence of the current rates of Ca loss, Federer et al. (1989) predict that plant biomass will decrease by 50% in the next 120 years in areas of high N deposition that are harvested on a 40-year cycle, and evidence of detrimental effects of base-cation depletion
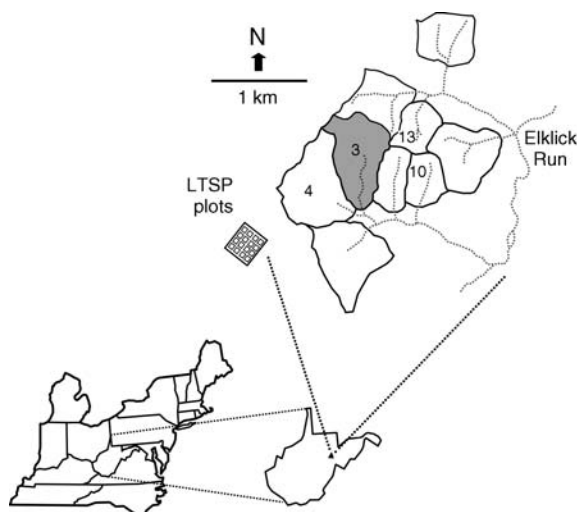
Fig. 1. Location of the Fernow Experimental Forest (in West Virginia, USA), the small watersheds (WS) we sampled, and the long-term soil productivity experiment (LTSP plots). Shading identifies WS 3, the watershed fertilized with 35.5 kg N·ha^{-1}·yr^{-1} as $(NH_4)_2SO_4$ since 1989. Typical stream-water nitrate concentrations are ~150 μmol/L for WS 3, ~55 μmol/L for WS 4, ~15 μmol/L for WS 10, and ~35 μmol/L for WS 13. Other watershed characteristics are given in Table 1.

on the growth of deciduous trees may already be appearing (Horsely et al. 2000, Bailey et al. 2004).

Since central Appalachia is considered to be a region that is susceptible to N saturation and its consequences (Adams et al. 2000), the purpose of this study was to examine the nature of nutrient limitation (N, P, and Ca) in different portions of a central Appalachian hardwood forest that have acidic forest soils, and that exhibit a wide range of N availability.

METHODS

To determine the nature of nutrient limitation (N, P, and Ca) in forest soils exhibiting differing N availability, we conducted three field studies in the Fernow Experimental Forest (West Virginia, USA). The first examined the relationship between N availability and P limitation by comparing indices of N availability to the activity of root-associated phosphomonoesterase (PME) at two spatial scales: small watersheds, and small plots within these watersheds. The second study used fertilized, root in-growth cores to assess the extent of N, P, and Ca limitation in the same small watersheds that were used in the first study. Finally, we tested the causality of any relationship between N availability and root-associated PME activity by measuring root-associated PME in the experimental plots of a long-term soil productivity (LTSP) experiment. The replicated plots in this experiment receive various combinations of nutrient additions and harvest treatments.

Study sites

The Fernow Experimental Forest is located near Parsons, West Virginia, USA (39°3′ 15″ N, 79°41′15″ S) in the unglaciated Allegheny Plateau (Fig. 1). It contains several small forested watersheds, four of which (WS 3, WS 4, WS 10, and WS 13) were selected for our studies because they differ greatly in stream water nitrate concentrations (Table 1).

All watersheds except WS 3 have not been cut since ca. 1905, are in close proximity (<2 km) to one another, have similar soils (loamy-skeletal, mixed, active, mesic Typic Dystrochrept), and typically receive 145 cm/yr of precipitation (Adams et al. 1994, 1997, Gilliam et al. 1996). WS 3 was cut 4 times since ca. 1905, and has been fertilized with 35.5 kg N·ha^{-1}·yr^{-1} as $(NH_4)_2SO_4$ since 1989. Cutting in WS 3 removed 13% of the basal area in 1958, 8% in 1963, 6% in 1968, and 100% between 1969 and 1972 (Kochenderfer 2006).

The ongoing LTSP experiment began in 1996 and is designed to assess the long-term effects (~80 yr) of biomass removal on forest soils and vegetation (Adams et al. 1999, 2004). This site is 1.6 km southwest of WS 3, has a slope of 15–31%, a southeastern aspect, and

TABLE 1. Site characteristics of the watersheds (WS) selected for this study at the Fernow Experimental Forest (near Parsons, West Virginia, USA).

Characteristic	WS 3	WS 4	WS 10	WS 13
Size (ha)	34.3	38.7	15.2	14.2
Aspect	S	ESE	S	NNE
Mean slope (%)	27	16	26	25
Stream flow (cm)	66.6	64.0	64.2	98.1
Stream water nitrate (μM)†	~150	~55	~15	~35
Dominant trees	*Acer rubrum, Betula lenta, Fagus grandifolia, Prunus serotina*	*Acer rubrum, A. saccharum, Betula lenta, Liriodendron tulipifera, Quercus rubra*	*Acer rubrum, Fagus grandifolia, Quercus prinus, Q. rubra*	*Acer rubrum, A. saccharum, Fagus grandifolia, Quercus rubra*
Land-use history				
Cut	ca. 1905, 1958, 1963, 1968, and 1969–1972	uncut since ca. 1905	uncut since ca. 1905	uncut since ca. 1905
Fertilized	Since 1989	never	never	never

† Stream water nitrate values represent the average of monthly, volume-weighted concentrations for January 1995–December 1999.

typically receives 145 cm/yr of precipitation. The site is divided into four blocks each containing one plot (0.2 ha with a 7.6-m buffer strip) for each set of four treatment combinations. The treatments include uncut and untreated control plots (CTL), plots that experienced whole-tree harvesting but were otherwise untreated (WT), plots that were whole-tree harvested and are fertilized with N (WT+N), and whole-tree harvested plots that receive additions of both N and dolomitic lime (WT+N+LIME). The N treatment consists of 36 kg $N \cdot ha^{-1} \cdot yr^{-1}$ as ammonium sulfate. The lime treatment consists of 22.5 kg $Ca \cdot ha^{-1} \cdot yr^{-1}$ and 11.9 kg $Mg \cdot ha^{-1} \cdot yr^{-1}$. Fertilization with N occurs three times per year (March, July, and November), and dolomitic lime is added once every other year. Prior to beginning the experiment, the trees growing on this site were dominated by *Acer saccharum*, *Quercus rubra*, and *Prunus serotina* (Adams et al. 2004).

The PME study

To assess P limitation in soils of the Fernow Experimental Forest, a prominent herbaceous-layer plant, *Viola rotundifolia*, was chosen for quantitative measurements of root-associated PME activity. Since *V. rotundifolia* is found throughout the Fernow Experimental Forest (Gilliam and Turill 1993, Gilliam et al. 1994, 1995, 1996), it allowed us to measure P limitation (as assessed by PME activity) in soils known to vary in N availability (Peterjohn et al. 1999, Christ et al. 2002), and to test the hypothesis that plants growing in soils with higher N availability will experience a greater P limitation as indicated by higher levels of PME activity.

In the summer of 2001 *V. rotundifolia* at 15 locations were sampled in WS 3, 4, and 10, and 13 locations were sampled in WS 13, for a total of 58 sites. Of these, 28 sites had been previously sampled for nitrate availability using anion-exchange membranes—9 sites in WS 4, 9 sites in WS 10, and 10 sites in WS 13. At each sampling location a minimum of five plants were collected within a 20-m radius and removed along with adjacent soil to prevent damage to the roots. The plants and surrounding soil (~top 5 cm) were sealed in plastic bags, transported to the laboratory on ice, and then refrigerated at 4°C for no longer than 72 h. Voucher specimens were also collected and compared to herbarium sheets to ensure the plants were properly identified. Identification as *V. rotundifolia* was confirmed when, in subsequent years, these plants were found in flower.

The PME activity associated with the root surface of *V. rotundifolia* was measured following published procedures (Tabatabai and Bremner 1969, Cumming and Weinstein 1990, Johnson et al. 1999). Freshly excised roots from the plants collected at a given location were submersed in 9.0 mL of a citric acid–sodium citrate buffer adjusted to a pH of 4 (a value typical for soils at our study sites). Next, 1.0 mL of 10 mmol/L *p*-nitrophenylphosphate (*p*-NPP) was added and the solution mixed for ~5 s. The compound *p*-NPP

acts as an artificial substrate for PME, which releases the colored compound *p*-nitrophenol (*p*-NP). After 1 h, the reaction was terminated by the addition of 2.0 mL of 0.5 mol/L NaOH. Color blanks were created from a composite sample of the *V. rotundifolia* roots collected in each watershed. These were processed in the same way as regular samples except the substrate was not added until after the addition of NaOH in order to prevent any release of *p*-NP. Standards consisted of *p*-NP in the following concentrations: 0, 50, 100, 150, and 200 μmol/L. Absorbance of solutions was measured spectrometrically at 410 nm using a Shimadzu UV 160U spectrometer (Shimadzu, Kyoto, Japan). After analysis of the PME activity, roots were dried (>48 h at 65°C) and weighed. Leaves from the same plants used in the PME assay were also dried at 65°C prior to analyzing them for their total N content by Dumas combustion (Bremner and Mulvaney 1982, Nelson and Sommers 1982) using a Carlo Erba NA 1500 N,C,S elemental analyzer (Carlo Erba, Milan, Italy).

At 28 of the locations where *V. rotundifolia* were sampled, nitrate availability has been previously measured using anion-exchange resin membranes (W. T. Peterjohn, *unpublished data*). Several times during the growing season (five times in WS 4 and 10, and once in WS 13), three strips of anion-exchange membrane (Dynambio, Madison, Wisconsin, USA) were inserted to a depth of ~7 cm at random locations within each plot. The plots were 10 m in radius and located at selected positions on a 100×100 m grid established in each watershed (Christ et al. 2002). Prior to deployment, anion exchange membranes (2×5 cm) were saturated with 1 mol/L NaCl and shaken 3 times for 15 min. Exchange membranes were then shaken and rinsed with distilled water several times, and left to dry overnight. In the field, membranes were attached by string to a small PVC (polyvinyl chloride) ring so they could easily be relocated. After 1 week, membranes were carefully removed from the soil, placed into pre-labeled plastic bags, and refrigerated until extraction. Upon returning to the laboratory, exchange membranes were rinsed with distilled water, and placed into pre-labeled 50-mL centrifuge tubes. Each membrane was extracted twice by shaking for 15 min in 25 mL of 1 mol/L KCl, and all extracts were refrigerated until nitrate concentrations could be analyzed by automated colorimetry (Hart et al. 1994) using a Lachat QuickChem AE autoanalyzer (QuickChem Methods 12-107-04-2-A and 1-B; Lachat, Milwaukee, Wisconsin, USA).

To determine the means, standard errors, and regression constants for both nitrate availability and PME activity, we used Statistica version 6.1 (StatSoft 2003). Regressions were used to examine relationships between PME activity and indices of N availability at two spatial scales: entire watersheds and 10-m-radius plots. At the watershed scale, stream-water nitrate concentrations were used as an index of N availability. At the scale of individual plots, both nitrate collected on

anion-exchange membranes and the average foliar N concentrations were used as independent measures of N availability. To determine if significant differences in PME activity occurred between watersheds, a one-way ANOVA was performed with SAS-JMP version 5.01 (SAS Institute 2002).

The fertilized root in-growth core study

The relative amount of root growth into microsites fertilized with different nutrients is a useful index of the degree to which a given nutrient limits growth in a plant community (Cuevas and Medina 1988, Raich et al. 1994). This index was used to assess the extent of N, P, and Ca limitation in the four watersheds being examined at the Fernow Experimental Forest. The response of root growth to microsites enriched with nutrients was measured using mesh bags (9×18 cm) made from fiberglass window screen (0.143×0.2 cm mesh). Each bag was filled with vermiculite and received either 55 mL of distilled water or 55 mL of a 0.1 mol/L solution of either NH_4Cl, Na_2HPO_4, or $CaCl_2$ (Cuevas and Medina 1988). Vermiculite was chosen because it has a relatively high cation-exchange capacity and low background levels of N, P, and Ca.

In June 2001 one set of four in-growth bags (one of each treatment type) were buried at 10 locations per watershed. Each bag was buried to a depth of 10 cm and in close proximity ($\sim$1 m) to the other bags in a given set. Each cluster of four bags was covered with wire mesh that was staked into the ground to prevent disturbance of the in-growth cores by animals. At the end of one year, all in-growth bags were retrieved by carefully slicing the soil around the core with a sharp knife. Each core along with any adhering soil was placed into a plastic bag and was frozen for future processing. To determine the amount of root material in each sample, the soil and roots were trimmed carefully from the outside of a thawed in-growth bag, and the contents sieved to obtain the larger roots. Smaller roots were collected after drying cores at 65°C and carefully removing the fine roots from the vermiculite. All root biomass was dried again at 65°C for at least 48 h and weighed.

To test for differences among the watersheds, the root growth data were used to calculate an index of growth stimulation that was analyzed using both a two-way nested ANOVA, and by separate one-way ANOVAs for each type of fertilizer addition. The index of growth stimulation we used was the ratio of root growth into the fertilized vs. unfertilized (water only) in-growth core at each sample site. This resulted in three response variables for each location—root growth stimulation due to N, P, and Ca additions. The statistical model for the two-way nested ANOVA included the following terms: watershed, plot (watershed), fertilizer type, and the various factor interactions. Of particular interest is the watershed × fertilizer interaction term because a significant interaction would indicate that the effect of

the type of fertilizer on root growth depends on the soil from a given watershed. Since the watersheds are known to vary in N availability, a significant interaction may support the hypothesis that either P and/or Ca are more limited in watersheds with greater N availability. All statistical analyses were performed using Statistica version 6.1 (StatSoft 2003).

The LTSP study

To test the causality of any association between N availability and P limitation (as assessed by root-associated PME activity), in the summer of 2005 we collected 10 individuals of *V. rotundifolia* from random locations within each of the 16 plots that comprise the LTSP experiment at the Fernow Experimental Watershed ($n = 160$ plants). All plant samples were collected, processed, and analyzed for root-associated PME activity using the same methods that were employed in our PME study using plants collected in the small watersheds during the summer of 2001 (see *Methods: The PME study* for details). If increased N availability causes an increase in P limitation, then those plots that have received annual additions of N since 1996 should have significantly greater rates of root-associated PME activity. To determine if significant differences in PME activity existed between the various treatment combinations, a two-way ANOVA without interaction was performed with Statistica version 6.1 (StatSoft, Inc.).

RESULTS

The PME study

Prior to all statistical summaries and analyses, two outliers were removed out of 58 samples. In addition, before running a one-way ANOVA all data were log transformed to normalize the distribution of the residuals. The root phosphomonoesterase (PME) activity (in micromoles of p-NP [p–nitrophenol] per gram of dry mass per minute) of *Viola rotundifolia* growing on the watersheds we examined were: 0.282 ± 0.058 for watershed (WS) 10, 0.416 ± 0.060 for WS 13, 0.488 ± 0.054 for WS 4, and 0.579 ± 0.56 for WS 3 (mean $\pm$ 1 SE). One-way ANOVA revealed that the watersheds were significantly different ($P = 0.001$) with respect to the root-associated PME activity of *V. rotundifolia* growing in the forest floor. A Tukey hsd multiple comparison showed that the PME activity was lower in WS 10 than it was in both WS 3 ($P = 0.001$) and WS 4 ($P = 0.026$), and PME activity in WS 13 tended to be lower than in WS 3 ($P = 0.070$).

At both of the spatial scales examined there were positive associations between root-associated PME activity and indices of N availability. When average stream-water nitrate concentrations for each watershed are used as an index of N availability, there was a strong, and seemingly curvilinear, relationship ($R^2 = 0.986$) between these values and the mean root-associated PME activity for *V. rotundifolia* growing in these watersheds (Fig. 2). At the spatial scale of
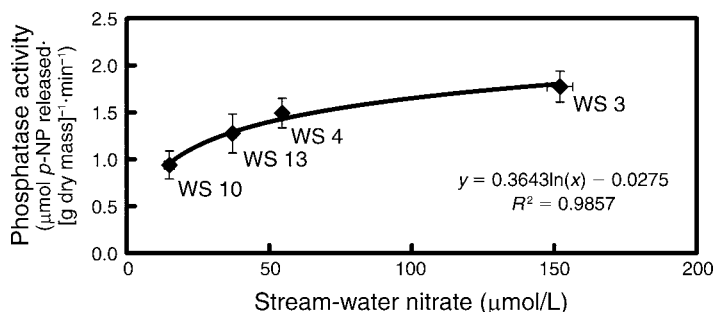
FIG. 2. Root-associated phosphatase activity of *Viola rotundifolia* growing in watersheds (WS) with different stream-water nitrate concentrations. Stream-water nitrate values are the average of monthly, volume-weighted concentrations for January 1995–December 1999. Data are means ± SE. Phosphomonoesterase (PME) activity is assessed by the release of *p*-nitrophenol (*p*-NP).

individual plots, there was also a positive, and apparently curvilinear, association ($R^2 = 0.425$) between root-associated PME activity of *V. rotundifolia* and nitrate availability when assessed by nitrate accumulation on buried anion-exchange membranes (Fig. 3). However, when foliar N concentrations were used as an index of N availability, the association between PME activity and N availability was not as strongly correlated ($R^2 = 0.291$) and less distinctly curvilinear (Fig. 4).

The fertilized root in-growth core study

All data were log transformed to normalize the distribution of the residuals and meet the assumptions of ANOVA. Three values were removed because root in-growth cores were either missing or damaged, and one outlier was removed because its value for a root growth stimulation ratio (20) was excessively large (>5 SD higher than the mean for that treatment type).

A two-way nested ANOVA found significant watershed ($P = 0.029$), plot nested within watershed ($P = 0.011$), and nutrient treatment ($P < 0.001$) effects. No support, however, was found for any interactive effect between the type of nutrients used and the watershed in which the fertilized in-growth cores were buried ($P = 0.750$). Regardless of the type of nutrient added to the in-growth cores, the stimulation of root growth was

greater, or tended to be greater ($P < 0.10$), when cores were buried in WS 3 and WS 4 compared to those buried in WS 10 and WS 13. Regardless of the watershed, the stimulation of root growth was greater in cores receiving P additions than those receiving N additions. There was also a trend ($P < 0.10$) toward the response of root growth being unique for each type of nutrient addition, with the growth response to P > Ca > N. Although no support was found for an interactive effect, it was clear that the differences between the watersheds were more pronounced for in-growth cores fertilized with P (Fig. 5). Indeed, when separate one-way ANOVAs were run for each nutrient treatment, significant differences between watersheds were found only for P-fertilized cores, and post hoc multiple comparisons (Tukey-Kramer hsd) indicated that P stimulated root growth more (or tended to stimulate root growth more) in WS 3 and WS 4. The significant effect of the sampling plots (when nested within a given watershed) indicates that it may be instructive to investigate patterns of between plot variability in more detail.

Regressions were used to examine the relationships between an index of N availability (leaf N concentration of *V. rotundifolia*) and the stimulation of root growth by the various nutrient additions. The foliar N concentrations of *V. rotundifolia* were measured as part of the PME study (see *Methods: The PME study*, above) in 33 of the 44 plots used in this study and are a useful index
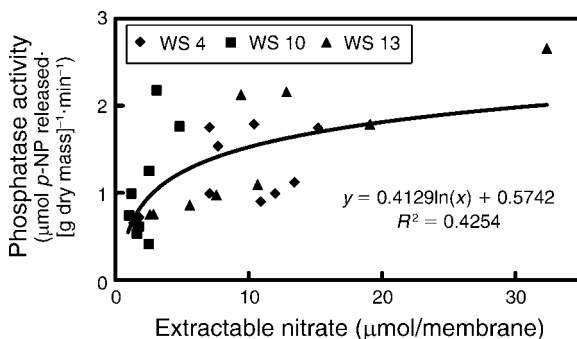

FIG. 3. Root-associated phosphatase activity of *Viola rotundifolia* growing in individual 10 m radius plots compared to nitrate availability in the same plots. Nitrate availability was assessed by the accumulation of nitrate on buried anion-exchange membranes (2 × 5 cm). Symbols represent the three watersheds (WS) with paired data. Phosphomonoesterase (PME) activity is assessed by the release of *p*-nitrophenol (*p*-NP).
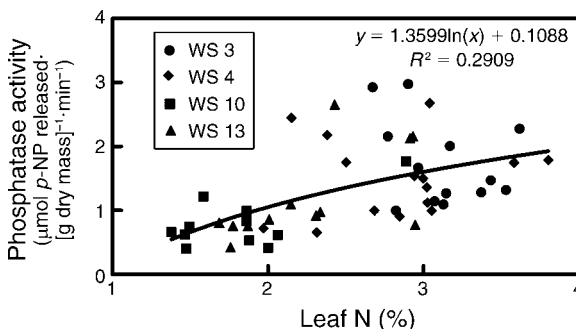

FIG. 4. Root-associated phosphatase activity of *Viola rotundifolia* growing in individual 10 m radius plots compared to the mean foliar N concentration of the same plants. Symbols represent the four watersheds sampled. Phosphomonoesterase (PME) activity is assessed by the release of *p*-nitrophenol (*p*-NP).
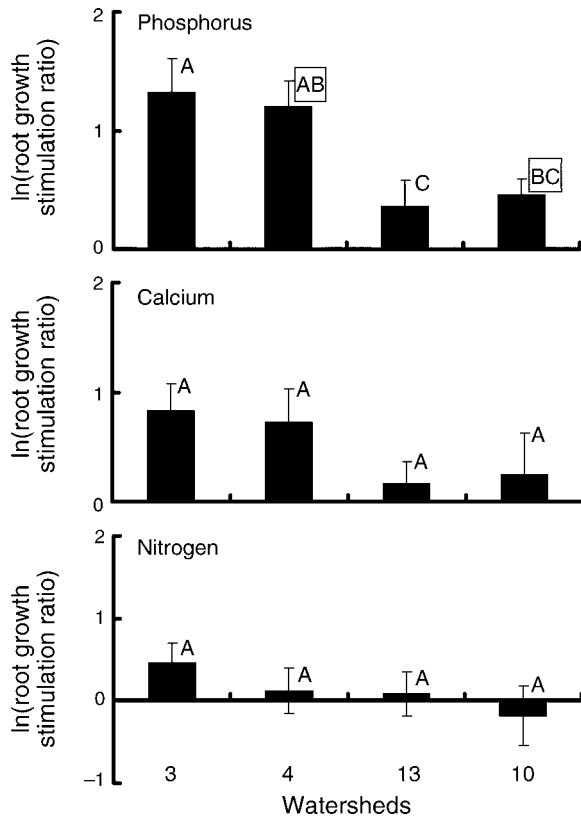
FIG. 5. Log-transformed root growth stimulation ratios for in-growth cores located in different watersheds of the Fernow Experimental Forest, West Virginia, USA. Bars with different uppercase letters were significantly different ($P < 0.05$). Letters with boxes have a trend toward being different ($P < 0.10$). Data are means ± SE. Watersheds are listed (left to right) in order of decreasing stream-water nitrate (Table 1).

of N availability in the surface soils at the Fernow Experimental Forest (Gilliam et al. 1996). Since foliar N concentrations were normally distributed and the stimulations of root growth by the various nutrients were log-normally distributed, log-linear regressions were performed. No association ($r^2 = 0.001$, $P = 0.840$) was found between N availability measured in this way and the stimulation of root growth by N additions (Fig. 6). However, there were stronger associations between N availability and the stimulation of root growth by both P ($r^2 = 0.142$, $P = 0.034$) and Ca ($r^2 = 0.100$, $P = 0.084$ with one outlier removed) additions (Fig. 6).

The LTSP study

All data were log transformed to normalize the distribution of the residuals and to meet the assumptions of ANOVA. A two-way ANOVA without interaction provided no support for any block effect ($P = 0.964$) and strong support ($P < 0.001$) for a significant treatment effect on root-associated PME activity. Post-hoc multiple comparisons (Tukey-Kramer hsd) indicated that the PME activity of *V. rotundifolia* roots was greater in plots

receiving N, and that PME activity was higher in plots that experienced whole-tree harvesting (Fig. 7).

DISCUSSION

The main findings of each of the three field studies all indicate that forests at the Fernow Experimental Forest (West Virginia, USA) become more limited by P as N availability increases. These findings include (1) a positive association between increasing nitrate availability and root-associated PME (phosphomonoesterase) activity at two spatial scales—entire watersheds and small plots within these watersheds; (2) a greater growth of roots into soil cores fertilized with P than into cores fertilized with either Ca or N (especially in watersheds and plots with higher N availability); and (3) significantly higher levels of root-associated PME activity in experimental plots that have received N additions for nine years. Since increases in PME activity are associated with enhanced P limitation, and root growth often responds to limiting nutrients, these results suggest that increasing N availability at this site will increase the demand for P by vegetation, and may lead to P limitation becoming an important control over forest growth.

One surprising result from our fertilized in-growth core study was that N-fertilized microsites in soils
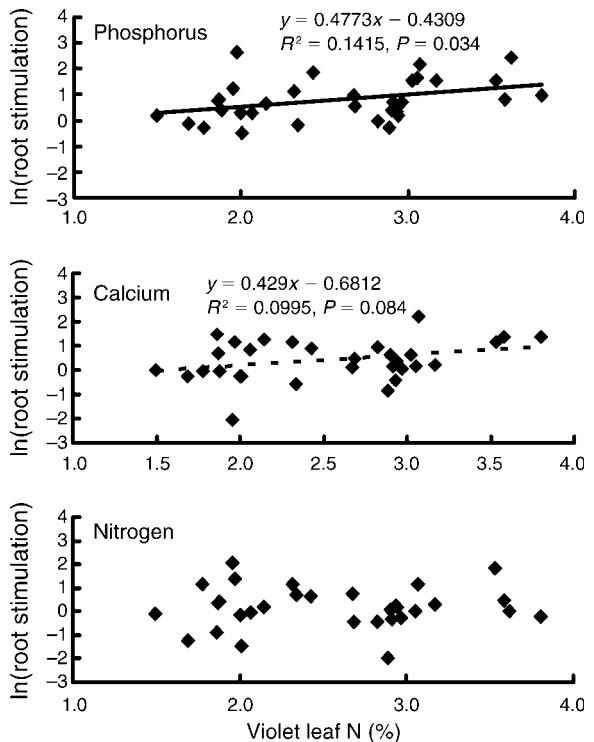

FIG. 6. A comparison of root growth stimulation by various nutrients with the mean leaf N concentrations of *Viola rotundifolia* growing in the same plots. The solid regression line indicates statistical significance ($P < 0.05$), and the dashed line represents a trend toward statistical significance ($P < 0.10$).
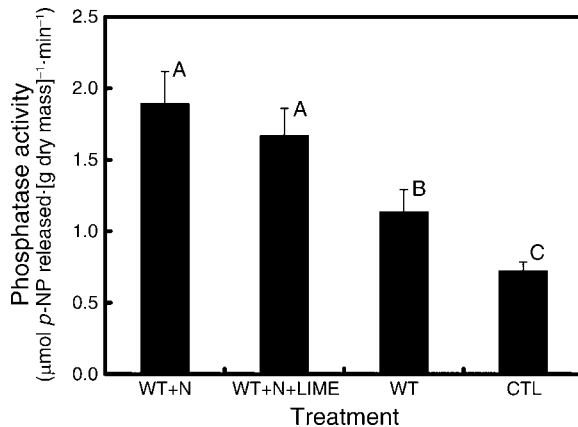
FIG. 7. Root-associated phosphatase activity of *Viola rotundifolia* growing in the various treatments of the LTSP (long-term soil productivity experiment) plots. The treatments include uncut and untreated control plots (CTL), plots that experienced whole-tree harvesting but were otherwise untreated (WT), plots that were whole-tree harvested and are fertilized with N (WT+N), and whole-tree harvested plots that receive additions of both N and dolomitic lime (WT+N+LIME). Bars with different uppercase letters were significantly different ($P <$ 0.05). Data are means + SE. Phosphomonoesterase (PME) activity is assessed by the release of *p*-nitrophenol (*p*-NP).

previously shown to have lower N availability (e.g., watershed [WS] 10) resulted in growth responses that were never larger than those due to the addition of other nutrients. This lack of preferential root growth into cores fertilized with N in WS 10 is difficult to understand. A previous study using in-growth cores positioned in WS 4 (high N availability) and WS 10 (low N availability) found a larger response of root growth to N additions in plots that exhibited several symptoms of N limitation, and a roughly equal response to added N or P in locations with high N availability (Foster 1997, Peterjohn et al. 1999). In contrast, our present study found a larger response of root growth to P vs. N additions regardless of the watershed being considered—although this preferential response of root growth for P vs. N was more pronounced in WS 3 and WS 4 (those with the highest stream nitrate concentrations), and roughly similar in WS 10 and WS 13. At the plot level, although there was a weakly positive association between N availability and the stimulation of root growth by P, a negative association was not detected between N availability and the stimulation of root growth by N addition. Many differences between these two field studies may contribute to their contrasting results. These differences include the material used to fill the in-growth cores (a calcined clay vs. our vermiculite), the length of burial at the field sites (5 months vs. 1 year), the number of locations in each watershed (4 sites vs. 10 sites), the amount of root material recovered per core (~0.30 g vs. ~0.05 g), and the year of the study (1996 vs. 2001). If both results are taken at face value they suggest that WS 4 may have shifted from N and P

co-limitation in 1996 to strong P limitation in 2001, whereas WS 10 has shifted from strong N limitation to co-limitation by N and P. However, a more likely explanation may lie in the fact that the two years had very different amounts of precipitation—1996 was extremely wet and 2001 was extremely dry. For the state of West Virginia the annual rainfall in 1996 was 153.2 cm, which makes it the second wettest year in the 110-year record (National Climatic Data Center 2005). In contrast, the annual rainfall in 2001 was 118.6 cm, making it the 25th driest year on record for West Virginia (National Climatic Data Center 2005). Measured precipitation at the Fernow Experimental Forest showed a similar pattern. The annual precipitation for WS 4 was 201.3 cm in 1996, and 133.0 cm in 2001.

Although not measured as part of this study, previous measurements of foliar N:P ratios at the Fernow Experimental Forest are consistent with the assertion that these forests are becoming more P limited as N availability increases. For example, Gilliam et al. (2001) found that the average, mass-based, foliar N:P ratio of *Viola rotundifolia* growing in WS 3 (the fertilized watershed) was 20.8, and significantly greater than the average ratio (16.1) measured for the same species in WS 4 (an unfertilized watershed). Furthermore, both values are in the range (>11) that appears to indicate P limitation for understory plants in upland ecosystems (Tessier and Raynal 2003).

As part of a larger study of watershed acidification, leaf samples from six tree species growing in WS 3 and WS 4 were collected in 1997 and 2002 (DeWalle et al. 2006). These data, however, were not used to calculate and compare N:P ratios. When we analyzed these data using separate, two-way ANOVAs for each species, two of the six species sampled (*Betula lenta* and *Liriodendron tulipifera*) had significantly greater mass-based, foliar N:P ratios (21.4 and 20.2, respectively) when growing in WS 3 (the fertilized watershed) than when they were growing in WS 4 (16.5 and 16.5, respectively). We also found no evidence that the differences between sites depended on the year in which the samples were collected. However, results from a separate study suggest that temporal variability may be important. In that study, May et al. (2005) compared the foliar nutrient dynamics of three tree species growing in WS 3 (*L. tulipifera*, *Acer rubrum*, and *Prunus serotina*) with those of the same species growing in a nearby, unfertilized stand of similar age but different cutting history. They collected foliar samples on four occasions (1992, 1997, 2000, and 2001) during a 10-year period, and found that foliar N:P ratios were elevated in the fertilized site (WS 3) for two of the species (*A. rubrum* and *P. serotina*), but only during some of the years when sampling occurred. Surprisingly, in 2001 the mean N:P ratios for all three species were lower in the fertilized site. Despite conflicting evidence about the importance of temporal variability, both studies found that the mean foliar N:P ratios for trees (regardless of species,

site, or sampling date) were higher than values typical of deciduous woody species worldwide (N:P = 13.9; Gusewell 2004), and were within the range of threshold values that indicate P limitation in other terrestrial ecosystems (Tessier and Raynal 2003).

In regions that receive high amounts of N deposition, reduced levels of soil base cations could also contribute to the limitation of plant growth in soils that develop from noncalcareous parent material. Results from our fertilized in-growth cores provide some evidence that Ca limitation may be enhanced in individual plots as N availability increases. More specifically, there was a trend ($P < 0.10$) towards a greater stimulation of root growth into Ca-fertilized microsites as N availability increased (Fig. 6), however this association was very weak ($r^2 = 0.100$, $P = 0.084$)—at least during the relatively dry year when our measurements were made. Given that the soils at our study sites are acidic (pH 3.9–4.5) and have moderately low base saturation (14–34%; Adams et al. 2006), the potential effects of base-cation depletion warrant further investigation under a broader range of environmental conditions.

In agreement with our main results, investigators working in other ecosystems have found that a sustained increase in N availability is associated with elevated levels of phosphatase activity, and other indices of P limitation. Experiments in a calcareous grassland found significant increases in root-associated PME activity in plants transplanted into soils that had received N additions for seven years (Johnson et al. 1999, Phoenix et al. 2003). A similar but shorter experiment, however, found that root-associated PME activity *decreased* in response to N additions suggesting that several years of chronic N additions are required to shift a plant community towards P limitation (Johnson et al. 2005). In a seasonal montane rainforest, Olander and Vitousek (2000) found that N additions significantly increased soil phosphatase activity in N-limited soils and, in the same location, Treseder and Vitousek (2001) found that N fertilization consistently increased root PME activity over a wide range of N availabilities. Using foliar N:P ratios as an index of P limitation, a recent study in the deciduous forest of the Catskill Mountains—an area experiencing high amounts of N deposition from the atmosphere—found that N:P ratios in understory plants were indicative of plants that were at, or near, P limitation (Tessier and Raynal 2003). They also found that experimental additions of N had no detectable effects, whereas P additions increased P concentrations and, in one case, increased biomass production. Thus, the collective results from the variety of ecosystems examined so far indicate that enhanced P limitation (as indexed by enhanced phosphatase activity or foliar N:P ratios) may be a general result of a prolonged increase in N availability.

The generality of this pattern, however, warrants further testing because the number of ecosystems examined so far is quite limited, and theoretically the emergence of strong P limitation will be constrained by factors that influence the supply of available P such as the degree of chemical weathering (Walker and Syers 1976), soil pH (Brady 1974), and geology. Given our current understanding, the extent of P limitation resulting from increased N availability should be the greatest in more weathered soils (e.g., unglaciated soils), more acid soils (due to greater P adsorption onto oxides of Fe and Al), and in soils derived from parent material with low levels of P-containing apatite (e.g., non-granitic bedrock). Such conditions are found in the sites we studied at the Fernow Experimental Forest, supporting our evidence for greater P limitation as N availability increases in these forests, and making these sites a valuable resource for improving, and testing, our understanding of how nutrient dynamics may change in the wake of N saturation.

Improving our understanding of the nature of nutrient limitation under conditions of high N availability is important because it will enhance our ability to manage these ecosystems, and predict how they will respond to altered environmental conditions. If, as appears to be the case at our study sites, a forested ecosystem shifts from N to P limitation, then the initial response might include changes in resource allocation by plants that preferentially improve the acquisition, or retention, of P. These changes could include an increased activity of root-associated phosphatase (Vance et al. 2003), a greater production of organic acids in root exudates (Marshner 1995), a shift in the type (or diversity) of mycorrhizal fungi that colonize plant roots in a way that increases P acquisition (Dighton et al. 1990, Dighton and Coleman 1992, Baxter and Dighton 2001), and a greater efficiency of P resorption from leaves prior to senescence (McGroddy et al. 2004). To the extent that the existing plant community can make these adjustments, the system should initially exhibit growth responses to both N and P additions. However, the response of ecosystem productivity to N additions may be misinterpreted because in reality the plants are metabolically adjusting to a greater demand for P—a case of P limitation in disguise (Vitousek and Howarth 1991). As N availability continues to increase, symptoms of "true" P limitation will emerge (growth responding only to P additions), and the composition of plant species should shift towards those that acquire, use, or retain, P more efficiently. Currently, few forest ecosystem models include P dynamics even though P is often considered to be the nutrient that will limit productivity under conditions of high N availability. As high atmospheric inputs of N to forested regions continue, it becomes increasingly important to incorporate P dynamics into ecosystem models if we hope to meaningfully interpret experimental results and predict the response of forest ecosystems to future changes in the environment.

If P limitation resulting from chronic N deposition becomes widespread in forest ecosystems, then amelioration of the potentially detrimental effects of N saturation might be achieved by additions of P, or by

the reduction of N inputs from the atmosphere. The potential effectiveness of P additions in reducing the symptoms of N saturation has been proposed (Fenn et al. 1998) and demonstrated at the local scale for at least one site (Stevens et al. 1993). However, P fertilization is unlikely to be an effective strategy for regional-scale mitigation efforts due to the limited quantity of inexpensive phosphate reserves, heavy metal contamination of the larger but more inaccessible phosphate deposits, and environmental problems associated with excessive P inputs (Smil 2000b, Tilman et al. 2001, Vance 2001). A more reasonable mitigation strategy for large regions is suggested by studies in Europe that have demonstrated that experimental reductions in N inputs can rapidly, and substantially, lower N availability (Quist et al. 1999, Corre and Lamersdorf 2004) and nitrate losses in soil leachate (Boxman et al. 1995, Bredemeier et al. 1995, 1998). However, recovery from any N-induced shift in species composition and tissue chemistry of the vegetation may take considerably longer (Boxman et al. 1995, Strengbom et al. 2001).

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