Manuscript title: Nitrogen and phosphorus regulation of soil enzyme activities in acid forest soils

Authors: Farrah R. Fatemi ^{1†}, Ivan J. Fernandez ², Kevin S. Simon³, David Bryan Dail⁴

† Corresponding author:

Farrah R. Fatemi

St. Michael's College

One Winooski Dr., Colchester, VT 05439

Email: ffatemi@smcvt.edu, phone: 802-654-2917

Author Affiliations:

¹ St. Michael's College, One Winooski Dr., Colchester, VT 05439

² School of Forest Resources and Climate Change Institute, University of Maine, Orono, Maine,
United States

³ School of Environment, The University of Auckland, New Zealand

⁴ Surface Water Quality Bureau, Santa Fe, New Mexico

Abstract

The activities of soil extracellular enzymes are important in governing rates of organic matter decomposition and nutrient cycling in forest ecosystems. Measurements of soil enzyme activities can provide insights on microbial function, in terms of how much energy microbial communities are investing to acquire particular nutrients from OM substrates. In ecosystems enriched with nitrogen (N), phosphorus (P) supply may play an important role in regulating microbial activity, enzyme production, and organic matter decomposition. The response of extracellular enzyme activity to chronic N-enrichment was assessed at a long-term paired watershed N-enrichment experiment, the Bear Brook Watershed in Maine (BBWM) in hardwood and softwood forest types. Specifically, we measured the ambient (extant) activity of C hydrolyzing β -glucosidase (BG) and xylosidase (XYLO), N hydrolyzing N-acetylglucosaminidase (NAG), and P hydrolyzing acid phosphatase (AP) in the watershed subjected to chronic N-enrichment, and in the reference watershed. Secondly, in a series of soil incubations, we characterized the extent to which microbial C and P acquisition were regulated by N and P availability. In these incubations, we measured BG and AP activity response to acute (high-dose fertilizer) nutrient amendments. We hypothesized that soil enzyme activities would respond more to enhanced P availability than N, particularly in the N-enriched watershed. Our results from extant enzyme activity measurements suggest that chronic N-enrichment inhibited rather than stimulated extant soil hydrolytic enzyme activities, which could reflect suppression of microbial biomass and activity. In the acute nutrient amendment incubations, our data indicate that inorganic P was more important than N in regulating soil microbial C and P acquisition in soils from both the N-enriched and reference watersheds. Our results also indicate that the extent to which P availability regulated microbial acquisition of P in O horizon soils was greatest in

softwood soils subjected to chronic N-enrichment. Findings from this study suggest that both forest type and soil inorganic P availability could be more important in influencing soil biological response to N pollution than previously recognized.

1. Introduction

Nitrogen pollution from human activities has dramatically enhanced N availability in terrestrial environments and shifted some ecosystems towards N saturation (Aber et al. 2003, Galloway 1998, Matson et al. 2002, Vitousek et al. 1997). Global rates of atmospheric N deposition are expected to increase in the coming decades (Dentener et al. 2006, Galloway et al. 2004) despite recent reductions in N deposition in parts of North America and Europe (Driscoll et al. 2003, Fowler et al. 2007). Although N in the environment can act as a pollutant, it is also a commonly limiting nutrient to terrestrial biota and thus, net primary productivity (LeBauer and Treseder 2008, Vitousek and Howarth 1991). Organisms require sufficient N supply to maintain a variety of physiological processes, including the synthesis of proteins, energetic nucleotides, pigments (chlorophyll), and nucleic acids. The classic paradigm in northern temperate forests is that N is the primary limiting nutrient to biological productivity. However, increases in the availability of N in these ecosystems could alleviate this limitation, and increase the relative importance of other essential nutrients such as phosphorus (P) (Gress et al. 2007, Elser et al. 2009, Phoenix et al. 2004, Vitousek et al. 2010).

Nitrogen-enrichment can alter important soil processes that are mediated by microbial communities, some of which can impact soil P cycling and availability. For instance, a meta-analysis of N-enrichment studies by Knorr et al. (2005) demonstrated that significant inhibition of litter decomposition can occur when N fertilization rates are 2-20x background rates of N deposition. Since litter is an important source of organic P in forest ecosystems, we can expect

that lower litter decomposition rates could result in less organic P substrates available for microbes to degrade. Other studies have suggested that N deposition can impede soil organic matter (SOM) decomposition by both suppression of microbial abundance and activities (DeForest et al. 2004, Ramirez et al. 2011, Treseder 2008) or inhibition of specific fungal communities (Frey et al. 2004, Wallenstein et al. 2006). Ecosystem N-enrichment can also enhance the activity of bacterial communities involved in N-mineralization (Aber et al. 1998, Jefts et al. 2004) by reducing C:N ratios in upper soil horizons; this could lead to an increase in N:P ratios in the soil matrix. Collectively, evidence suggests that N-enrichment can promote decomposition of more labile SOM fractions but impede decomposition of more recalcitrant fractions (Zak et al. 2008). These changes in decomposition caused by N-enrichment may stimulate or depress soil C storage, depending on litter quality characteristics (e.g., Waldrop et al. 2004), and the complex interactions among soil microbes, nutrients, or by multiple nutrient limitations.

Recent studies have demonstrated the importance of P in regulating microbial processes (Cleveland et al. 2002), and microbial activity (Liu et al. 2013) in tropical ecosystems, and microbial metabolic rates at the global scale (Hartman and Richardson 2013). However, the regulation of microbial processes by P in soils subject to high N loading in the Northeastern U.S. has not been well characterized. It is well recognized that chronic enrichment with N should enhance the role of P as a limiting nutrient for aboveground productivity in northern temperate terrestrial ecosystems (Gress et al. 2007, Vitousek et al. 2010). However, nitrogen deposition could also enhance the extent to which P is limiting to soil microbial communities by increasing N:P ratios in foliage (Braun et al. 2010, Bytnerowicz and Fenn 1996), leaf litter and SOM, thereby reducing the amount of P relative to N in substrates for microbial degradation. A recent

analysis of foliar chemistry data from 75 sites in the northeastern U.S. by Crowley et al. (2012) demonstrated that foliar N:P ratios increased with increasing N deposition in the Adirondacks, but not for the northeastern U.S. region overall. Furthermore, Naples and Fisk (2010) found that fine root growth responded positively to experimental P additions in mid-age forests in New Hampshire, but not in mature forests. They concluded that changes in nutrient demand with successional growth, and N deposition, can influence the extent of P limitation as stands age. For soil microbes subjected to high N loading, P availability could be more important than previously recognized in regulating microbial biomass and activity, and the rate at which SOM compounds are hydrolyzed.

Microbial production of C, N, and P degrading enzymes is energetically expensive (Allison 2005), and therefore resource allocation and energetic investment in C and/or P degrading enzymes should theoretically increase with enhanced N availability (Sinsabaugh and Moorhead 1994). In a meta-analysis of the response of soil phosphatase activity to N and P additions, Marklein and Houlton (2011) demonstrated that across a range of sites on several different continents, phosphatase activity typically increased under high N availability, suggesting accelerated P cycling under N-enrichment. However, the study by Marklein and Houlton only included two studies in temperate forests in the northeastern U.S., and among those two studies, an increase in phosphatase in response to N addition was only demonstrated in mature stands in one of these studies (Naples and Fisk, 2010). More information is needed on how a legacy of enhanced N loading to northeastern forests could influence the extent of P limitation on important ecosystem processes, such as microbial enzyme activity and SOM degradation. Furthermore, within northeastern forests, there is reason to expect that P limitation to microbial response to N-enrichment could be different among hardwood and softwood stands

because rates of nutrient cycling are generally lower in softwoods compared with hardwoods (e.g. Lovett et al. 2003). If increases in rates of N cycling are not matched by increases in P cycling, then P limitation could be strengthened by N-enrichment; this effect could be more pronounced in softwoods compared with hardwoods because of slower existing rates of P cycling.

We investigated the impacts of long-term N-enrichment on soil microbial enzyme activity in soils from hardwood and softwood soil types at the Bear Brook Watershed in Maine (BBWM). The BBWM is a long-term paired watershed experiment where forest ecosystem response to chronic N-enrichment and acidification has been studied for over two decades. At the BBWM, Mineau et al. (2013) explored indices of P limitation at watershed-scale using enzyme stoichiometry, and found no difference in stoichiometry to suggest N enrichment had altered soil microbial resource allocation strategies. However, the two forest types at BBWM have different litter quality that could influence microbial response to changes in nutrient availability, but forest type influences on enzyme activity at this site have not been characterized. We developed a set of experiments to determine how forest type and enhanced N and P availability influence soil microbial enzyme activity at the BBWM. To do this, we first measured the ambient (extant) soil enzyme activity from both softwood and northern hardwood soils in reference and treated watersheds. We hypothesized that long-term N-enrichment would accelerate microbial acquisition of C, N, and P compounds, resulting in higher hydrolytic enzyme activity in the N-enriched watershed. Secondly, in short-term soil incubation experiments, we used experimental high dose (acute) addition of inorganic P and N to soils from both the reference and N-enriched watersheds. These incubations were designed to test if P availability was more important than N availability in regulating the activity of C and P hydrolyzing enzymes in soils from both forest

types among the N-enriched and reference watersheds. Together, these experiments provide us with novel insights on the nature of nutrient regulation of microbial processes in acid forest soils affected by N pollution.

2. Material and Methods

2.1 Site description

The BBWM is a long-term paired watershed experiment located on the southeast slope of Lead Mountain (44° 52' N., 68° 06' W.) in eastern Maine. The treated watershed (West Bear; WB) is 11.0 ha and receives bi-monthly additions of (NH₄)₂ SO₄ above the forest canopy, adding 25.2 kg N ha⁻¹yr⁻¹ and 28.8 kg S ha⁻¹ yr⁻¹, compared with ambient levels of 3.2 kg N ha⁻¹yr⁻¹ and 2.4 kg S ha⁻¹yr⁻¹ as wet deposition at the nearest National Atmospheric Deposition Program site (Greenville, ME) in 2011. The adjacent untreated watershed (East Bear; EB) is 10.3 ha and serves as an ecological reference.

The BBWM climate is cool and temperate with a mean annual air temperature of 5.1 °C (Fernandez et al. 2007). Annual average precipitation is 1020 mm yr⁻¹; elevation ranges from 210-475 m. Soil parent material is stony Lodgement till, deposited during the late Wisconsinian age (ca. 13,000 ¹⁴C yr B.P, Thompson and Borns 1985). Bedrock is primarily composed of non-calcareous, non-sulfidic, meta-pelites and quartzites in upper elevations, and calc-silicate gneiss in lower elevations. Soils are commonly Haplic Podzols (Typic Haplorthods and Haplohumods), and average 0.9 m in thickness.

Overstory vegetation is dominated by American beech (*Fagus grandifolia* Ehrh.), sugar maple (*Acer saccharum* Marsh.), and red maple (*Acer rubrum* L.) of hardwood stands in the lower elevations of the watershed. Upper elevations in the watersheds are dominated by softwoods, primarily red spruce (*Picea rubens* Sarg.) with a minor presence of balsam fir (*Abies*

balsamea L. Mill.). Hardwood stands are developing from harvesting that occurred around 1945 (Norton et al. 1999), and softwood stands are estimated to be ≈ 30 years older than hardwoods.

2.2 Experimental design and sampling

We sampled soils from 10 5 x 5 m plots within each watershed (total=20); five plots each in hardwoods and in softwoods. These plots were located adjacent to long-term monitoring plots at this study site. These long-term plots were chosen to be approximately evenly distributed in each watershed, avoiding a 30 m buffer around watershed boundaries, and avoiding notable confounding conditions such as large rock outcrops, blowdowns, or notable mammal disturbances.

Soils were collected from measurement plots for three different aspects of this study. First, we made measurements of ambient enzyme activities to study the effect of whole watershed chronic N-enrichment with $(\text{NH}_4)_2\text{SO}_4$, on the activities of enzymes involved in microbial mineralization of P, along with enzymes also involved in C and N-mineralization. Secondly, we incubated soils with high doses (acute amendments) of P alone, or in combination with N to assess whether P or N availability regulated enzyme activity. The control soils in the in-situ incubations were used to characterize background rates of N-mineralization and soils amended with P alone were used to assess the influence of P availability on N-mineralization. Lastly, we conducted post-hoc measurements of microbial biomass to assess whether there could have been important differences in microbial biomass among the N-enriched and reference watershed that influenced the trends observed in either ambient enzyme activity, or enzyme response to acute nutrient amendments.

2.3 Extant enzyme activity sampling design

We measured ambient (extant) activities of four enzymes involved in the mineralization of C, N, and P, including β -glucosidase (BG), xylosidase (XYLO), N-acetyl-glucosaminidase

(NAG) and acid phosphatase (AP). Samples of the O and B horizons were collected on July 30, 2008 from all 20 plots, with four replicates per plot. A horizons are not present in these watersheds and E horizons occur inconsistently, therefore we only sampled the two predominant upper horizons, the O and the B horizons. Soils in O and B horizons were sampled within a 10 x 10 cm frame. Within each frame, coarse litter and the Oi horizon were removed and a trowel was used to collect a sample of 200-500 g (field-moist weight) from the top 10 cm of the Oa/e. Mineral samples were collected from the top 10 cm of the B horizon. Samples of O and B horizons were sieved through 6.3 mm and 2 mm mesh, respectively. Gravimetric moisture and % SOM by loss-on-ignition (LOI) were measured for each sample. Subsamples of soils were frozen for subsequent analysis of enzyme activity (described below).

2.4 Enzyme activity and net N-mineralization response to acute nutrient amendments

2.4.1 In-situ soil incubations

We measured enzyme activity and N-mineralization response to acute experimental increases in N and P availability in a series of incubations where soils were amended with N and P. Enzyme activity, $\text{NH}_4\text{-N}$, and $\text{NO}_3\text{-N}$ were measured approximately monthly, for three different time periods in 2008. To do this, soils were collected in July according to the same procedure described for measurements of ambient enzyme activities. After soils were sieved, 100g of soil was transferred to polyethylene plastic bags (Binkley and Hart 1989), and secured with a wire twist-tie. Soils in buried bags were returned to the field, buried in the horizon and plot from which they were originally collected and incubated for 28-38 days for three separate time periods in 2008: 1) July 16-August 22, 2) August 22-September 26, and 3) September 26-October 24.

Soils in bags were incubated with amendments of inorganic N (+N), inorganic P (+P), both inorganic N and P (+N+P), adjacent to samples that were incubated as controls (amended only with H₂O). Therefore, amendments were contained in the experimental units over the course of each incubation, and enzyme activities were measured on soil collected at the end of each incubation period. For these incubations, sieved soil was placed on wax paper, and fertilizer amendments were manually applied with plastic spray bottles. A standard amount of fertilizer was applied per gram of sieved, field-moist soil; to calculate application rates, we used bulk density data from O horizons from quantitative pit sampling at BBWM (SanClements et al. 2010). Soils were amended with N as NH₄NO₃ at a rate equivalent to 200 kg N ha⁻¹ (720 mg N kg⁻¹), inorganic P was applied as H₂O₄PNa·H₂O at a rate equivalent to 100 kg P kg⁻¹ha⁻¹ (360 mg P kg⁻¹). The N+P treatment was equivalent to 200 kg N ha⁻¹ and 100 kg P ha⁻¹, respectively.

After incubation, soils were divided into two additional sub-samples, one of which was frozen at -62 °C for analysis of the activity of BG and AP response to acute nutrient amendments, and one that was used for determination of gravimetric moisture content and LOI.

2.4.2 *Ex-situ* soil incubations

Ex-situ nutrient amendment experiments were conducted over a longer time period than in-situ incubations, approximately 6 months from late May until late October in 2009. In May, we sampled O horizons in four plots per study compartment for a total of 16 field plots. Three replicate samples were collected from each plot. Each sample was homogenized and split into four subsamples that were distributed among the four nutrient amendments as used in the *in-situ* experiment. The equivalent of 8 g oven-dry soil was incubated in aerobic chambers fitted with glass fiber filters. Soil moisture was maintained at ≈ 200%, which is a reasonable estimation of field capacity for BBWM O horizon soil types (Rourke 1994). Soils were incubated in the

laboratory at room temperature for a total of 182 days ($\approx$ 6 months). During the incubation period, soils were leached once per month with a micronutrient solution (after Knadelhoffer 1990) to prevent micronutrient deficiencies; P and N amendments were re-applied after each leaching. Inorganic nutrient application rates were the same as those described for *in-situ* experiments, but applications were made repeatedly at each month throughout the experiment to ensure that inorganic P and/or N availability remained high even after samples were leached. At 2 months, 4 months, and at the end of the experiment, 5 g of moist soil was retrieved from each chamber for analysis of extracellular enzyme activity.

2.4.3 Soil net N-mineralization, available PO_4 -P, and microbial biomass

Control samples from the *in-situ* incubation experiments were also used to determine extant rates of net N-mineralization and P availability prior to nutrient amendments. Furthermore, we assessed the response of net N-mineralization to P amendment by measuring NO_3 -N and NH_4 -N in the *in-situ* incubation. Prior to incubation, a set of sieved, field-moist subsamples were extracted in 1 M KCl (Robertson et al. 1999), and extracts were frozen until analysis for inorganic NO_3 -N and NH_4 -N. At the end of the *in-situ* incubation period described above, soils were retrieved from the field and extracted within several days of collection. All KCl extracts were filtered through Whatman™ 42 filter paper and then frozen at $\sim 20^\circ C$ until chemical analysis. Soil extracts were analyzed colorimetrically on an Alpkem OI Autoanalyzer using the cadmium reduction/sulfanilimide method 350.1 for NH_4 -N and the hypochlorite/salicylate method 353.1 for NO_3 -N (U.S. E.P.A. 1983), and for PO_4 -P on a Dionex 2000i/SP ion chromatograph using the ascorbic acid method.

Two years after we conducted measurements of extant enzyme activity and the *in-situ* incubations, we also made a one-time measurement of soil microbial biomass at BBWM in the

same plots that previous measurements of extant activity and N-mineralization had been measured. This one-time measurement was conducted in order to determine if there could be important differences in microbial biomass between the N-enriched and reference watershed soils that influenced the patterns observed in extant enzyme activities. In 2010, we collected samples from O horizons in 16 plots for measurements of microbial biomass C. In each plot, 4 replicates were collected. Samples were sieved through 6.3 mm mesh and replicates from each plot were homogenized and composited into one representative sample per plot. Microbial biomass C was measured on three replicates per composite. We used a chloroform-fumigation extraction method similar to that described by Paul et al. (1999), except that we used an extract: soil ratio of 12.5:1, as recommended by Needelman et al. (2001). Once extracted, solutions were filtered through 0.45 μm filter paper; extracts were frozen in a standard laboratory freezer until for dissolved organic carbon on an OI Analytical Wet Oxidation Total Organic Carbon Analyzer Model 1010. Microbial biomass C was calculated following Paul et al. (1999), using an extraction coefficient of 0.51 (Joergensen 1996).

2.5 Enzyme assays

Enzyme activity was measured using microplate assays with methylumbelliferone (MUB) labeled substrates (e.g. Carrierio et al. 2000). Following sieving, soil was frozen at -62°C until analysis, and thawed before assay preparation. After thawing, 0.5 g of soil was weighed into a 30 mL plastic centrifuge tube. Soil was suspended in 30 mL of 50 mM $\text{C}_2\text{H}_3\text{NaO}_2$ buffer (pH = 5). Centrifuge tubes were capped and the suspension was shaken vigorously by hand for 1 min, and then homogenized with a Power Gen 35 for 1 min. Seven mL of the soil slurry were diluted in 50 mL with acetate buffer on a stir plate; two hundred μL of the soil slurry were added to 96 well microplates. Fifty μL of a 200 μM substrate solution was added to each well and

fluorescence was measured at 25°C using a microplate fluorometer (Fluoroskan Ascent FL, Thermoelectron). The substrate concentration (200 µM) we used was well above the saturating concentration (135 µM) for BG in O horizon soils at this site (Stone et al. 2012) . We conducted post-hoc tests to determine if our substrate concentrations saturated activity of BG and AP in B horizon samples and AP in O horizon samples (Mineau et al. 2013). Our substrate concentrations (200 µM) were above saturation (< 80 µM) for O and B horizon soils for BG, and in the B horizon for AP, but our substrate concentration was about ½ the saturating concentration for AP in the O horizon. Consequently, our ability to detect treatment effects for AP in the O horizon should be considered conservative (German et al. 2011).

2.6 Statistical analyses

For the *in-situ* and *ex-situ* experiments, a nested hierarchical design was first used to test the effect of time (months in the *in-situ* and *ex-situ* incubations), watershed, horizon, and plot treatment on enzyme activities using the PROC MIXED statement in SAS version 9.2. Normality of all data residuals was assessed using the Kolmogorov–Smirnov test with an α level of 0.10; in cases where residuals were not normally distributed, log transformation was used to conform to the assumptions of normality of residuals. For *in-situ* and *ex-situ* experiments with a time component, enzyme activities were then averaged across time and plot, as the main effects of these factors were not significant. In order to compare the magnitude of nutrient enrichment effects in the experiments across watersheds, soil horizons, and treatments, we calculated nutrient response ratios (e.g. Elser et al. 2009). Response ratios were calculated as the enzyme activity for each individual nutrient-amended sample divided by the mean enzyme activity of the control samples. For *in-situ* and *ex-situ* enzyme activity data and net N-mineralization response to amendments in the *in-situ* component, least-squared means separation tests were used to

determine significant differences among response ratios from various amendments. For extant enzyme activity and net N-mineralization data, we used a two-way analysis of variance to test for the main effects of watershed (long-term N-enriched watershed versus reference watershed) and forest type, and the interaction of these effects on independent variables.

3. Results

3.1 Extant enzyme activities

The activities of AP, NAG, and BG were typically lower in the N-enriched WB watershed compared with the reference EB watershed. The activity of XYLO was similar between watersheds for both soil horizons (Fig. 1). Because enzyme activities were standardized to grams of SOM, enzyme activities in the O and B horizon were in most cases similar across horizons. Of the enzymes measured, there was a significant effect of forest type only on BG activity, which were 1.3-2.6 x higher in hardwoods compared with softwoods (not shown); this forest-type effect was not observed for the other enzymes studied, and therefore, results are shown aggregated across forest type.

3.2 Enzyme activity response to acute N and P amendments

In the *in-situ* and *ex-situ* acute nutrient amendment experiments, N amendment alone had little effect on AP and BG activities (Table 1), except in one instance where N stimulated AP in B horizon soils from softwoods in the treated WB watershed. Phosphorus amendment alone or in combination with N commonly suppressed AP. Phosphorus amendment typically enhanced BG activity, but less frequently than it suppressed AP activity. Phosphorus addition suppressed AP more consistently in O horizon soil than in B horizon soils.

To better address the magnitude of enzyme activity response to acute nutrient amendment we calculated response ratios (RRs) for enzyme activities (Fig.s 2 and 3). In theory, if microbial

enzyme activity was strongly regulated by inorganic N or P availability we could expect several types of responses for amended soils: 1) a downturn in AP activity with P amendment ($RR < 1$), 2) enhanced AP activity with N amendment ($RR > 1$), and 3) enhanced BG activity ($RR > 1$) with either N or P amendment. Data for AP RRs suggest a general reduction in AP activity ($RRs < 1$) by amendment with P across most soils in O and B horizons in hardwood and softwood forest types (Fig.s 2 and 3.). Response ratios for AP in soils amended with P and +N+P ranged from 0.36 - 0.89 and 0.38 - 0.97, respectively (Fig.s 2b, 2c). However, differences between RRs from the treated and reference watersheds do not suggest an effect of long-term N-enrichment on the magnitude of AP response to P addition alone or in combination with N, except in the case of O horizon soil from softwoods.

For O horizons under softwoods, the magnitude of AP response to P amendment was significantly greater in the N-enriched WB watershed (Average $RR = 0.37$ for *ex-situ* and 0.52 for *in-situ* in WBSW) compared with the reference watershed (average $RR=0.89$ for both *ex-situ* and *in-situ* samples in EBSW) (Fig. 2b). Therefore, data on AP activity response to P amendment suggest that N-enrichment enhanced the extent to which inorganic P availability regulated AP activity, but only in softwood O horizons. Soil BG RRs were typically >1 ; C hydrolyzing BG activity response ratios ranged from 0.76 - 2.5 (Fig.s 3a-3c). However, despite the fact that AP was most responsive to acute P addition in N-enriched softwood O horizons (WBSW), BG was least responsive to acute P addition in WBSW (Fig. 3b).

3.3 Soil N-mineralization, available PO_4 -P, and microbial biomass

Rates of net N-mineralization in O and B horizons were 40 - 260 % higher in soils from the N-enriched hardwoods compared to soils in the reference hardwoods (Table 3). Available PO_4 -P was also 2x higher in hardwood O horizons of the treated watershed than in the reference

watershed. Data from *in-situ* incubations suggest that net N-mineralization rates were stimulated by acute P amendment in hardwood soils from both the reference EB and N-enriched WB watershed (Fig. 4).

Soil microbial biomass C in O horizons was lower in the N-enriched watershed compared with the reference watershed by 17 - 25 %, although these differences were not statistically significant at the $\alpha = 0.05$ level (Table 2). Microbial biomass C, rates of net N-mineralization, and soil extractable $\text{PO}_4\text{-P}$ concentrations were lowest in the N-enriched softwoods compared with the other compartments we studied (N-enriched hardwoods and both forest types in the reference watershed) (Table 2).

4. Discussion

We hypothesized that long-term N-enrichment would accelerate microbial acquisition of C, N, and P compounds, resulting in higher hydrolytic enzyme activity in the N-enriched watershed; we expected this effect to be more pronounced in the hardwood forest type. Our data suggests that long-term N-enrichment at BBWM did not stimulate hydrolytic enzyme activity. Rather, our findings suggest that chronic N-enrichment generally suppressed the extant activity of key enzymes involved in the hydrolysis of cellulose, chitin, and P (Fig. 1) in both forest types. In nutrient amendment experiments, we predicted that P availability would be more important than N availability in regulating microbial acquisition of C and P. In these incubations, N amendment had little effect on AP and BG activity, neither in soils subjected to long-term N-enrichment in the treated watershed, nor soils from the reference watershed. Our results suggest that there was sufficient N without the N amendment for these microbial communities to produce AP and BG. Soil AP and BG activities were more responsive to acute P amendment alone, or in

combination with N, suggesting that inorganic P is more important in regulating the activities of these enzymes than inorganic N.

Our study revealed lower activities for most enzymes studied in the N-enriched WB watershed compared with the reference EB watershed, which is consistent with some studies that have demonstrated depression of soil microbial enzyme activity by N-enrichment (DeForest et al. 2004, Ramirez et al. 2012). Other studies, however, have reported that N addition stimulates enzyme activities, particularly for phosphatase (Chung et al. 2007, Gress et al. 2007, Johnson et al. 2010, Papanikolaou et al. 2010, Sinsabaugh et al. 2005, Stursova et al. 2006, Treseder and Vitousek 2001). Results from our one-time measurement of microbial biomass suggest a trend for lower microbial biomass C in the N-enriched watershed compared with the reference watershed. While we do not know whether the trend for lower microbial biomass in the N-enriched watershed was sustained over the growing season, this finding, along with the lower enzyme activities observed in the N-enriched watershed, is consistent with other studies that demonstrated N inhibition of microbial abundance or activity in other forest soils (Frey et al. 2004, Wallenstein et al. 2006,). Therefore, it is possible that N inhibition of microbial abundance and/or activity also accounts for the lower extant enzyme activities in the N-enriched watershed at the BBWM. Furthermore, an analysis conducted by Mineau et al. (2013) demonstrated that soil enzyme stoichiometric ratios were similar in the N-enriched WB and reference EB watersheds, suggesting that there was no difference in microbial nutrient acquisition strategies between the N-enriched and reference watershed. Together, the results from this study and the analysis by Mineau et al. (2013) suggest that there were no significant functional differences in microbial resource allocation strategies, but rather a general suppression of microbial activity by N-enrichment (Fig. 1). It is also possible that the lower enzyme

activities observed in our study in the N-enriched watershed reflect a difference in the composition of the microbial community in these watersheds that we did not measure.

The acidification that accompanied the N-enrichment in the treated watershed at BBWM also could have suppressed extant hydrolytic enzyme activity. Table 3 demonstrates that while bulk soil chemical properties in the O and B horizons of the N-enriched WB and reference EB watersheds were not significantly different, it is clear long-term N-enrichment has dramatically altered the acidity of soil solutions in WB. The treatment of WB has resulted in a progressive loss of soil base cations and an increase in acidity (Fernandez et al. 2003, Norton et al. 2004) that could influence the patterns observed in soil enzyme activities, because enzyme activity optima are pH-dependent (e.g. Turner 2010). Additionally, bacterial conversion of the NH_4^+ in BBWM fertilizer to NO_2^- in the first step of nitrification can further acidify soils through the release of H^+ into soil solution. Both N-enrichment and accelerated acidification could have been important factors that inhibited soil microbial enzyme activity and enzyme activity response to acute nutrient amendment.

Our results suggest that forest type could have an important influence on microbial enzyme activities in these soils, and the response of enzyme activities to changes in nutrient availability. For instance, although extant soil AP activities were typically lower in the treated WB watershed compared with the reference EB watershed (Fig. 1, Table 2), our analysis of extractable $\text{PO}_4\text{-P}$ in these soils (Table 2) demonstrated that $\text{PO}_4\text{-P}$ was higher in the N-enriched WB compared with reference EB only in the O horizons of hardwoods; this difference was not apparent between the watersheds in softwood stands. We hypothesize that the lower extant AP activities in the N-enriched watershed more likely reflects a general suppression of microbial biomass and activity, and not changes in the rate of microbial acquisition of P via AP activity.

The higher PO₄-P concentrations we observed in the N-enriched hardwood soils compared with reference hardwood soils could be attributable to a stimulation of nutrient cycling due to N-enrichment in the WB watershed. This stimulation of nutrient cycling could increase the pool of P available to plants and microbes that would otherwise been bound in litter and organic matter in the soil. We also observed some differences in BG activity and N-mineralization response to acute nutrient amendment; these differences could be attributed to variation in litter chemistry, SOM recalcitrance, and microbial biomass between the hardwoods and softwoods. For example, soil O horizon incubation results suggest that acute addition of P alone or in combination with N stimulated BG activity (Fig. 3) and net N-mineralization (Fig. 4) in N-enriched hardwoods but not in softwoods. The lack of response to acute P amendment in terms of N-mineralization and BG activity in N-enriched softwood soils could be attributed to lower microbial biomass in these soils (Table 2), from which an increase in rates of N-mineralization and C hydrolyzing enzymes may have been too small to detect. Acute nutrient amendment did not always stimulate BG activity, particularly in the N-enriched WB softwoods. This response could reflect a higher lignin content in the softwood litter type (e.g. Lovett et al. 2004), and potential inhibition of lignin degraders by the long-term N-enrichment (Gallo et al. 2004, Edwards et al. 2011), which could have limited the availability of cellulose substrate for BG degradation.

In the acute nutrient addition experiments designed to characterize microbial enzyme activity response to enhanced nutrient availability, we found that P addition alone or in combination with N commonly reduced AP activity, but not always (Table 1). In instances when AP activities were not significantly suppressed by acute P additions, constitutive enzyme concentrations and/or high extant variability in enzyme activities could have precluded the detection of an effect of acute nutrient amendment. Given the dose of P added in our acute P

amendment treatments (100 kg P ha⁻¹ equivalent), it is unlikely that inorganic P was still limiting to the microbial community. AP production is thought to be down-regulated by the availability of inorganic P through suppression of genes that enable P acquisition under high inorganic P availability (Dick et al. 2011). However, Jones and Oburger (2011) highlight that the influence of inorganic P (P_i) on the activity of phosphomonoesterases (PME: which includes AP) is rather poorly understood, because some studies in the literature have found that inorganic P concentration and PME activity are not necessarily strongly linked (see Jones and Oburger 2011 for a more complete discussion). For example, Allison and Vitousek (2005) found that inorganic P addition did not suppress AP activity in a short-term incubation experiment with Hawaiian rainforest soils, and hypothesized that constitutive enzyme concentrations and residual activity from enzymes bound to mineral surfaces could have precluded detection of a significant response, as could be the case in our study. However, more information is needed regarding how long enzymes stabilized in the soil persist, and rates of new enzyme production in order to more accurately assess enzyme activity response to experimental nutrient amendments. Collectively, findings from this study and the literature also raise the question: are PMEs sensitive indicators of inorganic P availability to microbes in acid forest soils such as those at our study site, given that that constitutive enzyme production could exert a strong influence on trends in enzyme activities?

5. Conclusions

Findings from this research suggested a general inhibition of the activities of several hydrolytic enzymes by two decades of chronic whole-ecosystem N-enrichment and acidification. Acute, short-term N amendment experiments suggested such suppression did not occur on short time scales (< 6 months). In this study, the form of added N could be important in regulating soil

enzyme activities. At our site, concomitant N-enrichment and acidification due to $(\text{NH}_4)_2\text{SO}_4$ treatment appears to have an inhibitory effect on hydrolytic enzymes activities. Ideally, biogeochemical models that include SOM dynamics should account for the potentially asynchronous response of hydrolytic enzymes to different forms of N deposition.

Results from short-term, acute nutrient amendment experiments suggested that inorganic P amendments had a more pronounced effect on microbial enzyme activity than inorganic N amendments. In acute nutrient amendment experiments, we found little evidence that chronic or acute N-enrichment led to elevated microbial acquisition of inorganic P, except in O horizon softwoods. However, inorganic P availability did appear to be important in regulating certain processes involved in C and N cycling in the hardwood forest type of both the reference and N-enriched watershed. Together, these findings suggest that inorganic P availability was more important in regulating enzyme activities in these soils than N availability, and this effect was different in the hardwood and softwood forest types.

These findings highlight the importance of forest composition in defining nutrient regulation of soil microbial community function, made even more important during periods of response and recovery to changing atmospheric N inputs. The challenge for future research is to understand the integration of processes governing nutrient regulation of microbial activity and enzyme production from the molecular to the ecosystem scale.

6. Acknowledgements

We wish to thank two anonymous reviewers, whose comments helped significantly improve this manuscript. We appreciate all the individuals that were involved in field and laboratory work on this project, including Cheryl Spencer, Mary Beth Parent, Dustin Johnson,

Shaughn Allen, Danielle Dutton, Abel Plaud, Jason Smith, Susan Devine, Benjamin Lynch and Anthony Drouin. This research was supported by grants from the National Science Foundation (DEB-0210257 and DEB-0414144). Additional financial support for this project was provided from University of Maine Department of Plant, Soil, and Environmental Sciences, the Maine Agriculture and Forest Experiment Station, the University of Maine Graduate School, the University of Maine College of Natural Sciences, Forestry, and Agriculture, and the New England Farm and Garden Association.

7. References

- Aber, J.D., Mcdowell, W., Nadelhoffer, K., Magill, A., Berntson, G., Kamakea, M., et al. 1998. Nitrogen saturation in temperate forest ecosystems - hypotheses revisited. *BioScience* 48, 921-934.
- Aber, J.D., Goodale, C.L., Ollinger, S.V., Smith, M.L., Magill, A.H., Martin M.E. et al. 2003. Is nitrogen deposition altering the nitrogen status of Northeastern forests? *BioScience* 53, 375-389.
- Allison, S. D., and Vitousek, P. M. 2005. Responses of extracellular enzymes to simple and complex nutrient inputs. *Soil Biology and Biochemistry* 37, 937-944.
- Binkley, D., and Hart, S. 1989. Components of nitrogen availability assessments in forest soils. *Advances in Soil Science* 10, 57-116.
- Binkley, D., Carter, R., and Allen, H.L. 1995. Nitrogen fertilization practices in forestry. In P.E. Bacon (Ed.) *Nitrogen fertilization in the environment*. Marcel Dekker, Inc, New York.
- Braun, S., Thomas, V.F.D., Quiring, R., and Flückiger, W. 2010. Does nitrogen deposition increase forest production? The role of phosphorus. *Environmental Pollution* 158, 2043-2052.
- Bytnerowicz, A., and Fenn, M.E. 1996. Nitrogen deposition in California forests: A review. *Environmental Pollution* 92, 127-146.
- Carreiro, M.M., Sinsabaugh, R.L., Repert, D.A., and Parkhurst, D.F. 2000. Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. *Ecology* 81, 2359-2365.

- Cleveland, C. C., Townsend, A. R., & Schmidt, S. K. 2002. Phosphorus Limitation of Microbial Processes in Moist Tropical Forests: Evidence from Short-Term Laboratory Incubations and Field Studies. *Ecosystems*, 5 (7), 680–691.
- Cleveland, C., and Liptzin, D. 2007. C:N:P stoichiometry in soil: is there a “Redfield ratio” for the microbial biomass? *Biogeochemistry* 85, 235-252.
- Chung, H. Zak, D.R., Reich, P.B., and Ellsworth, D.S. 2007. Plant species richness, elevated CO₂, and atmospheric nitrogen deposition alter soil microbial community composition and function. *Global Change Biology* 13, 980-989.
- Crowley, K., B. McNeil, G. Lovett, C. Canham, C. Driscoll, et al. 2012. Do Nutrient Limitation Patterns Shift from Nitrogen Toward Phosphorus with Increasing Nitrogen Deposition Across the Northeastern United States? *Ecosystems*, 15:940-957.
- DeForest, J.L., Zak, D.R., Pregitzer, K.S., Burton, A.J. 2004. Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forests. *Soil Science Society of America Journal*. 68, 132-138.
- DeForest, J. L. 2009. The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and l-DOPA. *Soil Biology and Biochemistry* 41, 1180-1186.
- Dentener, F., Drevet, J., Lamarque, J.F., Bey, I., Eickhout, B., Fiore, A.M. et al. 2006. Nitrogen and sulfur deposition on regional and global scales: A multi-model evaluation. *Global Biogeochemical Cycles* 20, GB 4003.
- Dick, C.F., Dos-Santos, A.L.A., Meyer-Fernandes, J.R. 2011. Inorganic phosphate as an important regulator of phosphatases. *Enzyme Research*, 2011, article ID 103980; doi:10.4061/2011/103980.
- Driscoll, C.T., Whitehall, D., Aber, J., Boyer, E., Castro, M. et al. 2003. Nitrogen Pollution in the Northeastern United States: Sources, Effects, and Management Options. *BioScience*, 53 (4), 357- 374.
- Edwards I.P., Zak D.R., Kellner H., Eisenlord S.D., Pregitzer K.S. (2011) Simulated Atmospheric N Deposition Alters Fungal Community Composition and Suppresses Ligninolytic Gene Expression in a Northern Hardwood Forest. *PLoS ONE* 6(6): e20421.
- Elser, J. J., Kyle, M., Steger, L., Nydrick, K., Baron, J.S. 2009. Nutrient availability and phytoplankton nutrient limitation across a gradient of atmospheric nitrogen deposition. *Ecology* 90 (11), 3062-3073.

- Fatemi, F.R., I. J. Fernandez, J.E. Szillery, S.A. Norton, and L.E. Rustad. 2012. Soil solution response to two decades of acidification at the Bear Brook Watershed in Maine. *Water, Air, and Soil Pollution* 223: 6171-6186.
- Fernandez, I. J., Rustad, L. E., Norton, S. A., Kahl, J. S., and Cosby, B. J. 2003. Experimental acidification causes soil base-cation depletion at the Bear Brook Watershed in Maine. *Soil Science Society of America Journal* 67, 1909-1919.
- Fernandez, I.J., Karem, J. E., Norton, S., and Rustad, L. 2007. Temperature, soil moisture and streamflow at the Bear Brook Watershed in Maine (BBWM). *Maine Agricultural and Forest Experiment Station Technical Bulletin* 196, The University of Maine, 32 pgs.
- Finzi, A. 2009. Decades of atmospheric deposition have not resulted in widespread phosphorus limitation or saturation of tree demand for nitrogen in southern New England. *Biogeochemistry* 92, 217-229.
- Fowler, D., Smith, R., Muller, J., Cape, J.N., Sutton, M., Erisman, J.W., et al. 2007. Long term trends in sulphur and nitrogen deposition in Europe and the cause of non-linearities. Pgs. 41-47 in Brimblecombe P., Hara H., Houle D., Novak M, eds. *Acid Rain - Deposition to Recovery*, Springer Netherlands.
- Fox, T.R., Allen, H. L., Albaugh, T.J., Rubilar, R., and Carlson, C.A. 2007. Tree nutrition and forest fertilization of pine plantations in the Southern United States. *Southern Journal of Applied Forestry* 31, 5-11.
- Frey, S.D., Knorr, M., Parren, J.L., Simpson, R.T. 2004. Chronic nitrogeN-enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. *Forest Ecology and Management* 196, 159-171.
- Gallo, M., Amonette, R., Lauber, C., Sinsabaugh, R.L., and Zak, D.R. 2004. Microbial community structure and oxidative enzyme activity in nitrogen-amended Northern temperate forest soils. *Microbial Ecology* 48, 218-229.
- Galloway, J.N. 1998. The global nitrogen cycle: changes and consequences. *Environmental Pollution* 102, 15-24.
- Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., et al. 2004. Nitrogen cycles: past, present, and future. *Biogeochemistry* 70, 153-226.
- German, D. P., Weintraub, M. N., Grandy, A. S., Lauber, C. L., Rinkes, Z. L., and Allison, S. D. 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. *Soil Biology and Biochemistry* 43, 1387-1397.
- Güsewell, S., and Gessner, M.O. 2009. N:P ratios influence litter decomposition and colonization by fungi and bacteria in microcosms. *Functional Ecology* 23, 211-219.

- Gress, S.E., Nichols, T.D., Northcraft, C.C., and Peterjohn, W.T. 2007. Nutrient limitation in soils exhibiting differing nitrogen availabilities: what lies beyond nitrogen saturation? *Ecology* 88, 119-130.
- Groffman, P.M., and Fisk, M.C. 2011. Phosphate additions have no effect on microbial biomass and activity in a northern hardwood forest, *Soil Biology and Biochemistry*, 43 (12), 2441-2449.
- Hartman W.H., and Richardson C.J. 2013. Differential Nutrient Limitation of Soil Microbial Biomass and Metabolic Quotients (qCO₂): Is There a Biological Stoichiometry of Soil Microbes? *PLoS ONE* 8(3): e57127.
- Herr, C., Chapuis-Lardy, L., Dassonville, N., Vanderhoeven, S., and Meerts, P. 2007. Seasonal effect of the exotic invasive plant *Solidago gigantea* on soil pH and P fractions. *Journal of Plant Nutrition and Soil Science* 170: 729-738.
- Hobbie, S., Eddy, W., Buyarski, C., Adair, C., Ogdahl, M., Weisenhorn, P. 2012. Response of decomposing litter and its microbial community to multiple forms of nitrogen-enrichment. *Ecological Monographs*, 82: 389-405.
- Hofmockel, K., Zak, D., & Blackwood, C. 2007. Does atmospheric NO₃⁻ deposition alter the abundance and activity of ligninolytic fungi in forest soils? *Ecosystems* 10, 1278-1286.
- Jefts, S., Fernandez, I. J., Rustad, L. E. and Dail, B. D. 2004. Decadal responses in soil N dynamics at the Bear Brook Watershed in Maine, USA. *Forest Ecology and Management* 189, 189-205.
- Joergensen, R. G. 1996. The fumigation-extraction method to estimate soil microbial biomass: Calibration of the kEC value. *Soil Biology and Biochemistry* 28, 25-31.
- Johnson, S.E., Loeppert, R.H. 2006. Role of organic acids in phosphate mobilization from iron oxide. *Soil Science Society of America Journal* 70: 222-234.
- Jones, D.L., and Oburger, E. 2011. Solubilization of phosphorus by soil microorganisms. In *Phosphorus in action*, Bunemann et al. (eds.); pgs. 169-198 DOI 10.1007/978-3-642-15271-9_7, # Springer-Verlag Berlin Heidelberg 2011.
- Johnson, D., Moore, L., Green, S., Leith, I.D., and Sheppard, L.J. 2010. Direct and indirect effects of ammonia, ammonium and nitrate on phosphatase activity and carbon fluxes from decomposing litter in peatland. *Environmental Pollution* 158, 3157-3163.
- Knadelhoffer, K. 1990. Microlysimeter for measuring nitrogen-mineralization and microbial respiration in aerobic soil incubations. *Soil Science Society of America Journal* 54, 411-415.
- Knorr, M., Frey, S.D., and Curtis, P.S. 2005. Nitrogen additions and litter decomposition: a meta-analysis. *Ecology* 86, 3252-3257.

- Liu L, Zhang T, Gilliam FS, Gundersen P, Zhang W, Chen H, et al. (2013) Interactive Effects of Nitrogen and Phosphorus on Soil Microbial Communities in a Tropical Forest. *PLoS ONE* 8(4): e61188. doi:10.1371/journal.pone.0061188
- LeBauer, D.S., and Treseder, K.K. 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89, 371-379.
- Lovett, G. M., Weathers, K. C., Arthur, M. A., and Schultz, J. C. 2004. Nitrogen cycling in a northern hardwood forest: Do species matter? *Biogeochemistry* 67: 289-308.
- Marklein, A. R., and Houlton, B. Z. 2011. Nitrogen inputs accelerate phosphorus cycling rates across a wide variety of terrestrial ecosystems. *New Phytologist* 193 (3), 696-704. DOI: 10.1111/j.1469-8137.2011.03967.x
- Matson, P., Lohse, K. A., and Hall, S. J. 2002. The globalization of nitrogen deposition: consequences for terrestrial ecosystems. *AMBIO: A Journal of the Human Environment* 31, 113-119.
- Mineau, M. M., Fatemi, F. R., Fernandez, I. J., Simon, K.S. 2013. Microbial enzyme activity at the watershed scale: response to chronic nitrogen deposition and acute phosphorus enrichment. *Biogeochemistry*.
- Naples, B., and Fisk, M. 2010. Belowground insights into nutrient limitation in northern hardwood forests. *Biogeochemistry* 97, 109-121.
- Needelman, B. A., Wander, M. M., and SHI, G. S. 2001. Organic carbon extraction efficiency in chloroform fumigated and non-fumigated soils. *Soil Science Society of America Journal* 65, 1731-1733.
- Norton, S., Kahl, J.S., Fernandez, I.J., Rustad, L., Haines, T. A., Nodvin, S., et al. 1999. The Bear Brook Watershed in Maine (BBWM) Environmental Monitoring and Assessment 55, 7-51.
- Norton, S.A., I.J. Fernandez, J.S. Kahl, and Reinhardt, R.L. 2004. Acidification trends and the evolution of neutralization mechanisms through time at The Bear Brook Watershed in Maine, U.S.A. *Water, Air, and Soil Pollution* 4, 289-310.
- Olander, L.P., and Vitousek, P.M. 2000. Regulation of soil phosphatase and chitinase activity by N and P availability. *Biogeochemistry* 49, 175-191.
- Paul, E.A., Harris, D., Klug, M., and Reuss, R. 1999. The determination of microbial biomass. p. 291-317. In G.P. Robertson et al. (ed.) *Standard soil methods for long term ecological research*. Oxford Univ. Press, New York.
- Papanikolaou, N., Britton, A.J, Helliwell, R.C, and Johnson, D. 2010. Nitrogen deposition, vegetation burning and climate warming act independently on microbial community

- structure and enzyme activity associated with decomposing litter in low-alpine heath. *Global Change Biology* 16, 3120-3132.
- Phoenix, G. K., Booth, R. E., Leake, J. R., Read, D. J., Grime, J. P., and Lee, J. A. 2004. Simulated pollutant nitrogen deposition increases P demand and enhances root-surface phosphatase activities of three plant functional types in a calcareous grassland. *New Phytologist* 161, 279-290.
- Ramirez, K.S., Lauber, C.L., Knight, R., Bradford, M.A., and Fierer, N. 2010. Consistent effects of nitrogen fertilization on soil bacterial communities in contrasting systems. *Ecology* 91:3463–3470. <http://dx.doi.org/10.1890/10-0426.1>
- Ramirez, K. S., Craine, J. M. and Fierer, N. 2012, Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. *Glob Change Biol*, 18: 1918–1927.
- Redding, E. 2011. Phenology at the Bear Brook Watershed in Maine: foliar chemistry and morphology (Master's thesis). University of Maine, Orono.
- Reinhardt, R. L., Norton, S. A., Handley, M., and Amirbahman, A. 2004. Dynamics of P, Al, and Fe during high discharge episodic acidification at the Bear Brook Watershed in Maine, U.S.A. *Water, Air, & Soil Pollution: Focus* 4, 311-323.
- Rourke, R.V. 1994. Chemical and physical properties of the Chesuncook, Colonel, Dixfield, and Telos Soil Map Units. Maine Agricultural and Forest Experiment Station, Technical Bulletin 155, 61 pgs.
- Robertson, G.P., Sollins, P., Ellis, B.G., and Laijtha, K. 1999. Exchangeable ions, pH, and cation exchange capacity. In G.P. Robertson et al. (ed.) *Standard soil methods for long term ecological research*. Oxford Univ. Press, New York.
- Saiya-Cork, K.R., Sinsabaugh, R.L., and Zak, D.R. 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. *Soil Biology and Biochemistry* 34, 1309-1315.
- SanClements, M.D., Fernandez, I.J., and Norton S.A. 2010. Soil chemical and physical properties at the Bear Brook Watershed in Maine, USA. *Environmental Monitoring and Assessment* 171, 111-128.
- Sinsabaugh, R. L., Moorhead, D. L. 1994. Resource allocation to extracellular enzyme production: A model for nitrogen and phosphorus control of litter decomposition, *Soil Biology and Biochemistry*, 26 (10),1305-1311.
- Sinsabaugh, R.L., Gallo, M.E., Lauber, C., Waldrop, M.P., and Zak, D.R. 2005. Extracellular enzyme activities and soil organic matter dynamics for northern hardwood forests receiving simulated nitrogen deposition. *Biogeochemistry* 75, 201-215.

- Sinsabaugh, R. L., Hill, B. H., and Follstad Shah, J. J. 2009. Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. *Nature* 462, 795-798.
- Sinsabaugh, R. L. 2010. Phenol oxidase, peroxidase and organic matter dynamics of soil. *Soil Biology and Biochemistry* 42, 391-404.
- Spohn M., and Y. Kuzyakov 2013. Phosphorus mineralization can be driven by microbial need for carbon. *Soil Biology and Biochemistry* 61, 69-75.
- Stone, M. M., Weiss, M.S., Goodale, C.L., Adams, M.B., Fernandez, I.J., German, D.P., Allison, S.D. 2011. Temperature sensitivity of soil enzyme kinetics under N-fertilization in two temperate forests. *Global Change Biology* 18 (3), 1173-1184. DOI: 10.1111/j.1365-2486.2011.02545.x
- Stursova, M., Crenshaw, C., and Sinsabaugh, R. 2006. Microbial responses to long-term N deposition in a semiarid grassland. *Microbial Ecology* 51, 90-98.
- Thompson, W.B., and Borns, H.W., Jr. 1985, *Surficial Geologic Map of Maine: 1:500,000*. Maine Geological Survey, Augusta, Maine.
- Turner, B.L. 2010. Variation in pH optima of hydrolytic enzyme activities in tropical rain forest soils. *Applied and environmental microbiology* 76 (19), 6485-6493.
- Treseder, K.K., and Vitousek, P.M. 2001. Effects of soil nutrient availability on investment in acquisition of N and P in Hawaiian rain forests. *Ecology* 82, 946-954.
- Treseder, K.K. 2008. Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. *Ecology Letters* 11, 1111-1120.
- Vadeboncoeur, M.A. 2010. Meta-analysis of fertilization experiments indicates multiple limiting nutrients in Northeastern deciduous forests. *Canadian Journal of Forest Research* 40: 1766-1780.
- Vitousek, P.M., and Howarth RW. 1991. Nitrogen limitation on land and in the sea: How can it occur? *Biogeochemistry* 13, 87-115.
- Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., and Tilman, D.G. 1997. Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Applications* 7, 737-750.
- Vitousek, P.M., Porder, S., Houlton, B.Z., Chadwick, O.A. 2010. Terrestrial phosphorus limitation: mechanisms, implications, and nitrogen-phosphorus interactions. *Ecological Applications* 20, 5-15.

761 Waldrop, M.P., Zak, D.R., Sinsabaugh, R.L., Gallo, M., and Lauber, C. 2004. Nitrogen
762 deposition modifies soil carbon storage through changes in enzymatic activity.
763 Ecological Applications 14, 1172-1177.
764

765 Wallenstein, M.D., McNulty, S., Fernandez, I.J., Boggs J, and Schlesinger WH. 2006. Nitrogen
766 fertilization decreases forest soil fungal and bacterial biomass in three long-term
767 experiments. Forest Ecology and Management 222, 459-468.
768

769 Wang, Z. and Fernandez, I. 1999. Soil type and forest vegetation influences on forest floor
770 nitrogen dynamics at the Bear Brook Watershed in Maine (BBWM). Environmental
771 Monitoring and Assessment 55, 221-234.
772

773 Weand, M., Arthur, M., Lovett, G., Sikora, F., and Weathers, K. 2010. The phosphorus status of
774 northern hardwoods differs by species but is unaffected by nitrogen fertilization.
775 Biogeochemistry 97, 159-181.
776

777 Zak, D.R., Holmes, W.E., Burton, A.J., Pregitzer, K.S., and Talhelm, A.F. 2008. Simulated
778 atmospheric NO_3^- deposition increases soil organic matter by slowing decomposition.
779 Ecological Applications 18, 2016-2027.

Table 1. Summary of acid phosphatase (AP) and β -glucosidase (BG) activity response to acute nutrient additions(-) is used to denote instances where enzyme activities were significantly lower than activities in control (un-amended) samples, while (+) denotes instances where enzyme activities were significantly higher than activities in control samples ($\alpha = 0.05$ for all comparisons).

AP	<u>O (in-situ)</u>		<u>O (ex-situ)</u>		<u>B (in-situ)</u>	
	+P	+N+P	+P	+N+P	+P	+N+P
Reference hardwoods	-	-		-	-	-
N-enriched hardwoods	-			-		
Reference softwoods		-		-	-	-
N-enriched softwoods	-	-	-	-	-	
BG						
Reference hardwoods				+		
N-enriched hardwoods		+		+		
Reference softwoods	+			+		
N-enriched softwoods					+	

Note: Blank cells indicate that average enzyme activities in amended soils were not significantly different from activities in control samples.

Table 2. The average rate of soil net N-mineralization (N-MIN) measured *in-situ* as mg N per gram of soil per day, extractable soil PO₄-P, and microbial biomass C (MB-C).

		N-MIN	PO ₄ -P	MB-C
		mg N g ⁻¹ d ⁻¹	mg/g	mg C g ⁻¹
O horizon	Reference hardwoods	3.3E+00 a	1.4E+01 a	3.4E+00 a
	N-enriched hardwoods	4.7E+00 b	2.8E+01 b	2.9E+00 a
	Reference softwoods	4.0E+00 ca	1.1E+01 a	2.7E+00 ab
	N-enriched softwoods	3.0E+00 a	1.1E+01 a	2.0E+00 b
B horizon	Reference hardwoods	2.0E-01 a	9.3E-01 a	--
	N-enriched hardwoods	7.0E-01 b	9.0E-01 a	--
	Reference softwoods	6.0E-01 b	1.1E+00 a	--
	N-enriched softwoods	6.0E-01 b	9.3E-01 a	--

Note: Letters are used to indicate significant differences in N-MIN or MB-C among the same soil horizon at the p=0.05 level.

Table 3. Soil chemical properties at the Bear Brook Watershed in Maine measured in bulk soil O horizons and B horizons from quantitative pit sampling as described by SanClements et al. 2010. Values for analytes measured in soil solutions collected from tension lysimeters reported by Fatemi et al. 2012 are also shown.

O-horizon	LOI (%)	H ⁺ (μmol/L)	N(g/kg)	C(g/kg)	C/N	P (mg/kg)
Reference hardwoods	6.8E+01	4.2E+02	1.9E+01	4.4E+02	2.3E+01	7.6E+01
N-enriched hardwoods	8.0E+01	5.9E+02	1.9E+01	4.1E+02	2.1E+01	7.1E+01
Reference softwoods	9.1E+01	1.3E+03	1.5E+01	4.8E+02	3.2E+01	4.9E+01
N-enriched softwoods	7.8E+01	8.5E+02	1.7E+01	4.4E+02	2.7E+01	4.0E+01
B-horizon	LOI (%)	H ⁺ (μmol/L)	N(g/kg)	C(g/kg)	C/N	P (mg/kg)
Reference hardwoods	1.6E+01	1.5E+02	3.8E+00	7.2E+01	2.0E+01	3.2E+00
N-enriched hardwoods	2.2E+01	1.7E+02	5.2E+00	1.0E+02	2.0E+01	4.5E+00
Reference softwoods	1.8E+01	2.0E+02	3.4E+00	8.1E+01	2.3E+01	3.9E+00
N-enriched softwoods	1.7E+01	1.5E+02	3.2E+00	7.0E+01	2.2E+01	2.7E+00
Soil solution	SO ₄ -S (μmol/L)	H ⁺ (μmol/L)	NO ₃ -N (μmol/L)	DOC (μmol/L)	Ca:Al (mol/mol)	PO ₄ -P (mg/L)
Reference hardwoods	7.8E+01	8.9E-01	8.4E-01	1.4E+02	7.8E+00	1.2E+00
N-enriched hardwoods	2.0E+02	2.0E+01	7.6E+01	1.2E+02	2.0E+02	3.5E+00
Reference softwoods	7.7E+01	2.4E+01	9.9E+00	5.3E+02	7.7E+01	2.1E+00
N-enriched softwoods	3.0E+02	4.6E+01	1.8E+02	3.8E+02	3.0E+02	1.3E+00

Note: Units of measurement are denoted in parentheses.

Fig. 1. Grand means by watershed for soil extracellular enzyme activities ($\mu\text{mol hr}^{-1} \text{g OM}^{-1}$) in O and B horizon soils aggregated across forest types. Samples were collected in July 2008 from the reference (EB) and treated (WB) watershed. BG= β -glucosidase, XYLO= xylosidase, NAG= N-acetyl-glucosaminidase, AP= acid phosphatase; (*) are used to distinguish means that are significantly different at the $\alpha = 0.05$ level between watersheds.

Fig. 2a - 2c. Response ratios for P-hydrolyzing acid phosphatase activities in nutrient amended soils. Ratios >1 indicate that activity response to amendment was positive, ratios <1 indicate that response was negative. Bars represent standard errors, and lower case letters indicate significant differences among the four compartments sampled within each horizon at the $\alpha=0.05$ significance level.

Fig. 3a - 2c. Response ratios for C-hydrolyzing acid β -glucosidase activities in nutrient amended soils. Ratios >1 indicate that activity response to amendment was positive, ratios <1 indicate that response was negative. Bars represent standard errors, and lower case letters indicate significant differences among the four compartments sampled within each horizon at the $\alpha=0.05$ significance level.

Fig. 4. Comparison of net N mineralization (N-MIN) rates from in-situ buried bag experiments for soils treated with acute P amendment and control soils. The asterisks indicate where there are significant differences in N-MIN rates using an $\alpha = 0.05$ level between the control soils and those amended with P.