

Effects of long-term nitrogen addition on phosphorus cycling in organic soil horizons of temperate forests

Christine Heuck · Georg Smolka · Emily D. Whalen · Serita Frey ·
Per Gundersen · Filip Moldan · Ivan J. Fernandez · Marie Spohn

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Abstract High atmospheric nitrogen (N) deposition is expected to impair phosphorus (P) nutrition of temperate forest ecosystems. We examined N and P cycling in organic soil horizons of temperate forests exposed to long-term N addition in the northeastern USA and Scandinavia. We determined N and P concentrations, enzyme activities and net N and P mineralization rates in organic soil horizons of two deciduous (Harvard Forest, Bear Brook) and two coniferous (Klosterhede, Gårdsjön) forests which had

received experimental inorganic N addition between 25 and 150 kg N ha⁻¹ year⁻¹ for more than 25 years. Long-term N addition increased the activity of phosphatase (+ 180%) and the activity of carbon (C)- and N-acquiring enzymes (cellobiohydrolase: + 70%, chitinase: + 25%). Soil N enrichment increased the N:P ratio of organic soil horizons by up to 150%. In coniferous organic soil horizons, net N and P mineralization were small and unaffected by N addition. In deciduous organic soil horizons, net N and P mineralization rates were significantly higher than at the coniferous sites, and N addition increased net N mineralization by up to 290%. High phosphatase activities concomitant with a 40% decline in P stocks of deciduous organic soil horizons indicate increased plant P demand. In summary, projected future global

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C. Heuck · G. Smolka · M. Spohn (✉)
Department of Soil Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER),
University Bayreuth, Dr.-Hans-Frisch-Str. 1-3,
95448 Bayreuth, Germany
e-mail: marie.spohn@uni-bayreuth.de

E. D. Whalen · S. Frey
Department of Natural Resources and the Environment,
University of New Hampshire, 114 James Hall, Durham,
NH 03824, USA

P. Gundersen
Section for Forest, Nature and Biomass, Department of Geosciences and Natural Resource Management,
University of Copenhagen, Rolighedsvej 23,
1958 Frederiksberg, Copenhagen, Denmark

F. Moldan
Swedish Environmental Research Institute (IVL),
Aschebergsgatan 44, 40014 Göteborg, Sweden

I. J. Fernandez
School of Forest Resources and Climate Change Institute,
University of Maine, Orono, ME 04469, USA

increases in atmospheric N deposition may induce P limitation in deciduous forests, impairing temperate forest growth.

Keywords Chronic nitrogen deposition · Exoenzymes · Long-term experiments · Nutrient mineralization · Phosphatase · Soil stoichiometry

Introduction

Future increases in atmospheric nitrogen (N) deposition are expected to alter not only N, but also phosphorus (P) cycling in temperate forest ecosystems. This trend is already apparent in decreased foliar P concentrations observed in many temperate forests over the last several decades (Houdijk and Roelofs 1993; Flückiger and Braun 1998; Duquesnay et al. 2000; Ilg et al. 2009; Jonard et al. 2015; Talkner et al. 2015). Although the implementation of air pollution control measures has reduced N emissions in Europe and the USA (Xing et al. 2013; Vet et al. 2014; Li et al. 2016b), the amount of N deposition is still well above pre-industrial levels in these regions (Galloway et al. 2004; Simpson et al. 2014) and global rates of deposition are projected to double by 2050 (Galloway et al. 2004; 2008). Temperate and boreal forests, which are often N-limited, are particularly sensitive to high N inputs (Aber et al. 1989; Vitousek and Howarth 1991). High N inputs might cause P limitation in temperate forests (Mohren et al. 1986; Tessier and Raynal 2003; Gress et al. 2007; Talkner et al. 2015) because N-induced forest growth can increase plant P demand (Gradowski and Thomas 2006, 2008; Li et al. 2016a). For example, P limitation of trees has recently been reported in hardwood forests of the northeastern USA (Goswami et al. 2018).

Net mineralization of N and P, defined as the difference between gross mineralization and net immobilization, makes these nutrients available for plant uptake (Schimel and Bennett 2004; Bünemann et al. 2007). The mineralization of organic P is catalyzed by phosphatases, which are exoenzymes released by plants and soil microorganisms. Nitrogen addition has been shown to elevate soil phosphatase activity, likely as a result of increased P demand by plants and microorganisms (Treseder and Vitousek 2001; Wang et al. 2007; Marklein and Houlton 2012;

Deng et al. 2017). The production of the N-rich phosphatases might be facilitated by high N availability (Treseder and Vitousek 2001; Wang et al. 2007; Marklein and Houlton 2012; Deng et al. 2017).

The activity of other enzymes besides phosphatases may also change in response to N availability. For example, the activities of the cellulose and the chitin degrading enzymes cellobiohydrolase and chitinase (β -1,4-*N*-acetylglucosaminidase) have been reported to increase (Weand et al. 2010a), whereas lignin degrading phenol oxidases are often inhibited by high N concentrations (Frey et al. 2004; Gallo et al. 2004; Waldrop and Zak 2006; Jian et al. 2016). Comparison of the activities of C-, N- and P-acquiring enzymes and their ratios can be used to identify the nutrient cycling processes in which organisms preferentially invest energy (Sinsabaugh et al. 2008, 2009; Sinsabaugh and Follstad Shah 2012; Herold et al. 2014). Such ratios might indicate, for example, whether microbial investment into P acquisition is increased compared to investment into N or C acquisition in response to long-term N addition.

Numerous studies have evaluated the effects of simulated N deposition in N addition experiments in forests of the temperate zones in North America and Europe (Dise and Wright 1992; Aber et al. 1993; Norton et al. 1999). Nitrogen addition has been shown to elevate N concentrations in foliage and soil organic horizons (e.g. Aber et al. 1993; Magill et al. 2004; Kjølneas and Stuanes 2008; Elvir et al. 2010), increase plant biomass (e.g. Aber et al. 1993; Magill et al. 1997; Lovett et al. 2013), stimulate nitrate leaching (e.g. Gundersen 1998; Jefts et al. 2004; Moldan et al. 2006; Lovett et al. 2013), and reduce soil cation concentrations (Currie et al. 1999; Moldan and Wright 2011). Further, net N mineralization often increases following N addition (Aber et al. 1993, 1995; Kjølneas et al. 1998; Jefts et al. 2004; Fatemi et al. 2016; Carrara et al. 2018). Despite this long history of research, the influence of chronic N deposition on P cycling is not well studied and the response of N:P ratios of vegetation and soils to long-term N addition is seldom reported (e.g. in Kjølneas et al. 1998; Kjølneas and Stuanes 2008; Weand et al. 2010a; Crowley et al. 2012). This underlines the need for further research of the influence of high N deposition on P cycling in temperate forests. Special consideration should be given to how element cycling processes in the organic horizon change upon increased N inputs because

organic horizons represent an important pool of P in forest soils that is rapidly mineralized by microorganisms and is very important for forest P nutrition (Ponge 2003; Huang and Spohn 2015; Spohn et al. 2018).

Our objective was to assess changes in N and P cycling and microbial nutrient acquisition in response to long-term N addition to temperate forests soils. Our hypotheses were that (i) N addition would increase N:P ratios of organic soil horizons, (ii) phosphatase activity would increase more strongly in comparison to the activities of C- and N-acquiring enzymes due to N addition and (iii) increased phosphatase activity would lead to increased net P mineralization. To test these hypotheses, we analyzed organic soil horizons of two deciduous and two coniferous long-term N addition experiments in the USA and Europe.

Materials and methods

Study sites and sampling

Two North American hardwood forest sites (Harvard Forest, Bear Brook) and two Scandinavian spruce forests (Klosterhede, Gårdsjön) exposed to long-term, experimental N addition were sampled. Harvard Forest (Massachusetts, USA, N42°32', W72°10') is located 330 m a.s.l., with mean annual precipitation and temperature being 1240 mm and 8.5 °C, respectively. The vegetation is dominated by black oak (*Quercus velutina*) and red oak (*Q. borealis*) mixed with other hardwood species including black birch (*Betula lenta*), red maple (*Acer rubrum*) and American beech (*Fagus grandifolia*). The soils are mainly Cambisols (Inceptisols; USDA Taxonomy), developed from sandy tills of the Gloucester series. The organic horizon has an average thickness of 5 cm and a bulk density ranging from 0.17 (N150) to 0.24 cm⁻³ (control), depending on N treatment. There are three N addition treatments with application rates (since 1988) of 0 (N0), 50 (N50) and 150 (N150) kg N ha⁻¹ year⁻¹ in the form of ammonium nitrate (NH₄NO₃). Each treatment plot has a size of 30 × 30 m. Ambient N deposition at the site is currently 8–10 kg N ha⁻¹ year⁻¹ (Aber et al. 1995; Magill et al. 2004; Schwede and Lear 2014).

Bear Brook is located in Maine, USA (N44°52', W68°06') and has a mean annual precipitation of 1400 mm and a mean annual temperature of 4.9 °C.

Its lower elevations, which were sampled in this study, are dominated by American beech (*Fagus grandifolia*), sugar maple (*Acer saccharum*) and red maple (*Acer rubrum*). The soils are mainly Podzols (Spodosols) developed from quartzite and gneiss covered by an organic horizon with an average thickness of 4 cm (bulk density: 0.09 g cm⁻³, both control and N addition plots). The Bear Brook experiment consists of two adjacent watersheds. The 11 ha watershed “West Bear” has received 25 kg N ha⁻¹ year⁻¹ in the form of ammonium sulfate ((NH₄)₂SO₂) since 1989 and the 10 ha watershed “East Bear” has served as a control. Ambient deposition is 3 kg N ha⁻¹ year⁻¹ (Norton et al. 1999; Fernandez et al. 2010; SanClements et al. 2010).

Klosterhede (Denmark, N56°29', E008°24') is situated at 27 m a.s.l., with a mean annual precipitation of 860 mm and mean annual temperature of 9 °C. A 108 year-old Norway spruce (*Picea abies*) plantation grows on Podzols (Spodosols) developed from glacial outwash sands. The organic horizon is on average 11 cm thick and has bulk densities of 0.10 (control) and 0.13 g cm⁻³ (N addition). The experiment was part of the “NITREX project” (Wright and van Breemen 1995) and consists of a 500 m² N-addition plot surrounded by three control plots. The N-addition plot has received 35 kg N ha⁻¹ year⁻¹ in the form of NH₄NO₃ in monthly doses since 1992; ambient N deposition is approximately 25 kg N ha⁻¹ year⁻¹ (Dise and Wright 1992; Gundersen and Rasmussen 1995; Gundersen 1998). Samples were taken from 15 × 15 m subplots.

The Gårdsjön experiment (Sweden, N58°04', E12°03'), which was also part of the “NITREX project”, is situated at 135–145 m a.s.l. with mean annual precipitation of 1100 mm and mean annual temperature of 6.4 °C. The dominant tree species is Norway spruce (*Picea abies*) with Scots pine (*Pinus sylvestris*) growing in drier areas. The main soil types are Orthic Humic Podzols and Gleyed Humoferric Podzols (Spodosols) developed from glacial till covered by an 8 cm thick organic horizon with a mean bulk density of 0.18 g cm⁻³ (both control and N addition). We sampled the catchment G2 (0.5 ha), which has received 40 kg N ha⁻¹ year⁻¹ in the form of NH₄NO₃ in small doses (128 mg N l⁻¹) along with each rain event since 1991, and the control catchment F1 (3.7 ha). Ambient N deposition at the site is 12 kg N ha⁻¹ year⁻¹ (Dise and Wright 1992;

Andersson et al. 1998; Moldan et al. 2006; Seftigen et al. 2013).

The organic horizon at Harvard Forest and Bear Brook was sampled in July 2016. Samples (20 × 20 cm) were collected from six replicate plots and divided into leaf litter (Oi horizon) and organic soil (Oe + Oa horizons). Samples were collected from Klosterhede and Gårdsjön in March–April 2017 following the same methods. Samples were stored at 4 °C and shipped immediately on ice to the University of Bayreuth, Germany. All Oe + Oa horizons were sieved to remove roots and stones and stored moist at 4 °C. They were characterized for total C, total and available N and P concentrations as well as microbial C, N and P concentrations, N and P stocks, pH, exoenzyme activity and net N and P mineralization rates. Leaf litters were homogenized by hand, dried at 60 °C, and subsequently analyzed for total C, N and P concentrations.

Soil characteristics

Gravimetric water content and maximum water holding capacity of the soils was determined following Naeth et al. (1991). Plastic tubes closed with a fine cloth at the bottom were filled with 1 cm of field-moist soil for two replicates from each site, weighed, and water saturated for 48 h. Samples were then drained for 48 h on a water saturated sand bath at 5 °C and weighed again. During draining, the tops of the tubes were covered with a wet cloth to prevent water loss via evaporation. Sample dry weights were determined after 48 h drying at 60 °C. The bulk density was calculated for the organic soil horizons of both N addition and control plots in six replicates.

Soil pH was measured with a gel electrode (WTW) in a suspension of moist soil and deionized water at a ratio of 1:5 (w/v). Total C and N concentrations were measured on dried (60 °C) and finely ground subsamples using a CN analyzer (Vario MAX, Elementar). Total soil P concentrations were measured with an inductively coupled plasma-optical emission spectroscopy (ICP-OES, Vista-Pro radial, Varian) after pressure digestion in concentrated nitric acid. Nitrogen and P stocks were calculated from total N concentrations (total P concentrations) and soil bulk density measurements.

Net mineralization

Net N and P mineralization were determined by incubating soil (ca. 50 g), adjusted to 60% water holding capacity, at 15 °C for 11 weeks. Net N mineralization was determined based on the increase in ammonium (NH_4^+) and nitrate (NO_3^-) concentrations extracted with cold water from 2.5 g dry-weight equivalents of moist soil in weekly (1st month) to biweekly intervals with a ratio of 1:20 (w/v). NH_4^+ and NO_3^- concentrations were measured with flow-injection analysis (FIA-Lab, MLE). The NH_4^+ -N + NO_3^- -N concentrations determined at the first measurement were defined as the available N concentration. In the same incubation, net P mineralization was determined based on the increase in phosphate (PO_4^-) concentrations in Bray-1 extracts (0.03 M NH_4F + 0.025 M HCl). For this purpose, 5 g dry-weight equivalent of moist soil were regularly extracted in a ratio of 1:10 (w/v). PO_4^- concentrations in the extracts were measured spectrophotometrically using a microplate reader (M200 pro, Tecan) according to the method of Murphy and Riley (1962). To prevent interference with the color formation of the assay, fluoride ions were neutralized with 0.1 M boric acid before addition of the molybdate blue reagent. The PO_4^- -P concentration determined at the first measurement was termed available P. Net N and P mineralization rates were calculated as the increase in NH_4^+ -N plus NO_3^- -N and PO_4^- -P concentrations, respectively, over time.

Soil microbial biomass

Soil microbial biomass C, N and P were determined by chloroform-fumigation extraction (Brookes et al. 1984, 1985; Vance et al. 1987) of subsamples (C and N: 5 g, P: 2.5 g) from the incubation experiment described above. Samples were fumigated for 24 h at room temperature. For microbial C and N, controls and fumigated samples were extracted in 0.5 M K_2SO_4 with a ratio (w/v) of 1:5 (Joergensen et al. 1995). The C and N concentrations in the extracts were determined with a CN analyzer (multi N/C 2100, Analytik Jena). For microbial P, controls and fumigated samples were extracted in Bray-1 solution (0.03 M NH_4F + 0.025 M HCl, Bray and Kurtz (1945)) in a ratio of 1:10. PO_4^- concentrations in the extracts were measured spectrophotometrically with a

microplate reader (M200 pro, Tecan) using the method of Murphy and Riley (1962) modified as above. The Bray-1 solution has been shown to be a very efficient extractant for microbial P (Khan and Joergensen 2012). Soil microbial biomass C, N and P were calculated as the difference of C, N and P concentrations in fumigated and control samples, and corrected by a factor of 2.22 for C and N and by 2.5 for P, respectively (Jenkinson et al. 2004).

Exoenzyme activity

The activities of cellobiohydrolase, chitinase and phosphatase were determined using the fluorogenic substrates 4-methylumbelliferyl- β -D-cellobioside, 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide and 4-methylumbelliferyl-phosphate according to Marx et al. (2001) and German et al. (2011). Briefly, 1 g moist soil was dispersed in 50 ml deionized water with ultrasound and 50 μ l of this soil slurry were transferred to black 96-well microplates in four analytical replicates. These samples were diluted with 50 μ l sterile deionized water and amended with 100 μ l of a 1 mM substrate solution. The plates were incubated at 30 °C for 210 min and fluorescence was measured after 30, 60, 90, 150 and 210 min. Fluorescence values were corrected for quenching of the soil, as well as for the fluorescence of substrate, and soil enzyme activity calculations were based on end-point measurements (German et al. 2011). The ratios of cellobiohydrolase-to-chitinase, cellobiohydrolase-to-phosphatase and chitinase-to-phosphatase were determined. For this calculation, the natural logarithms of the specific enzyme activities in μ mol g organic C⁻¹ h⁻¹ were used (Sinsabaugh et al. 2008; Herold et al. 2014).

Statistical analyses

Data were tested for significant site-specific differences between N addition treatments and controls. The normality of the data was examined visually by QQ-plots and analytically by Shapiro–Wilk tests, and the homogeneity of variances were checked with Levene's tests. In all cases in which normality assumptions were met, analyses of variance (ANOVA) followed by Tukey's multiple comparisons tests were used for Harvard Forest, which had three treatments, and t-tests were used for Bear Brook,

Klosterhede and Gårdsjön, which each had two treatments. Where assumptions of normality were violated, Kruskal–Wallis tests followed by multiple comparisons tests according to Dunn (Pohlert 2014) were calculated for Harvard Forest, and Wilcoxon rank sum tests were used for the other sites. The relationships between cellobiohydrolase, chitinase and phosphatase activity as well as net N and P mineralization and different fractions of soil C, N and P were analyzed with Spearman rank correlations. Where linear regressions were used, all residuals were checked for normal distribution and homoscedasticity. If assumptions for least-squares regression were not met, robust linear regression (Yohai 1987; Koller and Stahel 2011) was used with the R package “robustbase” (Rousseeuw et al. 2015). The influence of control and N addition treatments on regression analyses were examined with analyses of covariance (ANCOVA). All statistical analyses were performed in R version 3.2.2 (R Core Team 2015).

Results

N and P of leaf litters and organic horizons

The total N concentration of the leaf litter increased significantly at all sites due to N addition, on average by 17% (Table 1). The total N concentration of the organic soil horizon also increased at two of the four sites, on average by 42% (Table 2). The total N stock of the organic soil horizon increased significantly at Klosterhede (+ 15%), but decreased at Bear Brook (– 27%) in response to N addition (Table 2). The available N concentration in the organic horizon was significantly elevated at all sites upon N addition (Table 2).

The total P concentration of the leaf litter declined significantly at Gårdsjön, but increased at Bear Brook (Table 1). The total P concentration of the organic horizon decreased at Harvard Forest by 29%, but increased at Klosterhede by 20%. Total P concentrations of leaf litter and the organic soil horizon were significantly lower in the coniferous than in the deciduous forests ($P < 0.001$, Tables 1, 2). The total P stocks of the coniferous organic soil horizons were not affected by N addition. In contrast, P stocks were significantly reduced in the deciduous organic soil horizons due to N addition by 55 and 35% at Harvard

Table 1 Total C, N and P concentrations and C:N:P ratios of the leaf litter from control and N-addition plots at Harvard Forest, Bear Brook, Klosterhede and Gårdsjön

Site	Treatment	Total C (mg g ⁻¹)	Total N (mg g ⁻¹)	Total P (mg g ⁻¹)	C:N ratio	C:P ratio
Harvard Forest	Control	475 ± 04	16.7 ± 1.3	1.16 ± 0.04	32.9 ± 2.8	1039 ± 053
	+ 50 kg N	481 ± 03	18.9 ± 1.3*	1.24 ± 0.04	29.8 ± 2.1	1001 ± 037
	+ 150 kg N	493 ± 07***	17.8 ± 1.1	0.97 ± 0.10	32.3 ± 2.1	1324 ± 139*
Bear Brook	Control	464 ± 02	16.0 ± 1.1	0.84 ± 0.04	33.8 ± 2.2	1422 ± 056
	+ 25 kg N	469 ± 06	19.5 ± 1.3***	0.95 ± 0.04**	27.2 ± 2.1***	1265 ± 034**
Klosterhede	Control	477 ± 16	17.7 ± 0.5	0.82 ± 0.04	31.4 ± 1.2	1501 ± 060
	+ 35 kg N	496 ± 05*	20.0 ± 0.3***	0.76 ± 0.05	28.9 ± 0.4**	1642 ± 049**
Gårdsjön	Control	498 ± 03	13.5 ± 1.3	0.78 ± 0.11	43.2 ± 3.8	1665 ± 226
	+ 40 kg N	510 ± 09**	17.5 ± 1.6***	0.62 ± 0.13*	34.3 ± 3.8**	2220 ± 590

All ratios were calculated on a molar basis and all values are given as means and standard deviations. Asterisks mark the level of significance for all significant differences between the N addition treatment and the respective control (bold)

Levels of significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Forest and Bear Brook, respectively (Table 2). The available P concentrations of the organic soil horizon was elevated at Harvard forest by 67% due to N addition.

N:P ratios were increased in all leaf litters and organic soil horizons due to N addition, and the increases were significant for all sites except for Bear Brook (leaf litter) and Gårdsjön (organic soil horizon, Fig. 1). Overall, the N:P ratios of leaf litter and organic soil horizons were lower in the deciduous forests than in the coniferous forests (leaf litter: $P < 0.05$, organic soil horizons: $P < 0.001$).

Total C, pH and microbial biomass

Total C concentrations of leaf litter and organic soil horizons increased significantly under N addition at Harvard Forest, Klosterhede and Gårdsjön (Tables 1, 2). Nitrogen addition did not affect total C stocks of the organic soil horizons, with the exception of a significant increase at Gårdsjön (by 9%). Organic horizon soil pH was significantly reduced at Harvard Forest and Bear Brook in response to N addition. Microbial biomass C declined in response to N addition at Harvard Forest in the N150 treatment, but was not affected at the other sites (Table 2). Microbial biomass N and P concentrations and microbial biomass C:N, C:P, and N:P ratios were also unaffected by long-term N addition (Table 3).

Exoenzyme activities in organic soil horizons

Phosphatase activity in the organic horizon increased significantly in response to N addition at all sites, except for Klosterhede (Fig. 2c). Cellobiohydrolase activity increased significantly at Bear Brook and Gårdsjön, and chitinase activity increased significantly at Klosterhede and Gårdsjön upon N addition (Fig. 2a, b). On average, phosphatase activity increased more strongly due to N addition (260%) than chitinase (80%) and cellobiohydrolase activity (150%); however, ratios of phosphatase-to-cellobiohydrolase or chitinase activity were only significantly increased in response to N addition at Harvard Forest (Fig. 2).

Phosphatase activity was negatively correlated with total P concentrations ($R^2 = 0.46$, $P < 0.001$) and positively with total N concentrations ($R^2 = 0.26$, $P < 0.001$) and N:P ratios ($R^2 = 0.65$, $P < 0.001$; Fig. 3). An exponential model best described the negative relationship between phosphatase activity and P concentrations in the organic soil horizons. Phosphatase activity was also strongly positively correlated with N:P and C:P ratios and total C and N concentrations (Online Resource 1). Cellobiohydrolase exhibited similar correlations with these variables, while chitinase activity was only weakly positively correlated with them (Online Resource 1).

Table 2 Characteristics of organic soil horizons of control and N-addition plots at Harvard Forest, Bear Brook, Klosterhede and Gårdsjön

Site	Treatment	Total C (mg g ⁻¹)	Total N (mg g ⁻¹)	Total P (mg g ⁻¹)	Total C stock (kg m ⁻²)	Total N stock (g m ⁻²)	Total P stock (g m ⁻²)
Harvard Forest	Control	168 ± 29	08.3 ± 1.1	1.14 ± 0.10	1.63 ± 0.35	076.1 ± 08.3	11.93 ± 1.33
	+ 50 kg N	215 ± 58	11.5 ± 2.7	0.89 ± 0.04***	1.79 ± 0.30	096.7 ± 19.7	07.83 ± 2.38**
	+ 150 kg N	245 ± 60*	13.2 ± 3.2*	0.74 ± 0.08***	2.11 ± 0.88	111.6 ± 41.1	05.39 ± 0.88***
Bear Brook	Control	278 ± 25	14.8 ± 1.5	0.92 ± 0.18	1.10 ± 0.20	058.6 ± 12.1	03.20 ± 0.45
	+ 25 kg N	297 ± 15	16.7 ± 2.8	0.80 ± 0.10	0.83 ± 0.27	042.7 ± 13.2*	02.08 ± 0.74*
Klosterhede	Control	377 ± 26	12.1 ± 1.1	0.42 ± 0.05	4.99 ± 0.35	160.7 ± 14.3	05.56 ± 0.65
	+ 35 kg N	441 ± 24**	15.2 ± 0.3***	0.50 ± 0.02*	5.38 ± 0.30	185.3 ± 03.2**	06.08 ± 0.23
Gårdsjön	Control	450 ± 24	14.8 ± 2.6	0.58 ± 0.06	6.30 ± 0.34	207.3 ± 36.6	08.20 ± 0.89
	+ 40 kg N	491 ± 08**	17.4 ± 1.8	0.56 ± 0.04	6.87 ± 0.11**	243.8 ± 24.7	07.88 ± 0.53
C:N ratio	C:P ratio	Microbial C (mg g ⁻¹)	Microbial N (mg g ⁻¹)	Microbial P (mg g ⁻¹)	Available N (μg g ⁻¹)	Available P (μg g ⁻¹)	pH
23.6 ± 2.0	0380 ± 051	1.20 ± 0.11	0.20 ± 0.02	0.18 ± 0.04	066 ± 012	77.8 ± 29.8	4.7
21.7 ± 1.0	0629 ± 185*	1.18 ± 0.17	0.21 ± 0.05	0.20 ± 0.03	068 ± 012	37.9 ± 17.4**	4.4
21.8 ± 1.6	0877 ± 289**	0.94 ± 0.17*	0.15 ± 0.01	0.13 ± 0.04	097 ± 167**	12.8 ± 04.4***	3.5***
22.0 ± 1.8	0807 ± 167	1.63 ± 0.47	0.32 ± 0.10	0.34 ± 0.09	095 ± 021	39.0 ± 42.1	4.9
22.8 ± 2.8	1042 ± 100**	1.23 ± 0.19	0.23 ± 0.02	0.18 ± 0.07**	188 ± 081*	42.4 ± 20.4	4.0**
36.3 ± 1.6	2326 ± 141	1.18 ± 0.19	0.18 ± 0.05	0.13 ± 0.02	006 ± 002	04.8 ± 01.0	4.2
35.0 ± 1.5	2369 ± 139	1.23 ± 0.18	0.18 ± 0.04	0.14 ± 0.03	015 ± 005**	05.5 ± 01.0	4.1
38.3 ± 3.2	2011 ± 327	2.07 ± 0.29	0.41 ± 0.03	0.24 ± 0.06	003 ± 003	05.3 ± 01.7	4.4
32.2 ± 2.5**	2189 ± 139	1.85 ± 0.35	0.35 ± 0.05*	0.20 ± 0.07	054 ± 040**	04.1 ± 02.1	4.3

The stoichiometric ratios were calculated on a molar basis and all values are given as means with standard deviations. Asterisks mark the level of significance for all significant differences between the N-addition treatment and the respective control (bold)

Levels of significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Net N and P mineralization rates

Net N and P mineralization was substantially higher in deciduous forests than in coniferous forests ($P < 0.001$). Net N mineralization ranged between 0.09 and 0.56 $\mu\text{mol N g}^{-1} \text{day}^{-1}$ at the deciduous forest sites (Harvard Forest, Bear Brook) and between 0.02 and 0.05 $\mu\text{mol N g}^{-1} \text{day}^{-1}$ at coniferous forests (Klosterhede, Gårdsjön) across all treatments (Fig. 4). Net P mineralization was between 2.6 and 12.7 $\text{nmol P g}^{-1} \text{day}^{-1}$ in deciduous forests and 0.0 and 0.4 $\text{nmol P g}^{-1} \text{day}^{-1}$ in coniferous forests. Net N and P mineralization were only affected by N addition treatments in deciduous organic soil horizons. Net N mineralization increased significantly in the Harvard Forest N150 treatment (+ 290%) and at Bear Brook (+ 210%) compared to the control. Net P

mineralization increased significantly in response to N addition at Bear Brook (+ 400%), whereas it decreased significantly in the N50 treatment at Harvard Forest (+ 60%).

Net N mineralization was strongly correlated with several fractions of C, N and P and organic soil horizon C:N:P stoichiometry across all sites and treatments, whereas net P mineralization was only related to P fractions (Online Resource 1). Net N mineralization correlated strongly positively with dissolved organic N ($r = 0.89$), the N:P and C:P ratios ($r = 0.77$ and 0.78 , respectively), and total N concentrations of the organic horizons ($r = 0.59$). In addition, net N mineralization was strongly negatively correlated with total P concentrations ($r = -0.74$, Online Resource 1).

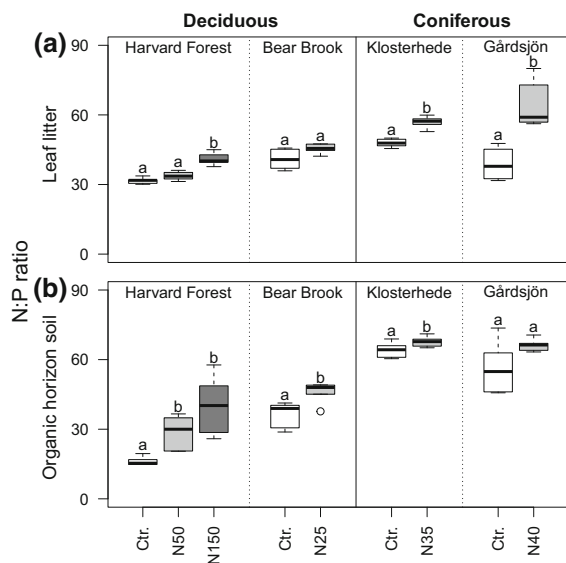


Fig. 1 N:P ratios of **a** leaf litter and **b** organic soil horizons of control and N addition treatments at Harvard Forest, Bear Brook, Klosterhede and Gårdsjön. Different lowercase letters indicate significant site-specific differences ($P < 0.05$)

Discussion

Long-term N addition significantly increased the N:P ratio and phosphatase activity in the organic horizon at three of the four forest sites. This response was particularly obvious in the deciduous forests where the P stock of the organic horizon was also significantly reduced in response to N addition. In the coniferous organic horizons, net N and P mineralization rates

were negligible and were not affected by N addition, indicating efficient microbial uptake of these nutrients. In the deciduous organic soil horizons, net N and P mineralization rates were significantly higher than in the coniferous forests and net N mineralization at these sites increased due to high chronic N inputs.

Exoenzyme activity

Phosphatase activity increased strongly in response to long-term N addition (Fig. 2c). This observation, in combination with the positive linear relationship between phosphatase activity and the total N concentrations of the organic soil horizons (Fig. 3), confirms previous findings (Naples and Fisk 2010; Weand et al. 2010b; Marklein and Houlton 2012). The observed relationship between total N concentrations and phosphatase activity may be due to an increased P demand of plants and potentially of microorganisms exposed to long-term N addition (Clarholm 1993; Olander and Vitousek 2000). The high N supply is likely beneficial for the synthesis of N-rich enzymes, including phosphatase (Allison and Vitousek 2005). Phosphatase activity was negatively correlated with total P concentrations and positively correlated with C:P and N:P ratios of the organic soil horizons (Fig. 3, Online Resource 1). This finding is in agreement with previous studies showing that the soil P concentration regulates phosphatase activity (Spiers and McGill 1979; Marklein and Houlton 2012), with declining activity where P is abundant (Juma and Tabatabai

Table 3 Molar microbial biomass C:N, C:P and N:P ratios in the organic soil horizons of long-term N addition experiments at the deciduous forest sites Harvard Forest and Bear Brook and the coniferous forest sites Klosterhede and Gårdsjön

Site	Treatment	C:N ratio	C:P ratio	N:P ratio
Harvard Forest	Control	7.3 ± 1.1	16.8 ± 2.2	2.3 ± 0.3
	N50	6.2 ± 0.4	15.1 ± 1.9	2.3 ± 0.5
	N150	6.6 ± 0.6	19.5 ± 5.9	2.9 ± 0.8
Bear Brook	Control	6.0 ± 0.4	12.6 ± 2.3	2.1 ± 0.5
	N	6.0 ± 0.5	15.7 ± 1.3	2.8 ± 0.4
Klosterhede	Control	8.0 ± 1.7	25.6 ± 2.7	3.3 ± 1.0
	N	7.9 ± 0.8	22.9 ± 3.1	2.9 ± 0.4
Gårdsjön	Control	5.9 ± 0.6	24.4 ± 5.2	4.2 ± 1.2
	N	6.2 ± 0.8	20.9 ± 2.7	3.4 ± 0.5

Values are given as mean with standard deviation (n = 6)

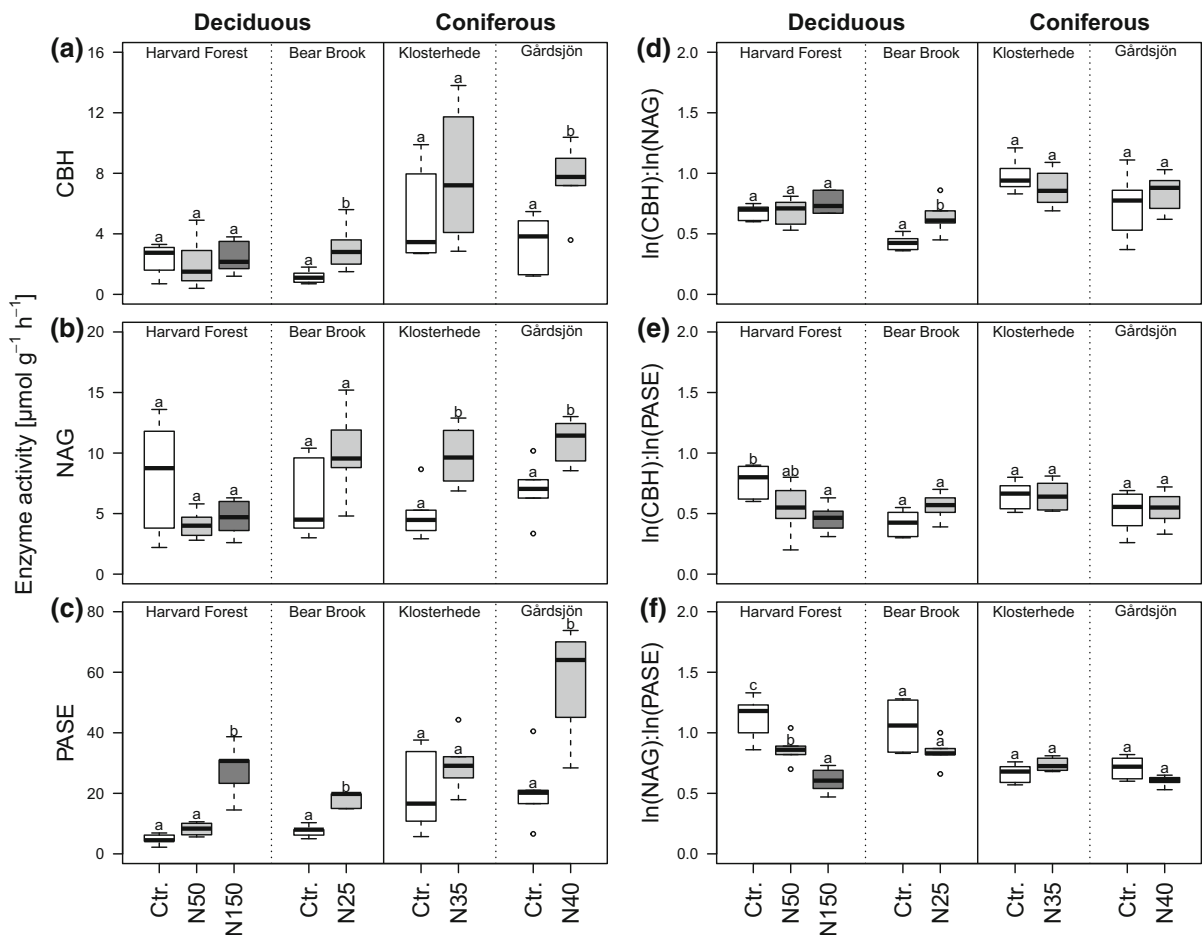


Fig. 2 Activities of the enzymes **a** cellobiohydrolase (CBH), **b** chitinase (NAG), **c** phosphatase (PASE) and ratios of the natural logarithms of specific enzyme activities, **d** CBH:NAG, **e** CBH:PASE, **f** NAG:PASE in the organic soil horizons of

1977, 1978; Olander and Vitousek 2000; Moscatelli et al. 2005; Marklein and Houlton 2012).

Cellobiohydrolase and chitinase activity also increased with N addition, mainly in the coniferous forests (Fig. 2a, b), suggesting that N addition at these nutrient poor sites led to increased microbial C demand and facilitated enzyme synthesis of N-rich hydrolytic enzymes. However, the increase in phosphatase activity in response to N addition was much larger than the increase in cellobiohydrolase and chitinase activity, indicating that long-term N addition predominantly increased P demand.

control and N addition treatments at Harvard Forest, Bear Brook, Klosterhede and Gårdsjön. Different lowercase letters indicate significant site-specific differences ($P < 0.05$)

Net N and P mineralization

Net N and P mineralization in the organic horizon were substantially higher in the deciduous forests than in the coniferous forests (Fig. 4). This is most likely because soil microorganisms experienced greater N and P scarcity in the coniferous forest soils compared to the deciduous forest soils, and therefore took up the available forms of the two nutrients almost completely. This is supported by the stoichiometry of the organic soil horizons. The C:N and N:P ratios in the coniferous forests were above the thresholds for net N mineralization in organic soils, which amount to 20–40 for the C:N ratio (Parton et al. 2007; Moore et al. 2011) and 60 for the N:P ratio (Heuck and Spohn

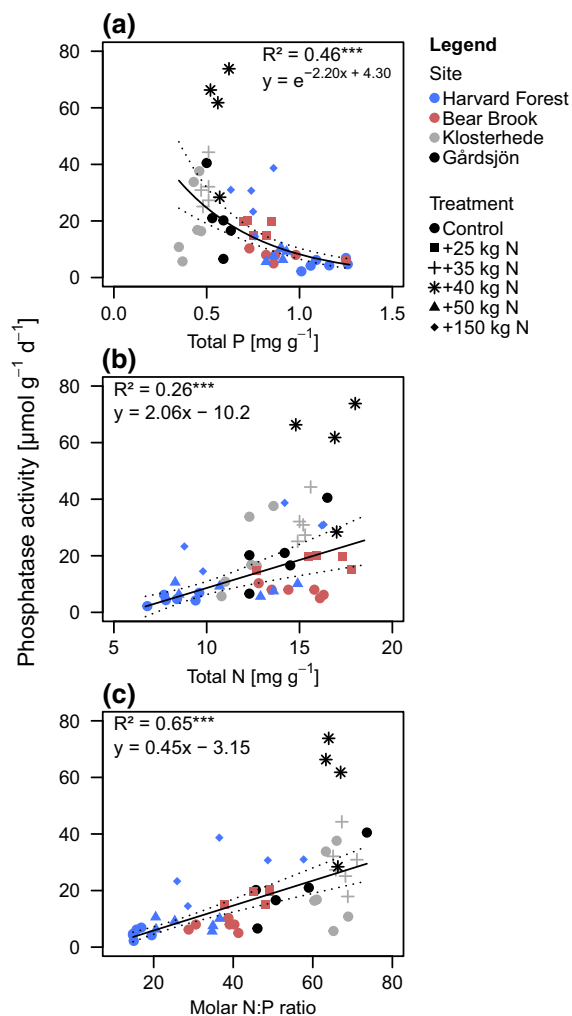


Fig. 3 Relationship between phosphatase activity and **a** total soil P concentration, **b** total soil N concentration and **c** molar N:P ratio of the organic soil horizons. Sites are distinguished by colors (blue: Harvard Forest, red: Bear Brook, grey: Klosterhede, black: Gårdsjön) and treatments are distinguished by symbols (control: circle, + 25 kg N: square, + 35 kg N: plus, + 40 kg N: star, + 50 kg N: triangle, +150 kg N: diamond). An exponential model was fitted for **(a)** and linear models for **(b)** and **(c)**. Fitted lines are presented with 95%-confidence intervals (dotted lines), R^2 and the regression equations. Levels of significance are: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

2016). Above these thresholds, N availability is so low that no net release of inorganic N occurs, and microorganisms immobilize all N when decomposing organic matter (Parton et al. 2007; Moore et al. 2011; Heuck and Spohn 2016; Spohn 2016). Analogously, net P mineralization was not observed in the coniferous forests, presumably because the threshold C:P

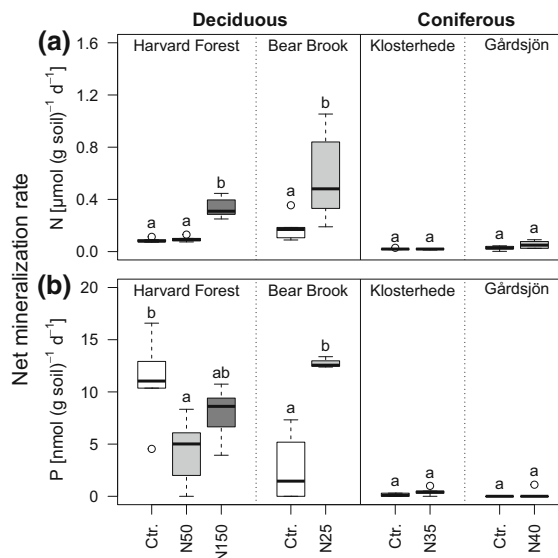


Fig. 4 Net mineralization rates of **a** N and **b** P in organic soil horizons of control and N addition treatments at Harvard Forest, Bear Brook, Klosterhede and Gårdsjön. Different lowercase letters indicate significant site-specific differences ($P < 0.05$)

(300–1700, Blair 1988; Moore et al. 2011) and N:P ratios (40, Heuck and Spohn 2016) for net P mineralization were exceeded. In the deciduous organic soil horizons, N addition increased microbial net N mineralization. This may have occurred because available N concentrations exceeded microbial N demand due to N addition, and thus microbes released surplus N in inorganic forms during organic matter decomposition (Prescott et al. 1992). This likely allowed the microbes to maintain their biomass C:N:P stoichiometry (Table 3) despite changes in organic matter stoichiometry (Table 2), and is in agreement with the theory of the microbial biomass stoichiometry being homeostatic (Cleveland and Liptzin 2007; Xu et al. 2013; Spohn 2016). In addition, variation in the microbial community composition of deciduous and coniferous organic horizons may have contributed to the differences in net N and P mineralization rates because of higher fungal:bacterial ratios in soils of temperate coniferous forests than in deciduous forest soils (Fierer et al. 2009).

Despite a consistent increase in phosphatase activity in response to N addition, we did not find a consistent increase in net P mineralization. In deciduous forests, net P mineralization both increased (Bear Brook) and decreased (Harvard Forest), whereas in the coniferous forests, net P mineralization rates were

very small across all treatments. Elevated phosphatase activity only increases net P mineralization if substrate concentrations are sufficiently high, and the microbial P demand is satisfied. This seemed to be the case at Bear Brook, but not at Harvard Forest. Another possible explanation for these divergent responses of the two deciduous sites is that different forms of N were added to soils at the two sites. Ammonium nitrate was added at Harvard Forest, whereas ammonium sulfate was used at Bear Brook. The added sulfate may have contributed to the additional P release, because sulfate might exchange with sorbed phosphate (Geelhoed et al. 1997).

Taken together, in deciduous organic soil horizons high rates of net N and P mineralization (Fig. 4) led to the formation of high concentrations of inorganic N and P (Table 2) that are potentially plant available. In contrast, in the coniferous organic soil horizons, net rates of N and P mineralization, and concentrations of plant available inorganic N and P were much lower (Table 2). Thus, in the deciduous but not in the coniferous forests, increased microbial net N and P mineralization may have facilitated increased plant nutrient uptake. The significant declines in P concentrations and P stocks in the deciduous organic soil horizons in response to long-term N addition (Table 2) most likely resulted from an increased P demand of the plants that led to increased P uptake. The increased P demand was likely caused by the high availability of N. This is supported by increased aboveground biomass and annual net primary productivity of trees in the N addition plots at Harvard Forest (Magill et al. 2004; Savage et al. 2013). Yet, aboveground biomass was unaffected by N addition at Bear Brook (Elvir et al. 2010), which might be due to the addition of sulfate together with N that may have led to desorption of adsorbed phosphate (see above). Substantial net P mineralization in the organic horizons of the deciduous forests likely allowed for transfer of inorganic P from the organic soil horizons into the vegetation by plant uptake or into the mineral soil by leaching. In the long-term, this may force plants to acquire P from the mineral soil, where P strongly adsorbs to minerals and therefore is more difficult to obtain.

Nutrient stocks of the organic horizon

The significant decrease in P stocks of the organic horizon in the two deciduous forests (Table 2) can

most likely be attributed to increased plant P uptake caused by the high N availability (see above). The organic horizon is an important pool of P for forest nutrition (Ponge 2003; Huang and Spohn 2015; Spohn et al. 2018), and a significant decline in P stocks of this pool might lead to plant P limitation in the long-term if N inputs remain high. Foliar P concentrations of several deciduous tree species are currently decreasing in Europe (Duquesnay et al. 2000; Ilg et al. 2009; Talkner et al. 2015). The reason for this decrease is not known. However, our study indicates that one possible explanation is atmospheric N deposition.

The N:P ratio of leaf litter and the organic soil horizon increased at most sites due to long-term N addition (Fig. 1), which is in agreement with previous results for foliage and the organic soil horizon of the site Gårdsjön (Kjønaas et al. 1998; Kjønaas and Stuanes 2008). The increase in the N:P ratios resulted from the decreased P stocks as well as from the large N additions that increased organic horizon N stocks either directly due to incorporation of added N in the organic horizon (Magill et al. 1997; Gundersen 1998) or indirectly due to increased foliar N concentrations (Aber et al. 1993; Gundersen 1998; White et al. 1999; Magill et al. 2004; Kjønaas and Stuanes 2008; Fernandez and Norton 2010).

Conclusions

Long-term N addition consistently increased phosphatase activities and N:P ratios of the organic horizons in both coniferous and deciduous temperate forests, indicating increased plant P demand, which confirms our first two hypotheses. In the deciduous forests, net N and P mineralization rates were substantially higher than in the coniferous forests. In contrast to our third hypothesis, high phosphatase activities caused by N addition did not consistently translate into high net P mineralization rates. This can be attributed to the organic horizon stoichiometry that differed among deciduous and coniferous forests, and to a very efficient immobilization of P by the microbial biomass in the coniferous organic horizons. We observed a decline in the P stocks of the organic horizons due to N addition in the deciduous but not in the coniferous forests. The decline in P stocks of the deciduous organic horizons indicates that in the long-term, high N inputs might lead to decreased plant P

uptake and even to plant P limitation in deciduous forests.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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