Received: 10 July 2020 | Accepted: 26 September 2020

DOI: 10.1111/1365-2435.13701

RESEARCH ARTICLE

Functional Ecology | BRITISH ECOLOGICAL SOCIETY

Long-term nitrogen enrichment does not increase microbial phosphorus mobilization in a northern coniferous forest

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Funding information
Svenska Forskningsrådet Formas; Kungl. Skogs- och Lantbruksakademien; Trees and Crops for the Future

Handling Editor: Carly Stevens

Abstract

1. Nitrogen (N) deposition can enhance carbon (C) capture and storage in northern coniferous forests but it may also enhance the demand for phosphorus (P). While it is well established that long-term N enrichment can decrease decomposition and enhance the accumulation of C in soils, it remains uncertain if a higher demand and acquisition of P influence soil C.
2. We studied microbial phosphorus mobilization and growth within a long-term N enrichment experiment in a Norway spruce forest, where N deposition was simulated by adding 0, 12.5 or 50 kg N ha^{-1} $year^{-1}$ for 21 years (n = 12), by incubating microbial ingrowth cores with needles and humus with low and high P content, and with sand with and without mineral apatite P.
3. Long-term N enrichment had no effect on microbial P mobilization in needles and humus and did not enhance the positive effect that apatite had on fungal growth. However, it consistently strengthened the retention of C in the soil by decreasing decomposition of needle and humus, both with low and high P content, and by increasing fungal growth in sand-filled ingrowth cores. Furthermore, we did not find any evidence that higher microbial P mobilization in response to N enrichment affected soil C storage.
4. These results show that long-term N enrichment in relatively young soils dominated by coniferous trees and ectomycorrhizal fungi can have relatively small impact on microbial P mobilization from organic sources and on the potential to mobilize P from minerals, and subsequently that elevated P demand due to N enrichment is unlikely to lead to a reduction in the soil C accumulation rate.

KEYWORDS
apatite, boreal forest, carbon sequestration, decomposition, ectomycorrhizal fungi, nitrogen deposition, soil phosphorus

1 | INTRODUCTION

During the past century, fossil fuel combustion and industrial production of fertilizers have contributed to a 3-fold to 5-fold increase in reactive nitrogen (N) emissions (Reay et al., 2008; Steffen et al., 2015). Short-term (<10 years) additions of N to ecosystems at northern latitudes can increase tree biomass production (LeBauer & Treseder, 2008; Nohrstedt, 2001; Tamm, 1991), and decrease mineralization of carbon (C; Fog, 1988; Janssens et al., 2010), and thus enhance their C storage (Gundale et al., 2014; Hyvönen et al., 2008; Maaroufi et al., 2015).

Thus, the increased input of N to northern coniferous forests during the past century may contribute to mitigate climate change by enhancing the C sink of ecosystems at northern latitudes (de Vries, 2009; Holland et al., 1997; Pan et al., 2011). However, although the input of N to northern forests is typically relatively low, long-term (>10 years) inputs may gradually shift limitations from N towards other factors. The impact of N deposition on the ecosystem C sink is therefore likely to depend on the availability of other growth-limiting resources, and how the acquisition of these other resources impacts the storage of C that is already present, particularly in soils where most of the C typically is stored (de Vries et al., 2014; Luo et al., 2016; Tarnocai et al., 2009).

After N, phosphorus (P) is the element that is required in the highest amount by forest vegetation and the demand for P is likely to increase as a consequence of long-term N deposition, and may even become limiting in some environments (Almeida et al., 2018; Harpole et al., 2011; Vitousek et al., 2010). In northern coniferous forests, P originates from minerals, mainly apatite that is mobilized by weathering, and once mobilized it is efficiently recycled via microbial decomposition and mobilization from plant litter (Stevenson & Cole, 1999). During the humification of plant litter, saprotrophic microbes and root-associated fungi such as ectomycorrhizal fungi (EMF) act in concert to mobilize N and P (Berg, 2014; Smith & Read, 2008). Supplied with labile C via tree roots, EMF is capable of extracting nutrients in the humus (Clarholm et al., 2015; Lindahl & Tunlid, 2015; Talbot et al., 2008). They become particularly prominent during late stages of decomposition (Berg & McClaugherty, 2014; Lindahl et al., 2007), and contribute to decomposition by producing enzymes and organic acids that can destabilize soil organic matter (Clarholm et al., 2015; Kuzyakov, 2010; Talbot et al., 2008). Whereas it is well known that short-term N enrichment of coniferous forests at northern latitudes can decrease EMF biomass and inhibit decomposition (Fog, 1988; Janssens et al., 2010; Treseder, 2008), it is not well understood whether N enrichment in the longer term (>10 years) influences the mobilization and supply of P. The inhibition of decomposition could potentially reduce P availability, but higher P demand could also trigger decomposition targeting P (Clarholm et al., 2015; Kuyper, 2017; Lambers et al., 2008). Thus, in addition to uncertainties regarding the supply of P as a growth-limiting resource, it is also uncertain how N may affect soil organic matter and the soil C sink when P becomes increasingly deficient (Luo et al., 2016; Wieder et al., 2015).

Several studies have found that P concentrations in plant tissues have declined in areas with high N deposition (Braun et al., 2010; Jonard et al., 2015), an effect that also can be generated experimentally by N addition (Almeida et al., 2018; Hedwall et al., 2017; Prietzel & Stetter, 2010; Tarvainen et al., 2016). Increased demand for P by vegetation could potentially constrain the C sink of forests in areas with elevated N deposition by limiting C uptake but could also affect the large pool of C present in soils, by increasing C allocation to nutrient acquisition in the organic layer and the mineral soil (Wieder et al., 2015). If N deposition enhances growth and the demand for P beyond the supply by mobilization by decomposition of litter and humus, further growth will ultimately be limited by the rate of mobilization of P through the weathering of primary minerals (Akselsson et al., 2008; Peñuelas et al., 2013).

Experiments growing tree seedlings at varying P availability in controlled laboratory environments have shown that trees allocate more photosynthate to root-associated fungi and that fungal weathering of apatite increases when the availability of P is low (Smits et al., 2012; Wallander, 2000). Similarly, field experiments have demonstrated that low foliage P concentrations correspond with a high fungal exploration of apatite in the mineral soil (Wallander & Thelin, 2008) and that the production of EMF biomass can be stimulated if N addition leads to increased P deficiency (Almeida et al., 2018; Wallander & Nylund, 1992). These observations indicate that long-term N enrichment may enhance C supply to roots and associated microbes involved in mobilizing P from soil organic matter, as well as from P in the mineral soil. It is, however, not well known if these effects occur also in relatively young coniferous forest soils on the northern hemisphere, where mineral P sources are still relatively rich. Moreover, it is not well understood how plant roots and EMF scavenging for mineral P sources influence the input and storage of C in the mineral soil.

The main aim of this study was to determine whether long-term N enrichment leads to an increase in P removal from P-rich organic matter, and an increase in fungal colonization of P-containing minerals; and further, to determine whether such N effects on P mobilization are associated with changes in organic matter accumulation. To test this, we used an experiment set up in a ca. 120-year-old Norway spruce (L.) Karst-dominated forest in northern Sweden, with long-term N additions consisting of control plots, and treatments where N had been added at a low (12.5 kg N ha^{-1} $year^{-1}$) and a high (50 kg N ha^{-1} $year^{-1}$) rate for 20 years. First, because long-term N enrichment would enhance forest P demand, we hypothesized that an increase in P removal from soil substrates would occur in response to N addition, particularly from P-rich litter and humus; and further, that EMF exploration of apatite P-rich mineral soil patches would increase. Second, we hypothesized that enhanced P acquisition in response to N enrichment would affect soil C, specifically by increasing mass loss of C in P-rich litter and humus as a result of enhanced decomposition, as suggested by Clarholm et al. (2015); whereas we expected increased C accumulation in P-rich mineral soil patches via microbial colonization and necromass inputs. To test these hypotheses, we created three types of P-rich and poor ingrowth cores (needles, humus and quartz sand). For the organic substrates, P-rich and poor materials were collected from control and P addition plots from a separate long-term P enrichment experiment in southern Sweden, whereas for the mineral soil substrate we created P-rich and poor substrates using quartz sand with or without apatite added. We incubated these substrates for 1 year in each of the N treatments plots, and measured changes in P, N and C content in needles and humus, and the fungal colonization and organic matter accrual in the sand ingrowth cores after the incubation. These measurements allowed us to test if N addition caused a shift in microbial P acquisition and investigate its consequences for the accumulation of C in northern coniferous forest soils.

2 | MATERIALS AND METHODS

2.1 | Field experiment

This study was conducted in one of the longest-running field experiments simulating anthropogenic N deposition in the boreal region, with N treatments established and maintained since 1996. It is set up in northern Sweden (64°14′N, 19°46′E) where the background N deposition is very low (<2 kg N ha^{-1} $year^{-1}$; Gundale et al., 2011, Pihl-Karlsson et al., 2013). The experiment consists of a Norway spruce (*Picea abies* (L.) H. Karst) dominated forest that naturally regenerated 120 years ago (From et al., 2016; Gundale et al., 2013) on glacial till deposited above the highest ice age coastline approximately 10,200 year bp (Stroeven et al., 2016). Nitrogen has been added as ammonium nitrate in May each year at a low (12.5 kg N ha^{-1} $year^{-1}$; 12.5N) and a high rate (50 kg N ha^{-1} $year^{-1}$; 50N) in a randomized complete block design, including paired control plots (0N). The 12.5N treatment level represents upper N deposition rates in the boreal region, while the 50N treatment serves as a useful comparison with several other N addition studies in the boreal region (Hyvönen et al., 2008). The experiment consists of plots of different sizes, with each plot size and N addition rate replicated six times. For this study, we used the two largest plot sizes (1,000 and 2,500 m^2) to reach a total of 12 replicates for each N addition rate (i.e. three treatment levels (0N, 12.5N and 50N) × 12 replicates = 36 plots in total).

During the first 7 years of the experiment, plant biomass production was strongly stimulated by N addition but has successively declined back to nearly the same level as before the N treatments started (From et al., 2016). In 2012 (the 16th year of annual N additions), the total soil N stocks in the 12.5N and 50N treatment was 10% and 33% higher, respectively, than the soil N stock in the control treatment (1,190 kg N/ha), and the C stock in the organic soil layer had increased by on average 10 kg C/kg N added (Maaroufi et al., 2015). In the 18th year, the basal area was 35% and 64% larger than the control treatment in the 12.5N and 50N treatment, respectively, and the 50N treatment had a basal area of 37 m^2/ha and a standing volume of wood of 380 m^3/ha (From et al., 2016). At this time, needle P content showed a near significant ($p = 0.054$) decline of 23% in the 50N treatment, compared to the control (Palmqvist et al., 2020), and below levels that have been suggested to be indicative of P deficiency (Figure S1; Thelin et al., 1998). In the 50N treatment, the organic soil layer P stocks have nearly doubled, an increase mainly explained by higher amounts of organic P (Palmqvist et al., 2020).

2.2 | Soil sampling

The organic layer between the fragmented litter layer and mineral soil was sampled in June and September 2016, that is, in the 20th year of annual N additions, 1 year before the installation of ingrowth cores. On each occasion, soil cores were collected from 30 locations spaced approximately 2 m apart in each plot by removing the intact litter layer and collecting the entire organic layer down to the mineral soil with a sharp 3 cm diameter steel corer. The mineral soil was discarded, and the remaining organic soil was pooled, homogenized, sieved (2 mm), placed in a cooler and then frozen the same day. Organic layer samples were then freeze-dried for chemical analyses as described below. Averages across the two sampling occasions were used for all analyses in this study.

2.3 | Ingrowth cores

We used ingrowth cores to measure decomposition and fungal growth. Ingrowth cores are simple tools that have been used extensively in ecosystem studies to measure the production of fine roots and fungal mycelium (Addo-Danso et al., 2016; Vogt et al., 1998; Wallander et al., 2012), and decomposition (Berg & McClaugherty, 2014). By choosing different substrates, the ingrowth core can provide valuable information about different ecosystem processes (Bödeker et al., 2016; Maaroufi et al., 2019). We constructed cylindrical 10 cm long by 2 cm wide ingrowth cores using fine nylon mesh (50 μm, SinTab), which prevents ingrowth of roots, including the majority of Ericaceous hair roots (Bonfante-Fasolo & Gianinazzi-Pearson, 1979). While the mesh excludes roots, they allow ingrowth and colonization by fungi and other microbes. To test if long-term N deposition stimulates microbial exploitation of P-enriched soil patches, we filled these ingrowth cores with three types of substrates, including two organic substrates (green needles or humus) and one mineral substrate (quartz sand). For each of these substrate types, we created a high and low P version.

The organic substrates (i.e. needles and humus) were collected in a P addition experiment in a Norway spruce (*Picea abies* (L.) H. Karst) forest (Almeida et al., 2018) located at Tönnersjöheden, in southern Sweden (56°41′N, 13°6′E). The experiment had added 200 kg P/ha in 2011 and 2012, which generated plant and soil material that was highly enriched in P (Table 1). Green needles directly from the branches and the lower part of the organic layer (i.e. the humus layer)

TABLE 1 The initial phosphorus (P), nitrogen (N) and carbon (C) content in % dry mass and element mass ratios in needle and humus used as substrates to assay microbial decomposition. The substrates were collected in control (low P) and treatment (high P) plots of a P addition experiment where a total of 400 kg phosphorus ha^{-1} $year^{-1}$ had been added 5 and 6 years earlier. Values refer to properties of the material collected and pooled across several replicates of the P addition experiment

	Substrate			
	Needle		Humus	
	Low P	High P	Low P	High P
Phosphorus (%)	0.068	0.208	0.068	0.215
Nitrogen (%)	1.09	0.98	1.19	1.34
Carbon (%)	52.8	51.8	30.9	40.0
P:C	0.00152	0.00294	0.00152	0.00289
N:C	0.0278	0.0251	0.0358	0.0325
N:P	18.2	9.1	24.6	13.2

in control and P-treated plots were collected in spring 2017, 5–6 years after the application of P, which provided needles and humus with P concentrations differing by a factor of 3 between substrates (Table 1). The needles and humus were dried (60°C) and ground (<2 mm) before they were filled into ingrowth cores as described below. In addition to the two organic substrates, the organic-free mineral substrate was obtained by acid washing (HCl, 2.5 M) and heating (550°C) quartz sand to remove organic material (Wallander et al., 2012). Phosphorus-enriched quartz sand substrates were made by adding apatite (0.05–0.63 mm) to a final concentration of 3 mass percentages in half of the quartz substrate whereas control substrates without P consisted of pure quartz sand (Krantz; Wallander et al., 2012; Wallander & Thelin, 2008).

Each ingrowth core was filled with approximately 7 g of the dried organic substrate (litter and humus enriched or not enriched with P) or 45 g of the acid-washed sand (enriched or not enriched with apatite P). Ingrowth cores with organic substrates were inserted diagonally in the organic layer, and those with the organic-free mineral substrates were inserted in the upper part of the mineral soil. Within each plot, we inserted six sub-replicate cores for each of the substrate types (i.e. 3 substrate types × 2 P levels × 6 sub-replicate cores × 3 N treatments × 12 replicate plots = 1,296 ingrowth cores in total). The incubation was initiated in October 2017 and terminated in October 2018, when all ingrowth cores were collected and immediately frozen. The mineral substrates were freeze-dried and the contents of the six sub-replicate cores per plot were pooled and homogenized. Ingrowth of fungi was measured as the concentration of the fungal biomarker ergosterol (described further below). The organic substrates were dried at 60°C, the ingrowth cores re-weighed to determine gravimetric mass loss during incubation, and plot-level averages were calculated for the six sub-replicate ingrowth cores of each plot. The content of the six sub-replicate cores of each substrate was then pooled, homogenized, ground and element concentrations measured, as described in Section 2.4.

2.4 | Chemical analyses

Phosphorus, N and C concentrations in the organic substrates before and after the incubation, and in the soil where they were incubated (see Section 2.2), were measured by mass spectrometry and element analysis (DeltaV; Thermo Fisher Scientific), whereas P concentrations were measured spectrophotometrically (Auto Analyzer III Spectrophotometer; Omniprocess) after digesting the samples in 8% H_2SO_4 (Twine & Williams, 1971).

Fungal biomass in the mineral soil ingrowth cores with and without apatite was measured as the concentration of the fungi-specific biomarker ergosterol (Nylund & Wallander, 1992; Yuan et al., 2008). Ergosterol was extracted as per Bahr et al. (2013) from 5 g of the freeze-dried and homogenized sand by vigorously shaking the sample in 2.7 ml MeOH (analytic grade 99.8%) for 3 min (Heidolph Multi Reax multivortex maximum speed). Extracts were then cleaned by centrifugation (Eppendorf Centrifuge 5810) for 5 min at 3,000 rpm to remove heavy particles from the extract and 0.9 ml of the supernatant transferred to opaque 1.5 ml plastic tubes (brown polypropylene SafeSeal, Sarstedt) and centrifuged at 13,000 rpm for 3 min to remove remaining particles. Of the supernatant, 600 µl was transferred to new tubes and evaporated and resuspended in a smaller volume to increase the ergosterol concentration in the final extract. The 600 µl extract and their standard ladder were evaporated under vacuum on a centrifuge at 1,500 rpm to prevent oxidation of ergosterol until completely dry and re-suspended in 200 µl MeOH for a theoretical concentration increase of 3X. To maximize resuspension capacity, the extract was shaken for 10 min (Heidolph Multi Reax, full speed), sonicated for 10 min and finally shaken again for 5 min. The resuspended extracts were filtered (45-µm Teflon syringe filters; Thermo Scientific) into autosampler vials for liquid chromatographic analysis (Shimadzu prominence HPLC). 100 µl of each extract was injected in MeOH (isocratic) at a flow rate of 1.5 ml/min. The extracts were separated on a reverse-phase column (Ascentis® Express C18, 2.7 micron) and ergosterol detected with an optical-ultraviolet detector (SPD-20A UV/VIS) after 3 min.

2.5 | Data analyses

To determine whether the organic matter in the substrates was rich or poor in P and N compared to the soils they were incubated in, we started by relating the four organic substrate combinations (substrate type × P amendment) with the three N treatments. We focused this first analysis on nutrient:C ratios to facilitate comparison of needle and humus substrates differing in ash content (>30% for humus vs. litter <5%). Then, in the second analysis, we calculated net mass loss or gain, that is, whether the elements were net mobilized or immobilized in the substrates to determine if the substrates acted as sources or sinks for N and P. This measurement enabled us to determine if microbes colonizing the substrates specifically extracted P or N to a higher degree than respiring and extracting C, that is, if the N treatments led to a greater decrease in P:C than N:C ratio, which would indicate that organic P is mobilized more rapidly due to N enrichment. We calculated this as the difference in initial mass of the substrate multiplied with its element concentration and the final mass multiplied with the final element concentration and the mass change for each element and finally expressed it as a percentage of the initial mass of the specific element. Thus, a value below zero indicates net mobilization of that element during the incubation, whereas a value above indicates net immobilization (Manzoni et al., 2010).

We set up linear mixed-effects models (ANOVA) to test whether N addition and substrate P content had an impact of the mass change of P, N and C in the organic substrates, or on the ergosterol and organic matter content in the mineral substrates. Nitrogen treatment (fixed factor, 2 *df*) was defined as a main plot factor within block (random factor, $n = 12$), and P amendment of the ingrowth substrates was defined as a sub-plot factor within each main plot (fixed factor,

1 *df*). The ergosterol data were log-transformed before statistical testing to meet the homoscedasticity assumption. All analyses were done in SPSS (IBM Corp., 2019).

Data are available from the Swedish University of Agricultural Sciences (SLU) Safe Deposit (https://www.safedeposit.se/projects/261).

3 | RESULTS

The N addition treatments increased the humus soil layer N:C ratio by +21%, from 0.0260 (C:N = 38) in the control treatment to 0.0315 (C:N = 31.7) in the 50N treatment ($p < 0.001$), whereas the P:C ratio was on average 0.0016 (C:P = 625), and did not differ significantly across the N treatments ($p = 0.167$; Figure 1). In comparison with the humus soil layer where the substrates were incubated, the needles had a lower N:C ratio than any of the N addition treatments, whereas the humus had a higher N:C ratio (Figure 1). The P:C ratios were close to those of the humus soil layer for both the low P needles and humus, whereas both substrates with high P had highly elevated P:C ratios (Figure 1). The needle and humus substrates, therefore, provided litter and humus that was highly enriched in P (P:C +93% and +90%, respectively) while they differed relatively little in N content (N:C −9.9% and −9.3% respectively; Table 1; Figure 1). This first analysis, therefore, revealed that the needle and humus provided a broad range of N and P concentrations relative to the soils in which they were incubated, and thus provided N-poor (needles) and N-rich (humus) substrates with a P content that was either similar to the surrounding soil (low P) or highly enriched (high P; Figure 1).

During the incubation of the P poor and enriched needles and humus, N:C and P:C ratios of both substrates generally converged with element ratios of the soil where they were incubated (Figure 1). The N:C ratio of the needles, which were more N poor relative to the soil, increased, whereas the N:C ratio of humus substrates, which was more N rich, decreased (Figure 1; Table S1). Similarly, the P:C ratios decreased in all substrates with a higher P:C ratio than the soil in which they were incubated, which it was for both of the P-enriched substrates as well as the low P humus, whereas the P:C ratio increased in the low P needles that had an initial P:C ratio lower than in the surrounding soil (Figure 1; Table S1).

Our second analysis, which focused on the net movement of elements into or out of the ingrowth cores, revealed that P was mobilized from both needle and humus, with a larger fraction of the initial P mobilized from substrates with high P content (Figure 2; Table 2). Nitrogen addition rate, on the other hand, did not impact the mobilization of P in either needle or humus substrates (Figure 2; Table 2). In contrast, N addition consistently increased N immobilization in all substrate and P amendment treatments. Specifically, substrates incubated at higher N addition rates also had higher final N mass by the end of the incubation indicating a net N immobilization in all substrates except for needles incubated in the 0N treatment (Figure 2; Table 2). The C always decreased in the needle substrate at all N addition rates and accumulated in the humus. Thus, the net movement of N and C changed consistently during the incubation in response

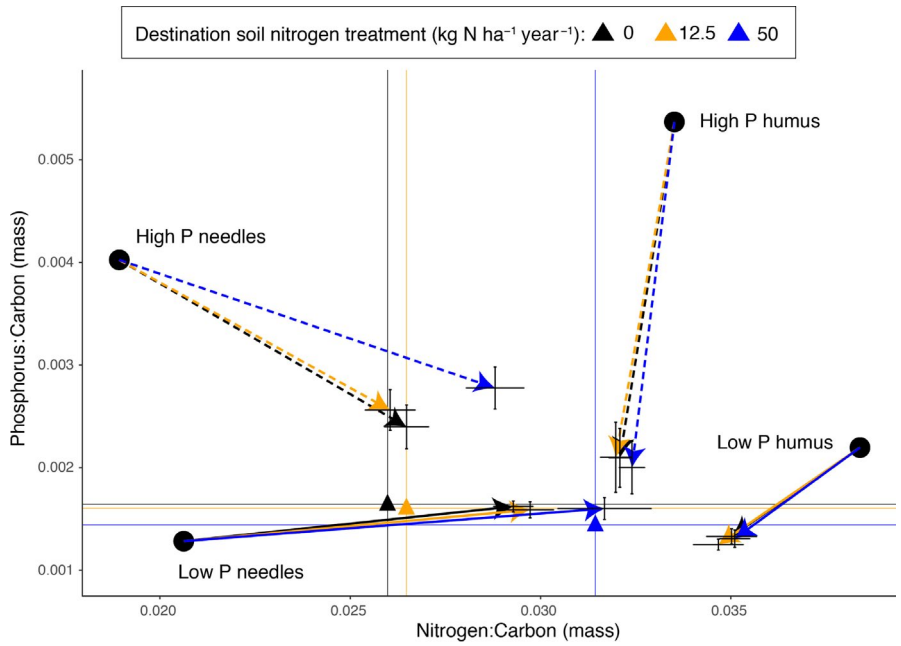

FIGURE 1 Overview of nitrogen and phosphorus to carbon content for phosphorus amended (dashed lines) and phosphorus un-amended (solid lines) needle and humus before (black dot) and after a 1-year incubation in one of three nitrogen treatments (point of arrow). Solid vertical and horizontal lines indicate the ambient soil nitrogen to carbon and phosphorus to carbon ratio at each of the three levels of nitrogen addition the substrates were incubated in. Colours refer to nitrogen addition treatments including a control receiving only ambient nitrogen deposition (2 kg N ha^{-1} $year^{-1}$, black lines), and a low nitrogen addition treatment (+12.5 kg N ha^{-1} $year^{-1}$, yellow lines), and a high nitrogen addition treatment (+50 kg N ha^{-1} $year^{-1}$, blue lines), which had been applied annually for 20 years at the time of the incubation. All values are mass element ratios and error bars denote 95% confidence intervals ($n = 12$)

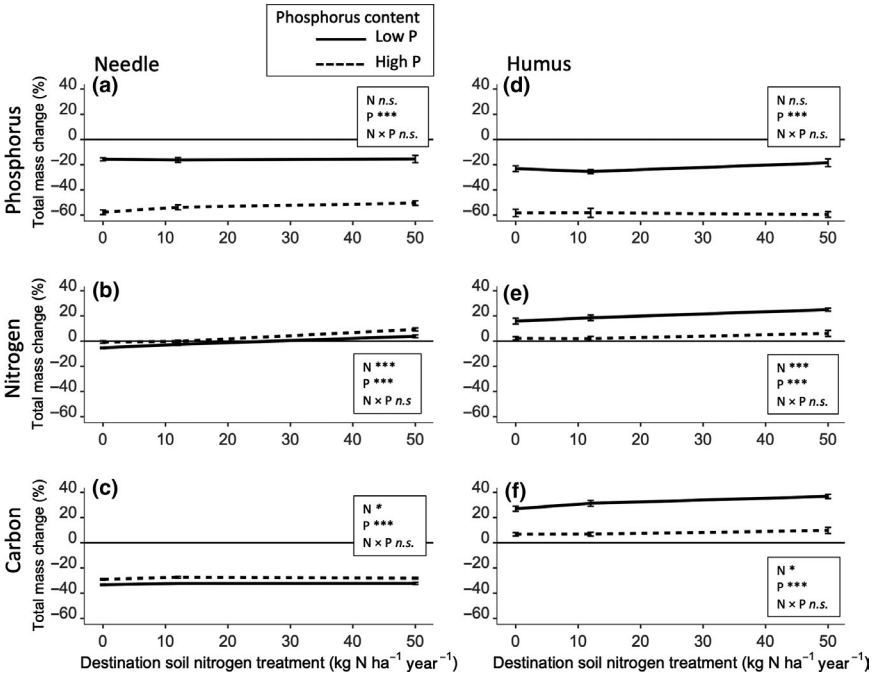

FIGURE 2 Percentage change in mass of phosphorus (a, d), nitrogen (b, e) and carbon (c, e) in needles (left) and humus (right) with and without elevated phosphorus content after a 1-year incubation at three levels of nitrogen treatments including a control, receiving nitrogen only at the ambient deposition rate (2 kg N ha^{-1} $year^{-1}$) and two treatments where nitrogen has been applied annually for 20 years (12.5 and 50 kg N ha^{-1} $year^{-1}$). Values are means of 12 replicate plots and error bars represent standard error of the mean (which may be too small in some panels to be visible). Inset boxes show the result of a mixed effect model test for direct and interactive effects of nitrogen addition (N) and phosphorus amendment (P) on mass loss of each element (Table 2) where $^{***}p < 0.001$, $^{*}p < 0.05$, n.s. if $p > 0.05$

TABLE 2 The direct and interactive effects of plot-level nitrogen (N) addition and substrate phosphorus (P) amendment on microbial decomposition and growth. Nitrogen treatments were a control receiving N only at the ambient deposition rate (2 kg N ha^{-1} $year^{-1}$), and two treatments where N has been applied annually for 20 years (12.5 and 50 kg N ha^{-1} $year^{-1}$) and substrate P amendment according to Table 1. Decomposition targeting P, N and carbon (C) were measured as the change in mass of each element in needles and humus. Growth was measured as the accrual of the fungal biomarker ergosterol and organic matter in ingrowth cores filled with organic-matter-free sand. All substrates were incubated for 1 year in the topsoil in each N treatment. The F and p values are derived from mixed-effects models ($n = 12$). The ergosterol data were log-transformed to meet the assumption of homoscedasticity. Significant ($p < 0.05$) effects are highlighted in bold

	Nitrogen addition (N)		Phosphorus amendment (P)		N × P	
Substrate	***F*-value**	***p*-value**	***F*-value**	***p*-value**	***F*-value**	***p*-value**
Needle						
Phosphorus	1.85	0.167	568	**0.001**	1.67	0.198
Nitrogen	42.2	**0.001**	56.8	**0.001**	2.95	0.297
Carbon	3.24	**0.010**	301	**0.001**	0.88	0.580
Humus						
Phosphorus	0.53	0.581	283	**0.001**	1.27	0.302
Nitrogen	6.39	**0.001**	196	**0.001**	1.65	0.297
Carbon	5.53	**0.011**	324	**0.001**	2.21	0.161
Sand						
Ergosterol	4.70	**0.02**	9.79	**0.004**	1.42	0.257
Organic matter	3.23	0.085	1.41	0.608	1.28	0.817

Note: The within-group degrees of freedom were 1 for P amendment (P), and 2 for nitrogen (N) addition and the N × P interactions. The error degrees of freedom were 66.

to N additions. Phosphorus enrichment also affected the movement of N and C, but in the opposite direction for needles and humus (Figure 2). Specifically, humus gained much less N and C during the incubation if it was amended with P, whereas needles consistently had slightly more N and C by the end of the incubation if they were amended with P. Despite clear direct effects of P amendment on mass change in all measured elements (Table 2), we did not find any interactive effects between N addition rate and P amendment on mass change in any of the elements ($p > 0.161$ for all interaction terms, Table 2).

In the mineral soil, both N addition and apatite P amendment had consistent effects on ergosterol concentrations (Figure 3; Table 2). Nitrogen addition increased the ergosterol concentrations significantly from 0.17 μg/g sand in the 0N treatment to 0.35 μg/g sand in the 50N treatment, and P amendment significantly ($p = 0.02$) increased ergosterol concentrations (Table S1). We did not find any

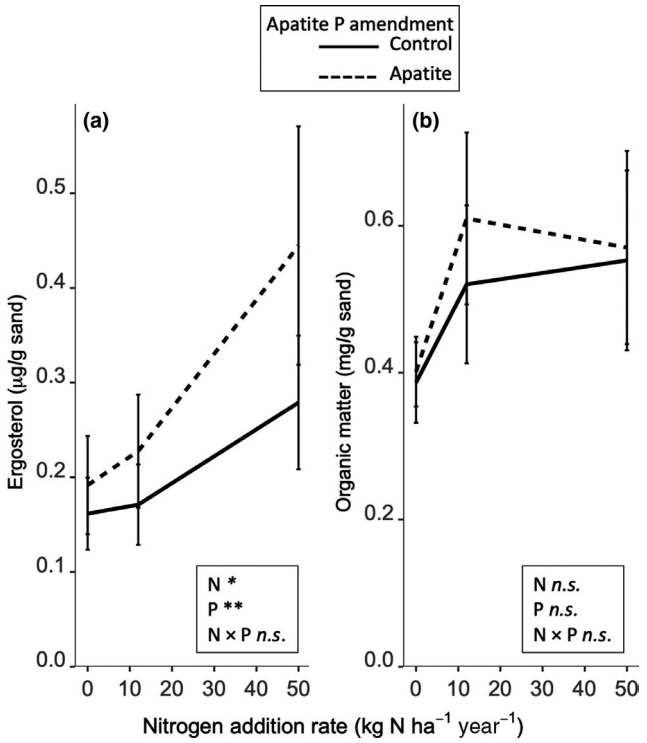

FIGURE 3 Production of fungal mycelium, measured with the biomarker ergosterol (a) and accumulation of organic matter (b) in ingrowth cores filled with initially organic-matter-free quartz sand with (dashed) and without (solid) amendment with the phosphorus-containing mineral apatite after a 1-year incubation in one of three nitrogen treatments. Nitrogen treatments are a control, receiving nitrogen at the ambient deposition rate (2 kg N ha^{-1} $year^{-1}$), and two treatments where nitrogen has been applied annually for 20 years simulating nitrogen deposition (12.5 kg N ha^{-1} $year^{-1}$) and a high nitrogen treatment (50 kg N ha^{-1} $year^{-1}$) serving as a reference point to other nitrogen addition experiments. Values are means and bars represents standard error of the mean ($n = 12$). Inset boxes show the result of a mixed effect model test for direct and interactive effect of nitrogen addition (N) and phosphorus amendment (P) on each response variable (Table 2) where $^{**}p < 0.01$, $^{*}p < 0.05$, n.s. if $p > 0.05$

significant effect of N addition or P amendment on the accrual of organic matter, and we did not find any support for a stronger positive effect of apatite at higher N addition rates for either ergosterol or organic matter (Table 2).

4 | DISCUSSION

The main aim of this study was to test whether N deposition in northern coniferous forests stimulates microbial mobilization of P and explore its impact on the soil C balance. We used a long-term experiment where N has been added at both low and high rates for two decades and measured P mobilization from needle and humus with different levels of P content, and fungal exploration of mineral P sources.

In contrast to our first hypothesis that N addition would enhance P demand and mobilization, we found that P was mobilized at a similar rate at all levels of N addition from both needles and humus, both with and without P amendment (Figure 2a,d; Table 2). Similarly, we found that apatite P amendment stimulated fungal ingrowth (i.e. ergosterol), but that the stimulating effect was similar for all N addition treatments (Figure 3; Table 2). Several experiments have demonstrated that apatite amendment enhances EMF growth, and that the stimulating effect diminishes after P additions, particularly when the foliage P levels are initially low (Almeida et al., 2018; Bahr et al., 2013; Hagerberg et al., 2003). The higher fungal ingrowth in the apatite amended ingrowth cores support the supposition that EMF plays a role in apatite weathering (Smits et al., 2012), but the lack of interaction with N addition implies that N enrichment has relatively minor impact on P acquisition from these minerals (Figure 3; Table 2). Together with the absence of direct effects of N, and interactive effects with P amendment on the mass loss of P in the organic substrates (Figure 2), our result shows that N deposition in a typical northern coniferous forest has little impact on microbial P mobilization.

The mobilization of P from organic substrates in our experiment, that is, 16 and 22% mass loss of the low P needles and humus within 1 year, respectively, was very high compared to N, which in contrast, were immobilized or stayed constant during the incubation (Figure 2; Table S1). Similar rates of P mobilization were recorded in a recent study in a nearby (<3 km) Scots pine forest where as much as 13% of the P was lost during a 1-year incubation in the absence of tree roots, compared to 17% in the presence of tree roots (Maaroufi et al., 2019), indicating that tree roots and their associated EMF play an important but not singular role in P mobilization. These effects may be unique to EMF dominated systems, where organic P is a major source of P, compared to systems dominated by arbuscular mycorrhiza (AM), as EMF to a higher degree use phosphatases to break ester bonds and release organic P, compared to AM fungi (Rosling et al., 2016). Similar rates were also observed by Bending and Read (1995) measuring P mobilization in humus colonized by different species of EMF fungi. They found that 22% of the P was lost after colonization by *Suillus bovinus*, whereas another species, *Thelephora terrestris*, did not affect P, indicating that the mobilization of P depends not only on the presence and absence of tree roots and EMF but may also be EMF species-specific. These differences may reflect differences in exudation of organic acids, where, for example, *Suillus* sp. produces high amounts of oxalic acids, whereas *Thelephora* sp. appears to lack these capabilities (Hobbie et al., 2009; Mahmood et al., 2001). We note that the P:C ratios of the humus layer in our experiment (Figure 1) are in the lower end of P:C ratios reported for similar forests, for example, by Wardle et al. (2016) and Vincent et al. (2013), indicating that our forest is not any less likely to be P limited compared to other similar forests in the region. Thus, our results suggest that the mobilization of P in the soil is a relatively rapid process even in the absence of N enrichment (Figure 2), which may reduce the likelihood of P limitations developing.

Second, we hypothesized that the acquisition of P would affect soil C. Specifically, we expected that the P amended needles and humus would decompose faster than the low P substrates, with the difference being greatest in N enriched plots; and further, that there

would be more fungal ingrowth and necromass accrual in mineral substrates amended with apatite P, especially in N-enriched plots. We found that P amended needles lost slightly less C than low-P needles (Figure 2c), which contradicts our hypothesis that microbial P mobilization would be associated with a loss of C. For humus, we found a net gain of C during the incubation, and consistent with our hypothesis, the P amended humus had less C by the end of the incubation than the low-P humus (Figure 2f). Our results show that the mobilization of P from needles and humus can occur without degradation of the surrounding C matrix, in contrast to what has been reported for the mobilization of N (i.e. via priming and nutrient mining; Craine et al., 2007; Kuyper, 2017; Talbot et al., 2008), and in contrast to suggestions that organic acids are used to destabilize soil organic matter to liberate P from supramolecular aggregates (Clarholm et al., 2015). For the sand ingrowth cores, we found that the accrual of organic matter (Figure 3b) tended to follow a similar pattern as the fungal biomarker ergosterol in response to N addition ($p = 0.085$) and that the presence of apatite enhanced fungal ingrowth (Figure 3a). Thus, our results indicate that the acquisition of organic P in needles and of apatite in the mineral soil increases the amount of C in both of these substrate types, regardless of the N environment where they are placed.

For the humus substrate, we observed a net C gain (Figure 2f), which is somewhat unusual in decomposition studies. However, humus has not been used as often as needles in decomposition studies, and previous studies using humus as a decomposition substrate have shown it is very resistant to decomposition, with mass loss typically below 10% per year (Bödeker et al., 2016; Maaroufi et al., 2019). In addition to the recalcitrance of the humus in general, the specific humus we used had a relatively high nutrient content compared to the surrounding soil matrix (Table 1; Figure 1), which may have stimulated fungal ingrowth causing this substantial C accrual. A similar degree of ingrowth was shown by Wallander et al. (2011), where sand filled ingrowth mesh bags amended with 1% maize compost gained as much as 1,400 kg C/ha of EMF origin over a 3-year incubation, and that between 15% and 30% of the C in the mesh bags at the end of the incubation were of EMF origin. Thus, the net accumulation of C in our humus ingrowth cores supports the suggestion by Clemmensen et al. (2013) that EMF contributes substantially to SOM formation in boreal forest soils.

Consistent with the positive impact of N addition on soil C accumulation recorded at this experimental site (Maaroufi et al., 2015), and in several other studies (Forsmark et al., 2020; Hyvönen et al., 2008; Janssens et al., 2010), we found that the addition of N enhanced C accumulation in both of the organic substrates (Figure 2) and tended ($p = 0.085$) to increase in the sand ingrowth cores (Figure 3). Nitrogen followed a similar trajectory as the C in both substrates, as expected from the tightly constrained stoichiometry of C and N in microbial biomass (Cleveland & Liptzin, 2007; Zhang & Elser, 2017). The similarities between the mass change of these elements provide further evidence that microbial ingrowth was likely the major driver of mass change of these elements in both needles and humus. In the mineral soil, we found that both N addition and apatite P amendments increased fungal growth. Thus, our findings are consistent with microbial growth and deposition of necromass as a driver of C accumulation (Kuzyakov, 2010; Liang et al., 2017), and that this mechanism may contribute to increasing soil C stocks in N-enriched forests (Cotrufo et al., 2013; de Vries et al., 2014; Pregitzer et al., 2008). However, as we do not find any support that N enrichment enhances P acquisition either in the organic or mineral soil (i.e. there were no interactive effects of N addition and P amendment (Figure 3; Table 2), our results imply that the acquisition of P as such is not a major mechanism driving soil C stock changes in N-enriched northern coniferous forests, relative to the direct effects of N itself.

The main finding of this study is that soil P mobilization was not enhanced by long-term N enrichment, indicated by the lack of main N effects and N by P interactions on P mobilization, which contradicts the view that N deposition in northern coniferous forests induces P limitations. In our study system, the tree growth response peaked in the 7th year of N addition and has since then declined to nearly the same level as in the control plots (From et al., 2016), indicating that the main growth limitation has shifted from N towards some other factor within one decade. A recent study from our experimental study system showed a downward trend in needle P content in the N addition plots (Figure S1; Palmqvist et al., 2020), to below levels considered to indicate P deficiency (Thelin et al., 1998). Significant shifts in P pools have also developed, most notably an increase in P stocks in the topsoil, mainly due to higher organic P content (Palmqvist et al., 2020). While these results indicate that N addition has affected some P pools and fluxes, recent profiling of enzyme activities showed that N addition had no effect on phosphatase activity, whereas both the oxidation of soil organic matter and acquisition of organic N have decreased (Forsmark, 2020). Phosphatase activity may be enhanced by short-term N addition due to N limitations on enzyme production (Allison et al., 2011), whereas the effect may diminish in the long term (Chen et al., 2020). Accordingly, shifts in P fluxes may have occurred during the initial phases of the N addition treatment, and this P capital may be subsequently conserved in the plant, soil and microbial biomass. Moreover, soil respiration and fungal biomass have remained low in the N treatments (Maaroufi et al., 2015), indicating that the addition of N has led to a persistent reduction in the allocation of C belowground, rather than to resumed belowground C allocation to stimulate P acquisition (Almeida et al., 2018; Wallander & Nylund, 1992). These previous studies and the result presented here indicate that below-ground P cycling is relatively unaffected by long-term N enrichment, and point towards factors other than P as limiting forest growth response to N enrichment, such as competition for light and water.

Understanding how various factors limit the capture and storage of C in forests in the northern hemisphere is critical to predicting their contribution to the global greenhouse-gas balance in the future (Ciais et al., 2019; Wieder et al., 2015). In contrast to plant-available forms of N that can be replenished from the atmosphere by natural and anthropogenic processes, the supply of P ultimately depends on the supply from weathering and mining of primary minerals, which could become increasingly limited in the future (Peñuelas et al., 2013) and may potentially constrain the positive feedback between elevated CO_2 and C capture (Jonard et al., 2015; Terrer et al., 2019). Using an N addition

experiment in a coniferous forest dominated by EMF on a relatively young soil, we do not find any evidence that 20 years of annual N enrichment enhances the mobilization of P from organic or mineral soil layers. Instead, we find a rapid baseline P mobilization, which may be enough to support the higher demand for P as N limitation is alleviated (Lang et al., 2016). These results are important because most previous work has been done on older soils in deciduous temperate or tropical forests where the soils have been subject to weathering and P depletion and where AM associations are more common (Braun et al., 2010; Prietzel & Stetter, 2010; Rosling et al., 2016). Our data instead suggest that P is likely to be sufficiently supplied by internal recycling in the relatively young and P-rich soils found in northern latitudes such as where our experiment is located; and further, that N deposition is unlikely to lead to widespread P limitations on C capture in these forests within the time scale of decades. Our results further show that N enrichment continues to increase soil C storage by decreasing decomposition and increasing the input of C to the mineral soil, and that nutrient mining targeting P is unlikely to greatly impact this trajectory of C accumulation.

ACKNOWLEDGEMENTS

The field experiment and support for our measurements were provided by the Swedish infrastructure for terrestrial ecosystem sciences (SITES). We thank Clydecia Spitzer and Paul Kardol for comments on an early draft. The project was funded by the Swedish Research Council for Environment, Agricultural Sciences, and Spatial Planning (FORMAS) and Trees and Crops for the Future (TC4F), and running costs were covered by the Royal Swedish Academy of Agriculture and Silviculture (KSLA).

AUTHORS' CONTRIBUTIONS

A.N. designed the original N addition experiment and B.F., H.W., A.N. and M.J.G. conceived the ideas and designed methodology for the current experiment; B.F. collected the data; B.F., H.W. and M.J.G. analysed the data; B.F. and M.J.G. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data from this article are available at https://www.safedeposit.se/projects/261

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Forsmark B, Wallander H, Nordin A, Gundale MJ. Long-term nitrogen enrichment does not increase microbial phosphorus mobilization in a northern coniferous forest. *Funct Ecol*. 2021;35:277–287. https://doi.org/10.1111/1365-2435.13701