

Decoupled stoichiometric, isotopic, and fungal responses of an ectomycorrhizal black spruce forest to nitrogen and phosphorus additions



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ABSTRACT

Many northern forests are limited by nitrogen (N) availability, slight changes in which can have profound effects on ecosystem function and the activity of ectomycorrhizal (EcM) fungi. Increasing N and phosphorus (P) availability, an analog to accelerated soil organic matter decomposition in a warming climate, could decrease plant dependency on EcM fungi and increase plant productivity as a result of greater carbon use efficiency. However, the impact of altered N and P availability on the growth and activity of EcM fungi in boreal forests remains poorly understood despite recognition of their importance to host plant nutrition and soil carbon sequestration. To address such uncertainty we examined above and belowground ecosystem properties in a boreal black spruce forest following five years of factorial N and P additions. By combining detailed soil, fungal, and plant $\delta^{15}\text{N}$ measurements with *in situ* metrics of fungal biomass, growth, and activity, we found both expected and unexpected patterns. Soil nitrate isotope values became ¹⁵N enriched in response to both N and P additions; fungal biomass was repressed by N yet both biomass and growth were stimulated by P; and, black spruce dependency on EcM derived N increased slightly when N and P were added alone yet significantly declined when added in combination. These findings contradict predictions that N fertilization would increase plant P demands and P fertilization would further exacerbate plant N demands. As a result, the prediction that EcM fungi predictably respond to plant N limitation was not supported. These findings highlight P as an under appreciated mediator of the activity of denitrifying bacteria, EcM fungi, and the dynamics of N cycles in boreal forests. Further, use of $\delta^{15}\text{N}$ values from bulk soils, plants, and fungi to understand how EcM systems respond to changing nutrient availabilities will often require additional ecological information.

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1. Introduction

Increased terrestrial N availability is a global issue with impacts extending beyond industrialized regions (Matson et al., 2002; Galloway et al., 2008). In most N-limited boreal forests anthropogenic deposition is less pronounced, but landscape modification and accelerated decomposition resulting from climatic warming can

increase *in situ* N mineralization and profoundly alter above and belowground ecosystem responses (Nadelhoffer et al., 1991; Hyvönen et al., 2007; Allison and Treseder, 2008; Aerts, 2010). Boreal ecosystems subjected to increased N availability may respond with greater carbon (C) fixation (Högberg et al., 2003), altered C and nutrient allocation patterns (Nadelhoffer, 2000; Mack et al., 2004; Vogel et al., 2008), and shifts in plant and fungal diversity, biomass, and elemental stoichiometry with uncertain functional consequences (Shaver et al., 2001; Nordin et al., 2005; Clemmensen et al., 2006; Treseder, 2008; Janssens et al., 2010; Wardle and Lindahl, 2014). Understanding how impacts of altered N availabilities will influence the function of boreal ecosystems requires assessment of multiple N cycling processes integrated through time. As such, stable isotope ratios of N (¹⁵N:¹⁴N represented as $\delta^{15}\text{N}$), as key integrative signals of the N cycle (Robinson, 2001), appear promising.

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Measurements of soil and plant $\delta^{15}\text{N}$ have been used to detect variations in N cycling due to climate (Amundson et al., 2003), disturbance (Pardo et al., 2002), reforestation (Davidson et al., 2007), deposition (Elliot et al., 2007), and the activity of ectomycorrhizal (EcM) fungi. This is due to key fractionation steps related to N loss-to-production ratios or shifts in the source or demand for N by host plants (Hobbie and Högberg, 2012; Mayor et al., 2015). A major limitation to interpreting plant $\delta^{15}\text{N}$ values as an indicator of altered N cycling arises from uncertainty in $\delta^{15}\text{N}$ values among forms of available N because bulk soil $\delta^{15}\text{N}$ is commonly the only pool measured (Craine et al., 2009; Pardo and Nadelhoffer, 2010). Measuring $\delta^{15}\text{N}$ of individual soil N forms permits assignment of plant $\delta^{15}\text{N}$ values as tracers of available N after accounting for intermediate sources of biological fractionation (Pardo et al., 2006; Templer et al., 2007; Kahmen et al., 2008; Mayor et al., 2012). For instance, once baseline ecosystem $\delta^{15}\text{N}$ values are established, both the source and proportional amount of EcM derived N can be better constrained (Hobbie and Hobbie, 2008; Yano et al., 2010) and the individual and interactive effects of N and P fertilization independently assessed (Mayor et al., 2014).

In order to assess the alteration of $\delta^{15}\text{N}$ sources and the activity of EcM fungi, we conducted a five year factorial N and P addition experiment in a mature black spruce forest of central Alaska. By combining detailed measurements of soil, plant, and fungal $\delta^{15}\text{N}$ with estimates of fungal biomass, growth, and activity, we sought to evaluate several hypothesized relationships governing plant and fungal nutrient limitations. By explicitly targeting the response of EcM fungi to altered soil fertility we aimed to better inform global change predictions regarding plant–soil functional interactions (Johnson et al., 2013; Deckmyn et al., 2014) and to elucidate understudied interactions with P availability in a putatively N-limited ecosystem.

Given that productivity of high latitude black spruce forests are considered N-limited, and N limitation of host plants is closely tied to belowground C allocation to EcM fungi (Högberg et al., 2010), we constructed the following hypotheses regarding expected responses of black spruce and associated EcM fungi to factorial N and P fertilization: (H1) N fertilization would increase black spruce [N] and ^{15}N content due to relief of growth limitation and uptake of ^{15}N enriched mineral N resulting from induced fractionation under N saturated conditions (Pardo et al., 2006); and, this would lead to (H2) a reduction in estimated plant dependency on EcM derived N. As such, lowered delivery of fungal N would lead to (H3) reduced relative $\delta^{15}\text{N}$ differences between EcM sporocarps and black spruce ($\delta^{15}\text{N}_{\text{fungi-plant}}$) because of less ^{15}N -retention by associated EcM fungi and less transfer of ^{14}N to host trees (Hobbie and Hobbie, 2008). Furthermore, we hypothesized that relief of tree N limitation through N fertilization would (H4) reduce fungal biomass and mycelial growth due to a reduction of belowground C allocation. In contrast, we expected P addition would only influence ecosystem properties when added with N due to an induced N/P co-limitation. As such, +N + P additions would: (H5) further decrease plant dependency on EcM derived N resulting in (H6) even smaller $\delta^{15}\text{N}_{\text{fungi-plant}}$ magnitudes relative to the addition of N, and (H7) the largest reduction in standing biomass and mycelial growth of EcM fungi.

2. Methods

2.1. Site description and experimental design

Boreal forest is the second largest terrestrial biome in the world and black spruce (*Picea mariana* [Mill.] BSP) dominated forest is the most abundant forest type in boreal North America (Vioreck and Johnston, 1990). Its success in the landscape is attributed to

extreme freezing tolerance, the ability to grow in shallow permafrost soils with impeded drainage, as well as the ability to grow on well-drained and more productive upland sites (Chapin et al., 2006).

The experimental site is located approximately 15 km south of Delta Junction AK, and consists of 16 plots arrayed in a factorial N \times P design consisting of four blocks of four $10 \times 10 \text{ m}^2$. Each treatment plot was fertilized annually in the early spring for 5 years prior to the 2007 growing season when sampling for this study was conducted. In 2002, each plot received single broadcast doses of pelletized $\text{NH}_4^+\text{NO}_3^-$ (+N), ortho- PO_4^- (+P), both together (+N + P), or none, at an initial level of 200 kg N and 100 kg P ha^{-1} in year 1 and 100 kg N and 50 kg P $\text{ha}^{-1} \text{ yr}^{-1}$ in subsequent years. Although these amounts of added N and P are unlikely to occur under natural conditions, they are of comparable magnitude to other boreal forest fertilization experiments that seek to relieve nutrient limitations (Högberg et al., 2006).

The forest is a mature (~80 years old) dry nonacidic black spruce forest (Hollingsworth et al., 2006) formed under low rainfall (~300 mm yr^{-1} MAP) and cold conditions ($-2 \text{ }^\circ\text{C}$ MAT) with a relatively shallow organic layer (6.3 cm O horizon). Soils are gelsols dominated by silt loams as described elsewhere (Treseder et al., 2004b). The forest canopy is dominated by black spruce with minor components of *Populus tremuloides* in two of the blocks. The understory vegetation consists of minor contributions from *Betula glandulosa*, *Salix* spp., *Vaccinium vitis-idaea*, *V. uliginosum*, and *Rhododendron groenlandicum* shrubs, with a 30–50% ground cover comprised of feather moss (mainly *Pleurozium schreberi* or *Hylocomium splendens*) and lichen (*Cladina*, *Cladonia*, and *Cetraria* spp.) (Treseder et al., 2004b; Mack et al., 2008).

2.2. Plant and fungal sampling

Needles from five, terminal, full sun branches were collected from the tops of five *P. mariana* trees in the canopy of each plot at peak of needle expansion, August 29–30, 2007, dried at $60 \text{ }^\circ\text{C}$, and composited by plot. Fine roots (<2 mm) were carefully excavated from three of these trees in each plot by tracing from trunk to terminal roots within the upper 6 cm of soil, composited by plot, and refrigerated until processing. The thin layer of secondary root tissue was carefully removed to prevent potential inclusion of fungal biomass in subsequent isotopic analyses although a minor component of EcM hyphae could be present as a Hartig net in the remaining root cortex (Högberg et al., 1996). Needle and root tissue were dried at $60 \text{ }^\circ\text{C}$ for 24 h, ground to a fine powder, and analyzed on a ThermoFinnigan continuous flow isotope ratio mass spectrometer coupled to a Costech C/N elemental analyzer at the University of Florida. Stable isotope abundances are reported as $\delta^{15}\text{N} = (\text{R}_{\text{sample}}/\text{R}_{\text{standard}} - 1) \times 1000$, where $\text{R} = ^{15}\text{N}/^{14}\text{N}$ and refers to the ratio of the sample and reference standard of atmospheric N_2 . Run standard error rates were typically less than 0.2%. Foliar P concentration (mg g^{-1}) was determined by combustion (1 h at $550 \text{ }^\circ\text{C}$) and dissolution of the ash in 10 mL of 1 M H_2SO_4 shaken for 16 h, filtered, and analyzed by automated colorimetry. Tree cores were obtained prior to and in year five of the experiment from all mature trees in each plot and growth rings quantified using WinDendro software. Growth responses were calculated as the change in ring width prior to and in the fifth year of fertilization.

Sporocarps were opportunistically collected during the 2005–07 growing seasons and as a result individual sporocarps may have experienced 3–5 years of nutrient enrichment. Sample sizes varied from 12 to 29 across treatments (individual plots represented by 3–11 sporocarps) with the fewest collected from the +N + P and +N treatments ($N = 12$ and 13 , respectively), and the most in the +P and control treatments ($N = 22$ and 29 , respectively).

Determination of the EcM nutritional habit for each fungal species was confirmed using a discriminant model of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values trained from a global dataset containing >800 identified sporocarps (Mayor et al., 2009). Phospholipid fatty acid analyses (PLFA) were performed on three 1–5 g (wet weight) frozen soil subsamples and standardized concentrations used as a metric of fungal biomass. This process involved an initial: lipid extraction, fractionation, and successive elution; followed by conversion of the methanol fraction into free methyl esters by mild alkaline methanolysis; and, analyses on a gas chromatograph with a flame ionization detector and a 50 m HP5 capillary column (Frostegård et al., 1993). The content of the specific PLFA 18:2 ω 6,9, standardized against mol% of a standard (PLFA 19:0), was regarded as a proxy of total fungal biomass (Frostegård and Bååth, 1996); a biomarker largely comprised of EcM-forming fungi in boreal spruce forest (Taylor et al., 2010).

In addition to standing fungal biomass, seven mycelial ingrowth mesh bags (52 μm mesh containing 7 cm^3 of acid washed quartz sand) were deployed throughout the 2007 growing season in each plot at the organic and mineral soil interface to measure actively growing fungal mycelia (Wallander et al., 2001). Three additional bags were inserted inside buried PVC collars to 15 cm depth in each plot to account for any saprotrophic fungal biomass; because negligible biomass was found corrective accounting was unnecessary. Ingrowth bags were removed from the field immediately prior to the first frost, refrigerated, and transported to UF where they were slowly dried, the sand suspended in water, and the degree of mycelial colonization scored under a dissecting microscope (1 = none, 2 = light and diffuse, 3 = extensive, 4 = extensive with few rhizomorphs, 5 = extensive with many rhizomorphs).

2.3. Soil nutrient sampling and $\delta^{15}\text{N}$ measurement

During the entire 2007 growing season, metrics of accumulated bioavailable $[\text{NH}_4^+]$, $[\text{NO}_3^-]$, and $[\text{PO}_4^-]$ were obtained from five field-incubated ion exchange resins bags (220 μm mesh) containing either 3 g of anion (Biorad®, AG 1-X8, #140-1421) or cation resins (Biorad®, AG 50W-X8, #142-1421) inserted into the upper 5–7 cm of soil in each plot. Resin bags were collected at the end of the growing season, carefully cleaned with deionized water, and refrigerated until extraction in an acidified salt solution (Giblin et al., 1994).

Extractable soil N forms for isotopic analyses were obtained either from the exchange resins (for mineral N) or 2 M KCl extractions of total dissolved N (TDN) made at the height of the growing season, early August 2007. In each plot, organic horizon soils from three cores were sampled to 10 cm depth and composited from within three zones of each $10 \times 10 \text{ m}^2$ plot with a volumetric corer (4.2 cm diameter). Green moss or lichens were removed from cores, depth recorded, and horizons separated. Each composited soil sample was stored on ice in the field and under refrigeration in the lab for approximately 24 h prior to salt extraction, filtration, and freezing. Concentrations of ammonium and nitrate from both KCl and resin extractions were analyzed colorimetrically as nitrate + nitrite, ammonium, and phosphate on a Lachat QuikChem 8500 (Hach Ltd., Loveland, CO, USA). Because 80% of black spruce roots are typically found in the organic horizons (Ruess et al., 2003) only the organic soils were extracted for $\delta^{15}\text{N}$ analyses. Total dissolved N and NH_4^+ extracts were oxidized with persulfate/thermodigestion and coupled to the highly sensitive denitrifier method (Sigman et al., 2001; Knapp et al., 2005) for $\delta^{15}\text{N}$ measurements as nitrate as detailed elsewhere (Mayor et al., 2012) and in the Supplementary File. The $\delta^{15}\text{N}$ value of dissolved organic N (DON) was calculated as the mass weighted difference between TDN, $\text{NH}_4^+ + \text{NO}_3^-$ and resin accumulated mineral $\delta^{15}\text{N}$ values using the following equation:

$$\delta^{15}\text{N}_{\text{DON}} = \left(\delta^{15}\text{N}_{\text{TDN}} \times [\text{TDN}] - \left(\delta^{15}\text{N}_{\text{NH}_4} - [\text{NH}_4^+] + \delta^{15}\text{N}_{\text{NO}_3} - [\text{NO}_3^-] \right) \right) / [\text{DON}] \quad (1)$$

where KCl extracts provided concentrations for all N forms and $\delta^{15}\text{N}_{\text{TDN}}$ measurements and cation and anion exchange cation and anion resins provided $\delta^{15}\text{N}_{\text{NH}_4}$ and $\delta^{15}\text{N}_{\text{NO}_3}$ values. Resin derived $\delta^{15}\text{N}$ values represent time integrated $\delta^{15}\text{N}$ measurements of the rapidly cycling low concentration ions of interest in sufficient masses necessary for the denitrifier method with no indication of isotope fractionation effects (Lehmann et al., 2001; Templer and Weathers, 2011).

2.4. Statistical analyses

Fertilizer treatment effects were analyzed using an ANOVA model testing N or P effects, their interaction, and block as main effects in accordance with the factorial design of the experiment. Parametric assumptions were assessed using Levene's test for homogeneity of variance and Shapiro–Wilk's test for normality ($\alpha = 0.05$). Response values were log transformed when necessary and fitted residuals visually assessed. Data were analyzed using JMP 11.1.1 (2013 SAS Institute).

2.5. Mass balance ^{15}N -based mixing models

The $\delta^{15}\text{N}$ value of black spruce represents the $\delta^{15}\text{N}$ value of N sources and the fractionation effects during their assimilation and transfer (Emmertson et al., 2001; Robinson, 2001). To estimate proportional contribution and pathway of N flux to black spruce across treatments we used the following $\delta^{15}\text{N}$ based mass balance mixing models developed for EcM and ericoid arctic tundra plants (Hobbie et al., 2009; Yano et al., 2010):

Three pool N source:

$$\delta^{15}\text{N}_{\text{available}} = f_{\text{DON}} \times \delta^{15}\text{N}_{\text{DON}} + f_{\text{NH}_4} \times \delta^{15}\text{N}_{\text{NH}_4} + f_{\text{NO}_3} \times \delta^{15}\text{N}_{\text{NO}_3} \quad (2)$$

$$\delta^{15}\text{N}_{\text{available}} = (1 - Tr) \times \delta^{15}\text{N}_{\text{fungi}} + Tr \times \delta^{15}\text{N}_{\text{transfer}} \quad (3)$$

Two pool plant mixture:

$$\delta^{15}\text{N}_{\text{plant}} = \delta^{15}\text{N}_{\text{available}} - \Delta_f \times (1 - Tr) \times f \quad (4)$$

Two pool fungal mixture:

$$\delta^{15}\text{N}_{\text{fungi}} = \delta^{15}\text{N}_{\text{available}} + \Delta_f \times Tr \quad (5)$$

where $\delta^{15}\text{N}_{\text{DON}}$ in Eqn. (2) is derived from a mass weighted equation based on the original 2M KCl concentration of N ions and $\delta^{15}\text{N}$ values measured from resin extracted ammonium and nitrate in Eqn. (1), as detailed elsewhere (Mayor et al., 2012, 2014). Equation (2) solves for the $\delta^{15}\text{N}$ value ($\delta^{15}\text{N}_{\text{available}}$) effectively available to plants and fungi based on proportionally weighted $\delta^{15}\text{N}$ values of the three soil N sources. In the remaining equations, Tr refers to the proportion of total fungal N that is transferred to host plants, $\delta^{15}\text{N}_{\text{transfer}}$ refers to the $\delta^{15}\text{N}$ value of the transfer compounds produced by ECM fungi, f refers to the proportion of plant N supplied by fungi, and Δ_f refers to the fractionation magnitude associated with transamination of soil N within ECM fungi during production of N transfer compounds (Hobbie and Hobbie, 2008).

We placed quantitative constraints on the source mixtures (DON, NH_4^+ , NO_3^-) and pathways (EcM vs. direct uptake) of N flux to black spruce. First, soil N mixtures were iteratively adjusted with 10% source contributions for each plot, constrained to within 0–100% of total tree N uptake. Next, fractionation magnitudes associated with the transformation of soil N to $\delta^{15}\text{N}$ -depleted transfer compounds by EcM fungi (Δ_f) were estimated at $\Delta 9 \pm 1\%$ based on laboratory and field analyses as described elsewhere (Hobbie and Hobbie, 2008). Lastly, based on extremely low $[\text{NO}_3^-]$ in Control and +P plots (e.g. <0.2% and <0.4% of TDN in C and +P treatments, respectively) we omitted $\delta^{15}\text{N}_{\text{NO}_3}$ values as a potential source. Inclusion of NO_3^- as up to 20% of source N did not change statistical findings. Similarly, $\delta^{15}\text{N}_{\text{DON}}$ was omitted as a potential N source in the +N and +N + P treatments owing to large proportional declines in DON (e.g. <13% of TDN), and highly variable $\delta^{15}\text{N}_{\text{DON}}$ values following N additions. Given that models were mathematically underdetermined by design, the results are presented as ranges of all possible soil N source mixtures in Supplementary Table S1.

3. Results

3.1. Response of soil properties

Soil organic horizon depth, bulk density, C/N, and pH were largely unaltered by nutrient additions. Only soil pH and C/N marginally increased in response to N and P additions, respectively (Table 1). As expected, soil extractable and resin accumulated mineral N (ammonium + nitrate) increased in response to N additions whereas resin accumulated phosphate increased in response to P additions (Table 1). The relative proportions of extractable TDN (DON + mineral N) were also significantly altered by N additions as DON comprised c. 96% of TDN in the –N treatments and mineral N comprised c. 86% of TDN in the +N treatments. This shift was due to both increases in mineral N and reductions of extractable [DON] in the +N treatments (Table 1). As a result, DON contributions in the +N treatments were inconsequential to mass balance mixing model solutions and the high proportional mineral N concentrations in five of the eight N addition plots prevented valid solutions and statistical testing of fertilizer effects on $\delta^{15}\text{N}_{\text{DON}}$ values (Tables 1 and S1). Despite five years of N additions, the $\delta^{15}\text{N}$ values of bulk soil organic matter (avg. = $0.5 \pm 0.1\%$; data not shown) and resin accumulated ammonium did not differ among treatments (Table S1). In contrast, N and P additions caused the $\delta^{15}\text{N}$ values of resin accumulated nitrate to be significantly ^{15}N enriched ($P = 0.0045$ and $P < 0.0001$, respectively). The substantial (17.9‰ relative to control) ^{15}N enrichment of soil nitrate in the +P treatment led to a significant

$\text{N} \times \text{P}$ interactive effect ($P = 0.0003$; Table S1) but no significant change in resin accumulated nitrate in the +P treatment (Table 1).

3.2. Response of black spruce foliar nutrients, foliar and root $\delta^{15}\text{N}$, and stem growth

Nitrogen additions significantly increased black spruce foliar [N] and P additions significantly increased foliar [P] ($P < 0.0001$ and $P = 0.0002$, respectively; Table 2). Nitrogen additions resulted in large increases in foliar N/P ratios ($P < 0.0001$), whereas P additions decreased foliar N/P ratios ($P = 0.0003$), and the intermediate values in the +N + P treatment led to an $\text{N} \times \text{P}$ interactive effect ($P = 0.018$; Fig. 1a). Foliar $\delta^{15}\text{N}$ values significantly increased in response to N additions ($P = 0.0012$; Fig. 1b; Table 2) but root $\delta^{15}\text{N}$ values were not significantly altered (Fig. 1b; Table 2). Phosphorus additions significantly decreased foliar [C] by c. 2% ($P = 0.0043$) and foliar $\delta^{13}\text{C}$ values exhibited an $\text{N} \times \text{P}$ interactive effect ($P = 0.034$) because N and P co-addition eliminated the minor (1‰) and marginally significant ($P = 0.087$) ^{13}C depletion seen in the +N treatment (Table 2). Black spruce basal area growth, as measured by tree ring increment change, was unaltered by N or P addition (data not shown).

3.3. Response of fungal biomass, mycelial ingrowth, and N delivery by EcM fungi

On average, EcM sporocarp $\delta^{15}\text{N}$ values were as low as 5.3‰ in the +N treatment, as high as 10.5‰ in the +N + P treatment, and intermediate in the Control and +P treatments (9.5 and 8.9‰). As a result of the +N+P enrichment there was a marginally significant overall P effect ($P = 0.056$) and an $\text{N} \times \text{P}$ interactive effect ($P = 0.021$; Table 2). The difference in fungal and foliar $\delta^{15}\text{N}$ values has been used as an indicator of fractionation associated with EcM N acquisition. The magnitude of ^{15}N differences between EcM fungi and black spruce ($\delta^{15}\text{N}_{\text{fungi-foilage}}$) was significantly reduced by N ($P = 0.0008$) and to a lesser extent P ($P = 0.044$) additions. As the +N + P treatment exhibited intermediate values there was a significant $\text{N} \times \text{P}$ interactive effect ($P = 0.015$; Fig. 1c; Table 2). Fungal biomass, as represented by the PLFA 18:2 ω 6,9 biomarker, was marginally lower in N treatments ($P = 0.052$) yet marginally greater in P treatments ($P = 0.071$; Fig. 1d, Table 2). Similarly, mycelial ingrowth was stimulated by P additions ($P = 0.028$; Fig. 1e, Table 2).

Plot-specific mass balance mixing models were used to estimate the proportion of black spruce N derived from EcM fungi (denoted as f in Eqn. 3). As a result of multiple nutrient addition effects on isotope end members (e.g. ^{15}N enrichment of soil nitrate, alteration

Table 1
Response of soil abiotic properties to five years of N and P fertilization. Nitrogen additions led to significant increases in all pools of extractable soil N. Phosphorus additions led to significant increases in soil phosphate. Bold values represent significant overall N, P, or combined $\text{N} \times \text{P}$ interactive effects. ANOVA derived F -values followed by significant P -values ($\alpha < 0.10$) in parentheses.

Soil property	C	+N	+N + P	+P	N effect	P effect	$\text{N} \times \text{P}$ effect
Organic soil depth (cm)	5.80 ± 0.52	6.31 ± 0.6	7.09 ± 1.1	5.97 ± 0.35	n.s	n.s	n.s
Bulk density (g cm^{-3})	0.16 ± 0.03	0.12 ± 0.02	0.14 ± 0.03	0.15 ± 0.01	n.s	n.s	n.s
C/N (organic soil)	25.0 ± 1.6	24.8 ± 0.94	25.4 ± 0.6	29.1 ± 1.28	n.s	4.1 (0.074)	n.s
pH (H_2O) ^a	4.75 ± 0.06	4.99 ± 0.06	4.87 ± 0.06	4.74 ± 0.08	3.9 (0.084)	n.s	n.s
KCl extractable N concentrations (org. soil)							
DON	314.0 ± 35.4	148.9 ± 10.0	135.9 ± 40.8	279.9 ± 19.7	7.7 (0.028)	n.s	n.s
NH_4^+ ^a	10.1 ± 0.9	660.0 ± 106.3	456.1 ± 67.5	8.62 ± 2.1	827.6 (<0.0001)	n.s	n.s
NO_3^-	0.79 ± 0.44	229.5 ± 78.1	169.6 ± 46.1	1.2 ± 0.4	20.9 (0.001)	n.s	n.s
Resin accumulated nutrient concentrations ($\mu\text{g g}^{-1}$ resin day ⁻¹)							
NH_4^+	1.08 ± 0.5	382.3 ± 96.7	168.1 ± 68.2	14.3 ± 8.0	32.5 (0.0003)	4.6 (0.061)	5.9 (0.038)
NO_3^-	0.08 ± 0.01	399.3 ± 130.2	463.7 ± 151.1	67.4 ± 29.2	14.4 (0.004)	n.s	n.s
PO_4^{3-} ^a	3.86 ± 1.9	3.19 ± 1.3	839.1 ± 539.7	1008.1 ± 378.6	n.s	130.9 (<0.001)	n.s

^a Statistical test performed on log-transformed variable.

Table 2

Response of black spruce foliage, EcM fungal biomass (PLFA) and hyphal ingrowth, their isotopic difference, and the modeled dependency (*f*) of black spruce proportional uptake of N to five years of N and P fertilization. Bold values correspond to significant overall N, P, and combined N × P interactive effects. ANOVA derived *F*-values followed by significant *P*-values ($\alpha < 0.10$) in parentheses.

Biotic property	C	+N	+N + P	+P	N effect	P effect	N × P effect
<i>Black spruce foliage</i>							
%C	48.0 ± 0.07	48.5 ± 0.14	47.7 ± 0.27	47.6 ± 0.16	n.s.	14.4 (0.0043)	n.s.
%N	0.77 ± 0.06	1.62 ± 0.09	1.43 ± 0.09	0.86 ± 0.06	87.5 (<0.0001)	n.s.	n.s.
%P	0.11 ± 0.004	0.10 ± 0.01	0.15 ± 0.00	0.19 ± 0.02	n.s.	34.7 (0.0002)	n.s.
δ ¹³ C (‰)	-28.5 ± 0.1	-29.3 ± 0.4	-28.4 ± 0.2	-28.6 ± 0.1	n.s.	3.7 (0.087)	6.3 (0.034)
δ ¹⁵ N (‰)	-4.4 ± 0.9	-1.7 ± 0.2	-1.1 ± 0.2	-4.5 ± 0.5	21.7 (0.0012)	n.s.	n.s.
<i>EcM fungi</i>							
δ ¹⁵ N (‰)	9.5 ± 1.1	5.3 ± 1.3	10.5 ± 1.3	8.9 ± 0.9	n.s.	4.8 (0.056)	7.8 (0.021)
PLFA 18:2ω6,9	3.66 ± 0.38	3.11 ± 0.21	3.62 ± 0.47	4.08 ± 0.22	5.0 (0.052)	4.2 (0.071)	n.s.
Hyphal ingrowth	2.14 ± 0.13	1.63 ± 0.17	2.91 ± 0.35	2.48 ± 0.44	n.s.	6.8 (0.028)	n.s.
<i>Plant–fungal interaction</i>							
δ ¹⁵ N _{fungi–foliage}	13.9 ± 0.2	6.98 ± 1.4	11.6 ± 1.3	13.4 ± 0.5	24.8 (0.0008)	5.5 (0.044)	8.9 (0.015)
EcM N in foliage ^a	0.29 ± 0.07	0.46 ± 0.13	0.14 ± 0.02	0.46 ± 0.14	n.s.	n.s.	6.1 (0.039)

^a Statistical test performed on log-transformed variable.

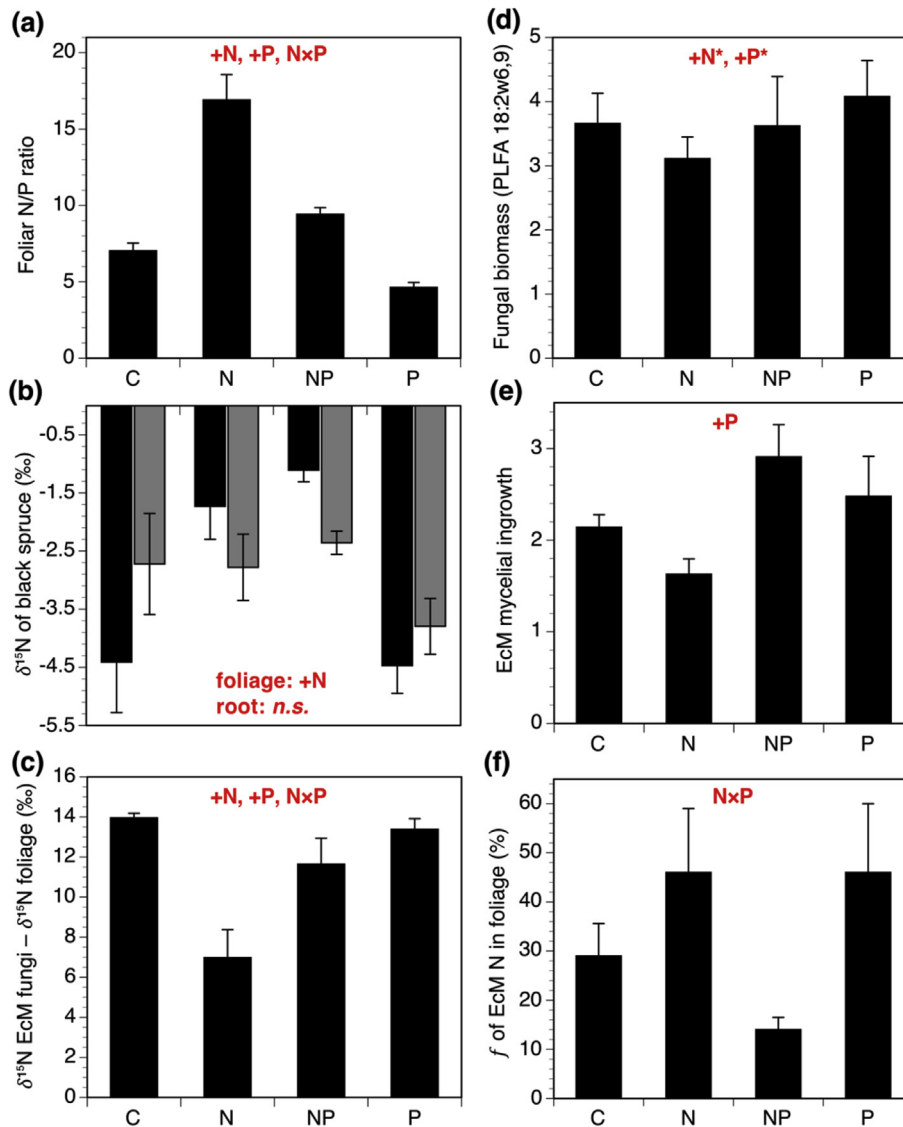


Fig. 1. Response of black spruce forest components to five years of N and P fertilization. Foliar N/P (a), foliar (black) and root (grey) δ¹⁵N values (b), δ¹⁵N differences between EcM fungi and black spruce foliage (c), fungal biomass (d), EcM mycelial ingrowth (e), and the modeled proportional (% of total foliar N) dependency (*f*) of black spruce on EcM derived N (f). Symbols represent significant N, P, or N × P interactive effects detailed in the text. * indicates a marginally significant effect ($\alpha < 0.10$).

of sporocarps $\delta^{15}\text{N}$, and ^{15}N enrichment of black spruce foliage), the amount of N derived from EcM fungi was significantly reduced in the +N + P treatment ($14 \pm 2\%$; $P = 0.039$ for N \times P interaction) relative to the average for the control ($29 \pm 7\%$; Figure 1f; Tables 2 and S1). There were also minor, yet statistically insignificant, increases in f estimates in both the +N and +P treatments ($46 \pm 13\%$). Mass balance mixing model solutions were not achievable in one of the 16 plots as available end members produced f estimates outside of valid solution space.

In order to explore controls over f estimates, regressions of this metric with potentially informative foliar ($\delta^{15}\text{N}$, N/P, and multiple enrichment [ϵ] factors) and soil metrics (both the concentration and $\delta^{15}\text{N}$ values of soil N and the biomarker and hyphal ingrowth metric of fungal biomass) were performed. Only two significant Pearson correlation coefficients were found; log-transformed f estimates were negatively correlated with the ammonium-based foliar enrichment factor ($\epsilon = \delta^{15}\text{N}_{\text{foliar}} - \delta^{15}\text{N}_{\text{NH}_4}$; $R^2 = 0.34$, $P = 0.023$; Fig. 2a) and EcM mycelial ingrowth ($R^2 = 0.51$, $P = 0.0027$; Fig. 2b).

4. Discussion

4.1. Alteration of soil N cycles and interactions with P availability

Five years of N additions caused large increases in extractable and resin accumulated mineral N. This N saturation led to a c. 50% decline in extractable [DON]. The decline of [DON] under N addition suggests sensitivity of enzyme activities to soil mineral [N] through either decreased production or increased consumption of labile DON. Added N has previously been shown to decrease lignolytic activity and increase proteolytic enzyme activity of fungi (Neff et al., 2002; Waldrop and Zak, 2006; Lucas and Casper, 2008; Allison et al., 2009). Such increases in mineral N/DON ratios, while likely beneficial to boreal forest tree productivity (Kranabetter et al., 2007; Mayor et al., 2012) may impact fungal community composition through reductions in taxa specializing on more recalcitrant N forms (Allison et al., 2007; Lucas and Casper, 2008).

The $\delta^{15}\text{N}$ values of total soil N and resin accumulated ammonium were unaffected by nutrient additions. This suggests that any small isotopic changes to organic matter inputs (plant and microbial residues) do not influence total soil $\delta^{15}\text{N}$ over such short time periods (Hobbie and Ouimette, 2009). Also, the use of ammonium-nitrate fertilizer rather than urea precluded potential

fractionation during ammonia volatilization (Bouwmeester et al., 1985; Mizutani and Wada, 1988). In contrast to the isotopic robustness of these two N pools, resin accumulated nitrate was significantly ^{15}N -enriched by both N and P additions. Such a response to N addition was expected based on field studies in lower latitude ecosystems (Houlton et al., 2006; Mayor et al., 2014) because stimulation of fractionating gaseous N (NO_x , N_2O , N_2) losses by nitrifying and denitrifying bacteria can lead to ^{15}N enrichment of remaining nitrate (Hobbie and Ouimette, 2009; Schlesinger and Bernhardt, 2013). As soil nitrate $\delta^{15}\text{N}$ values appear most sensitive to N saturation, studies that only measure total soil $\delta^{15}\text{N}$ may erroneously conclude that loss pathways or N sources were unaltered (e.g. Högberg et al., 2014).

Far less clear however, is the mechanism by which P addition, independent of N, led to ^{15}N enrichment of soil nitrate. Gaseous N losses are thought to be quantitatively unimportant in boreal soils owing to low soil anoxia and low mineral N abundance (Stehfest and Bouwman, 2006; Hobbie and Ouimette, 2009). However, soil nitrate in the +P treatment was markedly (17.9‰) more ^{15}N enriched than control soils, yet nitrate accumulation rates remained statistically equivalent to the Control. Further, the magnitude of ^{15}N enrichment observed in the +P treatment was reduced under N and P coaddition suggesting P specific stimulation of gaseous losses that are ameliorated by N/P coaddition (likely due to fertilizer dilution of residual nitrate). The mechanism by which P disproportionately stimulates fractionating losses is unclear, particularly in arctic soils (Chapin, 1996; Siciliano et al., 2009), although there is evidence from other ecosystems that elevated P availability stimulates nitrifying bacteria both directly (Mahendrappa and Saloni, 1982) and indirectly via pH influences on enzyme activity (Sinsabaugh et al., 2008). Phosphorus addition, however, did not alter soil pH in our experiment. Rather, several lines of evidence suggest P fertilization directly stimulated the activity of nitrifying and/or denitrifying bacteria. These lines of evidence include: a strong positive correlation between the denitrifier gene *nirK* and oxalate extractable P in a European spruce forest (Bárta et al., 2010); reductions in relative ^{15}N recovery in plant, microbial, and soil N pools in P amended moist soil incubations attributed to greater gaseous N losses (He and Dijkstra, 2015, and references therein); and, repeatedly observed increases in N_2O and NO emissions following P additions to forest (Mori et al., 2010; Fisk et al., 2014) and grassland ecosystems (Zhang et al., 2014). In contrast, chronic P additions did not lead to ^{15}N enrichment of soil

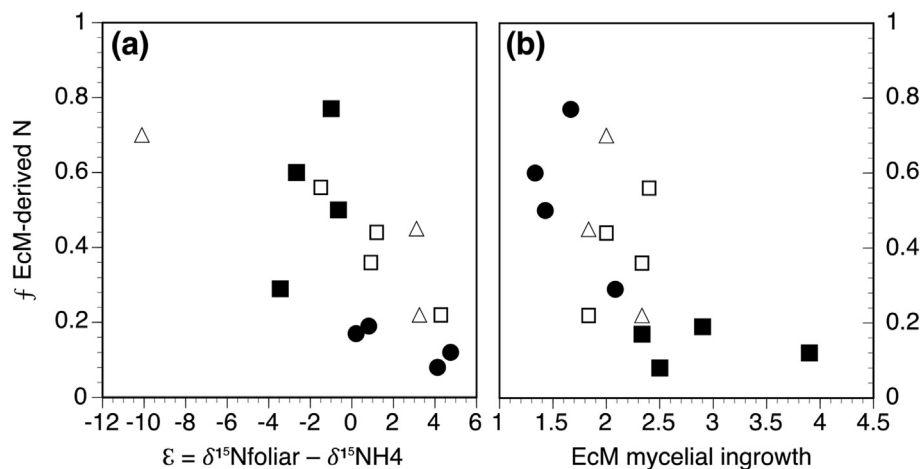


Fig. 2. Relationship between the modeled proportional dependencies of black spruce on EcM derived N (f) following five years of N and P fertilization. (a) The ammonium-based foliar enrichment factor (ϵ) was negatively correlated with f ($R^2 = 0.34$, $P = 0.023$; a) and (b) a metric of EcM mycelial ingrowth ($R^2 = 0.51$, $P = 0.0027$). Symbols correspond to treatment: +P open triangles, +N filled squares, +N + P filled circles, and control open squares.

nitrate in a mature tropical rain forest (Mayor et al., 2014). In conclusion, there appears to be strong evidence from this and other studies that nitrifying and/or denitrifying bacterial activity may be stimulated by P additions in many, but not all, ecosystems.

4.2. Response of fungal biomass and ingrowth

In partial support of our hypotheses that N additions would decrease fungal biomass and ingrowth due to relaxation of N demands by host plants (H4), there were marginal declines in fungal biomass in the +N treatment only. This was an expected outcome of a reduced below ground C allocation to EcM fungi following relief of N limitation to host plants – a pattern similar to that observed along N availability gradients in other high latitude ecosystems (Nilsson et al., 2005; Wallander et al., 2009; Högborg et al., 2010; Wardle et al., 2013). In contrast, P additions significantly increased mycelial ingrowth and marginally increased fungal biomass and no N × P interactions were observed. Increased mycelial ingrowth and fungal biomass suggests relief of P limitation to EcM fungi and is surprising given presumed N limitation of microbes in boreal soils (Hart and Stark, 1997; Li et al., 2014). Mycelial ingrowth was also stimulated in a N and P coaddition experiment in arctic tundra, a result attributed to both increased shrub (i.e. carbon) abundance and direct nutrient stimulation of mycelium (Clemmensen et al., 2006). Nutrient limitation to microbes appear to vary among regions in part due to differential nutrient limitation of above and belowground components (Treseder, 2008; Harpole et al., 2011), carbon and nutrient based interactions between them (Clemmensen et al., 2006), and the possibility of direct nutrient toxicity in some nutrient addition experiments (Wallenda and Kottke, 1998). These results further suggest that P availability is an under evaluated yet important driver of fungal growth and ^{15}N fractionation in boreal forests.

4.3. Alteration of black spruce foliar N/P ratios and foliar and fungal $\delta^{15}\text{N}$ values

Black spruce foliar [P] nearly doubled in response to P fertilization and increased by 50% in response to N and P coaddition. Similarly, and as hypothesized (H1), N fertilization also doubled foliar [N] across both N addition treatments. The use of foliar N/P ratios are often interpreted as a metric of relative N vs. P limitation (Ågren, 2008), although such ratios are typically more responsive to P than N availability given a general inability for plants to store N in excess of photosynthetic demands (Aerts and Chapin, 2000; McGroddy et al., 2004). Black spruce foliar N/P ratios were affected by all treatments – shifting from what can be considered non-optimal (<10) to relatively more optimal conditions (sensu Ågren, 2008) in response to N additions and to less optimal levels in response to P additions (c. 13 vs. 6 in the +N and –N treatments, respectively). Despite black spruce foliage achieving more optimal N/P ratios in response to N additions, growth rates based on tree ring increment widths did not indicate stimulation of growth in any treatment (M.C. Mack, unpublished data). Black spruce in these dry and cold sites are very slow growing, achieving average stem basal diameter of just 23 cm² (totaling 8 m² ha⁻¹) in the 80 or so years since the last stand replacing fire (Mack et al., 2008). The undetected growth response to nutrient additions could be due to a very slow stem response or to preferential investment of excess nutrients into the production of other plant tissues (foliage, roots, etc.).

Beginning with some of the first $\delta^{15}\text{N}$ measurements in agricultural systems it has been known that N fertilization causes plant $\delta^{15}\text{N}$ values to approximate the isotopic values of the fertilizer inputs, typically near the atmospheric standard of $0 \pm 2\%$ (Högborg et al., 1996; Vitoria et al., 2004). Black spruce is one of the most

^{15}N -depleted trees globally (Craine et al., 2009) and absorption of ^{15}N -enriched mineral N was therefore expected (H1) to cause ^{15}N enrichment (Högborg, 1991; Davis et al., 2004; Mayor et al., 2014). The ^{15}N enrichment of nitrate in the +P treatment, while large, did not appear influential over foliar $\delta^{15}\text{N}$ values due to its proportionally small contribution to the available N pools (0.4% of TDN, 12% of mineral N).

Unlike the increased [N] and $\delta^{15}\text{N}$ values in black spruce foliage, N source and contents to fine roots were statistically unaffected by nutrient additions. Although fine roots of *Pinus* and *Vaccinium* species have been observed to trace total soil $\delta^{15}\text{N}$ values following 20 years of N saturation in a Swedish boreal forest (Högborg et al., 1996), our study may have been of too short a duration for internal N reallocation to impact root $\delta^{15}\text{N}$ values (Kolb and Evans, 2002).

In agreement with hypothesis H3, $\delta^{15}\text{N}_{\text{fungi-plant}}$ values, a measure of fractionation associated with the EcM habit, were significantly lower (c. 50%) in the +N treatment, yet N and P coaddition did not further reduce $\delta^{15}\text{N}_{\text{fungi-plant}}$ values (H6). Depleted sporocarp and enriched plant $\delta^{15}\text{N}$ values in response to N additions theoretically represent reduced retention of ^{15}N during the transfer of ^{15}N depleted transfer compounds from EcM fungi to host plants. Such patterns were seen along successional chronosequences (Hobbie et al., 2005) and in N fertilized boreal forests (Hasselquist and Högborg, 2014). However, reliance upon EcM fungi for N in the +N treatment was not supported based on mass balance equations in our system. Only under N and P coaddition (discussed in Section 4.4 below) was a reduced reliance on EcM fungi for N indicated. Complicating our interpretation of these factors is the possibility that nutrient additions may have altered the fungal community (Lilleskov et al., 2001) and thereby the functional traits of distinct EcM fungal genera corresponding to exploration types, foraging depths, and preferred N forms which could contribute to variance in sporocarp $\delta^{15}\text{N}$ values (Trudell et al., 2004; Hobbie and Agerer, 2009).

The negative relationship between f and the $\delta^{15}\text{N}_{\text{foliage-NH}_4}$ in Fig. 2a indicates how black spruce dependency on EcM derived N diminishes as black spruce foliar $\delta^{15}\text{N}$ becomes more similar to or more ^{15}N enriched than the $\delta^{15}\text{N}$ value of soil NH_4^+ . This is an outcome of an increased modeled reliance upon soil NH_4^+ where both plant and NH_4^+ values become ^{15}N enriched.

4.4. Alteration of black spruce dependency on EcM derived N

There were non-significant increases in f in the +N and +P treatments due in part to high variability among plot solutions. For instance, f values for the +N and +P treatments ranged from 20 to 70% of black spruce proportional N uptake compared to just 8–19% in the +N+P treatment. The hypothesis that nutrient coaddition would diminish black spruce reliance upon EcM fungi for N (H5) was supported. This result ran counter to the observation that the smallest $\delta^{15}\text{N}_{\text{fungal-plant}}$ values, a metric of N processing in the EcM system, were observed in the +N treatment, followed next by the +N+P and +P treatments.

The question of what could cause such discrepancies among these metrics is an interesting one. Based on fertilization experiment along a P availability chronosequence in Hawaii (Treseder and Vitousek, 2001) N fertilization should have increased plant P demand and P fertilization should have further exacerbated plant N demand. If this prediction were correct then the proportion of plant N derived from EcM fungi would have ranked from high to low among treatments as P > C > N > N + P. Instead we found that the general ranking was P = N > C > N + P. However, another possibility is that increased P demands in the N treatment caused continued N delivery by EcM fungi due to an inability of EcM fungi to selectively deliver only the most limiting mineral nutrient at a time (e.g. a

mycogenic interpretation of the EcM symbiosis whereby plants can't choose to 'pay' photosynthate for only the most growth limiting nutrient). This latter interpretation can account for the observations that EcM-dependency declines when both N and P are added in combination, that EcM sporocarp $\delta^{15}\text{N}$ values were the most ^{15}N -enriched (a putative sign of greater N processing) in the +N + P treatment (in agreement with H5 and H6), and that f in the +P treatment was greater than the +N + P and Control treatments. This interpretation, however, cannot account for the relatively greater f value obtained in the +N treatment.

The reduced proportional dependency of black spruce for EcM derived N in the +N + P treatment does not correspond with the observation that fungal biomass and mycelial ingrowth were stimulated by P addition. Rather, the lowered N demand of black spruce in the +N + P treatment should have resulted in a decline in C supply, and hence EcM biomass and growth, as supported by the marginally significant decline in fungal biomass in the +N treatment. Instead both fungal biomass and ingrowth increased in response to P additions. In contrast, the +N + P treatment, with the lowest f values, also contained the highest mycelial ingrowth as shown in Fig. 2b. This suggests that high N and P availability may actually stimulate mycelial exploration of soil independently of host plant dependency on EcM derived N.

These responses indicate a partial decoupling between modeled plant N demands (which declined in the +N + P treatment but increased in the +N and +P treatments), ^{15}N -fractionation associated with the EcM symbioses (which declined in the +N treatment), and growth responses by the fungi themselves (increased in +P treatments). Such decoupled above and belowground responses suggest predicting the response of fungal growth using only foliar or sporocarp $\delta^{15}\text{N}$ values may not work in systems undergoing large changes to nutrient availability. This finding has implications for predicting input rates of recalcitrant carbon residues derived from EcM biomass as they have been shown to strongly influence soil [C] storage at local and global scales (Clemmensen et al., 2013; Averill et al., 2014) and calls into question the utility of plant and fungal $\delta^{15}\text{N}$ values to detect altered dependencies on EcM fungi in the absence of detailed measurements of individual soil $\delta^{15}\text{N}$ values, microbial responses to P availability, and possible changes to fungal community composition.

4.5. Conclusion

There were both expected and unexpected outcomes of five years of factorial N and P additions to an EcM black spruce forest. Nitrogen additions led to expected increases in foliar N/P ratios, reductions in $\delta^{15}\text{N}_{\text{fungi-plant}}$ values, and ^{15}N enrichment of soil nitrate. In contrast, P additions were surprisingly influential over the ^{15}N enrichment of soil nitrate and the biomass and ingrowth of EcM fungi. Further, N and P co-addition significantly decreased modeled dependency on EcM derived N despite increased mycelial ingrowth; findings seemingly in contrast to otherwise marginal increases in the modeled dependency on EcM derived N in single nutrient addition treatments, and intermediate increases in foliar N/P ratios and $\delta^{15}\text{N}_{\text{fungi-plant}}$ values in the +N + P treatment. Collectively, the unexpected responses of several ecosystem properties to P fertilization in a N-limited boreal forest suggest that even though most previous studies found N addition to suppress C allocation to EcM fungi, predicting the outcome of altered N availability in the EcM symbiosis, appears partially dependent upon relative P availabilities (Treseder et al., 2004a; Blanes et al., 2012; Deckmyn et al., 2014) thus complicating efforts to predict the response of high-latitude forest ecosystems to global change factors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2015.05.028>.

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