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Nitrogen Isotope Patterns in Alaskan Black Spruce Reflect Organic Nitrogen Sources and the Activity of Ectomycorrhizal Fungi

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Abstract

Global patterns in soil, plant, and fungal stable isotopes of N (δ^{15}N) show promise as integrated metrics of N cycling, particularly the activity of ectomycorrhizal (ECM) fungi. At small spatial scales, however, it remains difficult to differentiate the underlying causes of plant δ^{15}N variability and this limits the application of such measurements to better understand N cycling. We conducted a landscape-scale analysis of δ^{15}N values from 31 putatively N-limited monospecific black spruce (*Picea mariana*) stands in central Alaska to assess the two main hypothesized sources of plant δ^{15}N variation: differing sources and ECM fractionation. We found roughly 20% of the variability in black spruce foliar N and δ^{15}N values to be correlated with the concentration and δ^{15}N values of soil NH_4^+ and dissolved organic N (DON) pools, respectively. However, ^{15}N-based mixing models from 24 of the stands suggested that fractionation by ECM fungi obscures the ^{15}N signature of soil N pools. Models, regressions, and N abundance data all suggested that increasing dependence on soil DON to meet black spruce growth demands predicates increasing reliance on ECM-derived N and that black spruce, on average, received 53% of its N from ECM fungi. Future research should partition the δ^{15}N values within the soil DON pool to determine how choice of soil δ^{15}N values influence modeled ECM activity. The C balance of boreal forests is tightly linked to N cycling and δ^{15}N values may be useful metrics of changes to these connections.

Key words: 15N; black spruce; denitrifier method; dissolved organic nitrogen; ectomycorrhiza; isotope fractionation; mixing models.

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Introduction

The productivity and dynamics of many ecosystems are governed by nitrogen (N) availability; added N typically increases the biosynthesis of proteins and enzymes for photosynthesis (Galloway and others 2004; Howarth and Marino 2006) and results in greater productivity of terrestrial ecosystems

(Vitousek and Howarth 1991; Elser and others 2007). Because many human activities can increase N availability through deposition, or increase N-mineralization rates through climate warming and landscape modification, monitoring ecosystem responses to varying N availability is a research priority (Vitousek 1997; Mellilo and Cowling 2002). In N-limited boreal forests, slight changes in N availability can alter ecosystem productivity, carbon storage, and diversity of plants and microbes (Mack and others 2004; Toljander and others 2006; Treseder and others 2007). Assessing changes to ecosystem N availabilities and pathways of cycling, however, remains difficult because N cycling processes vary in both time and space (Binkley and others 2000).

Recent syntheses suggest that stable isotopes of N (represented as $\delta^{15}N$, a ratio standardized against atmospheric N) provide integrative measures of N cycling that can be used to infer processes at local, regional, and global scales (Amundson and others 2003; Craine and others 2009; Pardo and Nadelhoffer 2010). However, there are often multiple causes of $\delta^{15}N$ variability in plant–soil systems making definitive interpretation difficult (Högberg 1997). In soils, high N-mineralization rates often result in greater N losses from denitrification and leaching (Houlton and others 2006; Kahmen and others 2008) and this loss of ^{15}N-light N (for example, NO_3^-, N_2O, N_2) leads to ^{15}N-enrichment of remaining soil N pools (Piccolo and others 1994; Boeckx and others 2005; Pörtl and others 2007). Collectively the production, cycling rate, and demand for N would then determine the ''openness'' of the N cycle and the form of N capable of being lost from the ecosystem (Gebauer and Schulze 1991; Nadelhoffer and Fry 1994; Pardo and others 2006; Houlton and others 2007). In plants, preference of one isotopically distinct form of N over another could explain differences among co-occurring species (Schulze and others 1994; Michelsen and others 1996; McKane and others 2002; Houlton and others 2007). In addition, internal fractionation due to N processing could alter plant $\delta^{15}N$ values from their sources (Evans 2001).

An additional mechanism deemed important in many high-latitude ecosystems is the ^{15}N-fractionation that occurs during the delivery of N by ecto- and ericoid mycorrhizal fungi. These fungi modify plant $\delta^{15}N$ values through the production of ^{15}N-depleted N-transfer compounds (Michelsen and others 1998; Hobbie and Hobbie 2008). This process, scaled up globally, accounted for 29% of the variation in $\delta^{15}N$ values from more than 9,000 plants (Craine and others 2009). The corresponding retention of ^{15}N-enriched N is also seen to maintain a consistent divide among co-occurring ECM and saprotrophic fungi from around the world suggesting that this process is not limited to high-latitude forests (Mayor and others 2009).

To aid in the interpretation of black spruce $\delta^{15}N$ variability and boreal forest N cycling, this study focused on the simultaneous assessment of the two main causes of plant $\delta^{15}N$ variability: shifting $\delta^{15}N$ values of soil N and the activity of ECM fungi. We conducted our study in ECM-forming black spruce forests that exhibit a large range of foliar $\delta^{15}N$ values, aboveground biomass, and edaphic conditions. Because mineral N concentrations in high-latitude forests are at very low levels, we used the denitrifier method (Sigman and others 2001) to achieve ample within-plot replication of soil $\delta^{15}N$ values with minimal error. We then incorporated these detailed soil N measurements into previously established isotope mixing models designed to estimate the dependency of host plants on ECM fungi (Hobbie and Hobbie 2008). In this manner, we sought evaluation of how both the source (mineral or organic N) and pathway (ECM mediated vs. direct root uptake) of N cycling influences black spruce $\delta^{15}N$ values across 31 plots at the landscape scale.

Boreal black spruce forests were selected as an ideal species for this study because they form monospecific stands across a broad range of productivity-influencing topographic positions (Viereck and Johnston 1990; Chapin and others 2006; Hollingsworth and others 2006) and represent the lower third of foliar $\delta^{15}N$ values observed globally (Craine and others 2009). Based on previous isotope modeling in Alaskan tundra (Hobbie and Hobbie 2008; Yano and others 2010), we hypothesized that black spruce $\delta^{15}N$ would reflect variable dependency on ECM fungi for N delivery rather than simply altered soil N source $\delta^{15}N$ values. In addition, we sought to test if the proportional reliance of black spruce on ECM-derived N would be negatively correlated with proxies for soil N availability (both mineral and organic forms) and positively correlated with hyphal biomass and/or growth because of increased belowground C allocation under high plant N demand (Högberg and others 2010). Because the N cycle is closely coupled to C sequestration, providing baseline data and interpretation of ecosystem $\delta^{15}N$ patterns can inform the application of this foliar index to detect climatic-induced changes to high-latitude N cycles.

Materials and Methods

Soils, mature black spruce trees, and fungal sporocarps were sampled from 31 stands in central Alaska during the 2007 and 2008 growing seasons. These stands were selected from a pool of 90 potential 1 ha plots (Hollingsworth and others 2006, 2008) and encompassed an area of approximately 14,000 km^2. The primary filter for selecting plots was even representation of the full range of foliar $\delta^{15}N$ values observed during a pilot study (M. C. Mack, unpublished data). Sampling in each plot occurred along a 5 × 30 m belt transect centered on permanent markers and arrayed perpendicular to slope. Active layer depth, cation exchange capacity (CEC), soil moisture indices, stand diameter at breast height (dbh), and pH for these sites were previously obtained (Hollingsworth and others 2006) and, with the exception of dbh, reduced to a single principle component dimension for inclusion of a ''soil fertility'' metric in multiple regressions (Supplementary Information S1). Aboveground biomass was estimated using dbh-based allometric equations derived from 15 sites spanning the geographic range of our stands (Yarie and others 2007). In agreement with previous reports (Ruess and others 2003), black spruce fine roots were confirmed as extensively colonized by ECM fungi during soil and root sampling.

Foliar $\delta^{15}N$, %N, and %P were measured from terminal branch needles at the peak of needle expansion in 2007 (late August to early September) to reduce variability associated with changing N content during needle expansion (Chapin and Kedrowski 1983). Three full-sun branch tips were collected from three trees in each plot, composited by tree, and stored at 4°C for no more than 48 h before drying. Small diameter (≤ 1.5 mm) terminal root samples were excavated from the same trees and stored at 4°C for no more than 2 weeks before removal of the thin outer layer of secondary root tissue to prevent potential inclusion of adhered soil particles and fungal sheaths in subsequent elemental and isotopic analyses. Previous studies have shown that mycorrhizal sheaths can be from 2.4 to 6.4‰ more ^{15}N enriched than root cores (Högberg and others 1996). In most cases, root $\delta^{15}N$ values were composited by plot. Fungal sporocarps were collected and identified to genus when observed. Plant and fungal tissues were dried at 60°C for 24 h, ground to a fine powder, and analyzed on a ThermoFinnigan® continuous flow isotope ratio mass spectrometer coupled to a Costech® elemental analyzer at the University of Florida (UF). Stable isotope abundances are reported as $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$, where $R = {}^{15}N{:}{}^{14}N$ ratio of the sample or standard. Run error rates were better than 0.2‰ as compared to N3 NIST and, in the case of soil N extracts, USGS #32, 34, and 35 standards.

Five pairs of ion exchange resin bags were incubated throughout the growing season as metrics of bioavailable soil NO_3^-, NH_4^+, and PO_4^+ production (Giblin and others 1994) and time-integrated $\delta^{15}NH_4^+$ and $\delta^{15}NO_3^-$ values. Mesh resin bags (220 μm mesh, 6.25 $in.^2$) containing either 3 g of anion (Biorad®, AG 1-X8, #140-1421) or cation (Biorad®, AG 50 W-X8, #142-1421) beads were inserted below the sub-fibric organic soils (depths of 5–20 cm), an area coinciding with highest root densities (Ruess and others 2003), at five equidistant locations along belt transects. Resin bags were extracted with 100 ml of a weakly acidified 0.1 M HCl/2 M NaCl solution (Giblin and others 1994) and frozen prior to colorimetric analyses.

Assessment of the potential for fungal hyphal ingrowth was attempted with five sand filled nylon bags (52 μm mesh with ~7 cm^3 of acid washed quartz sand) deployed throughout the growing season at the organic–mineral interface (Wallander and others 2001; Nilsson and others 2005). Each bag was inserted along the same five equidistant locations on belt transects, opposite to the resin bag locations. Three additional bags were inserted inside buried PVC collars in each plot to account for saprotrophic fungal biomass, but because negligible biomass was found, corrective accounting was unnecessary. Ingrowth bags were removed from the field at the end of the growing season just prior to the first frost in early September, refrigerated, and transported to UF where they were slowly dried, the sand suspended in water, and the degree of hyphal colonization scored under a dissecting microscope (1 = none, 2 = light and diffuse, 3 = extensive, 4 = extensive with few rhizomorphs, 5 = extensive with many rhizomorphs). Visual examination has previously been shown to correlate well with weight and biochemical proxies of fungal biomass (Nilsson and others 2005). Hyphae of sufficient mass were composited by plot, dried, and prepared for isotopic analyses as above.

Potassium peroxodisulfate thermo-oxidation of total dissolved N (TDN) and NH_4^+ to NO_3^- (Cabrera and Beare 1993; Doyle and others 2004) was coupled to the denitrifier method to determine $\delta^{15}N$ values (Sigman and others 2001; Knapp and others 2005). The $\delta^{15}N$ value of dissolved organic N (DON) was calculated as the mass weighted difference between TDN and mineral N using the following equation:

$$\delta^{15}N_{DON} = (\delta^{15}N_{TDN} \times [TDN] - (\delta^{15}N_{NH_4^+} \times [NH_4^+] + \delta^{15}N_{NO_3^-} \times [NO_3^-]))/[DON] \quad (1)$$

The $\delta^{15}N$ values of mineral N were measured from resin extracts incubated throughout the growing season, and $\delta^{15}N_{TDN}$ and concentration values were determined from 2 M KCl extracts of organic soils made the day of sampling at the height of the growing season in mid-July 2007 (see Supplementary Information S1 for additional details on soil chemistry and isotope analyses).

To assess standing fungal biomass, phospholipid fatty acid (PLFA) analyses were conducted on 1–5 g subsamples of homogenized organic soils that were immediately frozen following the salt extraction mentioned above. All roots larger than 1 mm were removed prior to analysis. Two replicates were run for each plot and nine were run in triplicate to assess standard deviations. Measuring PLFA involved an initial lipid extraction, fractionation (Frostegård and others 1991), followed by conversion of the methanol fraction into free methyl esters by mild alkaline methanolysis, and analysis on a gas chromatograph with a flame ionization detector and a 30 m HP5 capillary column (Frostegård and others 1993). We used the total mol% of the PLFA 18:2ω6,9 to indicate the relative abundance of fungi across stands. 18:2ω6,9 has been regarded as an indicator of the relative abundance of fungi in general (Frostegård and Bååth 1996) and it has been confirmed to be largely comprised of ECM fungi in many coniferous forests (Högberg and Högberg 2002; Bååth and others 2004; Lindahl and others 2007). The relative dominance of ECM taxa has been further corroborated by molecular analyses in Alaskan black spruce forests including several stands in our analysis (Allison and others 2007; Taylor and others 2010).

Regression analyses and diagnostics were performed using the *R* statistical environment (2.11.1, The R® Foundation for statistical computing 2010) and JMP® 8.0.2 (SAS Institute Inc., Cary, NC, USA). Second-order bias-corrected Akaike information criterion ($\Delta_i = AICc_i - AICc_{min}$) was used to rank competing models (Burnham and Anderson 2004; Andersen 2008) designed to test hypotheses regarding controls over elemental, isotopic, and biomass patterns in black spruce forest (see Supplementary Information S1 for additional statistical detail).

Estimates of proportional N flux through ECM fungi was assessed with iterative models previously developed for arctic ecosystems (Hobbie and Hobbie 2006, 2008; Yano and others 2010):

$$\delta^{15}N_{available} = f_{NO_3^-} \times \delta^{15}N_{NO_3^-} + f_{NH_4^+} \times \delta^{15}N_{NH_4^+} + f_{DON} \times \delta^{15}N_{DON} \quad (2)$$

$$\delta^{15}N_{available} = (1 - T_r) \times \delta^{15}N_{fungi} + T_r \times \delta^{15}N_{transfer} \quad (3)$$

$$\delta^{15}N_{plant} = \delta^{15}N_{available} - \Delta_f \times (1 - T_r) \times f \quad (4)$$

$$\delta^{15}N_{fungi} = \delta^{15}N_{available} + \Delta_f \times T_r \quad (5)$$

where $f_{NH_4^+}$ and f_{DON} refer to the fraction of these soil N forms contributing to the available N pool, T_r refers to the proportion of total fungal N that is transferred to host plants, $\delta^{15}N_{transfer}$ refers to the $\delta^{15}N$ 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 (Hobbie and Hobbie 2008).

We quantitatively constrained the mass balance solutions in the following ways: (a) the fraction of plant N delivered by ECM fungi (f) cannot exceed 100% of the trees' N supply; (b) mixtures of the different N sources occur at 10% increments; and, (c) Δ_f was constrained to 8–10‰ based on laboratory, field, and meta-analyses described elsewhere (Hobbie and Hobbie 2006, 2008). Sensitivity of model outputs was assessed with regard to Δ_f magnitude, plant $\delta^{15}N$ end-member values, and the relative proportions of mineral versus organic N accessed.

Results

Black Spruce Elemental and Biomass Patterns

Foliar $\delta^{15}N$ values in black spruce trees varied by 6.5‰ (Supplementary Table S1). Foliar $\delta^{15}N$ was strongly correlated with both foliar N ($R^2 = 0.46$, $P < 0.001$) and phosphorus (P) concentrations ($R^2 = 0.51$, $P < 0.001$; Figure 1). Root $\delta^{15}N$ values were on average 2.4‰ more enriched than foliage and were positively correlated with foliar $\delta^{15}N$ values ($R^2 = 0.30$, $P = 0.002$). The difference between root and foliar $\delta^{15}N$ values was smallest at higher $\delta^{15}N$ values (Figure 2; $\delta^{15}N_{foliar} = 0.51 \times \delta^{15}N_{root} - 4.69$; range 0–5.5‰). Multiple regression models indicated foliar $\delta^{15}N$ values were best explained by the soil fertility principle component ($R^2_{adj} = 0.21$) and in two of the three most informative models a link to soil $\delta^{15}N$ values was suggested (Table 1). Foliar N concentrations were best explained by the DON content of organic soils

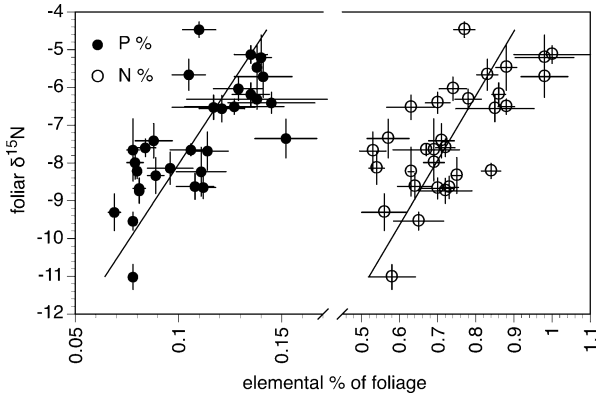

Figure 1. Relationships between $\delta^{15}N$ (‰) and N and P content (%) of full sun needles ($n = 3$) collected from black spruce (*Picea mariana*) trees in 31 plots in central Alaska. Phosphorus % (μg g^{-1}): $R^2 = 0.53$, $P < 0.001$; Nitrogen %: $R^2 = 0.46$, $P < 0.001$.

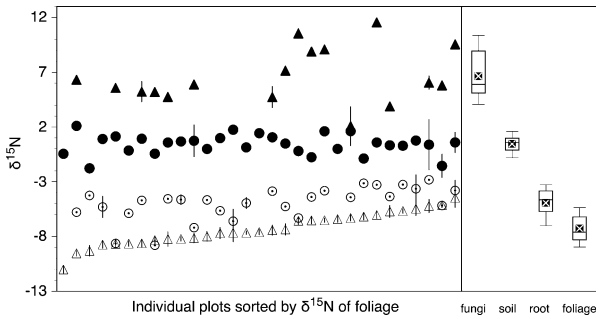

Figure 2. Average (±SE) black spruce full sun needle (*open triangle*), fine root (*open circle*), bulk organic soil (*filled circle*), and ECM sporocarp (*filled triangle*) $\delta^{15}N$ values across 31 plots in central Alaska. Box plots illustrate overall means (*times*), medians (*line*), and 95th percentiles. Points are arbitrarily arrayed according to average foliar $\delta^{15}N$ values to illustrate the lack of strong covariance among components. Only foliar $\delta^{15}N$ was significantly correlated with root $\delta^{15}N$ ($\delta^{15}N_{foliar} = -4.69 + 0.51\delta \times \delta^{15}N_{root}$, $R^2 = 0.30$, $P = 0.01$). The slope for foliar $\delta^{15}N$ (0.16) is nearly twice that of both the ECM sporocarps and black spruce fine roots (0.09), although not statistically different. Across all plots, ecosystem components were different from one another (paired t test, $P < 0.001$) and the mean isotopic differences between foliage and root, foliage and soil, and foliage and fungal $\delta^{15}N$ values were −2.4, −7.7, and −13.8‰, respectively.

(μg g^{-1} soil) either alone ($R^2_{adj} = 0.25$) or in combination with resin accumulated mineral N (ng N g^{-1} resin day^{-1}) and the soil fertility principle component ($R^2_{adj} = 0.31/0.32$; Table 1). Foliar P concentration was best explained by models containing resin accumulated PO_4^+ (ng P g^{-1} of resin day^{-1}, log-transformed) in conjunction with soil fertility or an interaction with hyphal ingrowth ($R^2_{adj} = 0.59$–0.60). The most parsimonious candidate model contained only the soil fertility principle component ($R^2_{adj} = 0.56$; Table 1).

Black spruce aboveground biomass averaged 33.5 Mg ha^{-1} but ranged widely, from 2.6 to 100 Mg ha^{-1} (Supplementary Table S1), in part reflecting the interaction of topography and paludification. Small biomass values were typical of low-lying stands with inundated root systems. The soil fertility principle component partially predicted black spruce biomass ($R^2_{adj} = 0.33$, $P = 0.003$), although post hoc examinations indicated much of the explanatory power was attributed to soil C:N values alone ($R^2_{adj} = 0.20$, $P = 0.008$). The inclusion of foliar N concentrations, arguably a proxy for plant N availability, was informative but did not increase the total explained variance (Table 1).

Fungal $\delta^{15}N$ and Biomass Patterns in Black Spruce Forest

Using a global discriminant analysis based on fungal $\delta^{15}N$ and $\delta^{13}C$ values, we confirmed that the 35 sporocarps collected from the plots were ECM with greater than 90% probability (Mayor and others 2009). These sporocarps belonged to the genera *Boletus*, *Cortinarious*, *Dermocybe*, *Gomphidious*, *Hebeloma*, *Laccaria*, or *Russula*. These genera are considered among the most commonly encountered ECM taxa in boreal forests (Lilleskov and others 2002; Tedersoo and others 2010). Due to the ephemeral nature of fungal sporocarps, these collections came from only 17 of the 31 sites. The inability to reliably measure each site's ECM fungal $\delta^{15}N$ value is not vital to the study as it is generally shown that even exhaustive multi-year sampling of fungal sporocarps represent only a small fraction of total belowground diversity (Koide and others 2005; Tedersoo and others 2010). For this reason, we used the average sporocarp $\delta^{15}N$ value (6.5‰) for each plot's ^{15}N mixing model as the best available integrative signal.

On average, ECM sporocarps were 13.8‰ more enriched than black spruce foliage (Supplementary Table S1); absolute values similar to ECM sporocarps observed elsewhere in Alaska (Clemmensen and others 2008; Hobbie and others 2009) and globally (Mayor and others 2009). Sporocarp $\delta^{15}N$ values were negatively correlated with DON concentrations (g m^{-2}) in organic soils ($R^2 = 0.38$; Figure 3) but unrelated to other soil fertility metrics. Diagnostics of the two influential values with high DON concentrations in Figure 3 did not suggest they were gross outliers (Cook's distance < 0.2, overall

Table 1. Multiple Regression Models from Central Alaskan Black Spruce Forests Ranked According to Bias-Corrected Aikake Information Criteria (Δ_i = AICc$_i$ − AICc$_{min}$)

Model/hypothesis	df	Δ_i	ω_i	$E_{i,j}$	R^2_{adj}
Foliar N ~ DON_{org}	29	0.00	0.38	1.00	0.25
Foliar N ~ DON_{org} + $MinN_{res}$	28	1.50	0.18	2.11	0.25
Foliar N ~ DON_{org} + fertility PC1	27	1.56	0.17	2.18	0.31
Foliar N ~ DON_{org} + $MinN_{res}$ + fertility PC1	26	3.25	0.07	5.09	0.32
Foliar P ~ fertility PC1 + log(PO_{4res})	27	0.00	0.30	1.00	0.60
Foliar P ~ log(PO_{4res}) × hyphae	27	0.00	0.30	0.98	0.59
Foliar P ~ fertility PC1	29	0.97	0.19	1.59	0.56
Foliar $\delta^{15}N$ ~ fertility PC1	28	0.00	0.38	1.00	0.21
Foliar $\delta^{15}N$ ~ fertility PC1 + $\delta^{15}N_{NH_4^+}$	27	1.23	0.21	1.85	0.22
Foliar $\delta^{15}N$ ~ fertility PC1 + $\delta^{15}N_{DON}$	27	1.33	0.20	1.94	0.22
Foliar $\delta^{15}N$ ~ fertility PC1 + $\delta^{15}N_{DON}$ + $\delta^{15}N_{NH_4^+}$	26	1.84	0.09	4.13	0.23
Spruce biomass ~ fertility PC1	28	0.00	0.59	1.00	0.33
Spruce biomass ~ fertility PC1 + foliar N	27	1.26	0.32	1.88	0.33
Mol% 18:2ω6,9 ~ C:N	28	0.00	0.63	1.00	0.52
Mol% 18:2ω6,9 ~ C:N + DON_{org}	27	2.66	0.17	3.78	0.51
Mol% 18:2ω6,9 ~ C:N + CEC	27	2.68	0.17	3.81	0.51

df = degrees of freedom, Δ_i = bias-corrected Aikake Information Criterion, ω_i = model probability, $E_{i,j}$ = evidence ratio, and R^2_{adj} = adjusted Pearson's correlation coefficient. Model variables predicting foliar N (g^{-1} N in black spruce needles) refer to DON_{org} = concentration of 2 M KCl extractable dissolved organic N from organic soils, $MinN_{res}$ = mineral N measured from ion exchange resins, fertility PC1 = first principle component axis containing soil fertility metrics without extractable N concentrations included. Model variables predicting foliar P refer to fertility PC1 = first principle component axis containing soil fertility metrics without extractable PO_4^- concentration included, log(PO_{4res}) = resin extractable PO_4^- concentration, hyphae = hyphal ingrowth biomass metric. Model variables predicting foliar $\delta^{15}N$ refer to fertility PC1 = first principle component axis containing soil fertility metrics without soil $\delta^{15}N_{NH_4^+}$ or $\delta^{15}N_{DON}$ values. Model variables predicting Spruce biomass refer to fertility PC1 = first principle component axis containing soil fertility metrics. Model variables predicting PLFA-based fungal abundance mol% 18:2ω6,9 refer to C:N = carbon to nitrogen ratio of organic soils, and CEC = cation exchange capacity of organic soils.

Leverage < 0.5) although their removal reduced R^2 to 0.17. Scatterplot matrices indicated no correlation with soil N $\delta^{15}N$ values in a univariate context.

The PLFA 18:2ω6,9-based estimates of the relative abundance of fungi were positively correlated with C:N ratios (%) of the organic soil (R^2_{adj} = 0.52, P < 0.0001; Figure 4) and were uncorrelated with hyphal ingrowth. More complex regression models containing DON (μg g^{-1} soil) and CEC (meq) were less probable (ω_i = 0.17) than the more probable parsimonious model containing only soil C:N ratios (ω_i = 0.63; Table 1).

Of the 180 hyphal ingrowth bags deployed throughout the growing season, only four plots produced enough composited tissue for $\delta^{15}N$ measurements. This was perhaps due to the lack of mineral nutrients contained in sand and the rela-

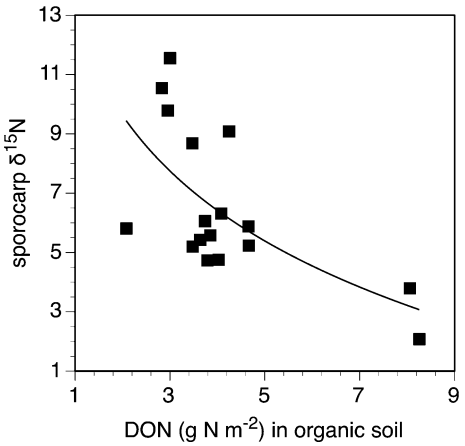

Figure 3. Relation among organic soil DON content and fungal sporocarp $\delta^{15}N$ values from 17 plots in central Alaska. R^2 = 0.38, $f(\chi) = -4.62 \times \ln(\chi) + 12.8$, P = 0.009.

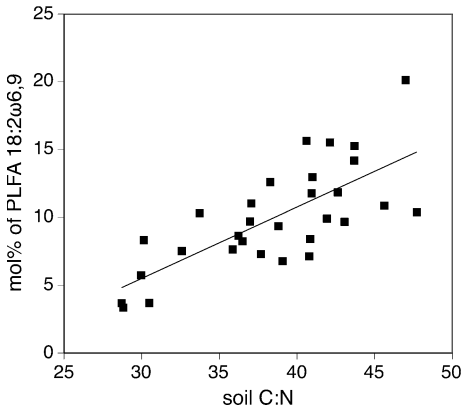

Figure 4. Relation among C:N ratios of the organic black spruce soils with the mol% phospholipid fatty acid (*PLFA*) 18:2ω6,9, a metric of relative fungal abundance (R^2 = 0.54, P < 0.0001).

tively short single-seasonal incubation period (Wallander and others 2004; Hendricks and others 2006). Measured $\delta^{15}N$ values of recovered hyphae were approximately 6‰ more depleted than sporocarps (mean $\delta^{15}N = 0.35‰$) and contained 50% less total N (1.8 vs. 3.65 %, respectively), presumably due to N being preferentially reallocated from ''evacuated'' hyphae to protein-rich sporocarps during development (Wallander and others 2004; Clemmensen and others 2006; Hobbie and Agerer 2009).

Patterns in Soil N across the Landscape

Salt extractable DON ranged from 148 to 603 μg g^{-1} in the organic horizons (Supplementary Table S1), a value nearly six times greater than in mineral soils (data not shown). Resin exchangeable NO_3^- and NH_4^+, proxies of mineral N bioavailability, ranged from 0 to 0.55 and 3.70 ng N g^{-1} resin d^{-1}, respectively (Supplementary Table S1). Resin exchangeable NO_3^- concentrations were detectable in only seven plots, requiring within-plot compositing for ^{15}N-measurements (detailed below). In contrast, NH_4^+ accumulated on exchange resins was detectable in all but two plots and on average was nearly an order of magnitude greater than NO_3^- (Supplementary Table S1). Resin exchangeable PO_4^--accumulation was ten times greater and 16 times more variable than that of NH_4^+ reflecting this ions greater availability yet lower mobility in boreal soil organic horizons (Supplementary Table S1).

Extractable $\delta^{15}N_{DON}$ values were significantly more depleted than $\delta^{15}N_{NH_4}$ ($N = 30$) and $\delta^{15}N_{bulk\ soil}$ ($N = 31$), which did not differ from one another (paired t tests, $P < 0.001$). None of the soil $\delta^{15}N$ values were correlated with one another and the few measurable $\delta^{15}N_{NO_3}$ values exhibited no ranking pattern (Figure 5). Enrichment (ϵ) factors based on soil $\delta^{15}N$ values ($\Delta\delta^{15}N_{plant-soil}$) were not clearly related to the ''N status'' of black spruce forests. Only an ϵ factor based on the difference between $\delta^{15}N_{foliar}$ and $\delta^{15}N_{mineral\ N}$ exhibited a weak positive relationship with resin accumulated NH_4^+ ($R^2 = 0.12$, $P = 0.05$). Overall, it is clear that neither ϵ factors nor the relatively common measurement of $\delta^{15}N_{bulk\ soil}$ in black spruce forests are good indicators of bioavailable soil $\delta^{15}N$ values.

ECM-Derived N-Transfer to Black Spruce

Using measured $\delta^{15}N$ values from black spruce roots, soil N forms, and average ECM sporocarps left only two factors to define in equations 2–5: the magnitude of the fractionation factor associated with fungal N processing (Δ_f) and the relative

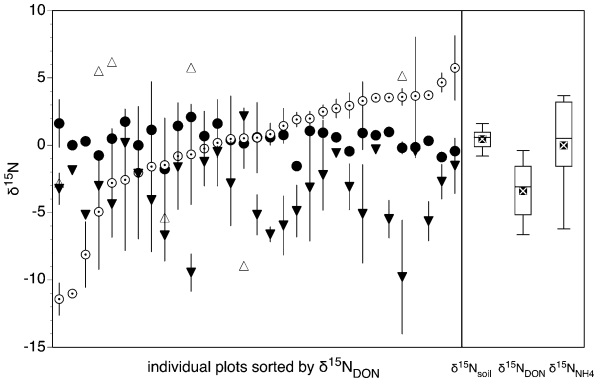

Figure 5. Mean ($n = 3$, ±SE) soil N $\delta^{15}N$ values from dissolved organic N (DON, *open circles*), ammonium (NH_4^+, *filled triangles*), and bulk organic soils (*filled circles*), and nitrate (NO_3^-, *open triangles*) across 31 plots in central Alaska. The $\delta^{15}N$ values are arbitrarily sorted by $\delta^{15}N_{DON}$ values to examine covariance among pools. The figure on the *right* is the mean (*times*) and median (*line*) ±SE of the isotope values used in our analysis.

proportions of NH_4^+ and DON that contribute to black spruce nutrition. Model results were estimated for Δ_f ranging from $\Delta = 8–10‰$ (Hobbie and Hobbie 2006, 2008) with smaller Δ magnitudes requiring greater N retention by fungi ($\Delta T_r \approx 5\%$). We evaluated the sensitivity of ECM dependency (f_{ECM}) to the full range of potential NH_4^+ contributions, from 0 to 100% of total tree N nutrition. Owing to the extremely low detection of NO_3^- in only seven plots, it was avoided as a potential N source in all but two plots where mass balance solutions supported small contributions of this end member (Supplementary Table S2).

Black spruce forests varied widely in their estimated dependency on ECM fungi (8–92% of their N requirements, avg. = 53%, $n = 29$; Supplementary Table S1). However, these averaged f_{ECM} values were uncorrelated with metrics of soil fertility (DON, NH_4^+, pH, moisture, CEC, active layer depth) or metrics of tree or fungal biomass. When viewed at the plot level, it is apparent that the range of f_{ECM} values is highly sensitive to two key model inputs: the proportion of mineral versus organic N and the plant $\delta^{15}N$ end member used. Only two plots were unsolvable with the assigned $\delta^{15}N$ end members although one of these was solvable if the leaf $\delta^{15}N$ end member was substituted for root tissue (plot J1 in Supplementary Table S2). In 83% of modeled plots, f_{DON} was positively correlated with f_{ECM} and in the remaining 17% they were negatively correlated. Omitting the unreasonably high slope in Plot 0225, the average slope of the relationship between f_{DON} and

f_{ECM} was 2.28 (n = 23) and the average negative slope was −2.25 (n = 5; Supplementary Table S2).

Estimates of f_{ECM} were also sensitive to the plant $\delta^{15}N$ end member used in the mixing models. Using root $\delta^{15}N$ end members, as mentioned in ''Materials and Methods,'' eliminated model dependency on varying internal fractionations observed in black spruce (Figure 2). However, the overwhelming majority of reported plant $\delta^{15}N$ measurements in the literature are from foliage. Had we used foliar measurements instead, then estimates of f_{ECM} would increase by 14% on average and f_{DON} would be slightly smaller in most cases (Supplementary Table S2).

Discussion

Black Spruce N-Limitations, DON Usage, and ^{15}N Fractionation

The average needle N:P mass ratio of 7 suggests that black spruce is growing under strongly N-limited conditions (Güsewell 2004) and yet the most informative regression models indicated that foliar P concentrations were strongly correlated with resin accumulated PO_4^+ concentrations. It is likely that the P accumulation is simply a case of luxury consumption (Bowman 1994; Van Wijk and others 2003). Support for this comes from a companion fertilization experiment where black spruce needle P concentrations were seen to double after 5 years of P fertilization (n = 4 plots; JR Mayor, unpublished data). Furthermore, N fertilization caused foliar N:P ratios to reach 17, a value approaching the range typically considered more P-limiting (Güsewell 2004), whereas P fertilization reduced N:P ratios to 4.6 (JR Mayor, unpublished data).

Regression models indicated a link between black spruce foliar N and soil N concentrations (Table 1). Foliar $\delta^{15}N$ values, in turn, were partially explained by soil fertility and the $\delta^{15}N$ values of soil N forms (Table 1). In accordance with biogeochemical theory, these findings suggest that the N content and $\delta^{15}N$ values of black spruce foliage partially reflect the availability and ^{15}N signature of extracted soil N. However, as greater than 75% of the variability in foliar N and $\delta^{15}N$ values remained unexplained by simple linear regressions, evaluation of the influence ECM fungi have on black spruce N dynamics is strongly warranted.

Plot-based mixing models indicated that greater dependency on ECM fungi generally corresponded with greater DON contributions to total N uptake. Although strict mineral N use is mathematically possible in 20 of the mixing models (f_{ECM} = 36%), it is mathematically improbable (Supplementary Table S2) and theoretically unreasonable to suspect that black spruce is not reliant on ECM fungi to meet annual N requirements. The question then becomes what proportion of DON and NH_4^+ are most likely. Previous researchers a priori assigned NH_4^+ contributions to be 10–50% of total N uptake based on the ratio of extractable inorganic to amino acid N present in their tundra soils, and that the 50% contribution of mineral N was considered a case of ''extremely high availability of inorganic N'' (Yano and others 2010). These authors used an even more depleted DON value than the average found in black spruce forests (−5.6‰ for hydrolysable amino acids) yet the value they estimated for f_{ECM} was 31–61% (avg. 51%), whereas the previous Hobbie and Hobbie (2006) estimate was 61–86% for tundra. Similarly constraining f_{DON} in our models results in average f_{ECM} estimates surprisingly similar to that obtained in tundra: 51 ± 6% constrained versus 53 ± 4% unconstrained (Supplementary Table S2). In summary, our estimates were surprisingly similar to the two previous attempts at estimating plant dependency on ECM-derived N and it is likely that DON comprises an appreciable portion of black spruce N.

Black spruce needles were more ^{15}N-enriched at greater N content (Figure 1)—a pattern also seen in several ECM tree species from high latitudes (Schulze and others 1994; Compton and others 2007; Kranabetter and MacKenzie 2010). Given the potential influence that ECM dependency has on foliar $\delta^{15}N$ values, the positive correlation between greater N concentrations and ^{15}N-enriched foliage may indicate conditions where dependency on ECM delivery of N is low. Evidence for this hypothesis is supported by laboratory (Hobbie and Colpaert 2003), greenhouse, and field observations along successional chronosequences (Hobbie and others 2005; Compton and others 2007; Hobbie and Hobbie 2008). However, similar patterns have been described from arbuscular mycorrhiza-forming tree species along gradients of tropical succession (Davidson and others 2007), precipitation (Schuur and Matson 2001), and soil age (Vitousek and others 1989) suggesting such patterns in relatively N-rich ecosystems are likely due to more closely coupled tracing of soil $\delta^{15}N$ values rather than fungal fractionation (Averill and Finzi 2011).

On average, the isotopic difference between root and needle tissues (Δ = 2.4‰) was similar to plants growing under mesic conditions (Högberg 1997; Evans 2001; Pörtl and others 2007). The observed differences were not consistent across plots; however, they ranged from 0 to 4‰ and converged

where tissue δ^{15}N values were more ^{15}N-enriched and N content was highest (Figure 2). This pattern suggests that sources of internal fractionation are only present under relatively greater N-limitation; the uncertainty of which justified the use of root rather than leaf δ^{15}N values in mixing models. It is important to consider internal fractionation because it could obscure the imprint of N sources (Evans 2001; Robinson 2001). One source of fractionation, resorption of foliar N prior to needle abscission, is not supported in black spruce (Kielland and others 1998; Kolb and Evans 2002), suggesting that other processes, such as N assimilation, could account for some of the variability. For instance, preferential mineral N assimilation in roots (Shearer and Kohl 1986; Evans 2001; Cambui and others 2011), organic N transported through xylem, or N leaked from roots have all been implicated in contributing to internal fractionation in plants (Dijkstra and others 2003; Yoneyama and others 2003).

Causes of Variability in Fungal Biomass and Sporocarp δ^{15}N Values

Soils with lower soil DON content harbored relatively ^{15}N-enriched sporocarps (Figure 3). One mechanism that could be responsible is greater N delivery to host plants under low soil DON conditions. This interpretation is a corollary of that invoked to explain ^{15}N-depletion of plants heavily dependent on N from ECM fungi (Högberg and others 1999; Hobbie and Colpaert 2003). However, there was no negative correlation among sporocarp and foliar δ^{15}N values as would be expected to corroborate this explanation (Figure 2). Such plot-level resolution may have offered additional insights into the effect of fungal delivery of N on sporocarp δ^{15}N values but was not possible due to the low number of sporocarps available during plot visits.

The average fungal δ^{15}N end-member value used in mixing models is representative of the functionally dominant and relatively ^{15}N-enriched group of ECM fungi present across sites. Most sporocarp collections belonged to the Cortinariaceae, a family demonstrated to dominate many central Alaskan black spruce forests based on environmental sequence data (Taylor and others 2010). These fungi also exhibit strong proteolytic capacities (Lilleskov and others 2011) that may have aided in the acquisition of organic N.

The positive relationships between fungal biomass and C:N ratios, as seen here (Figure 4) and in other ecosystems (Nilsson and others 2005; Smith and Read 2008; Wallander and others 2009), follow predictions based on increased belowground C allocation under strong N-limiting growth conditions (Figure 6). These patterns were driven by decreasing soil N content (data not shown) and could be due to the selective removal of N during decomposition of organic matter and colonization by ECM fungi (Lindahl and others 2007; Orwin and others 2011).

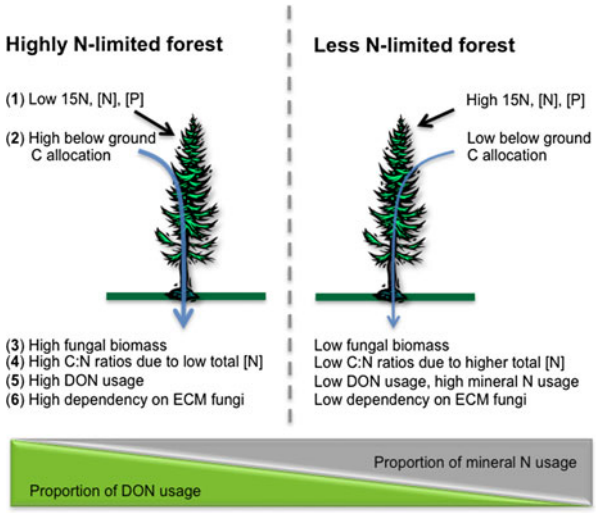

Figure 6. Summary of the relationship between black spruce δ^{15}N values, soil N status, and the abundance and activity of ectomycorrhizal (*ECM*) fungi. Highly N-limited forests: (*1*) exhibit low (for example, very negative) needle δ^{15}N values as well as N and P concentrations (Figure 1); (*2*) high belowground C allocation is presumed to be larger in these stands based on several studies in other boreal forests (Högberg and others 2003, 2010, 2011; Nilsson and Wallander 2003); (*3*) high relative fungal biomass follows the literature supported predictions (*2*) and is positively correlated with (*4*); (*4*) high C:N ratios were positively correlated with fungal biomass due to lower total (Figure 4) and dissolved N in organic soils; (*5*) high dissolved organic N (DON) is positively correlated with foliar N concentrations (Table 1) but negatively correlated with sporocarp δ^{15}N values (Figure 3) and corresponds to the mixing model estimations (Supplementary Table S2); and, (*6*) usage of DON requires a greater dependency on ECM fungi (Supplementary Table S2).

Causes of Variability in δ^{15}N Values of Soil N

The extractable DON pool is composed of numerous compounds with potentially distinct δ^{15}N values (Hobbie and Ouimette 2009) and this is likely attributing to the large variability observed in black spruce soils. These compounds must first enter the

rapidly cycling pool of low molecular weight amino acids prior to adsorption by roots and ECM hyphae (Talbot and others 2008; Näsholm and others 2009). Is the omission of this measurement vital to accurate modeling? Are amino acids the only organic N source used by black spruce (and ECM) and are they isotopically distinct from the larger DON pool? These questions are important because, if so, then this could influence mixing model estimations.

Literature reports suggest that individual amino acids can vary substantially, from −8.7 to +8.1‰ across ecosystems and environmental gradients (Melillo and others 1989; Silfer and others 1992; Ostle and others 1999; Bol and others 2008) but they are seldom measured within the same site. Yano and others (2010) measured acid hydrolyzable N pools of amino sugars and amino acids but not labile DON. Where similar pools have been measured, relative $\delta^{15}N$ rankings of soil N appear to vary among ecosystems (Houlton and others 2006; Houlton and others 2007; Pörtl and others 2007; Bol and others 2008), and perhaps even methods such as diffusion and hydrolyzed losses of NH_3^+ (Bol and others 2008) and salt versus resin N extractions (Koba and others 2003). Typical rankings observed are $\delta^{15}N_{DON} > \delta^{15}N_{amino} >$ mineral = bulk soil $\delta^{15}N$ (Takebayashi and others 2010; Yano and others 2010; Averill and Finzi 2011). If the forms of organic N accessed by ECM fungi were more ^{15}N-depleted than the total extracted DON pool then our estimates of f_{ECM} would be exaggerated. However, major portions of the DON pool, such as chitin, peptides, and proteins (Chalot and others 2002; Jones and Kielland 2002; Jones and others 2005), are readily accessed by many species of ECM-forming fungi (Abuzinadah and Read 1986; Abuzinadah and Read 1988; Lindahl and Taylor 2004; Read and others 2004) and there is little evidence suggesting fractionation during organic matter decomposition (Hobbie and Ouimette 2009). Because of this and the observation that black spruce forest soils have some of the highest protease activities of all taiga ecosystems (Kielland and others 2007) and the abundant Cortinariaceous fungi contain multiple enzymes and peroxidases (Nygren and others 2007; Bödeker and others 2009), we believe that the $\delta^{15}N_{DON}$ in black spruce forest is likely to match the cumulative $\delta^{15}N$ value of the numerous organic N compounds acquired by black spruce through their dependency on ECM fungi.

Summary

Stands with low foliar $\delta^{15}N$ values and low foliar N content also generally contain low total soil N contents, high soil C:N ratios, and a high dependency on DON rather than NH_4^+ as delivered by associated ECM fungi (Figure 6). Higher fungal biomass in these stands was corroborated by positive correlations with C:N ratios (Figure 6). In contrast, less severely N-limited stands are characterized by higher foliar $\delta^{15}N$ values, higher fertility, lower belowground C allocation, lower fungal biomass and soil C:N ratios, and a higher NH_4^+ usage (Figure 6).

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