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Article

Importance of Arboreal Cyanolichen Abundance to Nitrogen Cycling in Sub-Boreal Spruce and Fir Forests of Central British Columbia, Canada

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Abstract: The importance of N_2-fixing arboreal cyanolichens to the nitrogen (N)-balance of sub-boreal interior hybrid spruce (*Picea glauca* × *engelmannii*) and subalpine fir (*Abies lasiocarpa*) forests was examined at field sites in central BC, Canada. Host trees were accessed by a single-rope climbing technique and foliage as well as arboreal macrolichen functional groups were sampled by branch height in eight random sample trees from each of two high (High Cyano) and two low (Low Cyano) cyanolichen abundance sites for a total of 32 sample trees. Natural abundances of stable isotopes of N (^{15}N, ^{14}N) and carbon (^{13}C, ^{12}C) were determined for aggregate host tree and epiphytic lichen samples, as well as representative samples of upper organic and soil horizons (Ae and Bf) from beneath host trees. As expected, N_2-fixing cyanolichens had 2–6-fold greater N-contents than chlorolichens and a $\delta^{15}N$ close to atmospheric N_2, while foliage and chlorolichens were more depleted in ^{15}N. By contrast, soils at all trees and sites were ^{15}N-enriched (positive $\delta^{15}N$), with declining (not significant) $\delta^{15}N$ with increased tree-level cyanolichen abundance. Lichen functional groups and tree foliage fell into three distinct groups with respect to $\delta^{13}C$; the tripartite cyanolichen *Lobaria pulmonaria* (lightest), host-tree needles (intermediate), and bipartite cyanolichens, hair (*Alectoria* and *Bryoria* spp.) and chlorolichens (heaviest). Branch height of host trees was an effective predictor of needle

$\delta^{13}C$. Our results showed a modest positive correlation between host tree foliage N and cyanolichen abundance, supporting our initial hypothesis that higher cyanolichen abundances would elevate host tree foliar N. Further study is required to determine if high cyanolichen abundance enhances host tree and/or stand-level productivity in sub-boreal forests of central BC, Canada.

Keywords: sub-boreal forest nitrogen; $\delta^{15}N$; $\delta^{13}C$; arboreal lichens; cyanolichens; lichen epiphyte nitrogen; *Lobaria pulmonaria*

1. Introduction

Nitrogen (N) is commonly limiting to conifer forests in much of the Pacific Northwest, USA [1] as well as the central interior of BC, Canada [2]. Biological N_2-fixing species have been shown to contribute and mediate important inputs of N to these forests [3]. In central British Columbia, mature spruce and fir forests, particularly in the wetter ecological zones, can have well-developed and vertically stratified communities of lichen epiphyte, with N_2-fixing cyanolichens predominating (when present) in the lower canopy branches [4,5]. This stratification and interaction between upper and lower canopy zones can be further altered by vertical gradients in lichen epiphyte guild to create significant variation in N content [4] and $\delta^{15}N$ [6] across a vertical profile of a host tree. Although the presence and guild of epiphytic lichens have been shown to enhance certain aspects of N-cycling, empirical evidence for their forest level significance has been equivocal. For example, the removal of lichens from an oak forest system had no effect on tree growth [7]. Thus, while atmospheric N contributions from cyanolichens are observed in sub-boreal spruce and fir ecosystems [8], it is difficult to predict the nature of combined cyanolichen and host conifer tree ecological interactions (*i.e.*, mutualistic to competitive) over tree to forest spatial and as well as temporal scales.

Stable isotopes have been used experimentally to infer many properties about elemental cycles of important nutrients (most notably C and N) at a variety of temporal and spatial scales and ecological contexts. The ratio of the heavier less common stable isotope to that of the lighter abundant isotope (e.g., ^{15}N:^{14}N and ^{13}C:^{12}C) can be expressed as atom %, but are typically represented in delta (δ) notation, which is not the absolute isotope ratio, but the difference between the sample measurement and an internationally accepted reference standard [9] in parts per thousand or per mil (‰) [10]. In theory, natural abundances of isotopes can be used to make inferences about the contributions of N_2-fixing species to forest trees by exploiting the naturally occurring differences in ^{15}N:^{14}N ratios between plant-available mineral N sources in the soil and that of atmospheric N_2 utilized by N_2-fixing species [11]. Non-N_2-fixing plants receive their entire N supply from soil N pools and can be expected to be isotopically heavier or lighter than N_2-fixing plants and lichens in concordance with the soil $\delta^{15}N$. In either case, natural abundances of stable N isotopes have frequently been used to estimate direct or indirect contributions of N_2-fixation to plant N content [12].

Most terrestrial materials have $\delta^{15}N$ compositions ranging between −20‰ and +30‰ [13], but lichens have a more restricted range of ^{15}N compositions (−21.5‰ [14]) to (+18‰ [15]), and plants even moreso (e.g., −5‰ to +2.9‰ [16]). The natural abundances and ratios of stable isotope

compositions are potentially useful to the researchers quantifying these processes in that they can shift due to isotopic fractionation.

Isotopic fractionation can occur from a variety of processes. For example, N_2-fixation in organisms such as epiphytic cyanolichens does not discriminate between the ^{15}N and ^{14}N and therefore would normally represent N isotope ratios close to the atmospheric $\delta^{15}N$ standard of 0‰. By contrast, non-N_2-fixing elements and processes of forest systems such as ectomycorrhizae (ECM), mineralization of organic nitrogen, nitrification, microbial assimilation of inorganic N, and denitrification can fractionate stable isotopes of N belowground. Nutrient uptake in temperate and boreal trees is predominantly dependent on ECM hyphae growing into the soil from the mycorrhizal root tips [17]. Högberg *et al*. [18] found that ECM roots of Norway spruce (*Picea abies*) and beech (*Fagus sylvatica*) were 2‰ enriched in ^{15}N relative to non-mycorrhizal roots. The study also found ECM fungi were enriched in ^{15}N compared to their host plants, further suggesting that ^{15}N ECM discrimination was fungal in origin. ECM fungi were also more enriched in ^{13}C relative to total soil C [19], demonstrating ^{13}C ECM discrimination. Biologically mediated reactions that control elemental dynamics in soils can result in ^{15}N and ^{13}C enrichment or depletion, making inferences on forest ecosystem N- and C-cycling based on natural abundances of stable isotopes challenging.

Bulk soil $\delta^{15}N$ values are generally higher than atmospheric N (positive, ~5–10‰), a result of faster losses of the lighter isotope in soil N during decomposition [20], usually with increasing (more positive) deltas with increasing depth of organic ('L', 'F', and 'H') and mineral horizons [21]. Thus, fractionation of isotopes during litter decomposition in forests causes surface soils to have lower $\delta^{15}N$ values than deeper soil horizons [22]. Even slight fractionations occurring over decades of transfers of N from mineral soil to forest biomass can be sufficient enough to increase $\delta^{15}N$ of soil organic matter by ~6–8‰ [23]. Surface soils located beneath trees have also been found to have lower $\delta^{15}N$ values than those in open areas as a result of litter deposition [24], making decomposing epiphytic lichens an important factor influencing soil N isotope composition.

Nitrogen-fixing plants in western North American forest systems typically have more positive $\delta^{15}N$ values than non-N_2-fixing plants, and/or are closer to the of atmospheric N_2 [25,26]. The differences between these plant $\delta^{15}N$ values provide the basis of the ^{15}N natural abundance technique for estimating fixed-N contributions to terrestrial ecosystems [24]. However, attempting to trace fixed-N through ecosystems can be complicated by a myriad of fractionations, which are caused by numerous and often serial pathways of mineralization, nitrification, immobilization, and denitrification within the soil, plant root and mycorrhizal fungus mediation of soil N uptake [27].

Nutrient cycling processes in the soil are known to vary with plant community composition [28]. The processes associated with litter decomposition and enzymatic transformations of organic substrates in particular can vary because of differences in a variety of factors such as chemistry of the litter material, soil biota and soil chemistry associated with different plant species [29]. Both litter accumulation and stem flow can deliver N to epiphytes from various N pools [6], making the abundance and branch position of lichens on a tree significant. Lichens may play an important role in nutrient cycling in forest ecosystems, but the relative impact of lichens compared to other ecosystem components is not well understood. The effect of lichens on forest N-cycling could range from large in ecosystems where epiphytic lichens, especially cyanolichens are abundant, to insignificant where lichens are only present in low amounts [30].

In this study, we examine the importance of the vertical distributions of arboreal lichen biomasses [5] to the N-status of wet sub-boreal spruce and fir forest ecosystems of the central interior of BC, Canada. To do this, we measured the ^{15}N:^{14}N, %N of host tree (*i.e.*, conifer) foliage, host tree epiphytic lichen functional groups and organic and mineral soil horizons under the tree crowns at sites containing variable amounts of cyanolichen tripartite (*i.e.*, *L. pulmonaria)* and bipartite lichens from relatively low to high levels. We hypothesized that sub-boreal forest trees with high cyanolichen abundance should have higher needle %N reflecting the greater inputs of biologically fixed-N into these systems [8,31]. Knowledge obtained from this study should provide valuable information on the functional importance of epiphytic (N_2-fixing) cyanolichens to the N-status of wet sub-boreal conifer ecosystems of central BC.

2. Experimental Section

2.1. Study Area

The study area was described previously by Kobylinski and Fredeen [5]. In brief, this field study was carried out on the north side of the Fraser River near the town of Upper Fraser, BC, located approximately 70 km NE of Prince George, BC. All study sites were characterized by having relatively cool and moist summers and cold, snowy winters. Four mature sub-boreal forest sites were chosen based on having trees with predominantly high (High Cyano) or low (Low Cyano) epiphytic cyanolichen abundance and diversity, denoted as 'Upper Fraser' and 'Herrick' in previous work [5,32], respectively.

The two dominant tree species representing the vast majority of the canopy trees at all four sites were interior hybrid spruce (*Picea engelmannii* Parry ex Engelm. x *glauca* (Moench)) and subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.) High Cyano sites were in "sub-boreal spruce" (SBS) 'wk1' and the 'vk' subzones (BC Biogeoclimatic Ecosystem Classification scheme) with a mean elevation of 680 m above sea level (a.s.l.), mean summer temperature of 11.8 ± 5.3 °C and relative humidity of 78% [8]. Low Cyano sites had lower average cyanolichen abundances and diversities and were located within 10–20 km of the High Cyano sites at a slightly higher mean elevation of 850 m a.s.l. and similar mean summer temperature and relative humidity of 10.8 ± 5.3 °C and 78%, respectively [8]. Soils at all sites were Orthic Humo-Ferric Podzols formed from sandy-colluvial materials at the High Cyano sites and from sandy–skeletal glaciofluvial materials at the Low Cyano sites. Average precipitation in the ecotonal study area is approximately 897 mm per year [8].

2.2. Canopy Access and Biomass Sampling

Sample tree selection, canopy access and biomass sampling procedures were previously described in Kobylinski and Fredeen [5]. In brief, trees were selected randomly from High Cyano and Low Cyano sites, with the exception of the fact that trees adjacent to Sitka alder (*Alnus viridis*) were excluded from the study given that annual N-inputs from Sitka alder can be substantial [26] and could have confounded ^{15}N natural abundance interpretation. All study trees were canopy trees and in excess of 22 m in height and 20 cm in DBH. Needles and lichens were sampled from each canopy height zone at the highest accessible point of each of the 32 study trees. Access into canopies was achieved through

a single rope technique [33,34]. Selected trees were rigged, climbed and assessed vertically for epiphytic lichen biomass.

Lichen and needle sampling was described previously [5]. Briefly, epiphytic lichen functional group or species and host tree needle samples were collected over two summers: June to August 2008 and May to September 2009 at various heights within sample tree canopies. All cyanolichens in these mature forest tree sites were almost exclusively arboreal and on branches, with negligible cyanolichen occurring on the forest floor [35]. Bryophytes were essentially absent from all canopies, as is normal for these forest types [35]. Lichen biomasses were separated into five primary categories or functional groups based on biomass dominance by single species or functional group properties within different vertical canopy zones: *Alectoria sarmentosa* (Ach.) Ach., *Bryoria* Brodo & D. Hawksw. species, foliose chlorolichens, bipartite cyanolichens, and *Lobaria pulmonaria* (L.) Hoffm., the only tripartite cyanolichen at the site. The needle cohort was taken exclusively from the previous year's foliage to keep the age of the sampled needles constant. A processed and homogeneous sample for each functional group or needle was sent for isotopic analysis from the upper, middle and lower canopy height zones provided they were present in that zone.

2.3. Composite Soil Sampling

Soil samples were collected from the base of each sample tree between June and August 2009. We extracted soil cores using a soil auger (7.5 cm in diameter) by rotating the auger while applying downward force and lifting out the full blades. Soil core samples were extracted from a 1-m radius around each sample tree base in each of the cardinal directions. Samples were separated into three distinct layers: F-folic layer composed of organic matter rich in mycelia (~5 cm ± 2 cm deep), Ae-grayish surface soil layer (~2–10 cm thick); and Bf—yellowish brown to reddish brown subsoil layer (~10–20 cm). All layers were distinct and could be easily separated from each other; the soil auger was cleaned between cores. The three layers of the four subsamples were air dried for two days at room temperature (~22–25 °C), roots were removed and soil sieved through a 2-mm sieve. The F-layer samples were separated twice more (0.85 mm and 0.3 mm sieves) to remove small pieces of woody debris before composite samples were prepared. Composite samples were also made from the four Ae and Bf subsamples from each site. A dry weight of 2 g from each of the three layers of the four samples was mixed into one composite sample per soil layer (8 g dry weight), for a total of 96 soil samples.

2.4. Stable Isotope Analyses

Sample preparation of lichen, needle and soil samples took place at the University of Northern British Columbia (UNBC) before samples were shipped to the Stable Isotope lab at the University of Saskatchewan (U of S) in Saskatoon, Canada for analysis. Lichen and needle samples were oven dried for three days at 55 °C in paper bags. Samples were ball-milled (Retsch MM301, Hann, Germany) to a particle size of less than 250 μm and any fibrous matter or visible granules removed to improve precision of isotopic analysis. Ball-mill chambers were cleaned with deionized water and dried between samples. Samples (2 ± 0.2 mg) were stored in scintillation vials and encapsulated in 8 × 5-mm tin capsules (Catalog # D1008, Elemental Microanalysis Limited, Okehampton, UK) before combustion.

Soil, needle and lichen samples were analyzed using an elemental analyzer (Costech ECS4010, Valencia, CA, USA) coupled to a Delta V Advantage isotope ratio mass spectrometer with continuous flow (Conflo IV, Thermoscientific, Waltham, MA, USA) interface. The continuous flow gas isotope ratio mass spectrometer was used to measure % N, % C and the relative abundance of stable isotopes (^{15}N, ^{14}N, ^{13}C, and ^{12}C) in needle, lichen, and soil samples. Isotope ratio calibrations were performed with IAEA-N1, IAEA-N2, IAEA-NO-3, IAEA-CH6, and USGS-24 standards (International Atomic Energy Agency, 1995).

2.5. Natural Abundance Method

The δ values for ^{15}N and ^{13}C were calculated using equations 1 and 2, respectively.

$$(\delta\ ^{15}N\ (‰\ \text{relative to atmospheric}\ N_2) = (R_{sample}/R_{standard} - 1) \times 1000) \quad (1)$$

$$(\delta\ ^{13}C\ [‰\ \text{relative to V-PDB}] = (R_{sample}/R_{standard} - 1) \times 1000) \quad (2)$$

The value *R* is the ratio of heavy isotope (^{15}N and ^{13}C) to their lighter more abundant isotope.

2.6. Statistical Analyses

Data were analyzed using Sigma Plot 11 (Systat Software Inc., San Jose, CA, USA). Ordinary least square (OLS) multiple linear regression analyses were performed to address whether there were statistically significant differences in: (1) $\delta^{15}N$ and $\delta^{13}C$ between lichen functional groups and needles controlling for tree branch height, tree species, and lichen abundance; (2) $\delta^{15}N$ and $\delta^{13}C$ by tree branch height controlling for lichen functional groups and needles; (3) $\delta^{15}N$ and $\delta^{13}C$ between High and Low Cyano sites controlling for lichen functional groups, needles and soil; (4) $\delta^{15}N$ and $\delta^{13}C$ between tree species (spruce and fir) controlling for the lichen functional groups, needles and soil; and (5) foliar (needle) %N at sites with higher abundances of cyanolichen. The unstandardized coefficient (B) for OLS linear regression analyses provide an estimate of the change in the dependent variable associated with a one-unit change in the identified independent variable controlling for all other independent variables.

Six OLS regression models were created to predict: (i) $\delta^{15}N$ in lichen functional groups; (ii) $\delta^{15}N$ in needles; (iii) $\delta^{13}C$ in lichen functional groups; iv) $\delta^{13}C$ in needles; (v) $\delta^{15}N$ in soil; and (vi) $\delta^{13}C$ in soil. Correlation analyses were conducted between all the variables in each model to check for potential collinearity. Additional correlations were also conducted to look at the relationships between needle $\delta^{15}N$ and cyanolichen abundance, % N and cyanolichen abundance, and soil $\delta^{15}N$ and cyanolichen abundance.

Kolmogorov-Smirnov and Shapiro-Wilk tests were used to determine normality of data. Both tests showed lichen and needle $\delta^{15}N$ ($KS = 0.191$, $p < 0.001$ and $W = 0.886$, $p < 0.001$) and $\delta^{13}C$ ($KS = 0.122$, $p < 0.001$ and $W = 0.949$, $p < 0.001$) were not normally distributed. However, because of bimodal distributions of the dependent variable, data transformations were not used. The distribution of lichen and needle $\delta^{15}N$ and $\delta^{13}C$ (but not soil) showed some signs of heteroscedasticity and non-linearity across all independent variables.

3. Results

Nitrogen contents varied by over six-fold among lichen epiphytes sampled. At the extremes, *Alectoria sarmentosa* had the lowest % N (0.50 ± 0.13) and bipartite cyanolichens had the greatest %N (3.24 ± 0.52) on a dry weight basis (Figure 1). *Bryoria* spp. and foliose chlorolichens had very similar %N contents of 0.77 ± 0.18 and 0.73 ± 0.19, respectively. Needles had a higher %N content (1.11 ± 0.14) than hair lichens and foliose chlorolichens but not as high as *L. pulmonaria* (2.26 ± 0.30). The differences in mean %N between functional groups were significant ($F_{5,404}$ = 950.3, $p < 0.001$: Tukey test) except for *Bryoria* spp. and foliose chlorolichens (Figure 1).

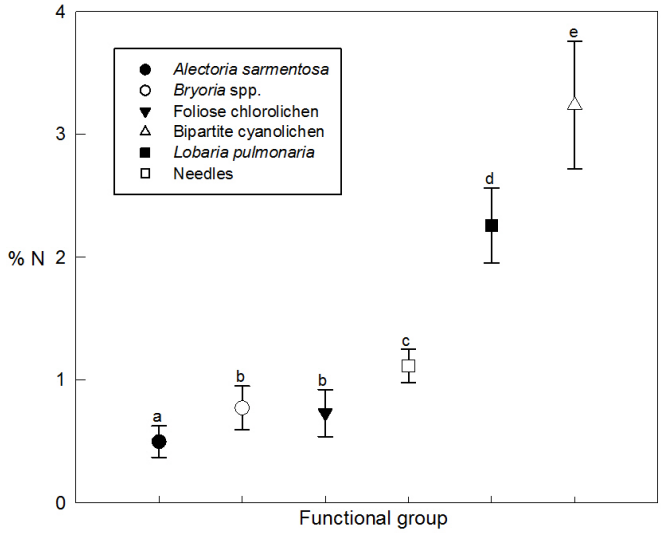

Figure 1. Mean %N (± SD) of lichen biomass in functional groups of lichen and foliage (needle) from 32 spruce and fir study trees with varying amounts of tree-level cyanolichen abundance. Different letters above error bars indicate significant ($p < 0.001$) differences between lichen functional groups (Tukey multiple mean comparison test).

All lichen functional groups or species and conifer foliage (needle) had mostly unique $\delta^{15}N$ signatures relative to their %N (Figure 2). *Bryoria* spp. hair lichens at the top of the canopy had the lowest $\delta^{15}N$, while the hair lichen *Alectoria sarmentosa* found lower in the canopy had slightly lower %N and higher $\delta^{15}N$ than *Bryoria*. Host tree needles and foliose chlorolichens had $\delta^{15}N$ values that were intermediate between hair lichens and cyanolichens but similar %N. While *L. pulmonaria* and bipartite cyanolichens both had $\delta^{15}N$ values close to atmospheric N_2 ($\delta^{15}N = 0$), *L. pulmonaria* had 1.5-times less N on average (Figure 2). Mean $\delta^{15}N$ isotope values were slightly higher at Low Cyano sites for all lichen functional groups and host tree needles, but there was no significant difference in the $\delta^{15}N$ values or %N of lichens or needles on trees from High Cyano (black symbols) *versus* Low Cyano (white symbols) sites (Figure 2).

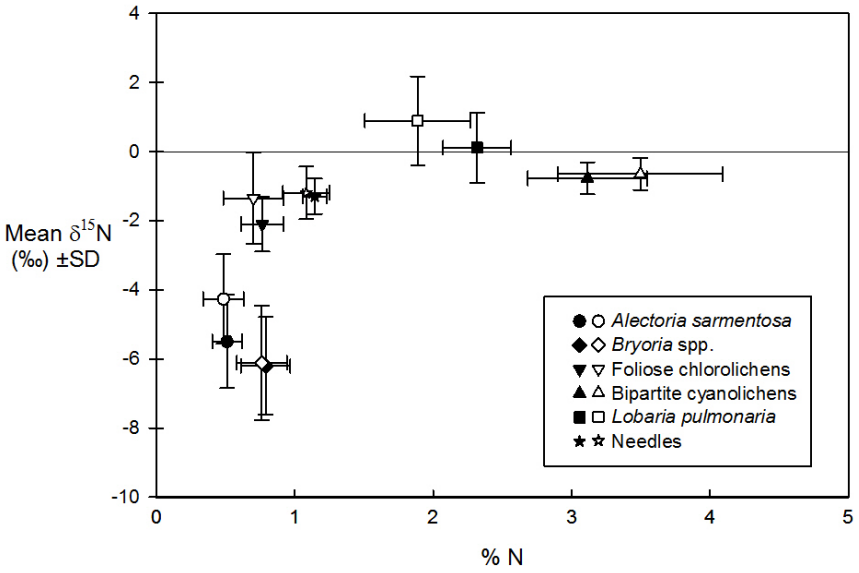

Figure 2. Mean (± SD) % N and $\delta^{15}N$ signatures of five epiphytic lichen and host tree foliage (needle) biomasses across four sites and two host tree species from sub-boreal spruce and fir forests in central interior BC. Half of the host trees were from high (High Cyano: black symbols) and the other half from low (Low Cyano: white symbols) cyanolichen abundance sites.

When we compared $\delta^{15}N$ of living conifer foliage (needle) on each of the 32 study trees to their actual cyanolichen abundance, there was a small, but ultimately not significant ($r = -0.16$, $p = 0.121$) negative correlation between mean needle $\delta^{15}N$ and increasing cyanolichen abundance (Figure 3a). Host tree foliage (needles) %N showed a significant but weak positive correlation to actual tree-level cyanolichen abundance ($r = 0.24$, $p = 0.019$; Figure 3b).

When lichen species, tree branch height, and tree species were controlled for, cyanolichen abundance in the canopy was a statistically significant but ineffective predictor of $\delta^{15}N$ ($B < 0.001$, $p < 0.001$, Table 1). The regression coefficients of the dependent variable $\delta^{15}N$ suggest that lichen species and cyanolichen abundance, but not tree species or tree branch height, were significant predictors of $\delta^{15}N$ in lichen functional groups. The regression analyses also show that *A. sarmentosa* ($B = -3.657$, $p < 0.001$), *Bryoria* spp. ($B = -4.838$, $p < 0.001$) and foliose chlorolichen ($B = -0.482$, $p = 0.002$) tree-level abundances were all negatively related to their $\delta^{15}N$ signatures. Conversely, bipartite cyanolichens ($B = 0.561$, $p < 0.001$) and *L. pulmonaria* ($B = 1.608$, $p < 0.001$) tree-level abundances were positively related to their $\delta^{15}N$ signatures. Both tree species ($B = -0.166$, $p = 0.095$) and tree branch height ($B = 0.010$, $p = 0.238$) were not significantly related to $\delta^{15}N$ levels in lichen functional groups (data not shown) and insignificant or with a very small coefficient for $\delta^{15}N$ in needles ($B = 0.028$, $p = 0.002$). Similarly, tree species ($B = -0.146$, $p = 0.267$) and cyanolichen abundance (data not shown) were not significant predictors of $\delta^{15}N$ in lichen or host-tree foliage.

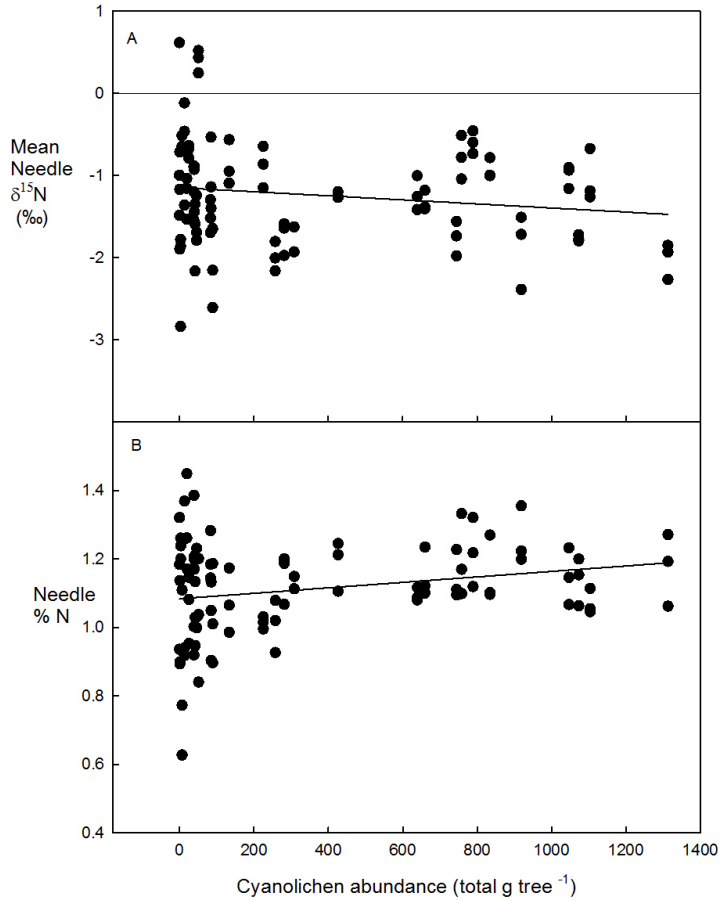

Figure 3. Mean (**a**) $\delta^{15}N$ and (**b**) %N of host tree foliage (needle) from low to high tree-level cyanolichen abundance (total g tree^{-1}).

Table 1. Linear regression (OLS) coefficients with standard error for the regression model predicting $\delta^{15}N$ of lichen functional groups (F = 220.501, $p < 0.001$, $R^2 = 0.817$) and host tree needles (F = 4.363, $p = 0.006$, $R^2 = 0.126$) based on tree species (spruce and fir), cyanolichen abundance, tree branch height, and lichen species or functional group.

	Lichen Functional Groups (Model i)		**Needles (Model ii)**	
	Unstandardized coefficient (B)	Std Error	Unstandardized coefficient (B)	Std Error
Intercept	−1.126 ***	0.165	−1.499 ***	0.171
Tree species (Spruce)	–0.166	0.099	−0.146	0.131
Tree branch height	0.010	0.008	0.028 **	0.009
Alectoria sarmentosa	−3.657 ***	0.168		
Bryoria spp.	−4.838 ***	0.183		
Foliose chlorolichen	−0.482 **	0.156		
Bipartite cyanolichen	0.561 ***	0.137		
Lobaria pulmonaria	1.608 ***	0.165		

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Soil horizon (F, Ae and Bf) $\delta^{15}N$ values were consistently higher at Low Cyano (means: F = 1.340, Ae = 5.130, Bf = 6.262) than at High Cyano (F = 0.782, Ae = 4.765, Bf = 5.383) sites, and decreased

with increasing cyanolichen abundance in all three horizons (Figure 4A–4C), respectively. The abundances of cyanolichens and $\delta^{15}N$ were negatively correlated in all three soil horizons, with correlations being nearly significant for the uppermost horizons (F: $r = -0.341$, $p = 0.056$; Ae: $r = -0.263$, $p = 0.146$), and significant for the Bf horizon ($r = -0.356$, $p = 0.045$). Total %N in the F-horizon decreased with increasing tree-level cyanolichen abundance (Figure 4D), but was relatively unaffected in Ae and Bf horizons (Figure 4E and 4F).

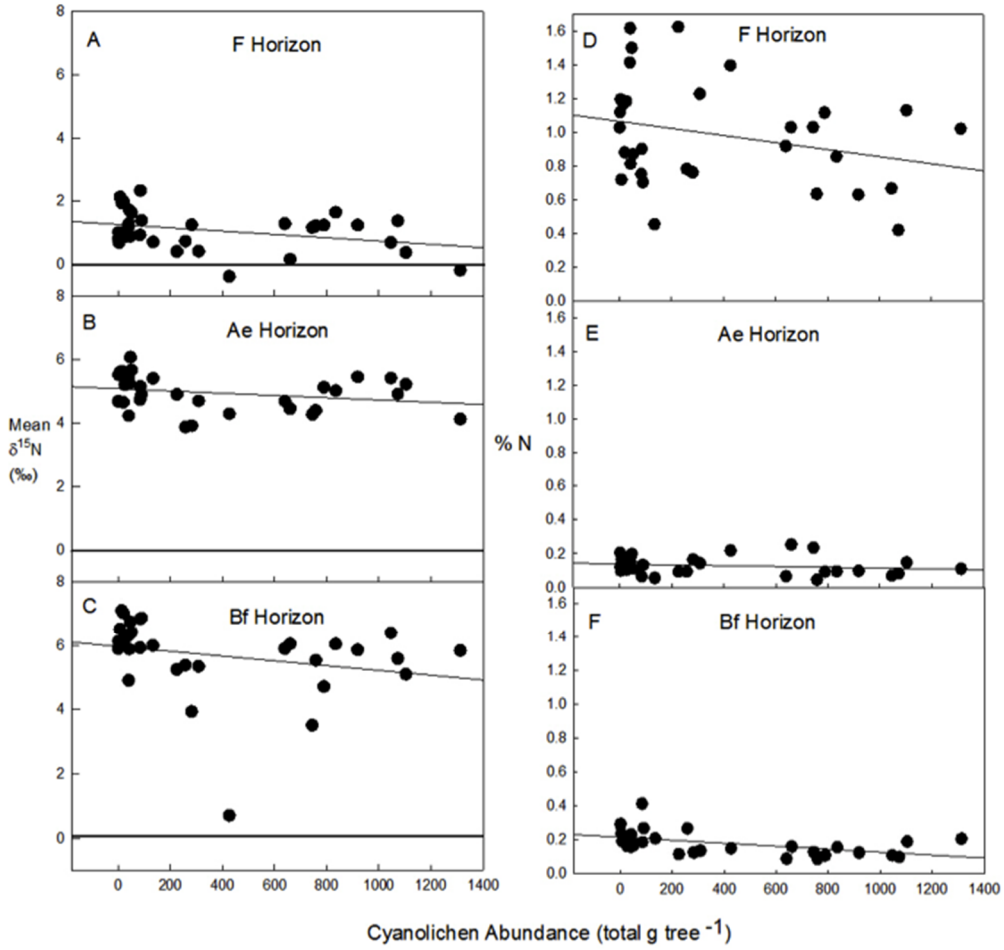

Figure 4. Mean $\delta^{15}N$ and %N of (A and B) the surface organic F horizons, (B and E) upper-most mineral (Ae) horizons and (C and F) lower mineral (Bf) horizons, respectively, beneath host trees with varying levels of cyanolichen abundance (total g tree^{-1}).

Tree species and both upper mineral soil horizons were all significant predictors and all contributed to the overall relationship of both soil $\delta^{15}N$ and $\delta^{13}C$ (Table 2). Cyanolichen abundance was a significant predictor for both soil $\delta^{15}N$ and $\delta^{13}C$, but coefficients were <0.01 and therefore not relevant (data not shown). There was also a statistically significant difference in soil $\delta^{15}N$ ($B = -0.525$, $p < 0.001$) and soil $\delta^{13}C$ ($B = -0.221$, $p = 0.009$) between host tree species (spruce and fir).

Table 2. Linear regression (OLS) coefficients with standard error for the regression model predicting $\delta^{15}N$ (F = 314.044, $p < 0.001$, $R^2 = 0.932$) and $\delta^{13}C$ (F = 117.897, $p < 0.001$, $R^2 = 0.838$) of soil based on tree species, and Ae and Bf soil horizons.

	$\delta^{15}N$		$\delta^{13}C$	
	Unstandardized coefficient (B)	Std. Error	Unstandardized coefficient (B)	Std. Error
Intercept	1.586 ***	0.140	−26.923 ***	0.094
Tree species	−0.525 ***	0.123	−0.221 **	0.062
Mineral surface soil (Ae)	3.887 ***	0.145	1.523 ***	0.097
Mineral subsoil (Bf)	4.777 ***	0.145	1.961 ***	0.097

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

When controlling for all independent variables, tree species and branch height were significant predictors of $\delta^{13}C$ of host-tree foliage (needles) and to lesser extents for lichen epiphytes (Table 3 and Figure 5A). Trends of increasing (less negative) $\delta^{13}C$ values with increasing branch height were evident for both *L. pulmonaria* and tree foliage (Figure 5A). There was evident clumping of $\delta^{13}C$ into three distinct groups (Figures 5A and 6). Specifically, $\delta^{13}C$ values of the lone tripartite cyanolichen (*L. pulmonaria*) were lower than host tree needles, which were in turn lower than hair lichens (*A. sarmentosa* and *Bryoria* spp.), bipartite cyanolichens and foliose chlorolichens.

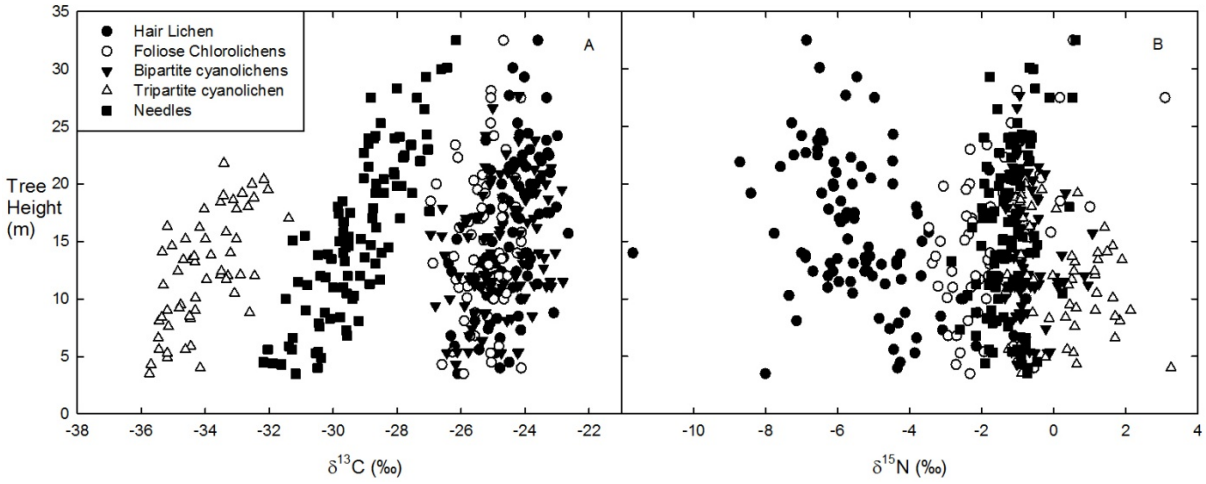

Figure 5. Variation in the natural abundances of (**A**) $\delta^{13}C$ and (**B**) $\delta^{15}N$ of five epiphytic lichen functional group and host tree foliage (needle) biomasses according to tree branch height for all sites.

Table 3. Linear regression (OLS) coefficients with standard error for the regression model predicting $\delta^{13}C$ in lichen functional groups (F = 711.517, $p < 0.001$, $R^2 = 0.942$) and needles (F = 79.545, $p < 0.001$, $R^2 = 0.724$) based on tree species (spruce), tree branch height and lichen functional groups.

	Lichen Functional Groups (Model iii)		**Needles (Model iv)**	
	Unstandardized coefficient (B)	Std. Error	Unstandardized coefficient (B)	Std. Error
Intercept	−30.523 ***	0.154	−31.868 ***	0.204
Tree species (Spruce)	0.048	0.089	0.622 ***	0.156
Tree branch height	0.093 ***	0.008	0.148 ***	0.011
Alectoria sarmentosa	4.899 ***	0.151		
Bryoria spp.	4.513 ***	0.164		
Foliose chlorolichen	3.969 ***	0.140		
Bipartite cyanolichen	4.324 ***	0.166		
Lobaria pulmonaria	−4.358 ***	0.149		

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Hair lichens *A. sarmentosa* and *Bryoria* spp. had uniformly negative $\delta^{15}N$ values that were also more negative than all other functional groups (Figure 5B), and trending lower with increase in branch height. The $\delta^{15}N$ of other lichen functional groups and host-tree foliage did not appear to be influenced by branch height.

Host tree needles had $\delta^{13}C$ values intermediate to *L. pulmonaria* and all other lichen functional groups (Figure 6), but at distinctly higher C concentrations than all lichen groups (Figure 7). Lichen functional groups, cyanolichen abundance, tree branch height, and the interaction between cyanolichens and site cyanolichen abundance (but not tree species) were significant predictors of $\delta^{13}C$ in lichen functional groups. The regression analyses showed that *A. sarmentosa* (B = 4.899, $p < 0.001$), *Bryoria* spp. (B = 4.513, $p < 0.001$), foliose chlorolichens (B = 3.969, $p < 0.001$), and bipartite cyanolichens (B = 4.324, $p < 0.001$) were positively related to $\delta^{13}C$ levels in lichen functional groups (Table 2). Conversely, *L. pulmonaria* (B = −4.358, $p < 0.001$) was negatively related to $\delta^{13}C$ levels in lichen functional groups. Both cyanolichen abundance (B = −0.001, $p < 0.001$) and tree branch height (B = 0.093, $p < 0.001$) were significantly related to $\delta^{13}C$ levels in lichen functional groups. There was also a statistically significant interaction between cyanolichens and site cyanolichen abundance and $\delta^{13}C$ increased slightly as site-level cyanolichen abundance increased (B = 0.001, $p = 0.005$, Table 2). There was no significant difference in $\delta^{13}C$ between sample tree species spruce and fir in lichen functional groups (B = 0.048, $p = 0.594$, Table 2). Alternatively, both tree species (B = 0.622, $p < 0.001$) and tree branch height (B = 0.148, $p < 0.001$), but not cyanolichen abundance (B < 0.001, $p = 0.942$), were significant predictors of $\delta^{13}C$ in needles (B = 0.028, $p = 0.002$).

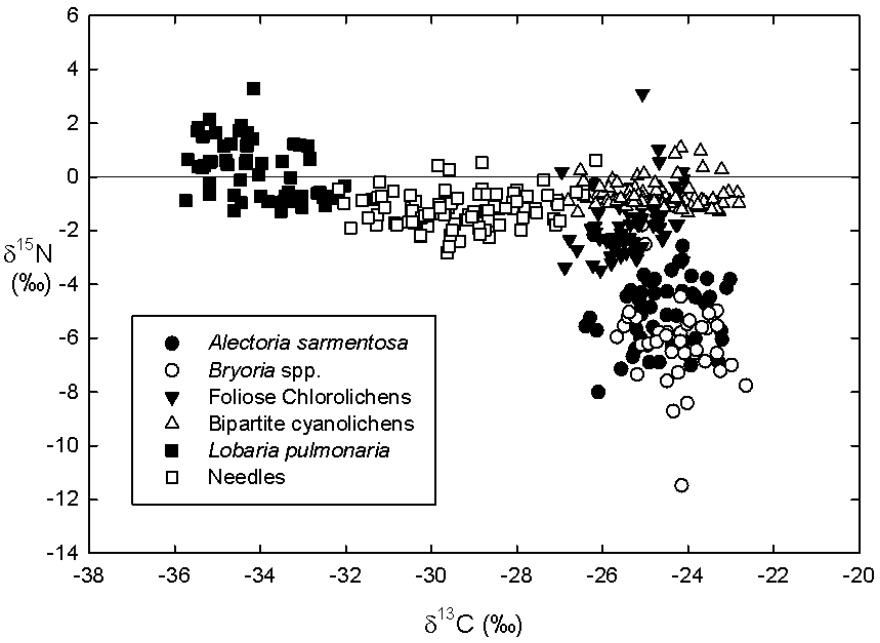

Figure 6. Dual natural abundance isotope plot of $\delta^{15}N$ *versus* $\delta^{13}C$ of the five epiphytic lichen functional group and host tree foliage (needle) biomasses across four sites and two host tree species.

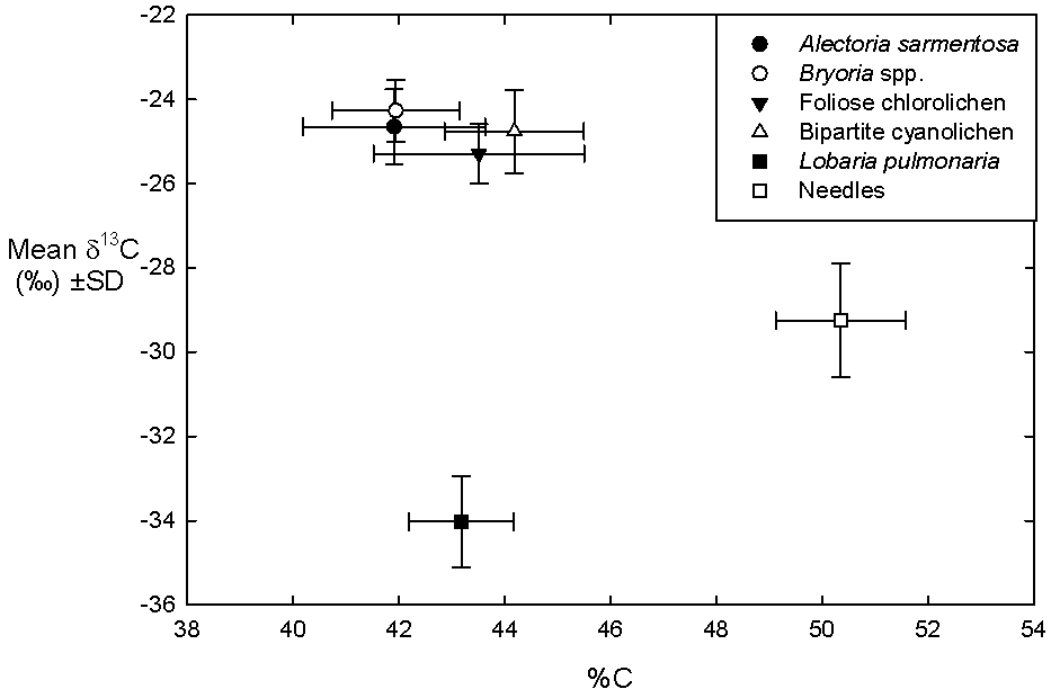

Figure 7. Mean (± SD) C concentrations *versus* $\delta^{13}C$ signals of five epiphytic lichen functional group and host tree foliage (needle) biomasses across four sites and two host tree species.

4. Discussion

Nitrogen-fixing cyanolichen epiphytes can be both diverse and abundant in conifer forests of central BC, and have been conjectured to be an important component of the N-cycle in these forest

locations [4,8,32,36]. Although lichen N pool sizes and decomposition rates in these studies were indicative of enhanced mineral N flux rates, as observed in other forests types [7], direct links between cyanolichen abundance and improved host tree N status have been elusive. In our study, gradients in cyanolichen abundance across host trees (interior hybrid spruce and subalpine fir) in two generally high cyanolichen abundance sites and two generally low cyanolichen abundance sites [8,32] provided us with an opportunity to examine the potential for enhanced cyanolichen N-inputs into these forests in central BC. We hypothesized that foliar (needle) N contents would be positively correlated with cyanolichen abundance, and in fact this was found to be the case, albeit the relationship was not strong (Figure 3b). We further sought to more directly link increased host tree foliar N with cyanolichen N using the natural abundances of ^{15}N and ^{14}N of forest tree, lichen and soil components of these systems.

Our results corroborate previous findings of $\delta^{15}N$ values in lichen functional groups and or species. It is already known that cyanolichens that fix N_2 typically have $\delta^{15}N$ values close to atmospheric N_2, *i.e.*, 0‰ [37,38]. We also found $\delta^{15}N$ values close to 0‰ for both bipartite and tripartite (*L. pulmonaria*) cyanolichens in our study. By contrast, other non-N_2-fixing arboreal macrolichens (hair and chlorolichen) as well as host-tree foliage all had $\delta^{15}N$ values that were negative and less enriched in ^{15}N than either cyanolichen functional group. Hair lichens had the lowest $\delta^{15}N$, while chlorolichens and host tree foliage were intermediate. Interestingly, *A. sarmentosa*, occurring lower in the canopy than *Bryoria* spp., and therefore overlapping with cyanolichen canopy zones [5], had $\delta^{15}N$ closer to atmospheric $\delta^{15}N$ (*i.e.*, 0) (Figure 2). This is consistent with *A. sarmentosa* receiving more leached-N from cyanolichens than *Bryoria* spp., and supported by the fact that an increase in the $\delta^{15}N$ of *A. sarmentosa* was observed in High Cyano sites, while relatively little change in $\delta^{15}N$ was observed for *Bryoria* spp. In both hair lichens, relatively low N contents and low $\delta^{15}N$ (~–6 ‰ in Low Cyano sites) were consistent with low atmospheric N inputs in central BC [39] and the negative $\delta^{15}N$ of precipitation N measured in other studies [31].

Although biological N_2-fixation may represent a significant N input into ecosystems, both via leaching (less well documented) and decomposition, it may be difficult to identify because of its small relative isotopic effect against the background of ecosystem component $\delta^{15}N$ signatures [12]. Nitrogen isotope signatures of epiphytes varied across functional groups and with canopy position, but the reason for lichen $\delta^{15}N$ variability with canopy position in most studies remains unexplained. The $\delta^{15}N$ of hair lichens, with single sources of N (*i.e.*, atmospheric fixed-N), are relatively easy to explain relative to other lichens (cyanolichen and chlorolichen) lower in the canopy where inputs and outputs are more complex. At least 10 processes have been identified that can alter $\delta^{15}N$ values, none of which can currently be separated out in field studies [9]. Key explanations for $\delta^{15}N$ variability in lichens are: (i) a preference for uptake of the lighter ^{14}N isotope which can then lead to $\delta^{15}N$ enrichment of outputs [40]; (ii) fractionation of N isotopes in gaseous phase ammonia is greater than in the liquid phase of nitrate; and (iii) transfer of organic N which can result in increased ^{15}N depletion of the photobiont and less depletion in the mycobiont [41]. In general, our results were in agreement with previous work showing that lichens with a green alga as their photobiont (all lichens except bipartite cyanolichens) showed greater relative ^{15}N depletion [42] (Figure 1).

Soil $\delta^{15}N$ values increased with soil depth in our study, consistent with results of Gebauer and Schulze [43]. In keeping with our expectations, we also observed decreasing trends in $\delta^{15}N$ with

increasing cyanolichen abundance in all soil horizons (Figure 4A–4E) equating to less positive soil horizon $\delta^{15}N$ values, more proximal to the $\delta^{15}N$ of cyanolichen biomasses which were uniformly close to zero. There are many reasons why soil $\delta^{15}N$ wouldn't necessarily be closely correlated with tree $\delta^{15}N$. First, measured $\delta^{15}N$ of soil pools may not represent the true isotopic composition of N available to plant roots, since most of the N in soils is bound in forms that are not immediately available to plants [25,44]. Second, only a few % of total soil N becomes available in a year [18], and symbiotic fungi (mycorrhizae) can alter the $\delta^{15}N$ of the N transferred from soil to host plant. Nevertheless, a similar downward trend in tree foliage $\delta^{15}N$ at our sites with increasing abundance of cyanolichen (Figure 3) was consistent with the downward trend in soil $\delta^{15}N$ (Figure 4A–4C). Explaining the increase in %N of host tree foliage with increased cyanolichen abundance is difficult to reconcile with total soil N. If greater cyanolichen inputs of N increased soil N concentrations, then a straightforward mass action of greater plant N uptake could have explained enhanced foliar %N. However, soil N decreased in all soil horizons, though not significantly, with higher cyanolichen abundance (Figure 4D–4F). Since total soil N does not in any way equate with amounts and forms of available inorganic N, it is possible that more readily available N fractions were more available at high abundance cyanolichen sites, even though total %N was not. Further work on soil N at these sites would be required to test this hypothesis.

Foliar (needle) $\delta^{15}N$ values in our host trees ranged between 0.6 and −2.8‰ and did not differ significantly between High and Low Cyano sites or tree branch height. However, there was a trend of decreasing foliar $\delta^{15}N$ with increasing cyanolichen abundance (Figure 3a). Gebauer and Schulze [43] reported lower, but overlapping, $\delta^{15}N$ values for conifer needles (between −2.5 and −4.1‰), which varied according to stand and age, with foliage from the healthiest site having the lowest $\delta^{15}N$. It is unclear why the range of $\delta^{15}N$ values observed by Gebauer & Schulze were more negative than ours, but one possibility would be the presumed greater atmospheric N inputs in these European forests when compared to the N-limited forests of central BC. Gebauer and Schulze [43] also reported a similar trend of $\delta^{13}C$ values of needles, which ranged between −26.2 and −32.2‰ and did not differ between lichen abundance sites but did change with canopy height. Similar to our study, needle $\delta^{13}C$ values were not significantly different between abundance sites (Table 3), were more negative in the lower canopy and increasing with branch height (Figure 5).

Lichen $\delta^{13}C$ values were previously found to vary widely over a large range of habitats and species [45]. Our lichen functional groups and tree foliage (needles) fell into three distinct groups with respect to $\delta^{13}C$ values: the tripartite (and only cephalodic) cyanolichen *L. pulmonaria* with the lowest $\delta^{13}C$ values (−36 to −31), host tree foliage with intermediate $\delta^{13}C$ values (−33 to −26), and all other lichens including bipartite cyanolichens, hair lichens and chlorolichens with the least negative $\delta^{13}C$ values (−28 to −23). Our values are entirely consistent with those of Maguas *et al*. [46], namely that tripartite lichen associations typically have the lowest and bipartite lichens the highest $\delta^{13}C$. Carbon isotope discrimination in lichens [42] has been attributed to the species of photobiont present. Carbon concentrations also clearly distinguished between conifer foliage (mean %C of ~50.5%) and all lichen functional groups (means of %C ranging from ~42%–45%). This range of values are in close agreement with %C values observed previously for epiphytic lichens in sub-boreal BC [8] and conifer foliage [47], respectively.

Interestingly, we observed positive relationships between tree branch height and $\delta^{13}C$ values for both *L. pulmonaria* and foliage. Possible causes that may be responsible for the observed height-specific differences in these $\delta^{13}C$ values are CO_2 source, light level and factors influencing diffusion resistance such as water availability. Carbon dioxide source influences the $\delta^{13}C$ value of lichens [48,49], and lichens growing in the canopy close to the forest floor (*i.e.*, *L. pulmonaria*) would more readily assimilate CO_2 enriched by soil respiration [46,50,51]. Light levels also influence photosynthesis and can alter the CO_2 gradient inside the lichen thallus. Inorganic carbon acquisition by lichen photobionts fluctuate greatly with moisture and light availability [52], and lichen hydration is known to affect $\delta^{13}C$ values. Increased $\delta^{13}C$ values have been reported for lichens in drier habitats [45] and on thinner branches, both being directly and indirectly associated with desiccation stress, respectively [53]. Water stress would be expected to increase with branch height. Therefore, differences in $\delta^{13}C$ likely exist due to biological traits (e.g., foliage (stomatal) *versus* lichen, cephalodic *versus* non-cephalodic lichen), different microclimatic conditions in the canopy and/or the uptake of isotopically different CO_2 sources. Irrespective of mechanism and ecological significance, the $\delta^{13}C$—in concert with the $\delta^{15}N$—are clearly useful in chemically distinguishing among epiphyte functional group and host tree foliage.

5. Conclusions

Stable isotope natural abundance techniques have been important tools for ecophysiology and ecosystem research for many decades now [10,18], but have infrequently been applied to determining the contributions that arboreal cyanolichens make to forest N-cycling. Despite the narrow geographic and temporal scope of this study, the results from our study support previous work at our high and low cyanolichen abundance sites [8,32], suggesting that trees with a high abundance of arboreal cyanolichens provide greater N-inputs into N-limited conifer forests of central BC. High cyanolichen abundance, typically coupled with higher overall arboreal lichen diversity, is usually constrained to mature and old-growth stands in drier parts of the central interior of BC. However, the reasons for the variability in cyanolichen abundance at the tree and site level, and the contributions that cyanolichen abundances make to tree and forest growth remain uncertain and are the topics of current and future research at our forest research sites. Low-input, extensive forestry practiced in central British Columbia could benefit from this increased understanding.

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Author Contributions

Ania Kobylinski is the lead author and researcher. She climbed nearly half of the experimental trees, conducted the field sampling, prepared samples for analyses, and collated and analyzed data. This work comprised one chapter in her M.Sc. thesis. Art Fredeen conceived of the fundamental research questions, acquired the research funding, helped in decision-making regarding sampling, analyses and data processing, and helped in the editing and/or writing of the thesis chapter and manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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