



# Atmospheric nitrogen enrichment changes nutrient stoichiometry and reduces fungal N supply to peatland ericoid mycorrhizal shrubs



Risto Vesala<sup>a,\*</sup>, Heikki Kiheri<sup>a,b</sup>, Erik A. Hobbie<sup>c</sup>, Netty van Dijk<sup>d</sup>, Nancy Dise<sup>d</sup>, Tuula Larmola<sup>a</sup>

<sup>a</sup> Natural Resources Institute Finland (Luke), Finland

<sup>b</sup> Department of Microbiology, Faculty of Agriculture and Forestry, University of Helsinki, Finland

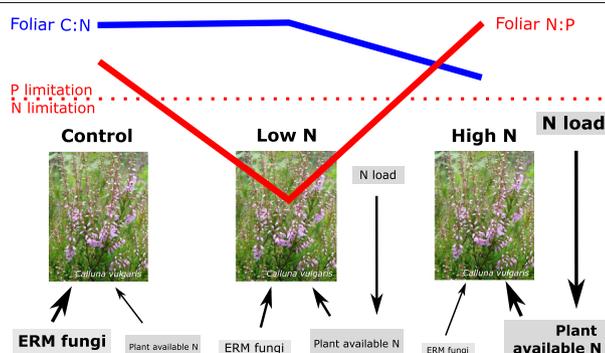
<sup>c</sup> Earth Systems Research Center, University of New Hampshire, United States

<sup>d</sup> UK Centre for Ecology & Hydrology (UKCEH), Edinburgh, UK

## HIGHLIGHTS

- Simulated N deposition decreased ericoid mycorrhizal activity in peatland shrubs.
- High-N treatments alleviated nitrogen and low-N treatments phosphorus deficiency.
- Decrease in ERM activity alter C/N cycling and may affect peat microbial communities.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Peatlands store one third of global soil carbon (C) and up to 15% of global soil nitrogen (N) but often have low plant nutrient availability owing to slow organic matter decomposition under acidic and waterlogged conditions. In rainwater-fed ombrotrophic peatlands, elevated atmospheric N deposition has increased N availability with potential consequences to ecosystem nutrient cycling. Here, we studied how 14 years of continuous N addition with either nitrate or ammonium had affected ericoid mycorrhizal (ERM) shrubs at Whim Bog, Scotland. We examined whether enrichment has influenced foliar nutrient stoichiometry and assessed using N stable isotopes whether potential changes in plant nutrient constraints are linked with plant N uptake through ERM fungi versus direct plant uptake. High doses of ammonium alleviated N deficiency in *Calluna vulgaris* and *Erica tetralix*, whereas low doses of ammonium and nitrate improved plant phosphorus (P) nutrition, indicated by the lowered foliar N:P ratios. Root acid phosphatase activities correlated positively with foliar N:P ratios, suggesting enhanced P uptake as a result of improved N nutrition. Elevated foliar  $\delta^{15}\text{N}$  of fertilized shrubs suggested that ERM fungi were less important for N supply with N fertilization. Increases in N availability in peat porewater and in direct nonmycorrhizal N uptake likely have reduced plant nitrogen uptake via mycorrhizal pathways. As the mycorrhizal N uptake correlates with the reciprocal C supply from host plants to the soil, such reduction in ERM activity may affect peat microbial communities and even accelerate C loss via decreased ERM activity and enhanced saprotrophic activity. Our results thus introduce a previously unrecognized mechanism for how anthropogenic N pollution may affect nutrient and carbon cycling within peatland ecosystems.

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\* Corresponding author at: Finnish Museum of Natural History, University of Helsinki, Finland.  
E-mail address: [risto.vesala@helsinki.fi](mailto:risto.vesala@helsinki.fi) (R. Vesala).

## 1. Introduction

Northern peatlands accumulate carbon (C) and nitrogen (N) in peat as microbial decomposition is largely suppressed by waterlogged conditions, lack of oxygen, and low pH and temperature (Clymo, 1984; Gorham, 1991; Moore et al., 2005; Kayranli et al., 2010). Nitrogen and other nutrients are tightly bound in organic compounds and thus peatlands are nutrient-poor environments for plants (Aerts et al., 1992; Bridgman et al., 1996, 1998). Ombrotrophic bogs are isolated from mineral soils and rely on N derived from atmospheric sources only, either via deposition or biological N<sub>2</sub> fixation. Similarly, phosphorus (P) originates solely from atmospheric deposition and often limits plant growth and ecosystem productivity together with N (Aerts et al., 1992; Bridgman et al., 1996; Wang et al., 2016). Additionally, living mosses effectively capture newly deposited nutrients, which then become available for vascular plants only after peat decays (Pastor et al., 2002; Limpens et al., 2006).

Many vascular plants growing in nutrient-limited environments can bypass N mineralization by assimilating organic N when available as free amino acids or short peptides (Kielland, 1994; Lipson and Näsholm, 2001; Näsholm et al., 2009; Tegeder and Rentsch, 2010). Evergreen shrubs of the Ericaceae use symbiotic ericoid mycorrhizal (ERM) fungi to access otherwise unavailable nutrient pools in soil organic matter (Smith and Read, 2008). ERM fungi can mobilize N from soil organic matter with their wide repertoire of extracellular proteases, lipases, and carbohydrate-active enzymes (Bajwa et al., 1985; Martino et al., 2018). Released simple peptides and amino acids are readily absorbed by the ERM mycelium and N is provided to host plants in exchange for photosynthesized carbohydrates (Stribley and Read, 1980; Smith and Read, 2008). In culture studies, ERM fungi also produce phosphatases and can transport inorganic phosphorus (P) to plant roots (Pearson and Read, 1973, 1975), indicating that ERM symbionts also improve P nutrition of their hosts.

During the last 150 years, anthropogenic N deposition has increased the availability of inorganic N (Vitousek, 1997; Galloway et al., 2004). This has changed nutrient limitations in many terrestrial and aquatic ecosystems (e.g. Aerts et al., 1992; Elser et al., 2009; Crowley et al., 2012; Chen et al., 2020). In ombrotrophic peatlands, increasing atmospheric N deposition is effectively buffered by *Sphagnum* mosses that intercept ammonium (NH<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) from precipitation (Aerts et al., 1992; Lamers et al., 2000; Chiwa et al., 2016) and assimilate newly deposited inorganic N into biomass. Thus, despite the increases in inorganic N inputs, nitrogen concentration in peat porewater at first remains largely unaffected (Lamers et al., 2000; Bragazza et al., 2005; Chiwa et al., 2016). If the amount of N deposition exceeds a certain level, the moss layer eventually becomes N-saturated. When the moss filtering fails, virtually all deposited N leaks to porewater. A critical deposition rate of 2.0 g N m<sup>-2</sup> y<sup>-1</sup> has been proposed for the *Sphagnum* layer to reach N saturation (Lamers et al., 2000; Harmens et al., 2014). Somewhat higher levels (up to 3.2 g N m<sup>-2</sup> y<sup>-1</sup>) were, however, suggested by Chiwa et al. (2016) based on 11 years of simulated N deposition in a long-term experiment at Whim Bog, Scotland (Sheppard et al., 2004, 2014).

Here, we used the Whim Bog N manipulation experiment to study how different levels of N additions and subsequent changes in porewater N concentrations are reflected in mineral nutrition and nutrient acquisition strategies of vascular plants. Our primary interest was in ericoid mycorrhizal shrubs, *Calluna vulgaris* (L.) and *Erica tetralix* (L.), as their metabolism is directly linked to below-ground carbon and nutrient cycling via symbiotic ERM fungi. Increasing N availability in peat porewater may reduce the need of plants to access soil organic N sources and decrease their dependence on ERM symbionts. This, in turn, may reduce the below-ground C investment of plants (Hobbie and Hobbie, 2008; Högberg et al., 2010) which, together with improved N availability, may alter competition among the peat microbial community, potentially leading to increased saprotrophic activity and

accelerated C loss (Gadgil and Gadgil, 1975, Högberg et al., 2003, Averill and Hawkes, 2016, Fernandez and Kennedy, 2016). Negative correlations between the strength of N deposition and growth or abundance of mycorrhizal fungi have been documented especially in many forest ecosystems dominated by ectomycorrhizal (ECM) fungi (e.g. Wallenda and Kottke, 1998; Nilsson and Wallander, 2003; Treseder, 2004).

We studied plant C:N:P stoichiometry to assess how 14 years of experimental N addition have changed the nutritional constraints of the ERM shrubs. Fertilization experiments in peatlands have shown a stronger response of shrub biomass production and peat decomposition to N + PK than N only addition (e.g., Bragazza et al., 2012; Kivimäki et al., 2013; Larmola et al., 2013), suggesting that the plants become P (or N/PK) (co-)limited under high atmospheric N deposition. This, in turn, may lead to upregulation of enzymes involved in P uptake, which we studied by comparing foliar N:P stoichiometry with root acid phosphatase activity. In addition, we analyzed the ratios of the naturally occurring nitrogen stable isotopes (<sup>15</sup>N:<sup>14</sup>N, expressed as δ<sup>15</sup>N) in plant leaves to study whether experimentally increased N deposition has decreased the dependence of ERM shrubs on their mycorrhizal symbionts in N acquisition. ERM and ECM fungi provide their host plants with nitrogen depleted in <sup>15</sup>N compared to the available soil N resources (Hobbie and Högberg, 2012), and due to this, plants colonized with ERM or ECM fungi have typically markedly lower foliar δ<sup>15</sup>N than co-occurring nonmycorrhizal plants in N-limited environments (Michelsen et al., 1996, 1998; Hobbie et al., 2005; Craine et al., 2009). Decreases in mycorrhizal N supply, and a concurrent increase in direct root N uptake from peat porewater, should thus increase foliar δ<sup>15</sup>N. Based on these premises we hypothesized the following:

1. Nitrogen fertilization decreases foliar C:N and increases foliar N:P ratios, reflecting improved N nutrition.
2. Root acid phosphatase activity increases with the increasing foliar N:P ratio, reflecting more severe P deficiency with N deposition.
3. Changes in foliar C:N:P stoichiometry are stronger with the increasing N dose and most prominent under high-N treatments.
4. Foliar δ<sup>15</sup>N of ERM shrubs exposed to N fertilization are higher than in untreated control plants, reflecting reduced N supply from mycorrhizal fungi and upregulated direct uptake of N.
5. Reduction in mycorrhizal N supply is strongest under the highest N additions and reflect root-accessible N in peat porewater.

## 2. Materials and methods

### 2.1. Study site and experimental setup

The study was carried out at Whim Bog (Scotland, UK; 55°46'N, 3°16'W) where an N manipulation experiment has continued since 2002. The area has relatively low annual atmospheric background deposition of approximately 0.8 g N m<sup>-2</sup>, a mean annual temperature of 7.9 °C and total precipitation of 1141 mm (Levy et al., 2019). The plots (12.8 m<sup>2</sup> each) established in a randomized block design are treated with different annual doses (0, 0.8, 2.4, or 5.6 g m<sup>-2</sup>) of N in addition to background deposition, supplied either as ammonium (NH<sub>4</sub>Cl) or nitrate (NaNO<sub>3</sub>). In addition to N-only treatments, half of the plots receiving the lowest and highest N doses are additionally fertilized with phosphorus and potassium supplied as K<sub>2</sub>HPO<sub>4</sub> (N + PK treatments, see Table 1). Fertilization is sprayed gradually during ca. 120 experimental precipitation events per year, simulating natural rainfall. Further details on the experimental design are provided by Sheppard et al. (2004, 2014).

### 2.2. Sampling of plant material and nutrient and isotope analysis

Foliar samples of the ericoid mycorrhizal shrubs *Calluna vulgaris* L. and *Erica tetralix* L. and the nonmycorrhizal sedge *Eriophorum*

**Table 1**

Different treatment combinations of N doses (in addition to the 0.8 g N m<sup>-2</sup> background) and sources (ammonium or nitrate, either alone or together with PK) at Whim Bog manipulation experiment. The experiment has been ongoing continuously since 2002.

N dose (g N m <sup>-2</sup> y <sup>-1</sup> )	Treatments with N alone (number of plots)	Treatments with N + PK (number of plots)
Control (0)	No added fertilizers (4)	–
Low N (0.8)	NH <sub>4</sub> <sup>+</sup> (4), NO <sub>3</sub> <sup>-</sup> (4)	NH <sub>4</sub> <sup>+</sup> + PK (4), NO <sub>3</sub> <sup>-</sup> + PK (4)
Medium N (2.4)	NH <sub>4</sub> <sup>+</sup> (4), NO <sub>3</sub> <sup>-</sup> (4)	–
High N (5.6)	NH <sub>4</sub> <sup>+</sup> (4), NO <sub>3</sub> <sup>-</sup> (4)	NH <sub>4</sub> <sup>+</sup> + PK (4), NO <sub>3</sub> <sup>-</sup> + PK (4)

*vaginatum* L. were collected in August 2016 (peak growing season) from 44 plots representing four replicate plots from the control, N alone and N + PK treatments with different annual N doses and N sources (Table 1). In addition, *Sphagnum* spp. shoots were sampled from the control and high N plots. A composite sample (1 g dry mass) of top canopy current-year leaves of each vascular plant or top 10 cm of green shoot of *Sphagnum* spp. were dried (+50 °C) and homogenized. Each homogenized sample was divided into two subsamples: one subsample (4 mg) was weighed in tin cups and analyzed for %C, %N, and <sup>15</sup>N content using a Costech 4010 Elemental Analyzer coupled to a Delta XP Mass Spectrometer with a precision on duplicate samples of 0.2‰ (University of New Hampshire, USA). Lab standards of sporocarps, tuna muscle, apple leaves (NIST1515), and pine needles (NIST1575a) were included with each run. The other parallel subsample (300 mg) was analyzed for phosphorus content using microwave acid digestion followed by ICP-MS elemental analysis (EPA method 3051) (University of Helsinki, Finland).

### 2.3. Measuring porewater N concentration

Peat porewater was collected monthly from each treatment plot during 2012–2015 using a syringe from dipwells installed vertically in the soil (70 cm long black plastic pipes with 40 mm inner diameter having 2 lines of 4 mm holes). The collected water samples were immediately filtered through a 0.45 μm membrane filter (Puradisc™, Whatman) and total N was analyzed by ANTEK. Concentrations of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> were analyzed by ion chromatography (Metrohm). The filtered samples were stored in the dark at +4 °C (for total N) or frozen for (NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>) until chemical analysis.

### 2.4. Data analysis

Differences among treatments in foliar C:N and N:P ratios were examined using one-way ANOVA and Tukey's HSD test. A factorial approach was used instead of treating N load as a continuous variable so as to detect also such changes in foliar nutrient stoichiometry that differ between low-N and high-N treatments. Each of the plant species was analyzed separately. In addition, data on root acid phosphatase activity of the shrubs *C. vulgaris* and *E. tetralix* from Whim Bog were compared with their respective foliar N:P ratios using linear regression to study whether root phosphatase activity was either up-regulated or down-regulated depending on plant nutritional status. Root acid phosphatase data were produced by Kiheri et al. (2020), using enzymatic assay described in Velmala et al. (2014), and were available from three control plots and three replicate plots representing each of the four high-N treatments (NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup>, with and without PK, see Table 1).

To eliminate δ<sup>15</sup>N variation caused by the isotopic composition of added fertilizers we normalized the foliar δ<sup>15</sup>N values of *C. vulgaris* and *E. tetralix* to that of nonmycorrhizal *Eriophorum vaginatum*. This was done for both shrubs by subtracting their δ<sup>15</sup>N from those of nonmycorrhizal *Eriophorum* growing in the same experimental plot. The obtained new variable Δ<sup>15</sup>N<sub>NON-ERM</sub>, describing the δ<sup>15</sup>N difference between the shrubs and *Eriophorum* in each plot, was compared with the two variables N dose (0, 0.8, 2.4 or 5.6 g N m<sup>-2</sup> y<sup>-1</sup>) and N source (NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup>) using general linear models. Plant species (*C. vulgaris*

or *E. tetralix*), and a dummy variable for whether the N was supplied alone or together with P and K, were included as additional fixed variables in the model to study their effects on Δ<sup>15</sup>N<sub>NON-ERM</sub> values of the shrubs. Analyses treated variable N dose either as a numeric or a factor variable, again, to find such differences in the response variable that differ between the low-N and the high-N treatments. However, as the two alternative models gave similar results, only those from the models with N dose treated as a numeric variable are reported. We also studied, using another linear model, the relation between the shrub Δ<sup>15</sup>N<sub>NON-ERM</sub> values and average dissolved total nitrogen (DTN) content in peat porewater to test whether changes in mycorrhizal N supply were directly related to peat porewater chemistry. Mean porewater DTN concentrations of different treatments were calculated separately based on winter measurements from October to April and on summer measurements from May to September. The latter mean was assumed to represent porewater concentrations during the active growth season.

All data were analyzed using R version 4.0.2 (R Core Team, 2019). Residuals of all applied linear models were normally distributed and did not show any patterns when plotted against fitted values.

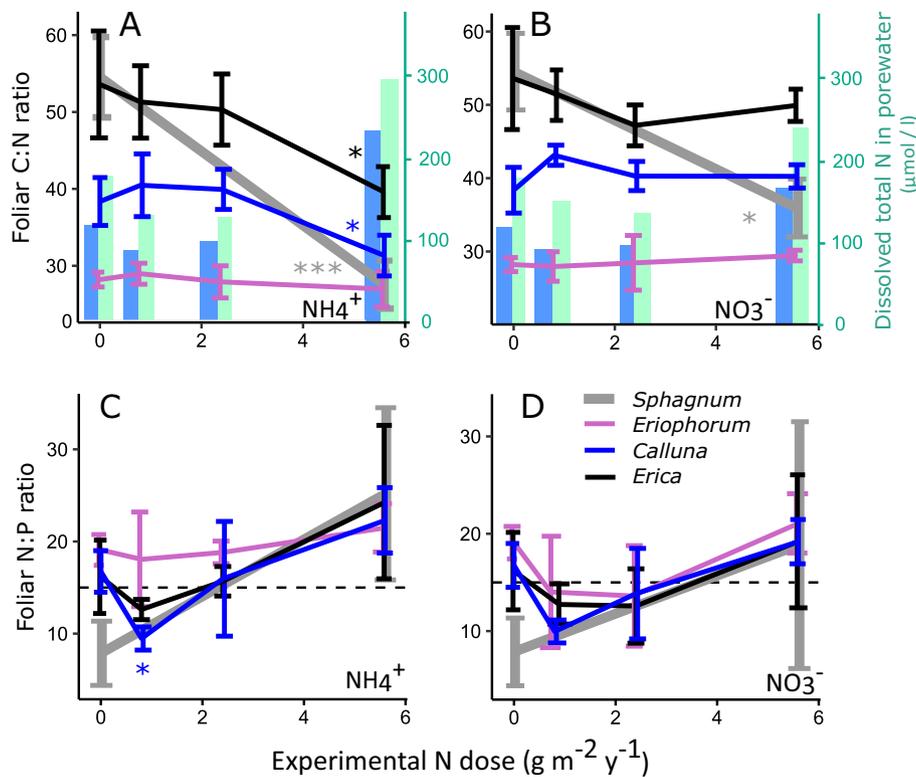
## 3. Results

### 3.1. Simulated N deposition and plant nutrition

Long-term fertilization treatments affected foliar nutrient stoichiometry but the effects varied between the different plant species and the two N sources. Foliar C:N ratios of *Calluna vulgaris* and *Erica tetralix* decreased significantly ( $p < 0.05$ ) under the high-N ammonium treatment but remained unaffected under other N fertilization treatments (Fig. 1 A–B). When N was added together with phosphorus and potassium (N + PK), foliar C:N ratios of *E. tetralix* also decreased significantly in high-N plots fertilized with nitrate, whereas foliar C:N of *C. vulgaris* only decreased in plots fertilized with ammonium (Fig. S1). C:N ratios of nonmycorrhizal *Eriophorum vaginatum* were always much lower than those of the studied shrubs and remained unaffected in all treatments (Fig. 1 A–B, Fig. S1). When N was supplied alone, C:N ratios of *Sphagnum* spp. were much lower in high-N treatments than in control plots, with the decline stronger in plots fertilized with ammonium (Fig. 1 A–B). However, in both N + PK treatments, C:N ratios of *Sphagnum* were not significantly affected (Fig. S1).

Compared to control plots, the N:P ratios of *C. vulgaris* and *E. tetralix* decreased in low-N plots without PK addition, although this decrease was only significant ( $p < 0.05$ ) in *C. vulgaris* in ammonium-treated plots (Fig. 1C). In contrast to low-N treatments, N:P ratios of shrubs in high-N treatments (without PK) were somewhat but not significantly elevated compared to control plots. Foliar N:P ratios of shrubs were significantly higher ( $p$  values ranging from 0.012 to <0.001) in high-N treatments than in low-N treatments with the same N source, except in nitrate-fertilized *E. tetralix* (Fig. 1 C–D). In *Eriophorum vaginatum*, N:P ratios tended to decrease (although non-significantly) in low- and moderate-N nitrate treatments (Fig. 1D) but remained unaffected in all ammonium treatments (Fig. 1C). Nitrogen to phosphorus ratios of *Sphagnum* spp. increased with N fertilization, but the differences relative to control plots were small in both ammonium and nitrate treatments (Fig. 1 C–D).

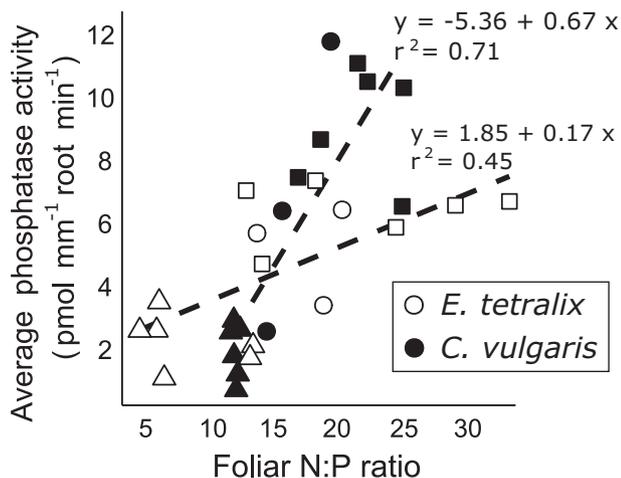
Average root acid phosphatase activities of *Calluna vulgaris* and *Erica tetralix* correlated positively with foliar N:P ratios (Fig. 2). The acid phosphatase activities of the two shrub species differed from each other, with *C. vulgaris* values significantly higher ( $p < 0.01$ ) than *E. tetralix*. The linear model explaining root acid phosphatase activity with foliar N:P ratios and species together with their highly significant interaction (i.e., root acid phosphatase activity = foliar N:P + species + foliar N:P × species) yielded an adjusted R<sup>2</sup> value of 0.63 ( $F(3, 26) = 17.46$ ,  $p < 0.001$ , see Table S1 for estimates and  $p$ -values for each variable). Regression equations and R<sup>2</sup> values are shown separately for *Calluna* and *Erica* in Fig. 2.



**Fig. 1.** Foliar carbon to nitrogen (A–B) and nitrogen to phosphorus (C–D) ratios of the studied plants across different levels of experimental N fertilization. *Sphagnum* mosses were studied only in control and high-N treatments. Each point with error bars represents mean value with standard deviation calculated from four composite plant samples originating from four parallel treatment plots (one sample per each plot). Horizontal dashed lines in panels C and D represent critical limit indicating whether the plant is nitrogen-limited (below the line) or phosphorus-limited (above the line) (Koerselman and Meuleman, 1996). Asterisks indicate statistically significant differences (Tukey's HSD test) between plants in fertilized and control plots: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . Bars in panels A and B represent average total N concentrations of peat porewater measured either during growing season (light green) or winter (light blue). Data from N + PK treatments are shown in supplementary Fig. S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.2. Simulated N deposition and foliar <sup>15</sup>N abundance

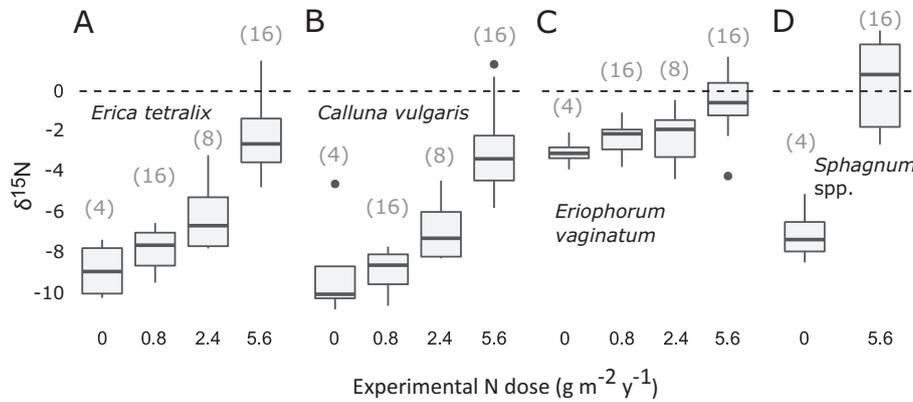
Foliar  $\delta^{15}\text{N}$  values increased in all plants as the experimental N dose increased (Fig. 3). The difference of  $\delta^{15}\text{N}$  values between the control plots and high-N plots was largest in *Sphagnum* spp. (Fig. 3D), intermediate in *Calluna vulgaris* and *Erica tetralix* (Fig. 3 A–B), and smallest in *Eriophorum vaginatum* (Fig. 3C).



**Fig. 2.** Linear regression between root acid phosphatase activity and foliar N:P ratios in *Erica tetralix* (open) and *Calluna vulgaris* (closed symbols). Circle = untreated control plots, squares = high N treatment without PK addition, triangles = high N treatment with PK addition.

Foliar  $\delta^{15}\text{N}$  values of the ERM shrubs *C. vulgaris* and *E. tetralix* were lower than those of nonmycorrhizal *Eriophorum vaginatum* within the same treatments (Fig. 3), except in two high N plots (one fertilized with ammonium and one with nitrate) where *Eriophorum* had lower  $\delta^{15}\text{N}$  than the shrubs. In control plots, the average  $\delta^{15}\text{N}$  depletion of both ERM shrubs to *Eriophorum* ( $\Delta^{15}\text{N}_{\text{NON-ERM}}$ ) was 5.9‰ (Figs. 3 and 4). The  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values of *C. vulgaris* and *E. tetralix* decreased as the experimental N dose increased (coefficient =  $-0.783$ ,  $p < 0.001$ ,  $R^2 = 0.47$ ; Fig. 4). Neither the form of added nitrogen (NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup>) nor the plant species (*C. vulgaris* or *E. tetralix*) significantly affected  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values when included in regression models with N dose, although *C. vulgaris* had slightly (0.8‰) higher  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values than *E. tetralix* ( $p = 0.053$ , see Table S2 for further details). Whether N was provided alone or together with phosphorus and potassium (N vs. N + PK treatments) was not a significant factor explaining  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values of the shrubs. We also analyzed the data without the four outlying datapoints having negative  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values (that is, data points originating from two exceptional study plots where *Eriophorum* had lower  $\delta^{15}\text{N}$  than shrubs) to assess their potential influence on results. Omitting outliers improved model fit (regression coefficient =  $-0.62$ ,  $p < 0.001$ , adjusted  $R^2 = 0.53$ ) and also made plant species a significant variable ( $p < 0.01$ , see Table S2 for comparison of the analysis with and without outliers).

The  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values of *C. vulgaris* and *E. tetralix* correlated negatively with the dissolved total nitrogen (DTN) in porewater (Fig. 5). This correlation was stronger in winter (coefficient =  $-0.028$ ,  $F(1, 86) = 57.45$ ,  $p < 0.001$ ,  $R^2 = 0.40$ , Fig. 5A) than during the growing season (coefficient =  $-0.020$ ,  $F(1, 86) = 38.21$ ,  $p < 0.001$ ,  $R^2 = 0.31$ , Fig. 5B). Plant species was not a significant factor in either regression. Additional analysis of the data without the outliers (four data points

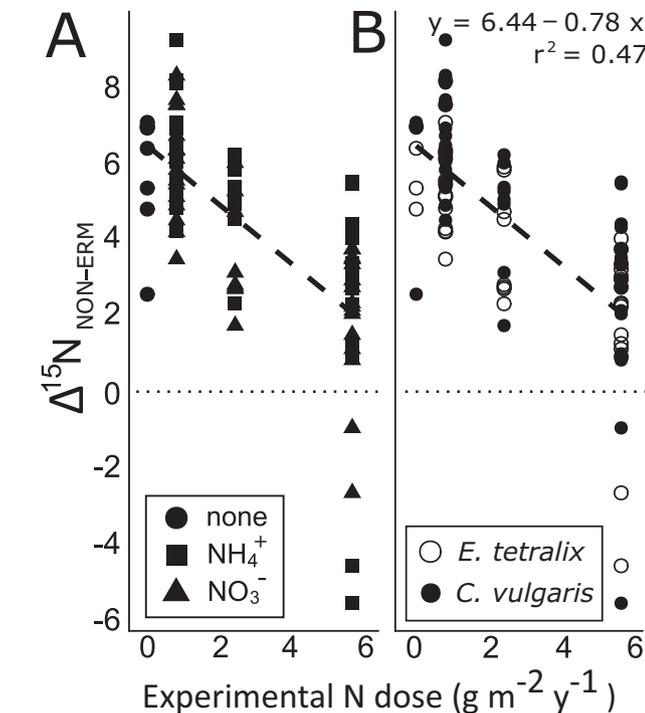
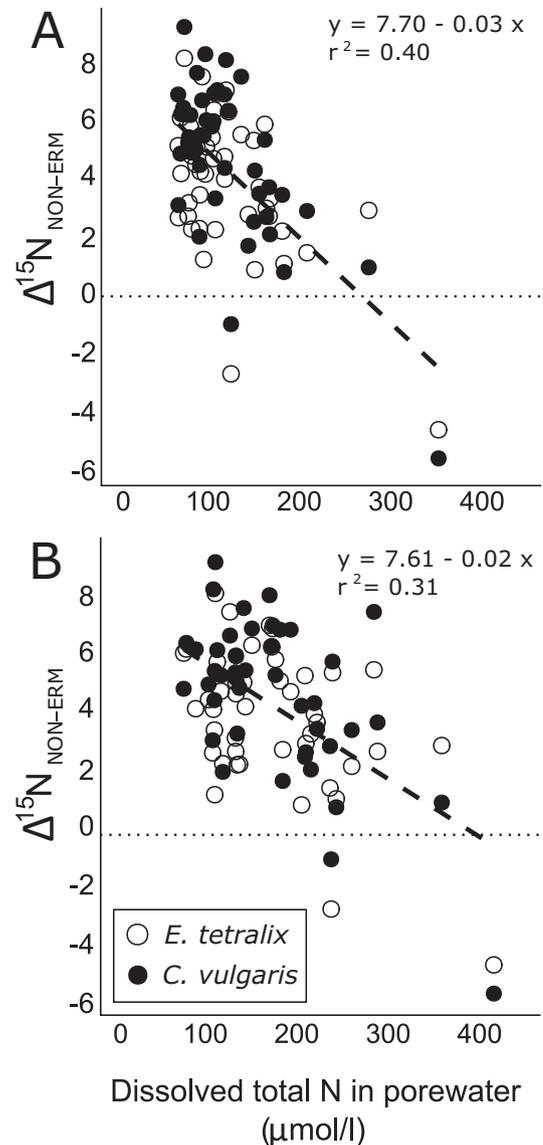


**Fig. 3.** Foliar  $\delta^{15}\text{N}$  values of ericoid mycorrhizal *Erica tetralix* and *Calluna vulgaris*, nonmycorrhizal *Eriophorum vaginatum*, and mosses (*Sphagnum* spp.) exposed to different N deposition levels for 14 years at Whim Bog. Data originates from all experimental plots (ammonium and nitrate treatments combined) including both N and N + PK treatments. Boxes represent the median and the upper and lower quartiles. Whiskers show 1.5 times the interquartile range and outliers are marked with dots. Number of observations (i.e. parallel treatment plots, one composite sample per each) is marked in parenthesis.

having negative  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values) resulted in shallower regression slopes and lower model fits, but the  $p$ -values were still highly significant (winter: coefficient =  $-0.021$ ,  $F(1, 82) = 24.49$ ,  $p < 0.001$ ,  $R^2 = 0.23$ ; summer: coefficient =  $-0.011$ ,  $F(1, 82) = 11.29$ ,  $p = 0.001$ ,  $R^2 = 0.12$ ).

**4. Discussion**

Our results demonstrate that elevated N deposition changes the nutrition and nitrogen sources of ericoid mycorrhizal shrubs in ombrotrophic peatlands. Both the C:N:P stoichiometry and  $\delta^{15}\text{N}$  in leaves of *Calluna vulgaris* and *Erica tetralix* were altered during the 14 years of N addition at Whim Bog. These effects were related to the level of N deposition: in agreement with our hypothesis, the foliar  $\delta^{15}\text{N}$  of shrubs increased linearly with increasing N deposition, but the



**Fig. 4.** Regression between  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values (i.e.  $\delta^{15}\text{N}$  difference to nonmycorrhizal *Eriophorum vaginatum*) of ericoid mycorrhizal shrubs and the annual N dose at Whim Bog. There was no significant difference between the two N sources (A) or species (B) although *Calluna vulgaris* tended to have slightly higher  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values than *Erica tetralix*. See results, for coefficients and  $R^2$  without negative outliers.

**Fig. 5.** Linear regression between foliar  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values of *Calluna vulgaris* and *Erica tetralix* and average dissolved total nitrogen (DTN) in porewater analyzed from the experimental plots in 2012–2015 either during winter (A) or summer (B). See results, for coefficients and  $R^2$  without negative outliers.

effects on nutrient ratios were more complicated. In contrast to our expectations, when N was added in low doses, the N:P ratios of *C. vulgaris* were lower than the control plants, suggesting improved P nutrition via upregulated P uptake. The positive correlation between root phosphatase activity and the foliar N:P ratios further supports this interpretation and additionally implies that the shrubs subjected to high-N treatments (without PK) had shifted from NP co-limitation towards P limitation as a result of long-term N manipulation.

#### 4.1. Nutrition of vascular plants under elevated N load

Partially in congruence with Hypothesis 1, *Calluna vulgaris* and *Erica tetralix* had significantly lower foliar C:N ratios in the high-N ammonium plots compared to controls (Fig. 1A). This suggests enhanced N acquisition as a result of long-term ammonium fertilization. In contrast, shrubs growing in nitrate-fertilized plots did not change C:N stoichiometry (Fig. 1B), except *E. tetralix* when nitrate was supplied together with P and K (Fig. S1). This demonstrates that the impact of elevated N deposition on foliar N:P stoichiometry depends on the form of nitrogen and may also be related to the availability of other nutrients. The foliar N:P ratios of both shrubs tended to be somewhat higher in high-N plots compared to controls (Fig. 1C), suggesting a shift from NP co-limitation towards P limitation in shrubs under high-N addition levels without added P and K.

Chiwa et al. (2016) showed that the *Sphagnum* layer at Whim Bog could retain added N of up to  $2.4 \text{ g m}^{-2} \text{ y}^{-1}$  above background deposition. The highest %N in moss capitula and highest inorganic N concentrations in peat porewater were in treatment plots receiving  $5.4 \text{ g N m}^{-2} \text{ y}^{-1}$  as ammonium (Sheppard et al., 2013; Chiwa et al., 2016). To effectively capture N from short rain pulses, *Sphagnum* mosses can rapidly take up inorganic N, particularly ammonium (Wiedermann et al., 2009; Fritz et al., 2014). Subsequent increases in intracellular ammonium levels affect moss growth negatively due to increased C demand of N assimilation and toxic effects of unassimilated ammonium (Nordin and Gunnarsson, 2000; Limpens and Berendse, 2003). Such declines in *Sphagnum* growth and simultaneous accumulation of excess amino acids have probably led to permanent N saturation in the high-N ammonium plots at Whim Bog. The much lower C:N ratios of mosses in the high-N ammonium plots versus the high-N nitrate plots support this idea. Different degrees of N saturation likely also reflect C:N ratios of ERM shrubs that differ between the high-N plots treated either with ammonium or nitrate (Fig. 1 A–B).

In contrast to ERM shrubs, foliar C:N ratios of the nonmycorrhizal sedge *Eriophorum vaginatum* remained at the same level in all treatments (including N + PK), but the level was lower than in either of the shrubs (Fig. 1 A–B, Fig. S1). Low C:N ratios, together with relatively high N:P ratios, suggest that *Eriophorum vaginatum* at Whim Bog is generally less N deficient than *Calluna vulgaris* or *Erica tetralix*. This might be due to its deeper root system (Wallén, 1992; Iversen et al., 2015) allowing *Eriophorum* to acquire N from potentially less utilized peat layers. Such a relationship between rooting depth and utilized N pools was demonstrated for *Phragmites australis* that effectively used inorganic N from deep peat layers down to 2 m that were inaccessible to other plants (Kohzu et al., 2003).

In contrast to treatments with the highest N doses (without PK), the lowest levels of additional N deposition decreased the foliar N:P ratios of the vascular plants (Fig. 1 C–D). Although significant only in *Calluna vulgaris*, changes in N:P ratios demonstrate that low-level and high-level N deposition may have different effects on plant nutrient stoichiometry, which falsifies Hypothesis 3. In *C. vulgaris*, foliar N:P ratios decreased from 16 in control plots to less than 10 in low-N ammonium treatments. This finding indicated paradoxically that, while the shrubs at Whim Bog might originally be limited more by P than N, small-dose N addition (without added P) shifted the plants towards N deficiency and alleviated P limitation. N fertilization often improves plant P uptake by enhancing phosphatase production (Marklein and

Houlton, 2012; Chen et al., 2020). As N-rich proteins, root phosphatases are a major investment for plants to produce, and individuals suffering from N deficiency often cannot afford their synthesis. Our results demonstrated that when N availability was experimentally increased, vascular plants and especially ERM shrubs invested the excess N to increased phosphatase activity, which effectively alleviated P limitation.

Interestingly, *C. vulgaris*, which had the strongest decline in foliar N:P ratios when subjected to low-N treatments, also showed much stronger response in root acid phosphatase activity in relation to changes in N:P stoichiometry than *E. tetralix*. Although root phosphatase data were not available from the low-N treatments, the steeper regression slope of *C. vulgaris* compared to *E. tetralix* in Fig. 2 suggests that the former upregulates root phosphatase activity more efficiently than the latter in response to small changes in N:P stoichiometry.

Wang et al. (2016) did not observe decreases in N:P ratios of shrub leaves in a corresponding long-term N fertilization experiment in Mer Bleue Bog (Canada) but soil phosphatase activity was significantly higher in fertilized plots than in control plots (Pinsonneault et al., 2016). The reason for these contrasting results from two different ombrotrophic peatlands might be related to the availability of N or P or to the water table level, which is much higher at Whim Bog than in Mer Bleue.

At Whim Bog, rates of N-acquiring enzymes in roots of peatland shrubs increased slightly along with increasing acid phosphatase activity, but the rates were not strongly coupled (Kiheri et al., 2020). The efficient uptake by mycorrhizal fungi and roots of P by peatland plants not only maintains plant stoichiometry but can be one reason for fast recycling of P from peat relative to C (Wang et al., 2015). This, in turn, may play a critical role in the observed narrow range of long-term C accumulation rates in northern peatlands (Wang et al., 2015; Schillereff et al., 2021 preprint).

When N was added together with P (N + PK treatments, Fig. S1), foliar N:P ratios declined in all plants, although the difference to controls was significant only in *C. vulgaris* subjected to low-N treatments. Phosphorus was supplied at a ratio of 14:1 N:P, corresponding to levels of nutrient stoichiometry measured from many wetland plants (Sheppard et al., 2004; Güsewell and Koerselman, 2002). These changes in foliar N:P ratios indicated that P limitation was lifted in all plants, the resulting level apparently representing the N:P ratio of the shrubs in conditions where neither N nor P limits plant growth. Both shrubs maintained their N:P stoichiometry at a constant level under all N + PK treatments despite the increasing doses of N (Fig. S1), which agrees with the findings of Wang et al. (2016) that peatland shrubs show strong C:N:P homeostasis. All these observations together underline how crucially N and P cycling are linked together in bog ecosystems, with availability of one also regulating availability of the other.

#### 4.2. Plant-mycorrhizal interactions under elevated N load

The nitrogen isotope composition of leaves can be used to assess N sources and N-acquiring pathways of plants. Foliar  $\delta^{15}\text{N}$  is affected by several factors, most importantly by the isotopic composition of soil nitrogen and root-microbial interactions (Högberg, 1997; Hobbie and Högberg, 2012). The  $\delta^{15}\text{N}$  values have vertical gradients in peat profiles, with an average increase of 1–4‰ from the peat surface to a depth of 50 cm being common in *Sphagnum*-covered ombrotrophic peatlands (Kohzu et al., 2003; Hobbie et al., 2017; Moore and Bubier, 2020). This is mainly due to differences in  $\delta^{15}\text{N}$  values between peatland shrubs and mosses (Moore and Bubier, 2020). While ericoid mycorrhizal shrubs rely mostly on N provided by their mycobionts, that is  $^{15}\text{N}$ -depleted relative to other soil N sources (putative mechanisms discussed below), the  $\delta^{15}\text{N}$  values of mosses are largely defined by the isotopic composition of precipitation (Bragazza et al., 2005; Zechmeister et al., 2008). Due to these contrasting N sources, *Sphagnum* mosses tend to be enriched in  $^{15}\text{N}$  compared to shrubs, as was also found at Whim Bog (Fig. 3). As the roots of vascular plants mostly occur in the surface

peat, and *Sphagnum* litter decomposes more slowly than vascular plant litter, peat  $\delta^{15}\text{N}$  values tend to approach those of *Sphagnum* as the depth increases (Moore and Bubier, 2020). Soil microbial processes, such as nitrification, denitrification, and N fixation (e.g., Elzen et al. 2018, Larmola et al., 2014) may additionally alter the  $\delta^{15}\text{N}$  differences among peat layers. Because of this vertical  $\delta^{15}\text{N}$  gradient, deep-rooted plants become more enriched in  $^{15}\text{N}$  than shallow-rooted plants (Kohzu et al., 2003; Moore and Bubier, 2020).

The rooting depth, however, is not the only factor that regulates foliar  $\delta^{15}\text{N}$  in peatlands. Ericoid plants show typically much lower foliar  $\delta^{15}\text{N}$  levels than other plants growing in same habitats (Michelsen et al., 1996, 1998; Nadelhoffer et al., 1996; Craine et al., 2009), which is thought to result mainly from the different N pools that the plants utilize (Emmerton et al., 2001; Hobbie and Högberg, 2012). Organic N in fresh litter is typically  $^{15}\text{N}$ -depleted compared to other soil N sources (Nadelhoffer & Nadelhoffer and Fry, 1988, Högberg, 1997). ERM fungi provide their host plants with access to this fresh N pool (Bajwa et al., 1985; Michelsen et al., 1998; Hobbie and Högberg, 2012), whereas nonmycorrhizal plants rely on older N pools that are typically higher in  $\delta^{15}\text{N}$  compared to fresh litter. Fungal  $^{15}\text{N}$  discrimination while transferring N to host plants, substantially affecting the  $\delta^{15}\text{N}$  levels in ECM plants (Hobbie and Colpaert, 2003; Hobbie and Högberg, 2012) but also demonstrated for ERM symbiotic *Vaccinium vitis-idaea* (Emmerton et al., 2001), might additionally decrease the plant  $\delta^{15}\text{N}$  levels.

According to Hypothesis 4, the mycorrhizal supply of N relative to direct root uptake in ERM shrubs was expected to decrease as a result of long-term N fertilization and subsequent increase in the concentrations of plant available N. This should be reflected in increased foliar  $\delta^{15}\text{N}$  as the proportion of fungal-derived nitrogen (low  $\delta^{15}\text{N}$ ) decreases in relation to nitrogen originating from inorganic N pools (high  $\delta^{15}\text{N}$ ). The applied fertilizers, however, may also have direct effects on plant  $\delta^{15}\text{N}$  levels reflecting their own isotopic composition. This was seen especially in *Sphagnum* that effectively takes nitrogen from precipitation. *Sphagnum*  $\delta^{15}\text{N}$  values increased from  $-7\%$  in control plots to  $+1\%$  in plots fertilized with the highest N doses (Fig. 3), suggesting that the  $\delta^{15}\text{N}$  values of the applied fertilizers were higher than any of the initial soil N pools at Whim Bog. We could eliminate this direct effect of fertilizers on foliar  $\delta^{15}\text{N}$  using another variable,  $\Delta^{15}\text{N}_{\text{NON-ERM}}$ , which compares the isotopic difference between the studied ERM shrubs and the identically treated nonmycorrhizal *Eriophorum vaginatum* growing in same experimental plots. In addition to  $\delta^{15}\text{N}$  variance caused by using N acquired either via mycorrhizal uptake or direct root uptake, the  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values still include the  $\delta^{15}\text{N}$  difference caused by the different rooting depths. This component, however, should not exceed the typical  $\delta^{15}\text{N}$  variance of 1–4‰ found within peat profiles of other comparable peatlands (Kohzu et al., 2003; Hobbie et al., 2017; Moore and Bubier, 2020). The  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values of both *Calluna vulgaris* and *Erica tetralix* decreased an average of 4‰ as a result of high-N fertilization treatments (Fig. 4). This decrease was likely caused by the decreased mycorrhizal N supply and the increased direct uptake of inorganic N in ERM shrubs but may also partially reflect  $^{15}\text{N}$  enrichment of surface peat and a subsequent decrease in the  $\delta^{15}\text{N}$  gradient with soil depth. However, as the vertical  $\delta^{15}\text{N}$  gradients in peat are largely caused and maintained by the constant supply of  $^{15}\text{N}$ -depleted nitrogen to ERM shrubs via mycorrhizal fungi, potential decreases in this component, as well, are likely to indirectly reflect lowered activity of mycorrhizal communities at a longer timescale. Thus,  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values may reflect relative plant N supply via mycorrhizal uptake vs. direct N uptake in ERM shrubs, with decreasing values suggesting reduced mycorrhizal activity. A model describing this interpretation is shown in Fig. 6.

The negative relationships between the  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values of *Calluna vulgaris* and *Erica tetralix* and the experimental N dose (Fig. 4) strongly suggests that the proportion of N acquired via mycorrhizal uptake was reduced due to long-term N fertilization, thus supporting Hypothesis 4. In sharp contrast, Kiheri et al. (2020) found that N fertilization increased root ERM colonization frequencies in both *C. vulgaris*

and *E. tetralix* at these same treatment plots at Whim Bog. These seemingly contradictory observations imply that root colonization frequencies do not necessarily reflect the functional status of the mycorrhizal interaction. Abundance of dark septate endophytes (DSE) may provide another explanation: while ERM abundance increased due to N fertilization, colonization frequencies of DSE fungi decreased dramatically (Kiheri et al., 2020). Although the ecology of these relatively common fungi is currently poorly known, there is some evidence that DSE fungi may contribute to plant nutrition by providing access to organic N and P sources (Mandyam and Jumpponen, 2005, Andrade-Linares and Franken, 2013).

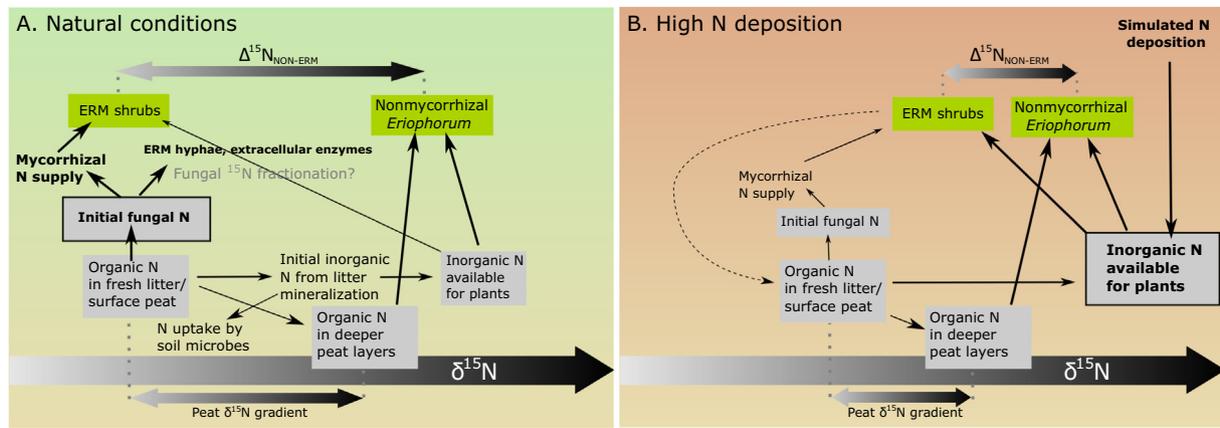
In Hypothesis 5, we proposed that the mycorrhizal N supply could be related to the concentrations of plant-available N in peat porewater. This relation was studied using dissolved total nitrogen (DTN) levels monitored at the treatment plots. DTN concentrations were used instead of inorganic N as both inorganic and organic N forms can be taken up directly by roots of most vascular plants (Sokolovski et al., 2002; Näsholm et al., 2009; Tegeder and Rentsch, 2010). The porewater DTN concentration and the  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values of both shrubs correlated negatively (Fig. 5), thus supporting the hypothesis. This relationship was stronger when the foliar  $\Delta^{15}\text{N}_{\text{NON-ERM}}$  values were compared with DTN concentrations monitored during winter (Fig. 5A) than during summer months (Fig. 5B), reflecting the seasonal variation in porewater DTN levels. Although the ammonium-fertilized plots were the most N-saturated plots with the highest inorganic and total N concentrations in porewater (Chiwa et al., 2016), we found no indication that the mycorrhizal N supply of ERM shrubs (i.e.  $\Delta^{15}\text{N}_{\text{NON-ERM}}$ ) would have decreased more in ammonium than in nitrate treatments (Fig. 4A). In addition to root uptake, some forest trees grown under high N deposition levels receive a substantial proportion of their total N via foliar uptake (Rennenberg & Rennenberg and Gessler, 1999, Gaige et al., 2007, Adriaenssens et al., 2011). Chiwa et al. (2019) demonstrated that canopies of *C. vulgaris* retained a significant proportion of N from the experimental N deposition at Whim Bog, suggesting that such foliar N uptake could also take place in peatland shrubs under high N load. If so, nitrogen uptake via leaves could reduce the dependence of ERM shrubs on their mycorrhizal fungi and on soil N resources.

## 5. Conclusions

Our results introduce a previously unrecognized mode of how anthropogenic N pollution may affect nutrient cycling within peatland ecosystems. The foliar N stable isotopes of two peatland shrubs, *Calluna vulgaris* and *Erica tetralix*, indicated a lowered N supply via mycorrhizal uptake as a result of 14 years of N fertilization at Whim Bog. Concurrently, the high-dose ammonium treatment significantly increased foliar N concentrations in relation to carbon in both shrubs, apparently through enhanced direct uptake of N, without mycorrhizal interactions. Low levels of N fertilization decreased N:P ratios of *C. vulgaris* and promoted P uptake, which underlines the crucial interactions between these two major nutrients. The observed reduction in mycorrhizal N supply, when associated with the suppressed reciprocal C fluxes from host plants, may impair the ability of peatland ericoid mycorrhizal fungi to compete with saprotrophic microbes not dependent on host carbon. This may ultimately accelerate peat C loss. Studies on fungal communities in peatlands subjected to long-term N manipulation experiments are needed to address these issues.

## CRedit authorship contribution statement

**Risto Vesala:** Conceptualization, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Heikki Kiheri:** Investigation, Writing – review & editing. **Erik A. Hobbie:** Conceptualization, Writing – review & editing. **Netty van Dijk:** Resources, Writing – review & editing. **Nancy Dise:** Resources, Writing – review & editing.



**Fig. 6.** Model of factors explaining the relative foliar  $\delta^{15}\text{N}$  levels of ERM symbiotic shrubs (*Calluna vulgaris* and *Erica tetralix*) and nonmycorrhizal *Eriophorum vaginatum* at Whim Bog under (A) natural conditions and (B) simulated N deposition. The  $\delta^{15}\text{N}$  increases from left to right. Gray boxes and arrows represent N pools and fluxes, respectively, with box size or arrow thickness roughly indicating the pool size or flux rate. Under natural conditions (A) *Eriophorum* has higher foliar  $\delta^{15}\text{N}$  than shrubs due to usage of N pools (organic and inorganic) in deeper peat layers enriched in  $^{15}\text{N}$  compared to fresh litter in surface peat. ERM shrubs, in turn, take their N mainly from fresh organic sources in topsoil (low  $\delta^{15}\text{N}$ ) via ERM fungi that may additionally discriminate  $^{15}\text{N}$  when transferring N to their hosts. Elevated foliar  $\delta^{15}\text{N}$  levels in plants under simulated N deposition (B) partially reflects the  $^{15}\text{N}$  enrichment of peat caused by the isotopic composition of the inorganic fertilizers (in this case enriched in  $^{15}\text{N}$  related to natural soil N pools). The  $\delta^{15}\text{N}$  values of ERM shrubs increase more than those of nonmycorrhizal *Eriophorum* ( $\Delta^{15}\text{N}_{\text{NON-ERM}}$  decrease) due to their reduced mycorrhizal N supply (low in  $^{15}\text{N}$ ) and increased direct N uptake from inorganic N pools (high in  $^{15}\text{N}$ ). This also leads to  $^{15}\text{N}$  enrichment of shrub-derived litter which subsequently increases the  $\delta^{15}\text{N}$  of surface peat (dashed arrow), thus decreasing the depth gradient in soil  $\delta^{15}\text{N}$ . This, in turn, affects  $\delta^{15}\text{N}$  of the organic N pool available for the ERM fungi and further decreases the  $\Delta^{15}\text{N}_{\text{NON-ERM}}$ .

**Tuula Larmola:** Conceptualization, Funding acquisition, Project administration, Investigation, Writing – review & editing.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2021.148737>.

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