

# Isotopic analysis of cyanobacterial nitrogen fixation associated with subarctic lichen and bryophyte species

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**Abstract** Dinitrogen fixation by cyanobacteria is of particular importance for the nutrient economy of cold biomes, constituting the main pathway for new N supplies to tundra ecosystems. It is prevalent in cyanobacterial colonies on bryophytes and in obligate associations within cyanolichens. Recent studies, ap-

plying interspecific variation in plant functional traits to upscale species effects on ecosystems, have all but neglected cryptogams and their association with cyanobacteria. Here we looked for species-specific patterns that determine cryptogam-mediated rates of N<sub>2</sub> fixation in the Subarctic. We hypothesised a contrast in N<sub>2</sub> fixation rates (1) between the structurally and physiologically different lichens and bryophytes, and (2) within bryophytes based on their respective plant functional types. Throughout the survey we supplied <sup>15</sup>N-labelled N<sub>2</sub> gas to quantify fixation rates for monospecific moss, liverwort and lichen turfs. We sampled fifteen species in a design that captures spatial and temporal variations during the growing season in Abisko region, Sweden. We measured N<sub>2</sub> fixation potential of each turf in a common environment and in its field sampling site, in order to embrace both comparativeness and realism. Cyanolichens and bryophytes differed significantly in their cyanobacterial N<sub>2</sub> fixation capacity, which was not driven by microhabitat characteristics, but rather by morphology and physiology. Cyanolichens were much more prominent fixers than bryophytes per unit dry weight, but not per unit area due to their low specific thallus weight. Mosses did not exhibit consistent differences in N<sub>2</sub> fixation rates across species and functional types. Liverworts did not fix detectable amounts of N<sub>2</sub>. Despite the very high rates of N<sub>2</sub> fixation associated with cyanolichens, large cover of mosses per unit area at the landscape

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scale compensates for their lower fixation rates, thereby probably making them the primary regional atmospheric nitrogen sink.

**Keywords** Interspecific variation ·  $^{15}\text{N}$  · Moss · Liverwort · Tundra

## Introduction

Through the quantification of interspecific variation in plant functional traits, comparative plant ecology has gained momentum in explaining species distributions, tradeoffs in biochemistry and physiology, as well as whole-ecosystem processes (Westoby and Wright 2006). Lavorel et al. (2007) considered vascular plant functional traits as predictors of key vegetation responses to environmental variation and vegetation effects on ecosystem functions at diverse spatial and temporal scales. In contrast to the effort put into developing extensive international methodological protocols and databases for quantitative analysis of vascular plant traits (Cornelissen et al. 2003), non-vascular plants such as cryptogams have remained mostly out of the scientists' trait research scope to date, even though the application of bryophyte and lichen traits as drivers of large-scale biogeochemistry holds much promise. Indeed, bryophytes and lichens are of particular importance in cold biomes such as the Arctic tundra (Longton 1997), where they are key contributors to green biomass and control soil hydrology, temperatures and chemistry (Cornelissen et al. 2007). One aspect of the latter is their ability to form symbiotic relationships with cyanobacteria (Dalton and Chatfield 1985; During and Van Tooren 1990; Henriksson et al. 1987). Most bryophytes have a high water retention capacity and thus provide a stable and favourable habitat for cyanobacterial growth and  $\text{N}_2$  fixation activity (Dickson 2000). In lichens, cyanobacteria are a symbiont which provide their fungal partner with nitrogen from fixed atmospheric  $\text{N}_2$  in exchange for physical protection and—in the case of tripartite lichens—from carbohydrates in the fungi (Nash 1996). Since nitrogen is a principal limiting environmental factor for plant growth and soil organic matter turnover in polar regions (Longton 1988) and biological nitrogen fixation there occurs mostly in cyanobacteria (Solheim et al. 1996), the interaction

between cryptogams and cyanobacteria forms an important field for ecological investigations.

A great deal of variation in nitrogen fixation of cyanobacterial bryophytes has been attributed to abiotic factors; Zielke et al. (2002) reported that in the High Arctic there is a strong dependency of fixation rates on site hydrology, temperature and light intensity. In regions with low precipitation during the growing season, for instance,  $\text{N}_2$  fixation is limited either to the period of snowmelt or to sites which stay wet during summer, e.g. peat bogs, fens, or dense moss mats (Zielke et al. 2005). Experimental long-term increase in UV-B radiation, simulating atmospheric ozone depletion, reduces  $\text{N}_2$  fixation activity in Arctic mosses, but does not affect it in sub-Arctic ones (Solheim et al. 2002, 2006). Fixation rates also depend on the successional age of boreal forests via differences in environmental moisture, nutrition and possible other factors (DeLuca et al. 2008; Lagerström et al. 2009; Zackrisson et al. 2004). In contrast to this emphasis on abiotic control over  $\text{N}_2$ -fixation, only a few studies have investigated the role of host specificity in the interaction between cyanobacteria and bryophytes (see During and Van Tooren 1990). For mosses the types of association probably range from simply fortuitous, through more or less specific epiphytism, to intracellular colonisation of dead moss cells or hollow hyaline cells (Solheim and Zielke 2002). Even though extensive information is available on cyanobacteria-plant symbioses (including therein liverworts and hornworts) (Rai et al. 2000), there is hardly any information on how moss-cyanobacteria associations are formed, what genes are involved if any, and the extent of nitrogen exchange between host and symbiont. At an ecosystem level, a single study seldom combines more than a couple of host species and those are often closely related phylogenetically. This poses problems when estimations of regional biological atmospheric nitrogen fixation are attempted. Even the widely applied technique for measuring  $\text{N}_2$  fixation rates by means of acetylene reduction capacity provides idiosyncratic data, unless calibrated for each analysis by means of  $^{15}\text{N}_2$  labelled gas (DeLuca et al. 2002).

This study is the first to examine the effects of cryptogam host specificity on rates of  $\text{N}_2$  fixation, independent of abiotic environmental factors. We hypothesised principal differences in  $\text{N}_2$  fixation rates (1) between lichens and bryophytes, and (2)

among bryophyte plant functional types. Thereto, measurements were done in monospecific cryptogam patches both in the field and in a common environment, using a standard method for  $^{15}\text{N}_2$  incubation. We surveyed a list of contrasting, abundant subarctic species to account for the phylogenetic, morphological and habitat diversity of hosts to cyanobacteria in the region. This host specificity could possibly arise from differences in surfaces available for bacterial colonisation, density of vegetation patches, and consequently in microhabitat moisture. On a larger scale, we compared the relative contributions of cyanobacterial bryophytes versus cyanolichens to the regional input of atmospheric nitrogen in the Subarctic. We hypothesised (3) that, because of the overall dominance in the regional vegetation cover, bryophytes could play an important role in overall  $\text{N}_2$ -fixation in the Subarctic. To test the latter hypothesis, we did not limit our analyses to comparisons on dry weight or area basis, but also included regional abundance.

## Methods

### Site description and plant material sampling

Field work took place in the summer of 2007 off the southern shore of Lake Torneträsk, North Sweden. Three distinct valleys in the vicinity of Abisko were considered for collecting cryptogams—four—one in Stordalen Nature Reserve and two in Abisko National Park. Each of the three locations included three vegetation types characteristic of the lowland Subarctic: nutrient poor birch forest with ericaceous undergrowth, oligotrophic bog dominated by *Sphagnum fuscum* hummock communities, and minerotrophic mire (fen). Thus each of the studied species was collected from three spatially separated habitats. In total 15 cryptogam species from different taxonomic groups, with varying morphologies and from contrasting habitats were considered for this study. These included 11 mosses—*Aulacomnium palustre* (Hedw.) Schwaeger., *Tomentypnum nitens* (Hedw.) Loeske, *Drepanocladus cossonii* (Schimp.) Loeske, *Paludella squarrosa* (Hedw.) Brid. Muscol., *Sphagnum lindbergii* Schimp. ex Lindb, *Sphagnum riparium* Aongstr., *Sphagnum fuscum* (Schimp.) Klinggr.,

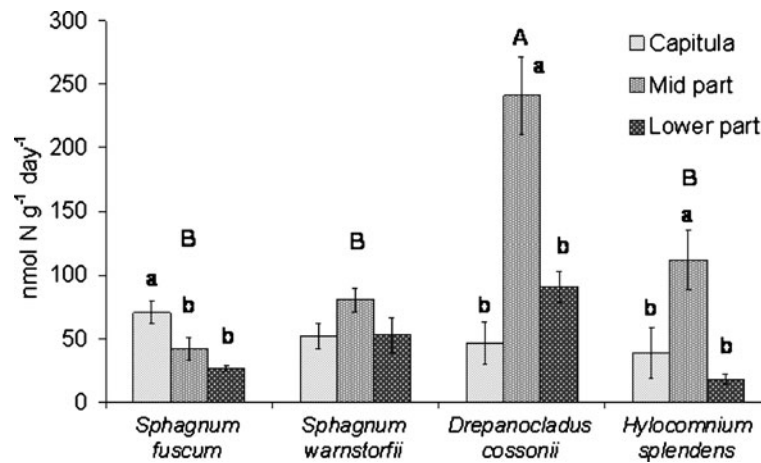
*Hylocomnium splendens* (Hedw.) B.S.G., *Pleurozium schreberi* (Brid.) Mitt., *Polytrichum strictum* Brid., *Polytrichum commune* Hedw.; two liverworts—*Lophozia lycopodioides* (Wallr.) Cogn., *Ptilidium ciliare* (L.) Hampe; and two cyanolichens—*Nephroma arcticum* (L.) Torss., *Peltigera aphthosa* (L.) Willd. Nomenclature followed recent checklists and identification guides: *Drepanocladus sp.* (Hedenäs 2003), Sphagna (Daniels and Eddy 1990), other musci (Hill et al. 2006), liverworts (Damsholt 2002), and lichens (Dobson 2005). We sampled monospecific patches of vegetation typical for the particular species' habitat. For each species four samples were gathered from each valley—two for incubation with  $^{15}\text{N}_2$  and two as controls. Collection from the same sites was performed twice, in mid-June and then in mid-July, to account for possible temporal variation through the growing season. However, due to logistic constraints, six of the moss species were collected and incubated only once, either during the first or the second round (for details see section “Statistical analysis”).

The bryophyte species were grouped into two functional types according to their shoot growth form, and mat architecture (Buck and Goffinet 2000): acrocarpous (erect shoots, growing in dense turfs) and pleurocarpous (crawling shoots, growing in mats).

In situ microhabitat moisture was recorded for each species, since we considered water availability a likely limiting factor for cyanobacterial activity. We monitored moisture availability in monospecific patches by means of air-dried wooden cylinders ( $r=1.25$  cm,  $h=7$  cm) nested for a week (July, 15–21) in the bryophyte turf at 5 cm depth. The cylinders were weighed before and after field placement to determine the relative increase in water content during a period without major rainfall.

### Sample processing and analysis of $\text{N}_2$ fixation

Pilot work determined the active part in  $\text{N}_2$  fixation along moss' shoots. Moss was cut into previous year's growth, older photosynthetically active part, and senesced part according to specific moss morphology and coloration. Segments below previous-year's growth were the most prominent in nitrogen fixation activity (see Fig. 1). Additionally, a Confocal Laser Scanning Microscopy, CLSM (Bio-Rad Radiance

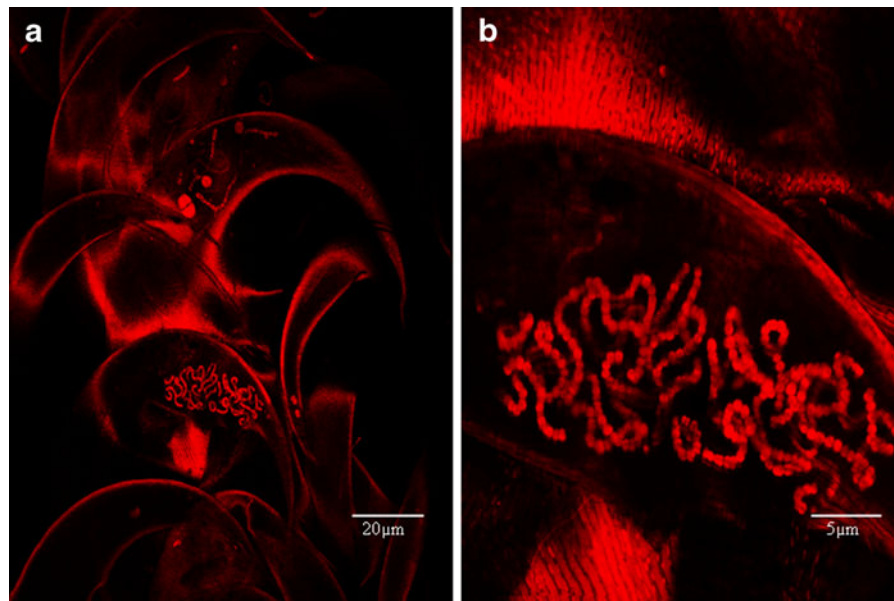


**Fig. 1** Daily N<sub>2</sub> fixation rates by shoot segments of four moss species. In the imbedded legend “capitula” refers to previous year’s growth, “mid part” to the photosynthetically active part below the capitula, and “lower part” refers to the senesced part.

Capital letters indicate overall statistical differences between species based on a Tukey post hoc test at the 0.05 significance level. Lower case letters indicate differences between moss segments in each respective species

2000), determined whether the measured rates of N<sub>2</sub> fixation corresponded to the degree of moss colonisation by cyanobacteria. The technique, described in detail by Solheim et al. (2004), is non-destructive and allows for optical cross-section observations of intact moss samples. Detected cyanobacterial filaments were found present in leaf groves and moss axils, with largest colonies observed along

the mid-length shoot segments (Fig. 2, see also the correspondingly high fixation rates for mid-part of *Drepanocladus cossonii* in Fig. 1). Field measurements of live (photosynthetically active) moss cushion thickness in the region (a minimum of 6 replicates per species) varied between 3.1 cm and 7.5 cm across moss species and were around 2.5 cm for leafy liverworts. Hence, to account for the contribution of



**Fig. 2** A red filter CLSM micrograph of a *Drepanocladus cossonii* shoot showing (a), a colony of filamentous cyanobacteria detected on top of a folded leaf (magnification  $\times 60$ ) and (b), the same view (magnification  $\times 200$ )

each species to the process of nitrogen fixation, we opted for a correspondingly large vial size for the main part of the experiment.

Collected plant material was temporarily stored in a dark climate chamber (5°C) and any living or dead material other than the target bryophyte or lichen carefully removed by hand. Mosses were cut to a maximal shoot length of 6 cm from the apex, liverworts were left intact, and for lichens three to four whole thallus lobes were used. Samples were saturated with water and placed in 135 ml glass vials. Right before incubation all samples were acclimated for 36 h outdoors in the experimental common environment, in a partly shaded spot just outside Abisko Research Station. Then the vials were sealed with rubber septa and in half of them 15 ml of headspace were replaced by 15 ml  $^{15}\text{N}_2$  (98% enriched, Cambridge Isotope Laboratories, Inc., U.S.A.), resulting in headspace enrichments of 10–19% depending on species [see Supplementary Materials S1 for note on calculation of final headspace enrichment]. The other half of the samples, the controls, was treated in the same manner except for the headspace substitution. All vials were placed randomly on three trays, representing statistical blocks (Quinn and Keough 2002), which corresponded to the three valleys the samples had been collected from. Incubation took 24 h during which the trays were rotated every 4 h to avoid possible unintended gradients in the common environment. Sunscreens were put up at noon to avoid direct sun illumination of the glass vials, in order to avoid any greenhouse effects (Zielke et al. 2002); this kept PAR at average levels of ca.  $75 \mu\text{Einstein m}^{-2} \text{s}^{-1}$  (ranging from 10 to  $240 \mu\text{Einstein m}^{-2} \text{s}^{-1}$ ). Additionally, temperature was recorded on Tinytag loggers coupled to probes inside and outside the vials and the data showed no significant difference within either of the incubation rounds (*T*-Test, for June  $P=0.90$ ; for July  $P=0.64$ ). June temperature averages inside and outside the vials were 18.0°C and 18.2°C respectively (13.5–22.7°C range). For July these averages were 10.5°C and 10.3°C (6.9–14.5°C range).

During each of the two phases of the experiment, samples from two moss species were nested in the field for acclimation and subsequent incubation in order to test for possible discrepancies between fixation rates in the experimental common environment and those in the field. We had indeed chosen

species from contrasting environments to account for the range of habitats in this study—*H. splendens* from a dry birch forest, *A. palustre* from wet depressions within a birch forest, *S. fuscum* from hummocks in an oligotrophic bog, and *D. cossonii* from water pools in a minerotrophic fen.

After the incubations all plant and lichen material was oven-dried for 48 h at 70°C, weighed, and powdered in a ball mill. Nitrogen concentrations and atom%  $^{15}\text{N}$  values were determined using an Elemental Analyser (NC2500, ThermoQuest Italia, Rodana, Italy) coupled to a Continuous-flow Isotope Ratio Mass Spectrometer (Delta Plus, ThermoQuest Finnigan, Bremen, Germany).  $\text{N}_2$  fixation was determined by the  $^{15}\text{N}$  increment over the corresponding controls' concentrations, corrected for each samples' headspace volume and expressed per unit dry weight [see Supplementary Materials S1 for details on calculations].

#### Estimation of regional $\text{N}_2$ fixation

In order to get a better understanding of the regional significance of  $\text{N}_2$  fixation associated with subarctic cryptogams, fixation rates were expressed on an area basis. To this aim, data on bryophyte cushion density and thickness per dry weight was gathered in the Abisko region with a minimum of six replicates per species. For lichens, oven-dry weight of individual thalli was expressed per unit surface area of fully expanded thalli (thallus specific weight) as in Dahlman and Palmqvist (2003). Variances of these measurements were incorporated in the final regional-scale  $\text{N}_2$  fixation budgets. The latter were consecutively upscaled to the landscape level by integrating species abundance and cover percentage data from former biodiversity studies in the Abisko region.

#### Statistical analysis

Data were analysed using the SPSS 13.0 software package. Rank transformations were carried out when both Levene's test and visual exploration of residual plots for homogeneity of variances violated the assumptions of the statistical tests used (Quinn and Keough 2002). A Repeated Measures ANOVA for the two consecutive measurements at each location could not be performed due to violations of the basic



assumptions of the test (homogeneity of variances and normality) and lack of useful biologically meaningful data transformations. Thus the two observations in time (in June and July) were treated as a nested factor within the main factor—species, to account for the fact that they were performed on the same plots. The local effect of valley location (sampling site), the effect of species and their interaction were tested. This analysis was performed on the reduced list of nine species which had been replicated over the two rounds of the experiment. Since no temporal variation was detected (see below), the whole list of 15 species (thus including *S. riparium*, *P. schreberi* and *P. strictum* from the first round; and *S. lindbergii*, *H. splendens*, and *P. commune* from the second round) was tested with One-Way ANOVA followed by a Tukey's test for differential effects of each species, and the two moss functional type groups (acrocarps and pleurocarps), respectively. Field measurements of nitrogen fixation were tested against common environment measurements on the same species coming from corresponding valleys and collection times by a Two-Way ANOVA model. At last, data on field

moisture levels for each species were compared to corresponding rates of N<sub>2</sub> fixation and analysed by means of a linear regression model.

## Results

### N<sub>2</sub> fixation across cryptogam species

Natural variation in amounts of stable isotope nitrogen in cryptogams proved to be considerable (average  $\delta^{15}\text{N}=0.68$ , range from  $-5.29$  to  $25.01$ ) and in some cases exceeded the enrichment after incubation (average  $\delta^{15}\text{N}=7.31$ , range from  $-5.49$  to  $86.63$ ). Hence, several control samples were richer in <sup>15</sup>N than their incubation counterparts and this rendered expressing daily species-specific N<sub>2</sub> fixation rates difficult (see Table 1, negative fixation rates). However, the intercept of our statistical model for differences in fixation rates (the one testing the effects of valleys (collecting sites), species, their interaction, and collection time as a nested factor in species) was significantly different from zero ( $P=0.005$ ) and

**Table 1** Daily N<sub>2</sub> fixation rates (average daily N<sub>2</sub> fixation rates of abundant sub-arctic cryptogam species, each collected from three distinct valleys in the region of Abisko, Sweden) of bryophytes and lichens averaged over two measurement days in June and July

Species	Dry weight estimates		Area estimates	
	$\mu\text{mol N kg}^{-1} \text{d}^{-1}$	SE	$\mu\text{mol N m}^{-2} \text{d}^{-1}$	SE
<b>Mosses</b>				
<i>Aulacomnium palustre</i>	32.35 <sup>a</sup>	15.57	32.53	19.43
<i>Tomentypnum nitens</i>	11.56 <sup>a</sup>	18.03	16.68	26.96
<i>Drepanocladus cossonii</i>	90.21 <sup>a</sup>	39.95	117.20	63.52
<i>Paludella squarrosa</i>	61.93 <sup>a</sup>	26.98	50.94	27.74
<i>Sphagnum lindbergii</i>	28.00 <sup>a</sup>	15.17	49.53	30.57
<i>Sphagnum riparium</i>	71.84 <sup>a</sup>	20.21	87.49	30.82
<i>Sphagnum fuscum</i>	13.33 <sup>a</sup>	13.17	21.70	23.05
<i>Hylocomnium splendens</i>	71.11 <sup>a</sup>	61.48	84.93	86.89
<i>Pleurozium schreberi</i>	10.17 <sup>a</sup>	26.37	13.24	35.37
<i>Polytrichum strictum</i>	3.03 <sup>a</sup>	25.10	8.16	68.78
<i>Polytrichum commune</i>	14.99 <sup>a</sup>	10.89	23.51	20.08
<b>Liverworts</b>				
<i>Lophozia lycopodioides</i>	-9.53 <sup>a</sup>	32.29	-5.94	19.76
<i>Ptilidium ciliare</i>	-41.52 <sup>a</sup>	44.77	-34.15	33.48
<b>Lichens</b>				
<i>Nephroma arcticum</i>	673.75 <sup>b</sup>	101.47	88.32	13.30
<i>Peltigera aphthosa</i>	3094.64 <sup>c</sup>	439.99	415.74	59.11

Superscripts indicate statistical differences between species based on a Tukey post hoc test at a 0.05 significance level

positive ( $B=57.0$ ). This provided evidence that incubation significantly increased the amounts of labeled  $N_2$  in the samples altogether. When tested separately, this scenario also held true for lichens ( $P=0.010$ ;  $B=2,631$ ) and mosses ( $P=0.044$ ;  $B=57.0$ ), but not for liverworts which showed no significant  $^{15}N$  uptake ( $P=0.75$ ;  $B=152$ ). We found no significant effects of valleys ( $P=0.17$ ) or collection time ( $P=0.47$ ) on the nitrogen fixation rates and concluded that within the studied region and time scale there were no consistent spatial or temporal variations in fixation rates. There was a highly significant effect of species ( $P<0.001$ ), but none of the species by valleys interaction ( $P=0.59$ ).

In order to include all of the species (together with the six species tested for  $N_2$  fixation only in one of the collection rounds) we had to average the two observations in time (June and July) for the rest of the species, which was justified based on the non-significant influence of time from the first test. The design included two factors—valleys and species, with a post-hoc test on species. Species had a significant effect ( $P=0.020$ ) on  $^{15}N$  accumulation, which was not influenced by valleys ( $P=0.409$ ). The only two cryptogam species that were fixing atmospheric nitrogen at significantly different rates from one another and higher than the bryophytes were the two lichens *P. aphthosa* and *N. arcticum* (see Table 1). Correspondingly, the tissue nitrogen contents of these two species (25.2 mg N g<sup>-1</sup> dry weight in *P. aphthosa* and 20.4 in *N. arcticum*) were also much higher than those of the mosses (a range of 3.4–9.6 mg N g<sup>-1</sup> dry weight for all the studied species).

For the four species nested in the field we found no significant differences in the pairwise comparisons between fixation rates in the experimental common environment and those in the ‘home site’ of each species ( $P=0.32$ ). Thus we concluded that exhibited rates of  $N_2$  fixation in the common environment were realistic field values.

The linear regression model of species  $N_2$  fixation rates and environmental moisture did not explain much of the observed variation ( $R^2=0.15$ ,  $P=0.24$ ). Whilst the higher fixation rates were mostly seen among the wetter species, there were also wet species with very low rates. Furthermore, grouping of bryophytes into functional types according to their morphology did not reduce the variation within mosses and liverworts (data not shown).

## Discussion

This study is the first to compare  $N_2$  fixation rates of a range of lichen and bryophyte species in a direct and standardised way in order to detect and quantify species contributions to ecosystem-level  $N_2$  fixation. It is also the first attempt at classifying cryptogams into functional types according to their potential to sustain colonies of symbiotic cyanobacteria. We have presented evidence for differential  $N_2$  fixation rates among high-level taxonomic lineages of Subarctic cryptogams and tested underlying patterns which might contribute to understanding the process. These include phylogenetic relationships among genera, extent of symbiotic interaction, growth form and morphology, and moisture. The study was carried out in standardised conditions for all species and, considering the large incubation vial size, provides relatively realistic estimates of the atmospheric nitrogen inputs to local ecosystems.

### $N_2$ fixation as a cryptogam functional trait?

We found significant differences in absolute rates of bivalent nitrogen fixation between bryophytes and lichens, and between the two lichens we considered in this study. Within bryophytes, mosses and liverworts showed a clear dissimilarity in their fixation rates as well. The results were highly variable from sample to sample, as also reported in studies from the high-Arctic (Solheim et al. 1996) and the Subarctic (Basilier et al. 1978), but we found no consistent temporal or spatial pattern within the range of our study. There were clear differences in the maximal amounts of fixed nitrogen among the considered species, but the within-species variation masked such patterns in the species means. The latter factor also hampered our attempt to report underlying patterns for the observed diversity in fixation rates. We found no correlation between growth form or habitat moisture and  $N_2$  fixation. Hence the only meaningful grouping factors were higher-level taxonomic lineages and specificity of the symbiotic relationship.

Clearly, lichens demonstrated outstanding capabilities of  $N_2$  fixation on a dry weight basis and this is possibly due to the symbiotic association between mycobiont and cyanobiont. It is interesting to note that the two lichen species showed also, correspondingly to their individual fixation rates, the highest

tissue N-content, which can be a result of intense incorporation of ammonium derived from cyanobacteria by the mycobiont (Rai 1988). They are both tripartite ones and the primary photobiont green algae form the main body throughout the lichen thalli. Cyanobacteria are the secondary symbiont and they are enclosed in cephalodia, where they behave as heterotrophs, providing nitrogen to their host in return for sugars (Nash 1996). This heterotrophic strategy allows for a much higher ratio of heterocysts (the actual site of nitrogen fixation) to vegetative cells in the cyanobacterial filaments. A study by Hitch and Millbank (1975) reports for *Peltigera aphthosa* and *Nephroma arcticum* 21.1% and 14.1% heterocyst frequencies respectively, whereas in bipartite cyanolichens heterocysts form on average 4% of the total algal cell population. Hence, it would be interesting to screen a wide range of bi- and tri-partite lichens and check whether these consistent morphological differences are manifested at the level of N<sub>2</sub> fixation rates.

Moss species in this study formed a group with rates of N<sub>2</sub> fixation one or two orders of magnitude lower than those in lichens. This corroborates findings of Solheim et al. (2002) who estimated fixation rates under natural conditions in Abisko at 2,760  $\mu\text{mol N kg}^{-1} \text{d}^{-1}$  for the cyanolichen *P. aphthosa* and 64  $\mu\text{mol N kg}^{-1} \text{d}^{-1}$  for the forest moss *H. splendens*. The differences we found in fixation rates among different moss species, although non-significant, appeared consistent. We expected these to be correlated with different hydrology regimes at a microhabitat scale for species specific patches. However the big variation in fixation rates within species did not allow us to build a strong regression model. Subdivision of mosses into groups based on their morphology or habitat did not succeed in explaining the observed N<sub>2</sub> fixation rate differences either, owing to the same complications.

The two liverworts in our study, *Lophozia lycopodioides* and *Ptilidium ciliare*, showed no fixation at all. They even showed negative values of fixation (see Table 1), as a likely artefact of our method of subtracting <sup>15</sup>N concentrations in control samples from those in incubated ones. Thus, in practice these values show a lack of nitrogen fixation activity. Despite the frequent abundance of the two birch forest species in the subarctic region, the results for degree of colonisation by cyanobacteria should not be surprising. Although in scientific literature close (internal) symbiotic relationships between a few

liverwort species and cyanobacteria have been reported (see Adams 2002), none of them listed *L. lycopodioides* or *P. ciliare*. Liverworts are known for rich composition of secondary compounds (Asakawa 1995, 2004; Mues 2000), many of which exhibit antimicrobial properties (Asakawa 2008). We speculate these compounds might also thwart colonization of liverworts by epiphytic cyanobacteria.

#### Methodological issues

Throughout the survey we experienced difficulties analysing the data on <sup>15</sup>N accumulation due to the great natural variation of this isotope in the Abisko non-vascular vegetation. A possible source of this signal noise (i.e. high <sup>15</sup>N levels in control samples) could have arisen due to the sampling approach. Moss was cut out to a depth of 6 cm below the surface, and thus potentially included old litter material, which might be enriched with the heavier nitrogen isotope as a result of discrimination during litter decomposition (Nadelhoffer et al. 1996). Nevertheless, we believe that the results shown here are robust and conclusive on the actual amount of atmospheric nitrogen fixation at the level of higher taxa. We consider techniques involving acetylene reduction activity (ARA) to be less laborious but also less informative especially when correlation to N<sub>2</sub> fixation and ammonia production is attempted. Theoretical stoichiometric ratio of ethylene to nitrogen (3:2) is rarely attained and must always be corrected by parallel uptake of labelled <sup>15</sup>N<sub>2</sub> for each organism and possibly location (Basilier 1980; Millbank 1981). Thus we advocate the use of labelled nitrogen gas in further studies and tentatively propose a more extensive sampling for natural abundance of <sup>15</sup>N isotope in the study sites.

#### Regional upscaling of the results

The process of bivalent nitrogen fixation associated with subarctic cryptogams affects nutrient cycling within the ecosystem in a number of ways, but based on our experimental study alone we cannot ascertain how much nitrogen enters the biocycle. Indeed, we need to account for the processes of denitrification, leaching, and relative contribution compared to nitrogen input from other sources (i.e. wet deposition, free-living cyanobacteria on bare soil, nodulated vascular plants). Since these are out of the scope of the current



study we remain cautious in extrapolating the results. Here we use former biodiversity studies in the Abisko region on species and vegetation type cover percentage estimations for the studied habitats. These data, nevertheless, are not meant to serve as a ground for total nitrogen input in the area, but rather as a means to estimate the relative spatial contribution of each of the life forms we investigated to  $N_2$  fixation. In order to achieve this we reported (Table 1) nitrogen fixation both on area and biomass bases, thus accounting for the high area-to-dry weight ratio of the lichens (Nash 1996). The information presented in Table 2 reflects the total nitrogen that enters a respective habitat per day, based on the ground cover of abundant habitat-characteristic species.

The two common cyanolichens we surveyed, *N. arcticum* and *P. aphthosa*, appear to contribute as much as the forest mosses to the total ecosystem input of atmospheric nitrogen into birch-heath woodland. Cyanolichens are virtually absent from the other two habitats studied, fens and bogs, where mosses fix substantial  $N_2$  on a landscape area basis. Thus we claim that mosses probably play a key role in the process of nitrogen fixation there. A similar pattern emerges from older studies done in the region on total yearly nitrogen input. The two cyanolichens *S. paschale* and *N. arcticum* fix  $0.38 \text{ g N m}^{-2} \text{ yr}^{-1}$  in similar Fennoscandian

woodland (Longton 1997) and *N. arcticum* alone fixes  $0.1 \text{ g N m}^{-2} \text{ yr}^{-1}$  in Fennoscandian tundra (Sonesson et al. 1975), whereas the two mosses *S. riparium* and *Drepanocladus exannulus* together fix as much as  $0.3\text{--}4.3 \text{ g N m}^{-2} \text{ yr}^{-1}$  in the Stordalen fen, Abisko (Basilier 1979). In comparison, atmospheric deposition levels of nitrate ( $\text{NO}_y$ ) and ammonia ( $\text{NH}_x$ ), as reported in a survey by the Swedish Environmental Research Institute, are less than  $0.4 \text{ g N m}^{-2} \text{ yr}^{-1}$  for the region of North Sweden (Binkley and Hogberg 1997). These data together ascertain the significance of biological  $N_2$  fixation to the N-limited vegetation in the Subarctic (Crittenden and Kershaw 1978).

In conclusion, our multispecies comparison of  $N_2$ -fixation rates of cryptogams, both under standardised conditions and in situ, applying a direct nitrogen detection method, has provided strong evidence for differential nitrogen input by cryptogam taxa in the Subarctic, and substantiates their importance to the nitrogen economy of these cold biomes. Furthermore we demonstrate that high rates of nitrogen fixation shown by cyanolichens on a small scale can indeed be moderate when extrapolated to an ecosystem level. We conclude that mosses, with their high biomass and overall abundance, are at least equally important drivers of large-scale atmospheric nitrogen input to the Subarctic.

**Table 2** Relative contribution of bryophytes and cyanolichens to the atmospheric nitrogen input to three representative habitats in the Fennoscandian Subarctic

Habitat	Fraction of total ground surface <sup>a</sup>		Estimate for relative N input <sup>b</sup> in $\mu\text{mol N m}^{-2} \text{ d}^{-1}$		Vegetation surveys
	Mosses	Cyanolichens	Mosses	Cyanolichens	
Bog	0.5	–	10.85	–	Abisko, Sweden— Sonesson (1967)
Fen	0.72–0.87	–	51.49	–	Stordalen, Sweden— Rosswall et al. (1975); Sonesson et al. (1975); Sonesson and Kvillner (1980); Wielgolaski (1972)
Birch forest	0.4–0.43	0.05	13.64	12.6	Kevo, Finland— Kallio (1975); Sonesson et al. (1975); Wielgolaski (1972)

<sup>a</sup> Presented fractions of ground cover are based on vegetation surveys for each of the specified habitats

<sup>b</sup> Extrapolated estimates of relative nitrogen input are derived from averaged species-specific N fixation rates per  $\text{m}^2$ . Dashes indicate absence from a particular habitat

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