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Nitrogen dynamics in boreal forests

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Nitrogen dynamics in boreal forests:

A feather moss' perspective

A thesis submitted to Bangor University by

Kathrin Ackermann

In candidature for the degree

Philosophiae Doctor

March 2013

School of Environment, Natural Resources and Geography

Bangor University

Bangor, Gwynedd

LL57 2UW

UK



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Thus, the task is, not so much to see what no one has yet seen;
but to think what nobody has yet thought, about that which everybody sees.

Erwin Schrödinger

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Abstract

The fixation of atmospheric nitrogen (N) is a major pathway for available N entering ecosystems. In N-limited boreal forests, a significant amount of N is fixed by cyanobacteria that live epiphytically on feather mosses. Despite the dominance of bryophytes in the boreal biome and their association with cyanobacteria, the role of feather mosses in boreal N cycling remains poorly understood. Further, evaluating abiotic controls on N₂ fixation in the feather moss-cyanobacteria association is a challenge yet to be addressed. Therefore, the aims of this thesis were to identify the main factors that drive N₂ fixation in the feather moss-cyanobacteria association, and to gain a deeper understanding of the feather moss' role for the N cycle in boreal forests.

The effects of natural and artificial N additions, N deprivation as well as the impact of drying and rewetting events on N₂ fixation rates in the ubiquitous- and cyanobacteria-hosting feather moss *Pleurozium schreberi* (Brid.) Mitt. were analyzed. Further, the ability of *P. schreberi* to take up N from soil was tested, assessing another possible pathway through which the moss can utilize N. The cyanobacterial contribution to the decomposition resistance of the moss was evaluated; and finally, when and how the fixed N enters the soil to become available for plants and microbes was assessed.

The results of my work demonstrated that N₂ fixation is negatively affected by natural as well as artificial N inputs, but the inhibition of N₂ fixation is dependent on the amount of added N and further, N₂ fixation seems to be resilient to N deposition as well as to drying-rewetting events indicated by the increase of N₂ fixation rates in the moss-cyanobacteria association upon removal of the stressors. *P. schreberi* was found to be able to take up organic and inorganic N from soil, but it accounted for a small fraction of total N demand. Cyanobacteria do not contribute to the moss' resistance towards decomposition by soil bacteria. Feather mosses effectively retain the acquired N, thus representing a short-term N sink.

By using lab as well as field-based approaches to identify factors that drive N₂ fixation in the feather moss-cyanobacteria association, I gained a deeper understanding of a major component of the N cycle in boreal forests. The moss was shown to have the ability to use soil-N and to retain N over several months, however, the moss likely represents a long-term N source to the boreal system, due to its association with N₂-fixing cyanobacteria. Nevertheless, assessing the transfer and exchange of N and carbon (C) between feather moss and cyanobacteria, and defining the interface of this, would improve our understanding of their role in ecosystem nutrient cycling and should be subject to future research.

LIST OF CHAPTERS

This thesis is based on the following articles and manuscripts:

- I. **Ackermann K, Zackrisson O, Rousk J, Jones DL, DeLuca TH** (2012) N₂ fixation in feather mosses is a sensitive indicator of N deposition in boreal forests. *Ecosystems* 15:986-998 doi: 10.1007/s10021-012-9562-y
- II. **Ackermann K, Jones DL, DeLuca TH**. Exposure to nitrogen does not eliminate N₂ fixation in the feather moss *Pleurozium schreberi* (Brid.) Mitt. in laboratory incubations. *Submitted for peer review (Plant and Soil)*
- III. **Ackermann K, Jones DL, DeLuca TH**. Recovery of N₂ fixation after drying and rewetting in an ubiquitous feather moss. *Submitted for peer review (Plant and Soil)*
- IV. **Ackermann K, Rousk J, Jones DL, Zackrisson O, DeLuca TH**. Feather moss nitrogen acquisition across natural fertility gradients in boreal forests. *Accepted for publication in Soil Biology & Biochemistry*
- V. **Ackermann K, DeLuca TH, Rousk J**. The cyanobacterial role in the resistance of feather mosses to decomposition - toward a new hypothesis. *Submitted for peer review (PLoS ONE)*
- VI. **Ackermann K, Jones DL, DeLuca TH**. Feather mosses in boreal forests: Nitrogen (N) sink or source? Insights from a ¹⁵N-application-experiment. *Submitted for peer review (Ecosphere)*

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1. Introduction

Boreal ecosystems are generally characterized by nitrogen (N) limitation (Tamm 1991) due to the accumulation of recalcitrant litter with high carbon (C)-to-N ratios and high lignin contents. A main source (> 50 %) of N for many ecosystems is the fixation of atmospheric N₂ performed by various bacteria, including both free-living and symbiotic cyanobacteria (Vitousek et al. 1997). Several species of N₂-fixing cyanobacteria have been found to colonize the feather moss *Pleurozium schreberi* (Brid.) Mitt. in pristine, unpolluted environments where the N₂ fixation of the feather moss-cyanobacteria association can contribute 50 % to the total N input (DeLuca et al. 2002). Feather mosses are a dominant feature of boreal forests, accounting for 60 - 90 % of the groundcover in pristine, mature forests (Oechel and Van Cleve 1986; DeLuca et al. 2002) and contribute significantly to biomass and primary productivity (Lindo and Gonzalez 2010; Turetsky 2003). However, N₂ fixation in the feather moss-cyanobacteria association has been found to be negatively affected by N inputs (DeLuca et al. 2008; Gundale et al. 2011) and therefore significant fixation only occurs in low N deposition areas. While it has been shown that artificial N additions in the laboratory and in the field inhibit N₂ fixation in the feather moss-cyanobacteria association, a more systematic assessment of the effects of N deposition along anthropogenic and natural N gradients on N₂ fixation has yet to be accomplished.

It has been hypothesized that, by maintaining stable environmental conditions for the cyanobacterial colonizer, the feather moss provides a favorable habitat for N₂ fixers. Nevertheless, besides N availability, the moisture status of the cyanobacteria-hosting feather moss seems to be an important driver of N₂ fixation, wherein moist conditions promote N₂ fixation (Jackson et al. 2011; Gundale et al. 2012). However, feather mosses experience large fluctuations in their hydrological status in boreal forests, undergoing significant natural drying and rewetting cycles over the course of only a few hours. While nutrient leaching upon rewetting of feather moss is documented (Carleton and Read 1991), the effect on N₂ fixation has not been tested so far.

Since feather mosses lack roots, they must satisfy their N demands elsewhere. For instance, feather mosses absorb and retain N from throughfall (Li and Vitt 1997; Kotanen 2002), and they relocate and recycle N from dead moss tissue via intracellular

and capillary movement (Aldous 2002). In addition to their main N source, absorption of N from dry and wet deposition, several studies have shown that various moss species are able to take up N from solution and from soil through root-like rhizoids (Ayres et al. 2006; Krab et al. 2008). Thus, mosses seem to use several pathways to access N: (1) throughfall N as their main N source; (2) recycling of N; (3) N uptake from soil, and (4) the possibility to gain access to fixed N_2 provided by associated cyanobacteria. However, no attempts have been made to qualitatively relate the various pathways feather mosses use to obtain N.

The feather moss-cyanobacteria association is assumed to be a mutualistic relationship, in which the partners exchange C and N with each other (Turetsky 2003). The bacterial partner retains only 20 % of the fixed N_2 , while the remaining portion is transferred as ammonium (NH_4^+) or amino acids to the host plant (Steinberg and Meeks 1991; Meeks and Elhai 2002). In addition, the plant partner is thought to provide protection and shelter. Further, it is possible that the cyanobacteria contribute to the unpalatable nature of the feather moss via production of toxic substances (Adams and Duggan 2008; Kaasalainen et al. 2012), which could explain the low utilization of feather mosses as a food source (Eskelinen 2002). However, while the plant partner in other documented symbioses commonly undergoes morphological changes in order to host the bacterial symbiont (Meeks 2009), the feather moss does not show obvious signs of adaptation to a cyanobacterial symbiont. Many studies have been investigating cyanobacteria associations with other plants, yet, none has explored the relationship between cyanobacteria and feather mosses. Thus, the relationship shared between feather mosses and cyanobacteria is unknown and remains unassessed to date.

Perhaps the most central question that remains unanswered is the fate of the fixed N_2 in feather mosses. The cyanobacteria are likely to leak NH_4^+ , but whether this N is transferred to the soil and if so, at which rates and timescales, is unknown. Mosses are known to be extremely efficient in retaining nutrients and water (Dickson 2000; Aldous 2002). This is not surprising given their dependence on nutrient and water supply through atmospheric deposition and the lack of roots. However, the pathway of N after fixation by cyanobacteria associated with feather mosses will characterize the N cycle of the boreal ecosystem as a whole, highlighting the question's relevance.

Given the global importance of the boreal biome, which accounts for 17 % of the earth terrestrial surface (DeLuca et al. 2008), and the dominance of feather mosses

in these N-limited ecosystems (Oechel and Van Cleve 1986), changes in N cycling processes due to fluctuations in N₂ fixation rates, are likely to have global implications.

I hypothesize that:

- (1) a) N₂ fixation in the feather moss *Pleurozium schreberi* is reduced by artificial as well as natural N inputs (*Chapter I, II, IV*),
b) Inhibition of N₂ fixation by high N inputs is reversible (*Chapter II*)
- (2) Cyanobacteria, although cell counts are below detection on moss from high N deposition areas, are constantly present on the moss leaves. Upon high N-demand (low N deposition), cyanobacterial population growth is induced which enables and triggers N₂ fixation; upon low N-demand (high N deposition), growth and N₂ fixation is inhibited – economize N use vs. N₂ reduction (*Chapter II*)
- (3) Cyanobacterial N₂ fixation rapidly recovers upon rewetting of desiccated moss (*Chapter III*)
- (4) *P. schreberi* is able to take up N from soil, both in organic and inorganic form (*Chapter IV*)
- (5) Cyanobacteria live in symbiosis with *P. schrebei*, providing not only N but also adding to the chemical protection of the moss towards decomposers and herbivores (*Chapter V*)
- (6) *P. schreberi* is highly conservative of N and does not readily lose N to the soil within short temporal scales (up to one year) (*Chapter VI*)

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2. Literature Review

2.1. The nitrogen (N) cycle and the boreal forest

Ecosystem nutrient cycles include the introduction of nutrients, their conversion, their transfer between organisms and their loss from the system. These processes are essential for the primary productivity on earth; and the first essential nutrient to grow short in supply will set the bar for that ecosystem's productivity. Boreal forests are considered to be limited in nitrogen (N) (Tamm 1991), due to the accumulation of recalcitrant litter and plant material with high carbon (C)-to-N ratios, leading to rapid immobilization of inorganic N and decreased net N mineralization rates (Keeney 1980; Scott and Binkley 1997). Further, there are few geological sources of N (Morford et al. 2011) in the European boreal forest. Therefore, boreal forest soils are characterized by a tight internal N cycle where immobilization processes dominate (Giesler et al. 1998; Schimel and Bennett 2004). Considering that the boreal biome accounts for 17 % of the Earth's land surface (DeLuca et al. 2008), the ability of this ecosystem to sustain productivity is important to consider for global biogeochemical budgets.

One main source of biological available N is the fixation of atmospheric N₂ performed by free-living or symbiotic bacteria and algae (Vitousek et al. 1997). This conversion of N₂ to ammonia (NH₃) is the initial step in the N cycle. While most well known symbioses are represented by legumes and associated root-bacteria, no legumes are found in northern boreal forest ecosystems. However, it has been shown that several moss species are colonized by N₂ fixing cyanobacteria that fix substantial amounts of atmospheric N (DeLuca et al. 2002; DeLuca et al. 2008). These moss-cyanobacteria associations contribute significantly to N input in boreal forests (Fig. 1), alleviating the pronounced N-limitation and thus, representing a vital feature for maintaining productivity in boreal ecosystems.

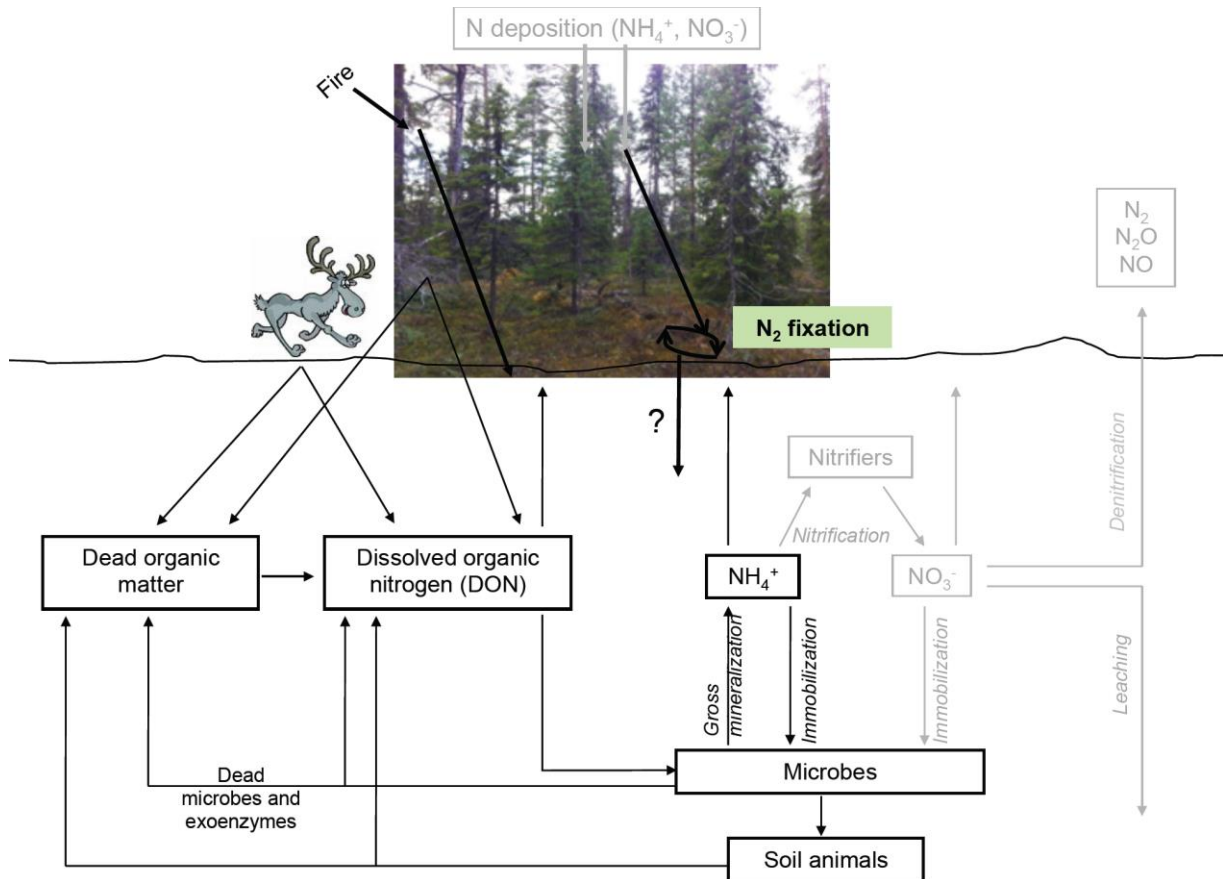


Fig. 1. Simplified schematic overview summarizing the N cycle in boreal forests, including N₂ fixation in feather moss carpets as a candidate for filling the N gap in boreal ecosystems. Boxes and arrows in grey indicate less common or less important pathways of N in the boreal forest.

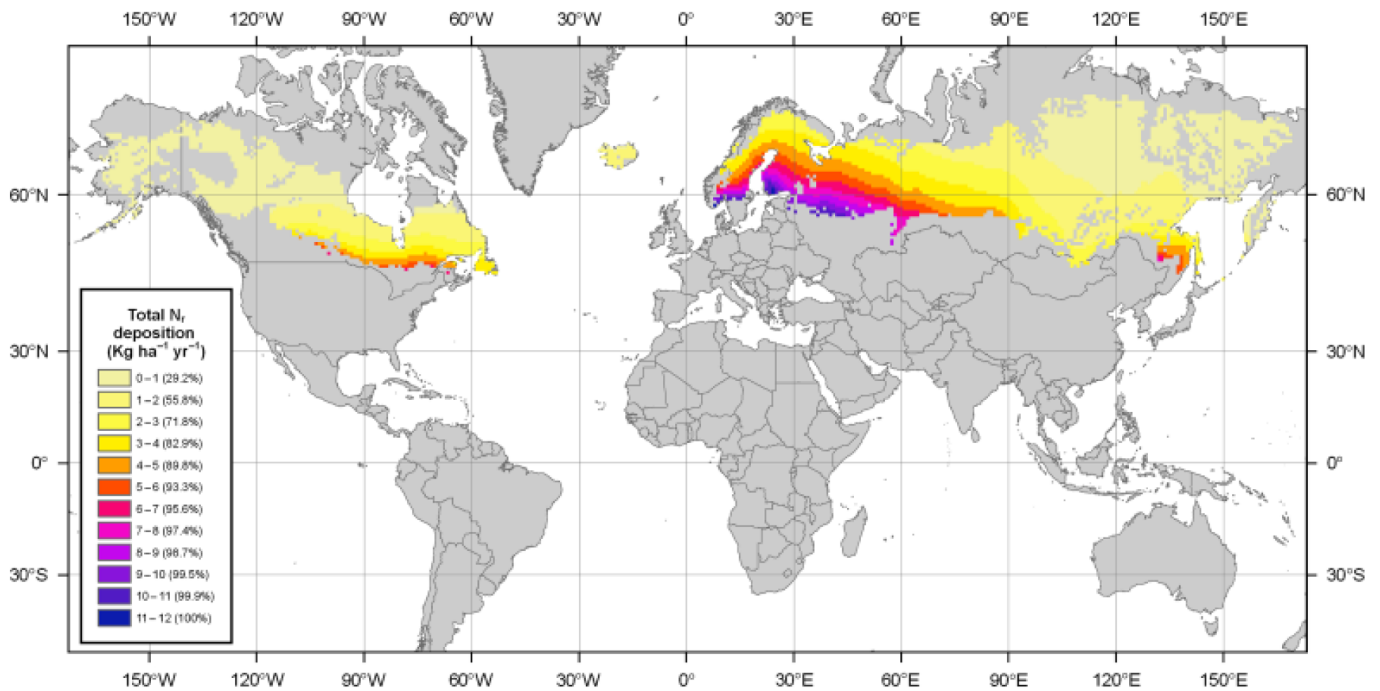


Fig. 2. The deposition of total reactive nitrogen (N_r) (NH_x and NO_y) ($kg\ N\ ha^{-1}yr^{-1}$) in the boreal biome. The colors correspond to $kg\ N\ ha^{-1}yr^{-1}$, with darker colors indicating higher N deposition (see legend). Values in parentheses are the portion of the boreal biome receiving N_r deposition at or below the given value (Gundale et al. 2011).

2.2. Boreal Forests - Feather mosses - Cyanobacteria

Boreal forests receive low amounts of background N deposition (Phil-Karlsson et al. 2009; Gundale et al. 2011) (Fig. 2). In addition, boreal forest soils are characterized by low concentrations of inorganic N, low pH and low temperatures (Read 1991), contributing to the N-limitation in these systems and suggesting a tight N-cycle. The vegetation in boreal forests consists of coniferous trees (*Pinus sylvestris* (L.), *Picea abies* (L. (Karst)), ericaceous shrubs (*Vaccinium vitis-idea* (L.), *V. myrtillus* (L.), *Empetrum hermaphroditum* (L.)), lichens (e.g. *Cladonia* sp.) and moss species (e.g. *Hylocomium splendens*, *Pleurozium schreberi* (Brid.) Mitt.).



Fig. 3. A late-succession boreal forest site in Northern Sweden with an open canopy and a moss-dominated understory.

Photo by K. Ackermann

Mosses likely play a crucial role in boreal forest ecosystems due to their contribution to habitat heterogeneity (Longton 1988), their influence on hydrology, temperature and chemistry of boreal forest soils (Cornelissen et al. 2007). For instance, summer soil temperature below moss carpets is lower compared to sites without moss cover (Bonan 1991; Startsev et al. 2007), leading to slower decomposition rates (Prescott et al. 1993). However, mosses release substantial amounts of nutrients (C, N, phosphorus (P)) upon rewetting of dried tissue, funnelling plant and microbial-available nutrients into the soil (Carleton and Read 1991). Further, mosses contribute fundamentally to the biomass and productivity in boreal forests, which can exceed tree biomass (Martin and Adamson 2001; Turetsky 2003; Lindo and Gonzalez 2010). The ubiquitous feather moss *P. schreberi* can account for up to 80 % of the ground cover in boreal forests (Oechel and Van Cleve 1986; DeLuca et al. 2002) (Fig. 3). In addition to its abundance, *P. schreberi* is colonized by N₂ fixing cyanobacteria (Fig. 4) in pristine, unpolluted environments where the cyanobacterial colonizers fix substantial amounts of atmospheric N₂ (DeLuca et al. 2002; Zackrisson et al. 2004). By buffering abiotic factors and exhibiting a high water retention capacity (Dickson 2000), feather mosses can provide a stable and favourable habitat for cyanobacteria, promoting N₂ fixation in N-limited ecosystems (DeLuca et al. 2002).



Fig. 4. A section of a *Pleurozium schreberi*-leaf at x 100 magnification under an UV-fluorescence microscope. Coiled chains of *Nostoc* are seen in bright red. Photo by K. Ackermann

To date, four cyanobacteria genera (*Nostoc*, *Stigonema*, *Calothrix*, *Cylindrospermum*) have been identified living epiphytically on leaves of *P. schreberi*. (Gentili et al. 2005; Ininbergs et al. 2011). Numbers of colonizing cells and N₂ fixation in *P. schreberi* show a linear relationship (DeLuca et al. 2007) (Fig. 5). Hence, cyanobacterial density on moss leaves can be used as an indicator for the reduction of atmospheric N₂ and vice versa.

N₂ fixation is commonly measured using a ¹⁵N₂-calibrated acetylene reduction (AR) assay (Schöllhorn and Burris 1967; Zackrisson et al. 2004), which takes advantage of the fact that the enzyme responsible for the reduction of N₂ (nitrogenase) preferably reduces acetylene (C₂H₂). The product of acetylene reduction by the cyanobacterial nitrogenase enzyme is ethylene (C₂H₄) (Dilworth 1966), which can be measured by gas chromatography. Several advantages nominate the AR technique as the preferable method for assessing N₂ fixation (Hardy et al. 1973): (1) the AR assay is 10³ - 10⁴ more sensitive than ¹⁵N methods, (2) the type of the electron acceptor (N₂ or acetylene) does not influence the rate of electron transfer, (3) ethylene is the sole product of AR, (4) ethylene does not inhibit N₂ fixation and is not reduced by nitrogenase, (5) no examples of non-N₂-fixing organisms have been found to reduce acetylene. Additionally, the activity of the nitrogenase enzyme is measured, not its concentration (Hardy et al. 1973). Therefore, the AR technique is a direct measurement of a function, which is an adequate approach to be able to understand ecosystem processes and factors influencing these. For the nitrogenase enzyme to work, ATP, a reductant (in this case acetylene) and anaerobic conditions are required (Hardy et al. 1973). In order to solve the problem of oxygen (O₂) sensitivity of the nitrogenase enzyme, cyanobacteria form specialized

heterocyst cells that restrict O_2 concentrations improving conditions for reducing reactions. Photosynthetic reactions are carried out in separate, vegetative cells; an extra wall layer composed of polysaccharides and glycolipids is deposited and aerobic respiration is increased to consume any O_2 that enters the cell (Wolk et al. 1994; Meeks 2009). These heterocysts are specialized for fixing N_2 , while another type of cells is specialized for movement via gliding (hormogonia; phototactic), in addition to the non-specialized, vegetative cells (Meeks and Elhai 2002).

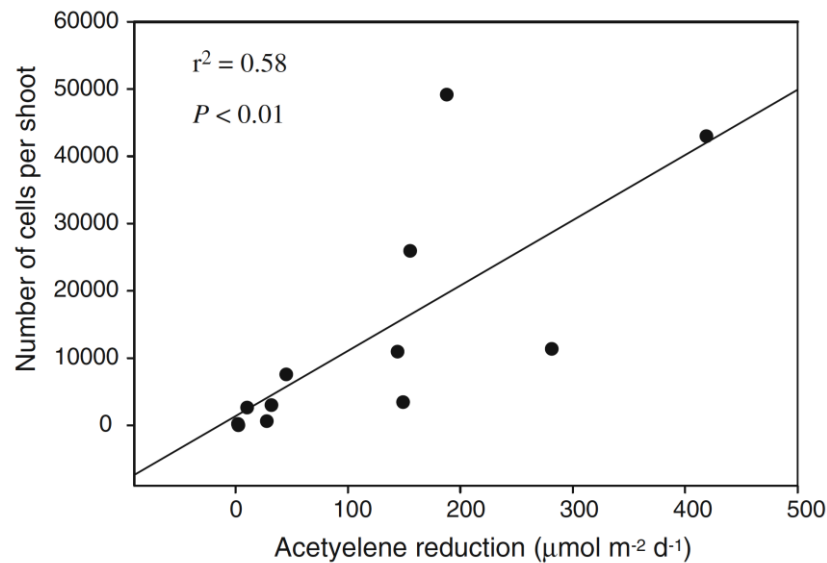


Fig. 5. Relationship between total numbers of cyanobacterial cells shoot⁻¹ and average acetylene reduction ($\mu\text{mol m}^{-2} \text{d}^{-1}$) as a measurement of N_2 fixation in the feather moss *Pleurozium schreberi*. From DeLuca et al. (2007).

2.3. Abiotic controls of N_2 fixation in the feather moss-cyanobacteria association

The association between feather mosses and cyanobacteria could play a fundamental role in the N cycling in N-limited boreal forests by contributing $2 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ via N_2 fixation to the N pool in mature forest ecosystems (DeLuca et al. 2002). This value is on par with the atmospheric N deposition in the boreal biome, which ranges between 1 and $2 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ (e.g. Gundale et al. 2011). Thus, the feather moss-cyanobacteria association can account for 50 % of the total N input in boreal forests (Cleveland et al. 1999; DeLuca et al. 2002; Zackrisson et al. 2004). However, moss biomass (Solga et al. 2005; Nordin et al. 2006), as well as biomass and activity (nitrogenase enzyme) of

cyanobacteria (DeLuca et al. 2007; DeLuca et al. 2008) are sensitive to N inputs, leading to drastic reductions in the abundance of dominant moss species and to significant reductions or total exclusion of N₂ fixation in feather moss-cyanobacteria associations (Zackrisson et al. 2004; DeLuca et al. 2008; Gundale et al. 2011).

Roads and road-derived pollutants represent a major cause of disturbance and pollution in many ecosystems (Gadsdon and Power 2009). Various effects of road derived N (NO_x, NH₃) on vegetation along road corridors have been observed including changes in plant growth (Bignal et al. 2008) and tissue N concentrations (Sheppard and Leith 2002). In particular, mosses seem to be sensitive to NO₂. Bell (1992) showed that road derived NO₂ emissions significantly inhibited shoot growth. In addition, roads also deposit heavy metals, dust and salt (Spellerberg 1998), leading to reduced photosynthetic activity in plants, accumulation of heavy metals in moss tissue and reduced plant growth along roads (Flückiger et al. 1977, 1978; Pearson et al. 2000). Heavy metal pollution along roads can be ascribed to tyre wear and car exhausts (Bakirdere and Yaman 2008), both of which have various effects on ecosystem function since the amount of input as well as the species of heavy metal that enters the system could have differently severe impacts.

Mosses effectively absorb nutrients and water from atmospheric deposition, which is their main source of N, making them extremely sensitive to increased nutrient and heavy metal inputs (e.g. Bengtsson et al. 1982). Therefore, subsequent effects of road derived N and heavy metal input on N₂ fixation in feather mosses growing along roads is likely. Given the pristine condition of boreal forests, even moderate road traffic is likely to affect ecosystem functioning along roads in these N-limited systems.

Artificial N additions *in-situ* have been shown to significantly decrease numbers of cyanobacterial cells on moss leaves at levels of only 3 kg N ha⁻¹yr⁻¹ coinciding with a significant reduction in N₂ fixation in the feather moss-cyanobacteria association (Gundale et al. 2011) (Fig. 6). Even 3 years after N additions (4.25 kg N ha⁻¹) in the field, N₂ fixation rates in feather mosses were still lower than in mosses not exposed to N additions (Zackrisson et al. 2004). However, the effects of road-derived N on N₂ fixation rates in mosses growing along roads has not been explored to date and further, the specific amount of N input that it takes to down-regulate N₂ fixation rates under controlled conditions has not been sufficiently investigated. Additionally, the potential of feather moss-cyanobacteria associations grown in high N deposition areas to fix N₂

has not been studied to date. Also, the capacity of N₂ fixation in the feather moss-cyanobacteria association to recover from high N loads remains unknown.

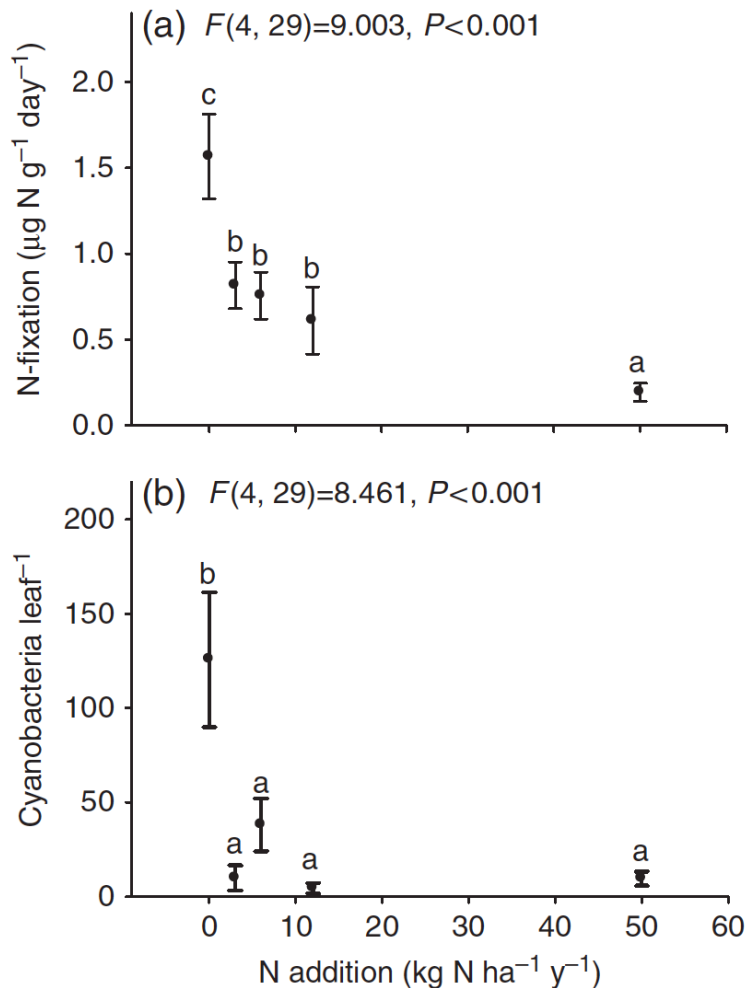


Fig. 6. Mean (\pm SE) of a) N₂ fixation per unit *Pleurozium schreberi* mass ($\mu\text{g N g}^{-1}\text{d}^{-1}$) and b) cyanobacteria density leaf⁻¹ of *P. schreberi* in response to N additions (0, 3, 6, 12, 50 kg ha⁻¹yr⁻¹) in the field. Significant differences are indicated with different letters (Gundale et al. 2011).

N₂ fixation performed by the feather moss-cyanobacteria association is affected by various abiotic factors, others than N, that influence the amount of N₂ that is fixed. For instance, the hydrological status of the moss seems to be a crucial factor driving N₂ fixation: moisture, as well as rainfall frequency positively affect N₂ fixation rates in feather mosses (Solheim et al. 2002; Gundale et al. 2009; Jackson et al. 2011; Gundale et al. 2012). This is not surprising, given the fact that mosses absorb water over their entire surface from the atmosphere and do not take up water from soil (Tyler 1990). Therefore, mosses readily lose water under dry conditions, which could in turn affect the activity of the cyanobacterial colonizer.

Moss growth as well as cyanobacterial activity peak in early spring after snowmelt (May-June) and in late summer (September), and drop in between (July-August) (Basilier and Granhall 1978; Zackrisson et al. 2004). This drop in moss growth and activity in summer could correspond to a period of dormancy in mosses as a result of dry conditions and photoinhibition (Sveinbjörnsson and Oechel 1992; Zackrisson et al. 2004). In the boreal forest, feather mosses are exposed to annual as well as extreme daily fluctuations in climatic conditions, leading to significant changes in the hydrologic status of the moss. Frequently, dry episodes are followed by heavy rainfall over the course of only few hours, resulting in high fluctuations in moisture content of the moss. Thus, feather mosses experience natural and intensive recurrent drying and rewetting events. Significant leaching of nutrients from mosses has been found upon rewetting of dried moss (Carleton and Read 1991), resulting in nutrient rich leachates available for soil biota. However, mosses are relatively desiccation-tolerant, they are able to withstand drying until no free water remains in the cells and quickly return to normal metabolism and growth upon rewetting (Proctor 2001). Also, many fundamental processes like photosynthesis, resume quickly after rewetting, with some moss species starting to fix CO₂ within minutes upon rewetting (Proctor et al. 2007).

P. schreberi is a ubiquitous, widespread species, found in various habitats that exhibit many different climatic characteristics (Tesky 1992). *P. schreberi* is classified as being poikilohydric (the state of hydration is controlled by the environment) (Glime 2007) and is supposed to be tolerant to desiccation due to enacting cellular repair (Glime 2007). However, moisture condition of mosses could change the nutrient supply and nutrient exchange between mosses and associated cyanobacteria. Scott (1960) suggested that not only nutrients, but also light and moisture could affect the balance and rates of nutrients exchanged between the symbiotic partners in lichen symbioses, upsetting the relationship between the mycobiont and the photobiont.

The recovery of nitrogenase activity in free-living cyanobacteria after desiccation is supposed to be slower than the recovery of photosynthesis because *de novo* protein synthesis is required for N₂ fixation (>24 h vs. 4 h for N₂ fixation and photosynthesis, respectively) (Hawes et al. 1992) (see also Fig. 7). However, lichen species that live in symbiosis with cyanobacteria (e.g. *Nostoc sp.*) as photobionts (cyanolichens) seem to be more tolerant to drying than lichen species with other photobionts than cyanobacteria, suggesting that cyanolichens are tolerating more severe drying events since they are dependent on liquid water for activity (Gauslaa et al. 2012).

Also, cyanobacteria can form dormant cells during dry conditions and quickly resuscitate upon rewetting (Kaplan-Levy et al. 2010) to resume fixing N_2 as opposed to first recolonize and regrow a population. However, the effects of drying and rewetting on N_2 fixation rates in the feather moss-cyanobacteria association has not been explored to date. Further, the ability of the nitrogenase activity in cyanobacteria associated with feather mosses to recover upon rewetting of dried moss remains unknown.

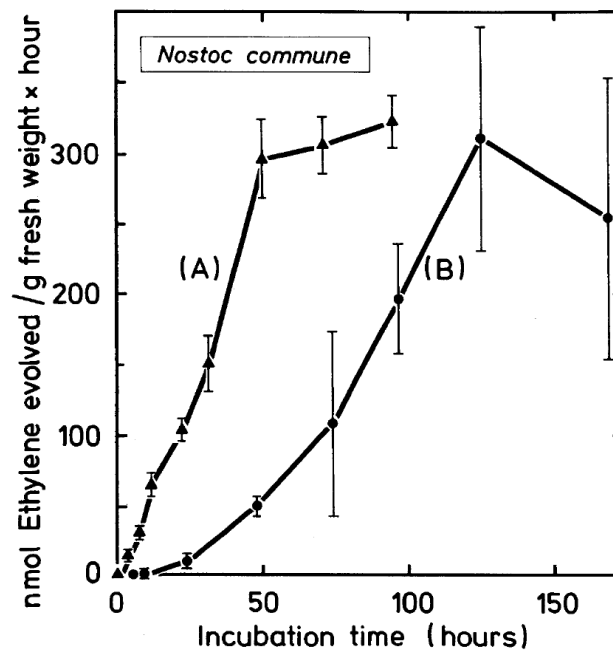


Fig. 7. Nitrogenase activity as nmol ethylene produced $g\ fw^{-1}h^{-1}$ of free-living *Nostoc commune* upon rewetting after a 2-day dry period (A) and after a 2-year dry period (B). From Scherer et al. (1984).

2.4. Nitrogen (N) utilization by *P. schreberi*

Nitrogen is an essential nutrient for plants, animals, and microbes; however, the boreal forest is considered to be N-limited (Tamm 1991), resulting in slow N turnover rates (Read 1991). In most high latitude or high altitude ecosystems, inorganic N fluxes are found to be insufficient to cover the N demands of plants (e.g. Kielland 1994). In addition, organic N is often found in higher concentrations than inorganic N in soil solution, especially in soils with low pH and low inorganic N availability (Kielland 1994; Kielland 1995; Nordin et al. 2001; Finzi and Berthrong 2005). Thus, N demand

of plants has to be satisfied by a combination of sources and pathways (Jones et al. 2005). There is accumulating evidence that, besides the uptake of mineralized, inorganic N (ammonium (NH_4^+), nitrate (NO_3^-)), plants possess the capacity to take up organic N in the form of amino acids, urea, polyamines and small polypeptides (Kielland 1994; Schimel and Bennet 2004; Persson and Näsholm 2008; Krab et al. 2008; Hill et al. 2011) (see Fig. 1). However, the uptake capacity of plants is restricted to these low molecular weight dissolved organic nitrogen (DON) compounds (DiTomaso et al. 1992; Bush 1993). Several decades ago, Newton (1974) documented that mycorrhizal and non-mycorrhizal roots could take up intact amino acids (see also Watson and Fowden 1975; Kielland 1994). Since then, a vast amount of studies have been conducted dedicated to prove that plants are able to take up organic N directly, short-circuiting the N cycle (Schimel and Bennett 2004). For instance, Näsholm et al. (1998) and Persson and Näsholm (2008) showed that many boreal forest and taiga plant species have the ability to take up amino acids directly from soil pools. Therefore, DON may represent a significant and directly useable pool of N for taiga forest plant species (Jones and Kielland 2002). In contrast, other studies showed that organic N represents only a minor source for plants to cover their N needs. For instance, Hodge and co-workers (2000) showed that 26 % of added organic N was captured by plants, but this accounted for only 1 % of the total N in plants. Further, amino acids are removed rapidly from the soil-N pool via microbial activity, resulting in fast turnover rates of amino acids in soils (Jones and Kielland 2002; Rousk and Jones 2010).

Competition for low molecular weight DON between microbes and plants is likely to occur especially in the rhizosphere, where root exudation of low molecular weight compounds including amino acids, sugars and organic acids is high (Curl and Truelove 1986). Although microbes seem to be superior competitors for inorganic and organic N in soils, plants have the advantage that root turnover times are slower than turnover times of microbial cells and thus, may be better competitors over longer time periods (Hodge et al. 2000).

Not surprisingly, mycorrhizal plants seem to be better competitors than non-mycorrhizal plants for organic N in the rhizosphere (Jones et al. 2005). In addition to vascular plants, mosses have been found to take up amino acids from solution and directly from soil (Ayres et al. 2006; Krab et al. 2008; Hill et al. 2011) (Fig. 8). Further, it has been suggested that feather mosses are associated with fungi (Kausarud et al. 2008; Davey et al. 2009), which could enhance the uptake of N from soil. Feather

mosses, however, are thought to cover most of their N demands via absorption of N originating from atmospheric deposition, leaching and throughfall (Li and Vitt 1997; Kotanen 2002). Another source of N for mosses is the efficient relocation and recycling of nutrients along the moss-profile, from dead moss tissue to the growing parts at the apex (Aldous 2002). In addition, feather mosses possess an endogenous N supply due to their association with N₂ fixing cyanobacteria, which fix substantial amounts of atmospheric N in pristine environments (DeLuca et al. 2002). Thus, feather mosses are able to gain N via various sources and pathways. However, no attempts have been made to qualitatively relate one source of N with another source of N for mosses. For instance, combining measurements of N₂ fixation rates in feather mosses with their ability to take up N directly from soil and with that, to explore their role as a net N source due to their cyanobacterial associates or as a N sink due to their ability to take up N from soil, and to retain and capture N from throughfall, could help to fill crucial knowledge gaps. Further, analysing soil-N cycling processes in relation to varying N₂ fixation rates could help to answer the question of what role the feather moss-cyanobacteria association play in the boreal N cycle.

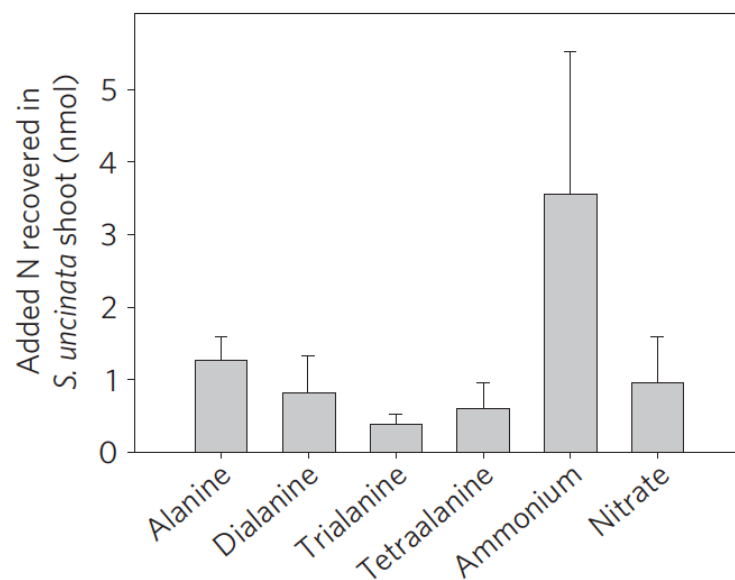


Fig. 8. Recovery of added N in moss shoots of *Sanionia uncinata* 1 h after injection of amino acid, peptide or inorganic N into the soil below the moss. Values are mean \pm SE. From Hill et al. (2011).

2.5. Ecology of the feather moss-cyanobacteria association – What relation do they share?

The term symbiosis (*Symbiotismus*) was first introduced in 1877 by A.B. Frank, who described it as a case in which two different species (symbionts) live in or on one another, irrespective of the role of the individuals. The very source of our existence is the result of two fundamental symbioses between prokaryotes, in which one partner formed the vital cell organelles chloroplasts or mitochondria. Probably the most well known group of bacteria living in symbioses with plants are cyanobacteria, descendent from the bacteria that colonized eukaryotic cells to form the plant characterizing chloroplasts (Mereschkowski 1905). Cyanobacteria are an ancient, diverse and widespread group found as free-living cells and colonies as well as living in symbiosis with higher plants, lichens and bryophytes (Rai et al. 2000; Adams and Duggan 2008; Meeks 2009). Cyanobacteria are facultative autotrophs, they possess the ability to fix C as well as N, which allows the establishment of the cyanobacteria-plant symbioses in various ecosystems. Since the symbioses are favored under N-limiting conditions, their importance in N-limited ecosystems should not be underestimated (Rai et al. 2000). In their free-living state, cyanobacteria retain the ability to fix both essential nutrients (C, N). However, when living in association with a plant partner, cyanobacteria commonly stop fixing C and instead receiving C from their symbiotic partner in exchange for fixed N₂ (Meeks 2009). The plant partner receives N as NH₄⁺ or amino acids from the cyanobacteria and in return provides carbohydrates, shelter and protection (Meeks et al. 1985; Steinberg and Meeks 1991), suggesting that the cyanobacteria down-regulate their own photosynthesis (Meeks and Elhai 2002; Adams and Duggan 2008). Given that N₂ fixation is a highly energy demanding process (Scherer and Zhong 1991; Turetsky 2003; Houlton et al. 2008), living in association with a symbiotic partner could also have an economic advantage. While studies on bryophyte-cyanobacteria associations are scarce, similar mechanisms and principles are assumed to take place in the bryophyte-cyanobacteria relationship (Rai et al. 2000; Turetsky 2003): the bryophyte offers protection, carbohydrates and receives fixed N₂ in return.

However, in the lichen symbiosis for instance, the balance between the exchange of nutrients seems to be not always exclusively beneficial, but rather depends on the nutrient demands of the partners (Johansson et al. 2011). Scott (1960) already noticed that variations in the supply of nutrients, light and moisture could upset the symbiotic

balance between the mycobiont and photobiont in lichen symbioses. The growth of both symbionts is controlled by N, C and moisture levels, resulting in a delicate balance between the partners (Scott 1960).

Given the significant N-limitation of boreal ecosystems (Tamm 1991), the cyanobacteria could provide crucial nutrients to the ecosystem, affecting the productivity and the biogeochemical cycles. The abundant and ubiquitous feather moss *Pleurozium schreberi* lives in association with various cyanobacteria (Gentili et al. 2005; Ininbergs et al. 2011) that fix atmospheric N₂. Although feather mosses are a characteristic and dominant feature of boreal forests, they are consumed by very few herbivores (Prins 1982). Furthermore, the few herbivores that do consume mosses appear to specifically avoid *P. schreberi* and *Hylocomium splendens* (also colonized by cyanobacterial N₂ fixers) (Eskelinen 2002). Moss litter is known to be recalcitrant and a poor N source for microbes, resulting in slow to no detectable decomposition rates in high latitude ecosystems (Hobbie 1996). Also, mosses produce inhibitory compounds like phenols, which add to the recalcitrant nature of moss litter. In addition, moss-specific secondary metabolites (oxylipins) could contribute to the decomposition resistance of the moss (Croisier et al. 2010; Matsui 2006) and could also be used to regulate the colonization by cyanobacteria. Nevertheless, given the low density of easily decomposable plants in boreal ecosystems, the low litter quality of moss (Prins 1982; Hobbie 1996) seems to be an insufficient explanation for the lack of herbivory of this plentiful plant material.

Cyanobacteria are known to produce toxins (e.g. microcystins) (Cox et al. 2005; Adams and Duggan 2008; Kaasalainen et al. 2012). Microcystins are highly toxic, small, cyclic peptides produced by cyanobacteria in freshwater systems (predominantly strains of the genus *Nostoc*) that have been found responsible for animal poisoning (Sivonen 2009). *Nostoc* has been found to also produce the toxin when living in symbioses with lichens (Kaasalainen et al 2009; Kaasalainen et al. 2012). Reindeers, thus, avoid eating cyanolichens, even during periods of starvation (Storeheier et al. 2002; Rai 2002). Given that the feather moss is colonized by strains of *Nostoc* as well, it is likely that toxic substances produced by the cyanobacterial colonizer provide protection and would explain the moss' resistance to herbivory and decomposition. This would add to the cyanobacteria's contribution to the feather moss-cyanobacteria relationship, which would be another dimension of the proposed mutualistic symbiosis between cyanobacteria and its feather moss-hosts.

2.6. Where does the nitrogen (N) go?

In northern boreal forests, atmospheric N deposition ($1 - 2 \text{ kg N ha}^{-1}\text{yr}^{-1}$) and total N input is low (Tamm 1991; Gundale et al. 2011). Since feather mosses host N_2 fixing cyanobacteria, they likely represent a major N source in N-limited ecosystems (DeLuca et al. 2002, Gundale et al. 2011). Given the abundance and biomass of feather mosses in boreal forests (Oechel and Van Cleve 1986), their N input could be crucial for the N cycle. Mosses lack roots and their connection to soil is limited to their rhizoids, reaching only the surface layers of soil and most often only the humus layer. Mosses have, however, been found to be associated with a variety of fungal species (Kauserud et al. 2008; Davey et al. 2009), which could account for N translocation from deeper soil layers to the moss and vice versa. Also, increased microbial activity has been found below moss carpets in boreal forest ecosystems (Jackson et al. 2011), which could be attributed to nutrient leaching after rainfall events (Carleton and Read 1991; Jackson et al. 2011).

Since mosses capture and retain significant amounts of N from throughfall (Fig. 9) and host N_2 fixing cyanobacteria, it has been suggested that forest ecosystems are dependent on N release from moss carpets (Weber and Van Cleve 1984; Oechel and Van Cleve 1986; Carleton and Read 1991), especially in low-N deposition areas where N_2 fixation rates in mosses are high (Gundale et al. 2011). Thus, the main N input to the system is via the moss layer (Li and Vitt 1997), making the moss the rate-limiting step. However, little is known about the fate of the N_2 that is fixed by cyanobacteria associated with mosses. To date, no knowledge exists of the fixed N_2 , whether it is transferred to the soil and with that, if it is available for microorganisms and plants. Assuming the fixed N_2 is transferred to the soil, questions about the amount, extent and rates of the transferred N emerge. Thus, the role and importance of feather moss-cyanobacteria associations for the boreal N cycle and as a significant N source remains unclear and is in need of systematic study.

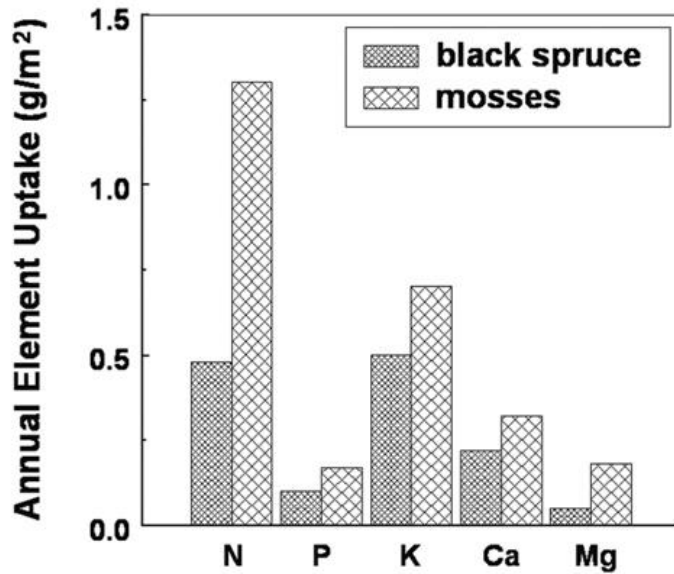


Fig. 9. Comparison of annual nutrient uptake by mosses and black spruce (*Picea mariana*). Figure based on Oechel and Van Cleve (1986).

2.7. Objectives

- (1) Identify the drivers of N_2 fixation in the feather moss-cyanobacteria association (*Chapters I, II, III, IV*):
 - ⇒ How do “artificial” and “natural” N inputs affect N_2 fixation? What is the threshold above which N deposition inhibits N_2 fixation?
 - ⇒ How does water stress affect N_2 fixation?
 - ⇒ Is N_2 fixation in the feather moss-cyanobacteria association able to recover from stress factors such as increased N deposition, desiccation and subsequent rewetting?
- (2) Evaluate the relationship between feather mosses and its colonizing cyanobacteria: do the cyanobacteria contribute to the moss’ unpalatable nature? (*Chapter V*)
- (3) Evaluate the role of *Pleurozium schreberi* as a N source or sink for the boreal forest (*Chapter IV, VI*):
 - ⇒ Is the feather moss able to take up N from soil, and if so, in what form? Can we relate N uptake from soil to N_2 fixation rates?
 - ⇒ Is the fixed N_2 transferred from moss to soil? If so, when is it detectable in the soil?

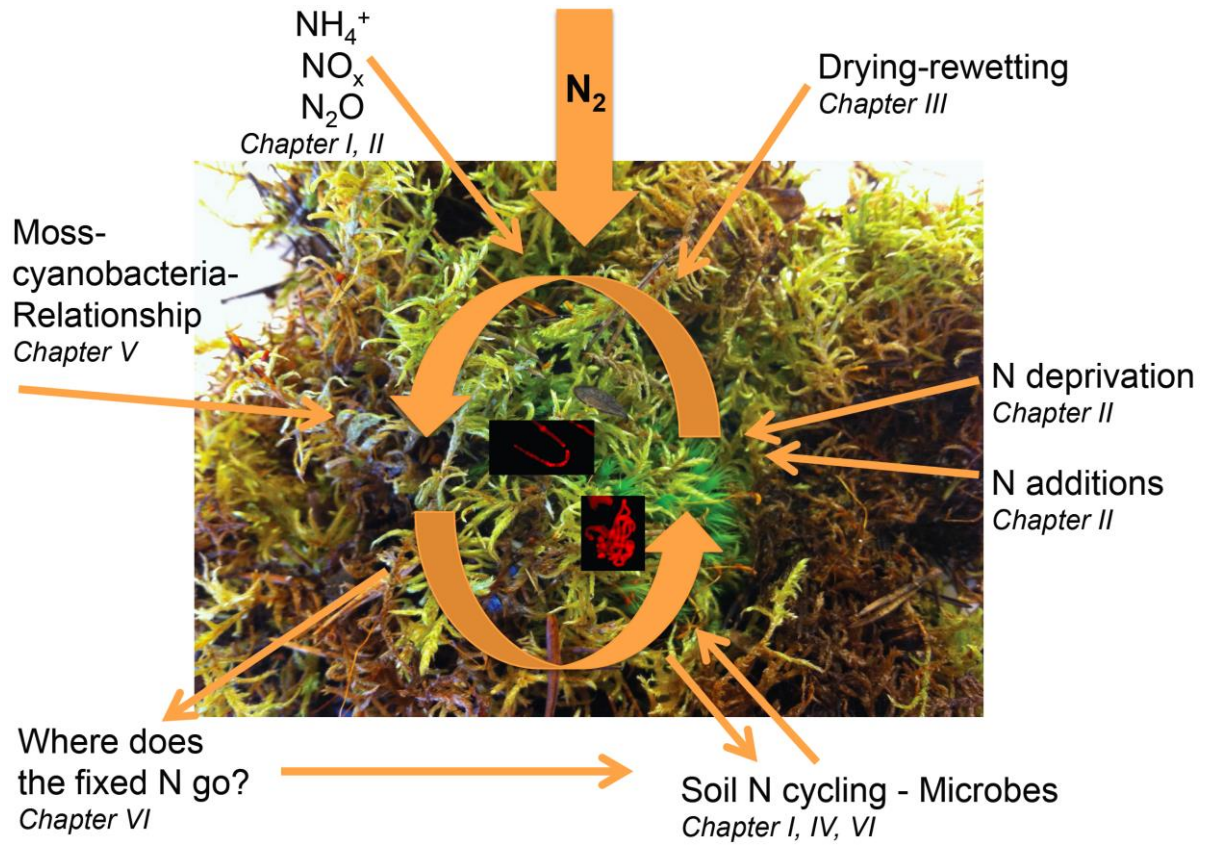


Fig. 10. Schematic summary of the main aspects of my PhD project with the abiotic factors that drive N_2 fixation in the feather moss-cyanobacteria association and the influence on the soil and the soil biota.

2.8. References

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I.

N₂ fixation in feather mosses is a sensitive indicator of N deposition in boreal forests

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KA performed the lab analyses. OZ and THD performed the nitrogenase analyses. JR conducted the PLFA analyses. KA analyzed the data. KA wrote the first draft, with all authors contributing to the final manuscript.

II.

Exposure to high nitrogen (N) does not eliminate N₂ fixation in the feather moss *Pleurozium schreberi* (Brid.) Mitt. in laboratory incubations

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Exposure to nitrogen does not eliminate N₂ fixation in the feather moss *Pleurozium schreberi* (Brid.) Mitt. in laboratory incubations

Kathrin Ackermann, Davey L. Jones, Thomas H. DeLuca

Abstract

The feather moss *Pleurozium schreberi* (Brid.) Mitt. has commonly been found to be colonized by cyanobacteria in pristine environments. These moss-associated cyanobacteria fix substantial amounts of atmospheric N₂ in N-poor ecosystems, but are inhibited by N deposition. Although feather mosses are a common and dominant feature in many ecosystems, the threshold of N input that leads to the inhibition of N₂ fixation has not been adequately investigated. Further, the ability of N₂ fixation to recover in mosses from high N deposition areas has not been studied to date. Herein, we report results from two laboratory studies in which we (1) applied a range of concentrations of N as NH₄NO₃ to moss from Northern Sweden with a low N deposition history, and (2) we took mosses from a long-term high N deposition area (Wales, UK) and deprived them of N to test their ability to recover N₂ fixation. Higher addition rates (up to 10 kg N ha⁻¹) did not systematically inhibit N₂ fixation in *P. schreberi*. Conversely, by inducing N deprivation in mosses from a high N environment, N₂ fixation rates increased after several weeks and reached values found in moss from the low N deposition areas. We estimate that the threshold of total N deposition to inhibit N₂ fixation in *P. schreberi* is likely to be higher than 10 kg N ha⁻¹. Further, the feather moss and its associated cyanobacteria is able to recover from high N inputs and is able to fix atmospheric N₂ after a period of N deprivation.

Keywords: Acetylene reduction, boreal forest, bryophytes, cyanobacteria, N deposition, nitrogenase activity

Introduction

Most high latitude ecosystems like boreal forests are characterized by nitrogen (N)-limitation (Tamm 1991), whereas many other ecosystems have experienced increased N inputs due to anthropogenic activity (e.g. Galloway and Cowling 2002). We can therefore distinguish between areas with high levels of atmospheric N deposition (through industry, traffic, etc.) and areas with low levels of atmospheric N deposition, such as northern boreal, Arctic and Antarctic ecosystems (e.g. Shaver and Chapin 1980). These ecosystems receive a large proportion of total ecosystem N via fixation of atmospheric N₂ by free-living or symbiotic cyanobacteria (DeLuca et al. 2002; Zackrisson et al. 2004; Smith 1984; Stewart et al. 2011). The ubiquitous feather moss *Pleurozium schreberi* (Brid.) Mitt. is colonized by several species of cyanobacteria in pristine, unpolluted areas, where they fix substantial amounts of atmospheric N₂ (DeLuca et al. 2008; Gundale et al. 2011). This association was found to contribute significantly to the N cycle in northern boreal forests by accounting for 50 % of the total N input (Cleveland et al. 1999; DeLuca et al. 2002; Zackrisson et al. 2004). In these mid to late succession forests, feather mosses are found in dense carpets, contributing significantly to the biomass and primary productivity (Lindo and Gonzalez 2010; Turetsky 2003). Hence, their association with several species of cyanobacteria could fill the N gap in N poor systems by funneling available N to the system. However, it has been shown that moss biomass (Solga et al. 2005; Nordin et al. 2006), the presence of cyanobacteria on moss leaves (DeLuca et al. 2007), as well as the activity of the nitrogenase enzyme catalyzing the fixation of atmospheric N₂ (DeLuca et al. 2008) are all highly sensitive to N inputs, leading to the reduction or complete exclusion of N₂ fixation in moss experiencing high N inputs (Zackrisson et al. 2004; Gundale et al. 2011; Ackermann et al. 2012). However, the precise amount of N input that leads to reduced N₂ fixation rates remains poorly understood. Further, the potential of N₂ fixation by feather moss-cyanobacteria associations in high N deposition areas has not been explored to date.

Herein, we report results from an N addition experiment and from an N deprivation experiment, which add to the growing understanding of the feather moss-cyanobacteria association. To determine the N addition threshold for nitrogenase activity in cyanobacteria, we applied N as NH₄NO₃ in various concentrations over a period of four weeks on *P. schreberi* and its associated cyanobacteria collected from

mid to late succession forests in Northern Sweden where atmospheric N₂ deposition is low (Phil-Karlsson et al. 2009) and therefore, N₂ fixation is high (DeLuca et al. 2008; Ackermann et al. 2012). By using a laboratory approach, we were able to apply various amounts of N and to monitor N₂ fixation rates over several weeks under stable conditions. Also, by measuring N₂ fixation rates after only few weeks of N addition, we would be able to detect short-term effects of N additions, which could be relevant when N inputs are fluctuating, for instance, in periods of elevated N concentrations in rainfall (industry highs, traffic, etc.). Considering previous studies on the sensitivity of N₂ fixation in the feather moss *P. schreberi* to external N inputs (DeLuca et al. 2008; Gundale et al. 2011), we expected N₂ fixation to decrease at an addition of about 6 - 10 kg N ha⁻¹.

We also compared N₂ fixation rates in *P. schreberi* from low deposition areas in Northern Sweden with rates from a high N deposition area in Wales, UK. Further, we complemented the N-addition experiment with a mirrored “deprivation” approach. Here, we deprived the moss from N to induce N₂ fixation in the “Welsh” moss and thus determine whether moss and cyanobacteria are able to recover from increased N loads and start fixing atmospheric N₂. We hypothesize that the moss constantly hosts cyanobacteria, but the numbers of colonizing cells differ depending on the levels of N input. Further, we postulate that cyanobacteria in mosses from high N deposition areas are capable of colonizing and fixing N₂ after a period of N depletion.

Materials and Methods

Study sites and sampling

For the N addition experiment, we collected shoots of *P. schreberi* from four mid to late succession forest sites in Northern Sweden (“Dötternoive”, “Björknäs”, “Laddok”, “Kuottavare”) in August 2011. The sites were located between latitude 64-65 °N and longitude 18-19 °E and have been described previously (DeLuca et al. 2002; Zackrisson et al. 2004). Vegetation at all sites was composed of Scots pine (*Pinus sylvestris* L.), Norway spruce (*Picea abies* L. (Karst)), feather moss carpets of *P. schreberi* and *Hylocomium splendens*, shrubs like *Vaccinium vitis-idea* (L.), *V. myrtillus* (L.) and *Empetrum hermaphroditum* (Hagerup).

For the N deprivation experiment, we used shoots of *P. schreberi* from “Dötternoive” and “Björknäs”, representing low N deposition areas (1 - 2 kg N ha⁻¹ yr⁻¹), as well as from a 64 year old Scots- and Corsican Pine (*P. sylvestris*; *P. nigra* L.) forest at Newborough, Wales, UK (53° 9265 N, 4° 2340 W), where N deposition is high (> 12 kg ha⁻¹yr⁻¹, Jones et al. 2004). Additionally, samples of the N₂ fixing lichen *Peltigera* spp. (Willd.) were taken from the same location in Wales to be able to compare N₂ fixation in a mutualistic symbiosis with N₂ fixation in the *P. schreberi*-cyanobacteria association, which has not been characterized so far. *Peltigera* spp. is colonized by the same species of cyanobacteria (*Nostoc* sp.) and is known to fix substantial amounts of atmospheric N₂ (Alexander and Shell 1973; Hitch and Milbank 1975). Mean annual temperature and precipitation were 9.8 °C and 1044 mm at the UK site, respectively. The understory vegetation in Newborough forest was composed of *P. schreberi*, *H. splendens*, *Cladonia* spp., *Peltigera* spp. and *Deschampsia cespitosa* (Beauv.).

Nitrogen addition experiment

Three replicates for each N addition (0, 3, 5, 10 kg N ha⁻¹) per sampling site (4 forest sites) were used. One replicate consists of 10 moss shoots. These were placed in 50 ml centrifuge tubes and kept in a growth chamber at 10 °C, 70 % humidity and 16/8 h light/dark cycles during the course of the experiment. N was applied as NH₄NO₃ at 4 different concentrations: 0, 3, 5, 10 kg N ha⁻¹ over a four week period with a volumetric addition of 5 ml week⁻¹. Distilled water was used as control treatment (0 kg N ha⁻¹). One quarter of the aimed N concentrations were applied every week so that the final N concentrations were reached after four weeks. We did not apply the total N concentrations at once in order to prevent damage of the moss. A stock solution of 100 mg N l⁻¹ of NH₄NO₃ was diluted to yield 3 kg, 5 kg and 10 kg N ha⁻¹ solutions. The centrifuge tubes were perforated at the bottom to ensure through-flow of N and to prevent accumulation of N in the tubes.

Nitrogen deprivation and cyanobacteria counts

Four replicates (10 shoots each) of *P. schreberi* from each of the two Swedish forest sites and from the Welsh site as well as small parts (2 – 3 cm²) of *Peltigera* spp. from the Welsh site were transferred into 50 ml centrifuge tubes. The samples were kept in the growth chamber at constant 10 °C, 70 % humidity and 16/8 h light/dark cycles

during the course of the experiment. Moss shoots and lichen samples were rinsed with 5 ml sterile, distilled water every second week to induce N deprivation. The centrifuge tubes were perforated at the bottom to ensure water flow. Cyanobacteria on moss leaves were counted at the beginning of the experiment, after 16 weeks and at the end of the experiment (24 weeks) using a Zeiss Axiophot fluorescence microscope, with 20 x magnification. Twenty-five moss leaves per replicate were screened for cyanobacterial presence. Cyanobacteria possess auto-fluorescent phycobiliproteins to capture light; therefore no additional staining was required for visualization.

N₂ fixation analyses

Nitrogen fixation analyses were performed using a ¹⁵N₂-calibrated acetylene reduction assay (Schöllhorn and Burris 1967) as described by Zackrisson et al. (2004). Briefly, 10 moss shoots from each site were placed in 20 ml glass vials, sealed and 10 % of the headspace was replaced with acetylene. Moss samples were incubated for 24 h in the growth chamber at 10 °C. Ethylene generated in the headspace by the cyanobacterial nitrogenase enzyme was measured by gas chromatography equipped with a Flame Ionization Detector (Varian, Santa Clara, USA) using an automatic headspace sampler (Quma, Wuppertal, Germany). N₂ fixation in the feather moss was measured before the N additions (t0) and after 1.5, 3.5, 5.5, 11, 13, 15, 17 and 19 weeks after the first N addition (15 weeks after the last addition). N₂ fixation in the N-deprived moss was measured with the same protocol before the experiment and at 2, 4, 6, 8, 10, 12, 16, 18, 20, 22 and 24 weeks after the start of the experiment. Data are presented as the amount of acetylene reduced to ethylene in $\mu\text{mol m}^{-2} \text{d}^{-1}$, where 10 moss shoots of *P. schreberi* represent an area of 2.8 cm². The surface area of the lichen samples ranged between 2 and 3 cm².

Statistical analyses

Differences in acetylene reduction between N additions, time and sampling sites were tested with analyses of variance (ANOVA) approaches with repeated measurements. The same approach was used to detect differences in acetylene reduction between sampling sites and time in the N deprivation experiment. Relationships between acetylene reduction, N addition, cyanobacteria counts and time were tested with linear

regression analyses. All analyses were performed using R 2.14 (R Development Core Team 2011).

Results

Nitrogen addition experiment

Nitrogen fixation in moss from all sites showed a similar trend (Fig. 1 A-D): Immediately (four days) after the last N addition, with the final N concentrations, N₂ fixation increased slightly followed by a steep decline two weeks after the last N addition in all samples and all treatments. However, N₂ fixation in all moss samples recovered after they reached the lowest levels but decreased again by the end of the experiment. ANOVA with repeated measurements showed no differences in N₂ fixation between the sites, but differed significantly in the different N additions ($F = 4.24$; $p = 0.006$) and over time ($F = 40.24$; $p < 0.001$). No correlation was found between N addition and N₂ fixation when data from all sites were combined (Fig. 2). Thus, no consistent pattern in maximum and minimum N₂ fixation rates could be identified due to N additions. For instance, moss from “Laddok” had highest as well as lowest levels of N₂ fixation rates in the 10 kg N ha⁻¹ treatment (see below).

Mean N₂ fixation in moss from Björknäs was 31.9 $\mu\text{mol m}^{-2} \text{d}^{-1} \pm 6.5$ ($\pm\text{SE}$) (acetylene reduced) before the first N addition (Fig. 1 A) and ended with a mean of 10.5 $\mu\text{mol m}^{-2} \text{d}^{-1} \pm 5.1$ after 19 weeks (Fig. 1 A). Lowest values were found two weeks after the last N addition in the 10 kg N ha⁻¹ treatment (1.3 $\mu\text{mol m}^{-2} \text{d}^{-1}$ acetylene reduced) and highest values after 17 weeks in the control treatment (487.1 $\mu\text{mol m}^{-2} \text{d}^{-1}$), thus differed significantly between the N additions ($F = 13$; $p < 0.001$). Here, N₂ fixation was negatively correlated with N additions ($r^2 = 0.07$; $p = 0.002$; $df = 34$). N₂ fixation in moss from Dötternoive started with a mean of 56.8 $\mu\text{mol m}^{-2} \text{d}^{-1} \pm 13.5$ and by the end of the experiment, mean N₂ fixation was 10.4 $\mu\text{mol m}^{-2} \text{d}^{-1} \pm 2$ (Fig. 1 B). Lowest levels of N₂ fixation were reached at two weeks after the last N dose in the 5 kg N ha⁻¹ treatment (1.2 $\mu\text{mol m}^{-2} \text{d}^{-1}$) and highest after 15 weeks in the 10 kg N ha⁻¹ addition (382 $\mu\text{mol m}^{-2} \text{d}^{-1}$) (Fig. 1 B). N₂ fixation was not correlated with N additions. N₂ fixation in *P. schreberi* from Kuottavare had an average of 43.9 $\mu\text{mol m}^{-2} \text{d}^{-1} \pm 9.9$ at the start of the experiment and 21.1 $\mu\text{mol m}^{-2} \text{d}^{-1} \pm 3.5$ at the end of the experiment (Fig. 1 C). Values were lowest at two weeks after the last N addition in the 10 kg N ha⁻¹

treatment ($1.6 \mu\text{mol m}^{-2} \text{d}^{-1}$) and highest immediately after the last N addition in the 3 kg N ha^{-1} treatment ($284.3 \mu\text{mol m}^{-2} \text{d}^{-1}$). Here, N addition had no effect on N_2 fixation. N_2 fixation was high in moss from Laddok at the beginning of the experiment (mean $65.9 \mu\text{mol m}^{-2} \text{d}^{-1} \pm 19.8$), ended with $16.9 \mu\text{mol m}^{-2} \text{d}^{-1} \pm 3.4$ (Fig. 1 D). The lowest activity was measured two weeks after the last N addition in the 10 kg N ha^{-1} addition ($1.3 \mu\text{mol m}^{-2} \text{d}^{-1}$) and highest 15 weeks after start also in the 10 kg N ha^{-1} additions ($1065 \mu\text{mol m}^{-2} \text{d}^{-1}$). No differences in N_2 fixation between the N additions were found.

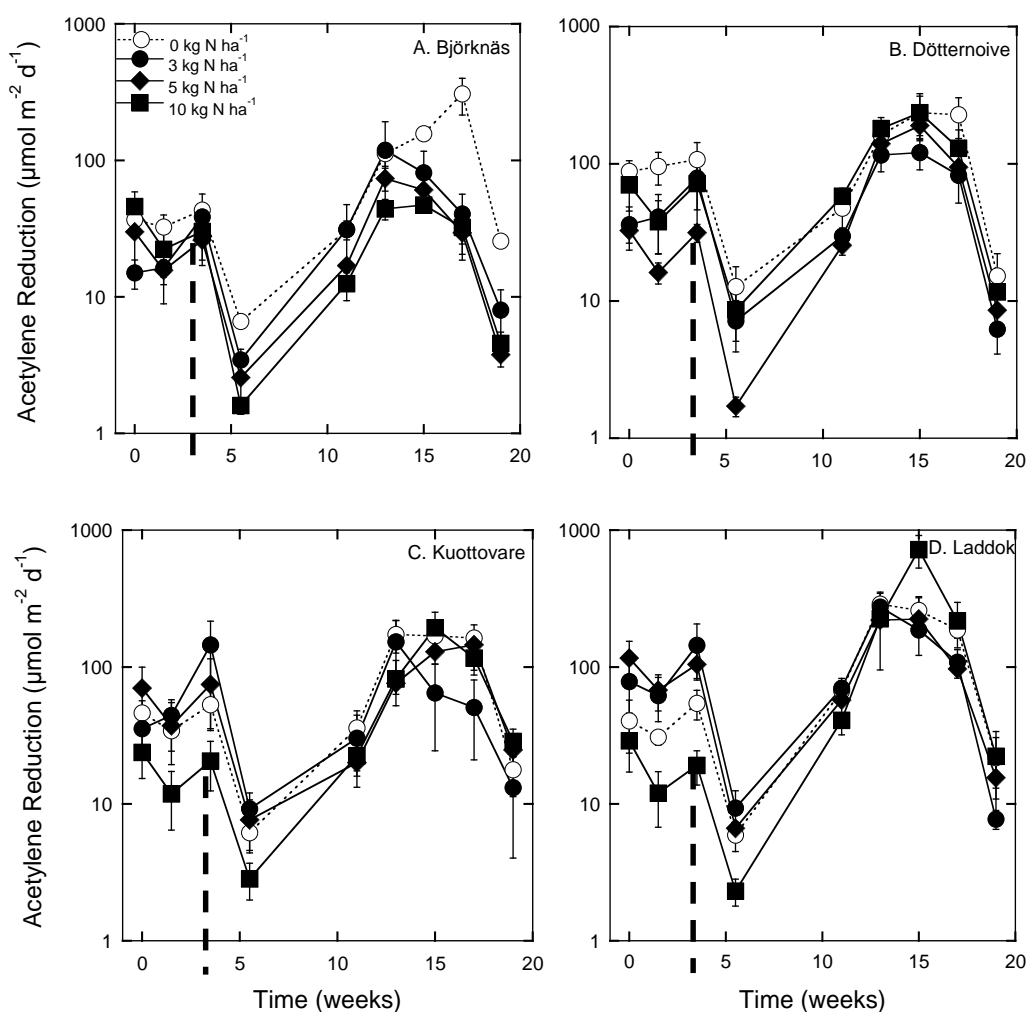


Fig.1. N_2 fixation (acetylene reduction ($\mu\text{mol m}^{-2} \text{d}^{-1}$)) in *P. schreberi* collected from four N-poor forest sites (Björknäs; Dötternoive; Kuottavare; Laddok) in Northern Sweden over the course of the N addition experiment (0 - 19 weeks). Shown are mean values ($n = 3$) \pm SE in moss treated with different N additions (0, 3, 5, 10 kg N ha^{-1}). Vertical dashed lines indicate the last N additions (receiving the final N concentrations (3, 5, 10 kg N ha^{-1})). Open circles represent the control treatments (distilled water = 0 kg N ha^{-1}); filled circles the 3 kg N ha^{-1} additions, filled rhombi the 5 kg N ha^{-1} additions and filled squares represent the 10 kg N ha^{-1} additions. Note the logarithmic Y-axis.

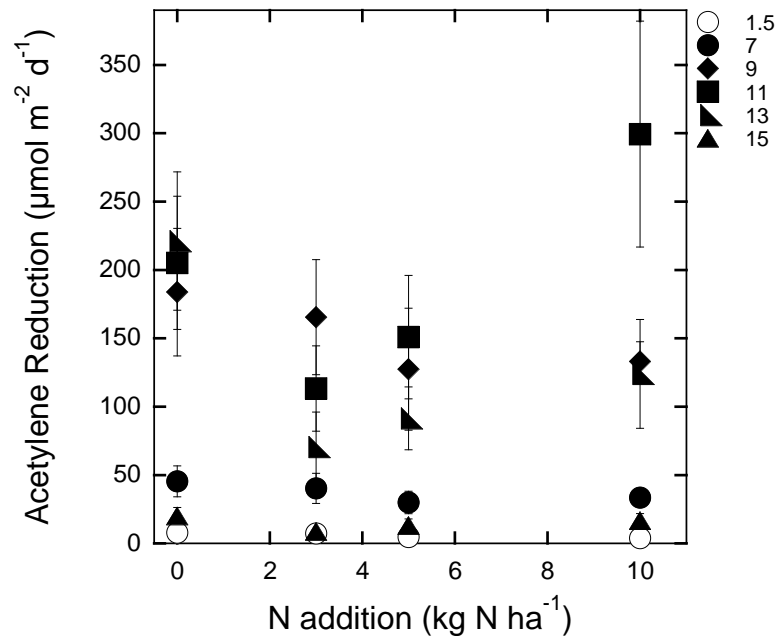


Fig. 2. N₂ fixation (acetylene reduction (μmol m⁻² d⁻¹)) in *P. schreberi* in relation to the N additions (0, 3, 5, 10 kg N ha⁻¹) measured at various time points after the last N addition. Shown are mean values ±SE of the four forest sites in Northern Sweden over the course of the experiment. Different symbols represent the weeks (1.5 - 15) after the final N additions. No correlation between AR and N addition was detected.

Nitrogen deprivation experiment

Nitrogen fixation in the N-deprived moss showed variation between measurements, but the pattern seemed similar between the different sites (Fig. 3). Moss from the high N deposition area (Newborough, Wales) started with no detectable N₂ fixation rates (0 μmol acetylene reduced m⁻² d⁻¹), but increased over time and reached N₂ fixation levels similar to moss samples from Northern Sweden after 22 weeks of N deprivation (430.7 μmol m⁻² d⁻¹) (Fig. 3). Thus, N₂ fixation rates increased significantly with time in the absence of N ($r^2 = 0.07$; $p = 0.04$; $df = 46$). N₂ fixation rates in moss from low N deposition areas started higher than the “Welsh” moss (Björknäs: 3.1 μmol m⁻² d⁻¹; Dötternoive: 1.4 μmol m⁻² d⁻¹) and reached highest levels after 6 weeks of N deprivation (Björknäs: 3853 μmol m⁻² d⁻¹; Dötternoive: 1743 μmol m⁻² d⁻¹). No significant correlation with time was found. The lichen samples from Newborough had highest N₂

fixation rates of all samples at the beginning of the experiment ($28.8 \mu\text{mol m}^{-2} \text{d}^{-1}$). And rates were highest after 6 weeks of N deprivation ($5503 \mu\text{mol m}^{-2} \text{d}^{-1}$) (Fig. 3).

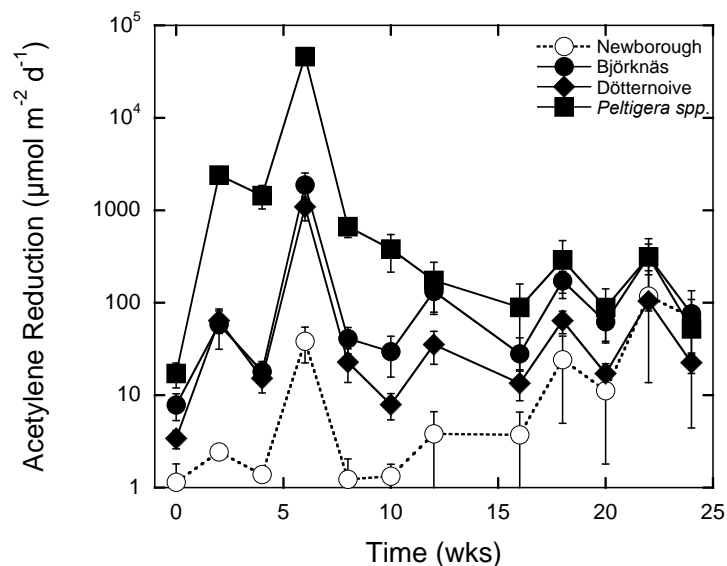


Fig. 3. N_2 fixation as acetylene reduction ($\mu\text{mol m}^{-2} \text{d}^{-1}$) in the feather moss *P. schreberi* from a high N deposition area (Newborough, UK; open circles) and two low N deposition areas (Björknäs (filled circles), Dötternoive (filled rhombi), Sweden) and N_2 fixation in the lichen *Peltigera spp.* from a high N deposition area (Newborough, UK; filled squares). Shown are means ($n = 4$) \pm SE. SE are sometimes hidden behind symbols. Note the logarithmic Y-axis.

Table 1. Number of cyanobacteria leaf⁻¹ and acetylene reduction ($\mu\text{mol m}^{-2} \text{d}^{-1}$) in *P. schreberi* at 3 time points (0, 16, 24 weeks) during the N deprivation experiment from one high N deposition area (Newborough, UK) and two low N deposition areas (Björknäs and Dötternoive, Northern Sweden). Values are mean (n = 4) and \pm standard error (SE).

| Time | Sample | # Cyanobacteria | Acetylene Reduction ($\mu\text{mol m}^{-2} \text{d}^{-1}$) |
|------|-------------|-----------------|--|
| 0 | Newborough | 0 \pm 0 | 1.14 \pm 0.66 |
| 16 | Newborough | 1 \pm 1 | 3.72 \pm 2.85 |
| 24 | Newborough | 43 \pm 32 | 69.75 \pm 65.33 |
| 0 | Björknäs | 14 \pm 9 | 7.84 \pm 2.53 |
| 16 | Björknäs | 14 \pm 5 | 28.10 \pm 13.69 |
| 24 | Björknäs | 60 \pm 15 | 76.08 \pm 32.62 |
| 0 | Dötternoive | 31 \pm 11 | 3.40 \pm 0.76 |
| 16 | Dötternoive | 27 \pm 14 | 13.43 \pm 4.7 |
| 24 | Dötternoive | 23 \pm 6 | 22.42 \pm 5.28 |

Numbers of cyanobacteria in moss samples from Newborough increased from undetectable numbers of cyanobacteria at the beginning of the experiment to a maximum of 140 cells leaf⁻¹ (43 \pm 32) (mean \pm SE) by the end of the experiment (Fig. 4; Table 1). In moss from Björknäs, numbers increased from 14 \pm 9 leaf⁻¹ at the start to 60 \pm 15 leaf⁻¹ at the end of the experiment. Numbers of cyanobacteria in moss from Dötternoive remained similar over the course of the experiment (31 \pm 11 leaf⁻¹ at the beginning and 23 \pm 6 leaf⁻¹ at the end of the experiment). Acetylene reduction and cyanobacterial colonization showed a linear correlation ($r^2 = 0.87$, $p < 0.001$) (Fig. 5).

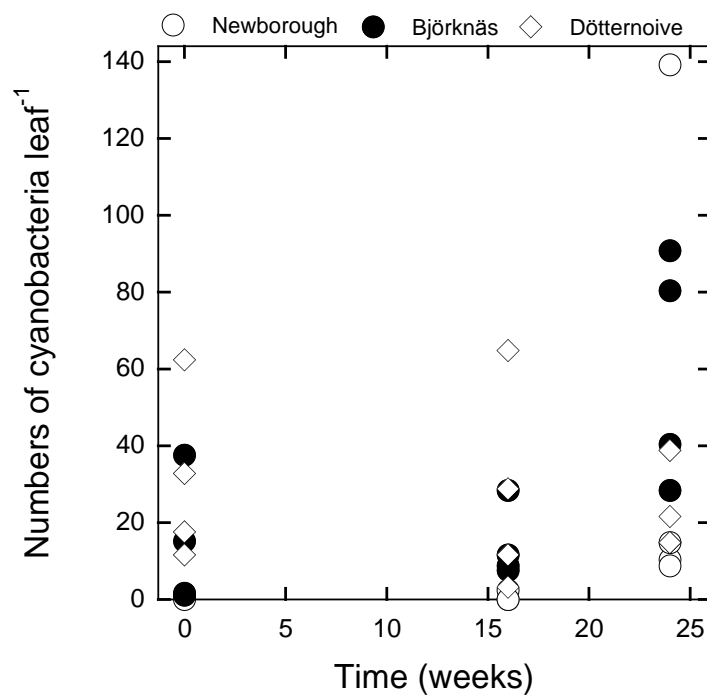


Fig. 4. Numbers of cyanobacteria leaf⁻¹ in *P. schreberi* over the course of the N deprivation experiment (at the beginning, after 16 and 24 weeks of N deprivation). Shown are total cyanobacterial numbers in replicate moss samples from Newborough (high N deposition area; open circles), Björknäs (low N deposition area; filled circles) and Dötternoive (low N deposition area; open rhombi). Twenty-five leaves per replicate were screened for cyanobacteria.

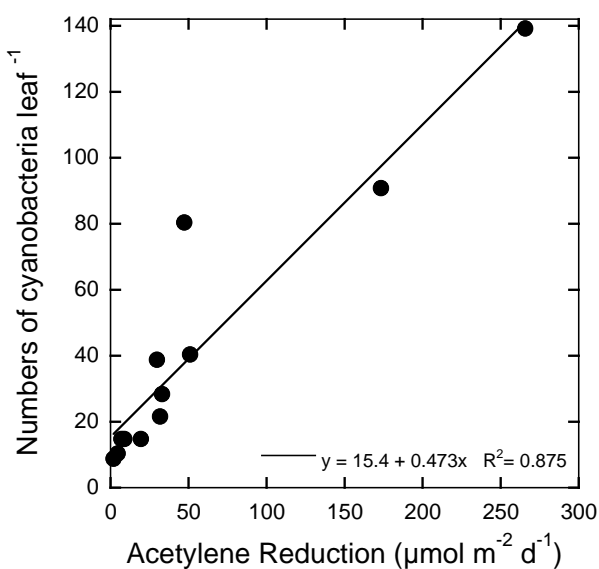


Fig. 5. Numbers of cyanobacteria on *P. schreberi* leaves after 24 weeks of N deprivation. Shown are total counts leaf⁻¹ from all sample sites (n = 12). Regression equation, regression line and r^2 value are given.

Discussion

Nitrogen addition experiment

The response of N₂ fixation to inorganic N additions in our experiment was similar in moss samples from all forest sites and also in the different N addition rates. We found a sharp decline in all treatments two weeks after the last N addition, when the N application had been applied in full. However, N₂ fixation seemed to recover after several weeks. We cannot conclude that the decrease in acetylene reduction was due to N additions as we found a decrease in all treatments (see Fig. 2). The observed decline in N₂ fixation was rather caused by a different factor. For instance, moisture content of moss has been shown to positively affect N₂ fixation in mosses, whereas dry conditions have been observed to reduce N₂ fixation dramatically (Solheim et al. 2002; Gundale et al. 2009). Further, N₂ fixation is not only promoted under moist conditions, but also, frequency and volume of water input influence N₂ fixation (Jackson et al. 2011), with frequent watering appearing to be more important than the volume of water added (Jackson et al. 2011; Gundale et al. 2012a). We watered the moss by adding 5 ml of the N additions in the first four weeks of the experiment. As the moss seemed optimally moist when kept in the growth chamber at low temperature and high humidity, we did not apply additional water between the acetylene reduction runs. Also, the recovery of N₂ fixation after week six suggests that cyanobacteria were permanently present on the moss leaves, but could have been in a dormant state during the course of the experiment when environmental conditions have been unfavourable (e.g. dry moss shoots). The cyanobacteria could have become active again when conditions improved (Kaplan-Levy et al. 2010) since cyanobacteria are known to be able to form dormant cells under harsh conditions like desiccation (Potts 1994; Kaplan-Levy et al. 2010).

Light intensity is a factor that can affect N₂ fixation in the moss-cyanobacteria association (see e.g. Gundale et al. 2012b) and could explain fluctuations in the measurements. However, we kept the samples in the growth chamber at constant day/night cycles over the course of the experiment, excluding the effects of light intensity on N₂ fixation in our experiment. In addition, DeLuca et al. (2007) found no effects of different light conditions on N₂ fixation rates in feather mosses.

The results reported herein contrast with previous field studies, which showed that long-term external N additions of 6 kg N ha⁻¹ of prilled fertilizer decreased N₂ fixation in feather moss carpets (Gundale et al. 2011). Further, low additions of only 3

kg N ha⁻¹, which are much lower than background N deposition rates in mid Europe (Stevens et al. 2004), decreased moss mass and density of cyanobacteria colonizing the moss (Gundale et al. 2011). Additions of only 4.25 kg N ha⁻¹ in the field decreased N₂ fixation in feather mosses for over two years (Zackrisson et al. 2004). However, it should be emphasized that N additions in the growth chamber eliminate background rates of atmospheric and throughfall N deposition which are likely to range from 6 to 8 kg N ha⁻¹ yr⁻¹ (DeLuca et al. 2008) creating, for example, a 10.25 – 12.25 kg ha⁻¹ total N deposition rate in the Zackrisson et al. (2004) field experiment. Further, measurements in these previous studies were performed many weeks after the N additions (14, 16, 17, 64 weeks) and additionally after 3 years, which could represent long-term effects in contrast to our experiment in which we aimed to test immediate effects. Also, the flow through system we used here to prevent accumulation of N in the tubes could have led to insufficient exposure of N to the moss. Only moss samples from “Björknäs” seem to follow our expectations; here, we found highest N₂ fixation rates in the control treatments and lowest in the 10 kg N ha⁻¹ addition. However, 10 kg N ha⁻¹ might not be an unusual amount of N entering the system via deposition and throughfall and thus, might not be sufficient to inhibit nitrogenase activity completely. Even in the boreal, mosses can experience high N inputs in the range of 0 – 20 kg N ha⁻¹yr⁻¹ (Co-operative Programme for Monitoring and Evaluation of the Long-range Transmission of Air pollutants in Europe, EMEP: www.nilu.no/projects/coc/index.html) via canopy throughfall, leaching and wet and dry deposition. In addition, 50 % of the total wet N deposition in boreal forests is supposed to enter the system via snow melt (Gundale et al. 2011), which could have a completely different effect on N₂ fixation in feather mosses than directly applied N and washed through N solutions. This suggests that the way N enters the system, and also in which form (e.g. organic or inorganic), could affect nitrogenase activity differently, this is an area that warrants further investigation.

To reiterate, we found no N₂ fixation in the feather moss from Wales, UK, where N deposition is estimated to be > 12 kg N ha⁻¹yr⁻¹ (Jones et al. 2004), and nitrogenase activity remained high following 10 kg N ha⁻¹ applied to mosses from Sweden that were otherwise treated with distilled water. These results taken together suggest that (1) there is a N-inhibition threshold of N₂ fixation and (2) this threshold likely lies between 10 and 15 kg N ha⁻¹. The critical load for total N deposition is defined as “A quantitative estimate of an exposure to deposition of N as NH_x and/or

NO_y below which empirically detectable changes in ecosystem structure and function do not occur according to present knowledge” (Hornung et al. 1995). Our estimate of the critical load for N deposition for N₂ fixation in moss (10 - 15 kg N ha⁻¹) matches exactly the recommendation for understory vegetation in boreal forests (10 and 15 kg N ha⁻¹yr⁻¹; Achermann and Bobbink 2003) and falls within the range of other estimates for similar N-poor ecosystems (e.g. Arctic heath: 5 –15 kg N ha⁻¹ yr⁻¹; Hornung et al. 1995).

Nitrogen deprivation experiment

At the start of the N deprivation experiment, we found neither cyanobacteria nor detectable N₂ fixation rates in moss from Newborough, but we observed detectable numbers of cyanobacteria as well as N₂ fixation rates in mosses from Sweden. Thus, our results indicate that higher rates of atmospheric N deposition in Wales has led to a reduction or total exclusion of N₂ fixation in these feather mosses (see also DeLuca et al. 2008). Further, we found a clear and linear relationship between numbers of cyanobacteria and N₂ fixation rates in moss. N₂ fixation in *P. schreberi* from Wales increased with time when exposed to watering with no N addition, thus, the longer the moss is experiencing N deprivation, the higher the N₂ fixation rates. This suggests that the moss permanently hosts cyanobacteria (see above), but their numbers are reduced (DeLuca et al. 2007) and activity is truncated when N availability is high, as N₂ fixation is energy expensive (Turetsky 2003) and is not required when N is available. The moss samples in our experiment had no contact to soil and were kept in the growth chamber during the course of the experiment, thus colonization of the moss by cyanobacteria after the start of the experiment is unlikely to have occurred, supporting the idea of permanent existent cyanobacteria on the moss. As soon as N availability is decreasing, cyanobacteria might become reactivated, growing, and start fixing N₂. Thus, both, moisture and N availability seem to be important drivers of N₂ fixation in the moss-cyanobacteria association.

We have to keep in mind, however, that the moss samples from Wales were also exposed to salt input from the nearby sea, which could have influenced the N₂ fixation capacity of the moss. Nevertheless, since it has been repeatedly found that N₂ fixation in the moss is strongly inhibited by N deposition (e.g. DeLuca et al. 2008; Gundale et al. 2011), we assume this is also applicable for the same moss species growing in Wales.

Nitrogen fixation in the lichen *Peltigera spp.* from Newborough was higher than in the Swedish moss despite the high atmospheric N loads in Wales. The fact that *Peltigera spp.* was fixing N₂ in the high deposition area and the feather moss did not fix N₂ in the same area, suggests that the association between the mycobiont (fungus) and the photobiont (green algae or cyanobacterium) in the lichen is certainly more obligate and better protected than the association between moss and cyanobacteria. Lichens represent a classic obligate symbiosis in which both partners benefit and are dependent on each other (Schwendener 1867; Cooke 1977). In contrast, the feather moss-cyanobacteria association is non-obligate: the moss can receive all required nutrients from dry and wet deposition and is independent of the cyanobacteria. Cyanobacteria are facultative autotrophs; thus, are not dependent on the plant partner either, as they are able to fix both carbon (C) and N (Rai et al. 2000; Turetsky 2003). Additionally, N₂ fixation in the lichen was similar over the course of the experiment, with only one exception when all samples showed high activity (6 weeks), supporting the idea that N₂ fixation in lichens might be less influenced by external factors than N₂ fixation in feather mosses.

The feather moss may control colonization by cyanobacteria through the release of chemo-attractants when N availability is low to promote colonization and thus, to gain an endogenous N supply by hosting N₂ fixers (Rai et al. 2000). When N availability or N input is high, hosting N₂ fixers to keep an endogenous N supply is no longer necessary.

Despite increasing N loads in an N enriched world, there is hope: N loads as high as 10 kg N ha⁻¹ do not necessarily inhibit N₂ fixation in the feather moss-cyanobacteria association. Further, after a period of N deprivation, cyanobacteria associated with moss with a “N-history” recommence activity and fix atmospheric N₂ (again). Thus, feather mosses could function like a N rheostat, turning on N₂ fixation when N deposition is low and turn it off when N deposition is high. Our results show that components of ecosystems that have been exposed to high N loads are able to recover and could return to their pristine functioning when N deposition is reduced.

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III.

Recovery of N₂ fixation after drying and rewetting in an ubiquitous feather moss

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Author contributions: KA designed the experiments. TD collected the samples. KA performed the experiments KA analyzed the data. KA wrote the first draft, with all authors contributing to the final manuscript.

Recovery of N₂ fixation after drying and rewetting in an ubiquitous feather moss

Kathrin Ackermann, Davey L. Jones, Thomas H. DeLuca

Abstract

The feather moss *Pleurozium schreberi* is ubiquitous and dominant in boreal ecosystems where it is exposed to large daily and yearly fluctuations in sunlight, temperature, precipitation and humidity and thus, undergoes significant shifts in its moisture status. *P. schreberi* is colonized by cyanobacterial nitrogen (N) fixers which contribute to the N input in N-limited boreal forests. While the effects of drying and rewetting on nutrient leaching and photosynthesis in mosses have been studied, few investigations have effectively evaluated the effect on N₂ fixation. Here, we present results from a drying-rewetting experiment using *P. schreberi* and its colonizing cyanobacteria as an indicator for periods of moisture stress and subsequent rewetting on N₂ fixation in boreal forests. Drying of mosses almost completely eliminated N₂ fixation, but rates slowly recovered after rewetting, reaching N₂ fixation levels of moist (non-water stressed) moss 5 days after rewetting. Thus, recovery of N₂ fixation in moss is possible, but short and recurrent cycles of intensive drying and rewetting events could potentially eliminate N₂ fixation in the moss-cyanobacteria association. However, *P. schreberi*, with its efficient water holding structure, seems to provide a highly suitable habitat for cyanobacteria in the boreal forest where summers can create extremely dry short-term and unfavorable conditions.

Keywords: Acetylene reduction, boreal forest, drought, N cycle, nitrogenase

Introduction

Nitrogen (N) fixation performed by cyanobacteria that colonize feather mosses in pristine boreal forests contributes about 2 kg N ha⁻¹ yr⁻¹ to N-limited late succession forests (DeLuca et al. 2002a; Gundale et al. 2011). Various abiotic factors influence the amount of N₂ that is fixed. For instance, moisture (Gundale et al. 2009), N limitation in late succession (Zackrisson et al. 2004; DeLuca et al. 2008), and rainfall frequency (Jackson et al. 2011; Gundale et al. 2012) have been found to positively affect N₂ fixation in the feather moss-cyanobacteria association. However, in the boreal forest,

feather moss carpets can become extremely dry during long summer days and then rewet during subsequent heavy rainfall events, thus, experiencing natural and intensive drying and rewetting events. While it has been shown that nutrient leaching and nutrient release from moss carpets after drying and rewetting is reasonably high (Carleton and Read 1991), the effects of rewetting on N₂ fixation in feather mosses has not been explored to date.

It has been shown that mosses can withstand drying until no free water remains in the cells and they quickly return to normal metabolism and growth upon rewetting (Proctor 2001). Additionally, the recovery of important biological processes (e.g. photosynthesis) is supposed to be rapid after rehydration in desiccation tolerant mosses, with some species starting to fix CO₂ immediately after rehydration (Proctor et al. 2007). However, recovery time is highly dependent on species and habitat (Proctor 2001).

One of the most abundant feather mosses in boreal forests is *Pleurozium schreberi* (Brid.) Mitt., which can account for 60 - 90 % of the ground cover in mature boreal forests (Oechel and Van Cleve 1986; DeLuca et al. 2002b). *P. schreberi* is colonized by several species of cyanobacteria (Gentili et al. 2005), fixing substantial amounts of atmospheric N₂ (DeLuca et al. 2002a; Zackrisson et al. 2004). Although *P. schreberi* is poikilohydric (the state of hydration is controlled by the environment) (Glime 2007), it can be found in dry habitats (Tesky 1992) and is supposed to be desiccation tolerant due to its ability to enact cellular repair (Glime 2007). However, dry mosses are inactive and it can take up to 24 h after rehydration until full repair of damaged membranes occurs (Proctor 1981; Oliver and Bewley 1984). Thus, frequent and short sequences of desiccation seem to be more damaging than long periods of desiccation (Oliver and Bewley 1984).

Cyanobacterial nitrogenase catalyzes the reduction of atmospheric N₂ in the *P. schreberi*-cyanobacteria association. It has been shown that the recovery of nitrogenase activity in free-living cyanobacteria after desiccation is slower than the recovery of photosynthesis after desiccation due to *de novo* protein synthesis required for N₂ fixation (> 24 h vs. 4 h for N₂ fixation and photosynthesis, respectively) (Hawes et al. 1992). However, the rate of recovery of N₂ fixation in desiccated feather mosses after rewetting is currently unknown.

N₂ fixation is commonly measured with the acetylene reduction (AR) assay (Schöllhorn and Burris 1967). This assay is based on the reduction of acetylene (C₂H₂)

to ethylene (C_2H_4) performed by the cyanobacterial nitrogenase enzyme, which reduces N_2 as well as acetylene (Dilworth 1966). The production of ethylene in moss samples is usually measured after 24 h of incubation with acetylene, assuming a linear increase of N_2 fixation with incubation time (Zackrisson et al. 2004).

Although the AR assay has previously been shown to be linear with time through 24 hours, there has been no attempt to test the linearity of the AR assay in longer incubations. We propose that the AR assay is sensitive to long-term incubations (greater than 24 hours). Therefore, incubation time could bias the results from the AR assay, which could lead to under- or overestimation of ecosystem N_2 fixation rates and with that, ecosystem N budgets. To assess the relationship between AR and incubation time, we incubated samples of *P. schreberi* with acetylene and measured AR at various time points after incubation over a 65 hour period.

We then used our knowledge of the linearity of AR to examine the influence of extreme drying and rewetting events on N_2 fixation in feather mosses and to estimate the amount of time needed for the cyanobacterial nitrogenase enzyme to recover from desiccation and to recommence fixing N_2 . In our experiments, moss samples were kept in the same glass vials over the course of the experiments to reduce disturbances to a minimum to be able to ascribe changes in N_2 fixation to the drying and rewetting events. We specifically aimed to answer the following questions: (1) how does N_2 fixation in *P. schreberi* change with incubation time? (2) How quickly and completely does the *P. schreberi*-cyanobacteria association reach N_2 fixation rates that are comparable to rates in constant moist moss after rewetting of air-dried moss? These collective findings could have implications for studies aiming to estimate ecosystem- N_2 fixation with the AR technique and may account for some of the spatial and temporal variability previously observed in the field (DeLuca et al. 2002a; Zackrisson et al. 2004).

Materials and Methods

N₂ fixation time series and drying-rewetting experiment – sampling and experimental set up

Moss shoots from *P. schreberi* were collected at a late succession forest (299 years since last fire; Zackrisson et al. 1996) in northern Sweden (“Dötternoive”; 65.4375°N, 18.4440°E) in August 2011. The site has been described previously (Ackermann et al.

2012) and moss samples from this site have been shown to be sufficiently active to be used in our experiments (Ackermann et al. 2012). Moss samples were transported in sealed plastic bags and brought to Bangor via airplane. Upon arrival in Bangor, moss samples were kept in a growth chamber at 10 °C, 16/8 h light/dark cycles until the start of the experiment to ensure nitrogenase activity.

For the N₂ fixation time series experiment, as well as for the drying-rewetting experiment, 10 moss shoots per time point with three replicates each were placed in 20ml glass vials and kept at room temperature (~ 20 °C) over the course of the experiments. AR was measured at 2, 4, 8, 17, 24, 40, 48 and 65 h after incubation with acetylene to analyze the relationship between incubation time and N₂ fixation in the moss.

For the drying and rewetting experiment, half of the glass vials with moss shoots were used as control samples and kept moist over the course of the experiment. The second half was subjected to desiccation by air-drying. For that, the vials were placed in a warm greenhouse (approximately 28 °C) for 72 h un-lidded and with constant airflow induced by a fan. The control samples were placed in the same greenhouse but kept moist by leaving the vials lidded. Moisture in the control samples was maintained with distilled water as necessary. AR was measured in all samples at the start of the experiment, when all samples were still moist. Wet weight of moss samples was determined prior to drying. When the desiccated moss was below 9 % moisture, AR was measured again in the dried and control samples. The dried moss was rewetted with distilled water to the starting wet-weight and AR was measured at 2, 4, 8, 16, 24, 32, 48 and 120 h after rewetting in the dried-rewet and in the control samples. Due to the required incubation prior to ethylene measurement, we used different moss samples at each time point after rewetting with three replicates each. After each measurement, moss samples were dried at 80 ° for 3 days and weighed.

N₂ fixation analyses

N₂ fixation analyses were performed using a ¹⁵N₂-calibrated AR assay (Schöllhorn and Burris 1967) as described by Zackrisson et al. (2004). Briefly, 20 ml glass vials with moss shoots were sealed and 10 % of the headspace was replaced with acetylene. Moss samples were incubated for 24 h at room temperature (~ 20 °C) in the drying-rewetting experiment. Various incubation times were used in the N₂ fixation time series experiment (see above). Ethylene generated in the headspace by the cyanobacterial

nitrogenase enzyme was measured by gas chromatography equipped with a flame ionization detector (Varian, Santa Clara, USA) using an automatic headspace sampler (Quma, Wuppertal, Germany). Data are represented as AR in $\text{nmol g}^{-1} \text{DW}$ or $\mu\text{mol m}^{-2}$ where 10 moss shoots of *P. schreberi* represent an area of 2.8 cm^2 (Zackrisson et al. 2004)

Statistical analyses

To test for differences in AR between the incubation times in the time series experiment, Analysis of Variance (ANOVA) approaches were used followed by Tukey's Post Hoc Test. The relationship between AR and incubation time was analyzed with linear regression. The cumulative AR was best described with a power fit equation calculated in KaleidaGraph 4.1.3. (Synergy Software). Differences in AR and weight between the dried moss and the constant moist moss was analysed with ANOVA followed by Tukey's Post Hoc Test. Linear regression analyses were used to estimate the relation of AR with time after rewetting. T-Tests were used to test for differences in AR before and after drying. Cumulative AR values are presented. All analyses were performed using R 2.14 (R Development Core Team, 2011).

Results

N₂ fixation time series experiment

Acetylene reduction per unit time was found to increase significantly with incubation time ($r = 0.88$; $p = 0.003$; $df = 6$) (Fig. 1). Acetylene reduction rates were linear over a 40 hour period after which they increased exponentially. Mean AR in moss before the start of the experiment was $728 \text{ nmol g}^{-1} \text{DW} \pm 243$ ($\pm\text{SE}$). During the experiment, the lowest AR was measured after 2 h incubation ($0 \text{ nmol g}^{-1} \text{DW}$) and the highest after 65 h of incubation with acetylene ($4264 \text{ nmol g}^{-1} \text{DW}$).

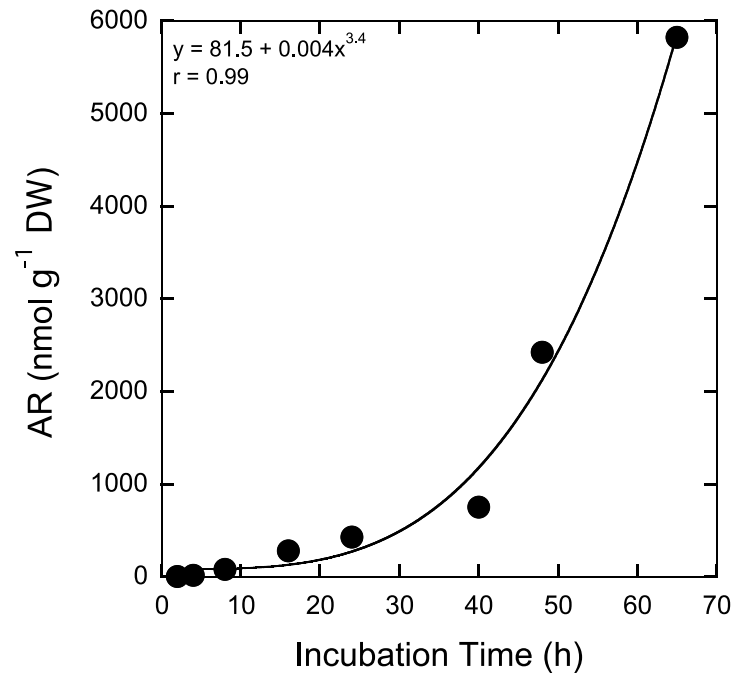


Fig. 1. Cumulative N₂ fixation (nmol acetylene reduced g⁻¹ DW) in *Pleurozium schreberi* measured at different times (2 - 65 h) of incubation with acetylene. Power Fit equation and r-value are given.

Drying and rewetting experiment

Wet and dry weights of moss shoots of *P. schreberi* were similar between the two different treatments (dried-rewet moss and continuously moist moss) (Table 1), thus, moss mass was not likely to bias the comparisons of AR between the treatments.

Table 1. Wet and dry weight (g) of 10 shoots of *Pleurozium schreberi* used in the experiments. Given are mean values \pm SE (n = 24). Wet weight for all moss samples was determined before the experiment, oven-dry weight was determined after the experiment (80 °C, 3 days). Also given are means \pm SE for the air-dried moss (~ 28 °C, 72 h in the green house) as well as moisture content (%) on a fresh weight basis.

| | Wet weight (g) | Oven-dried (g) | Air-dried (g) |
|------------------|------------------|------------------|------------------|
| Control moss | 3.12 \pm 0.14 | 0.16 \pm 0.01 | – |
| Dried-rewet moss | 3.12 \pm 0.09 | 0.14 \pm 0.005 | 0.27 \pm 0.04 |
| | (100 % moisture) | | (8.6 % moisture) |

There was no significant difference in AR between the mosses before the drying: mean AR in the moss before drying was 50.1 $\mu\text{mol m}^{-2} \pm 4.7$ (SE) and in the control moss 61.4 $\mu\text{mol m}^{-2} \pm 6.8$. After drying, AR was significantly reduced in the dried moss compared to AR in the same moss before drying (3.0 $\mu\text{mol m}^{-2} \pm 0.7$; $t = -9.9$; $p < 0.001$), but remained similar in the continuously moist moss (97.6 $\mu\text{mol m}^{-2} \pm 19.6$) and thus, was significantly different in the two treatments before rewetting ($t = -4.8$; $p < 0.001$).

After rewetting, clear differences between treatments were observed (Fig. 2). The continuously moist moss showed more than 10-fold higher AR levels measured at the same time intervals throughout the experiment than the dried and rewetted moss ($F = 1.19$; $p < 0.001$). AR in both treatments showed a similar shape of cumulative AR levels after rewetting with a linear increase until 40 h after rewetting (Fig. 2). But after 48 h, AR in the continuously moist moss did not increase further in contrast to the dried-rewet moss (Table 2). Nevertheless, AR was positively correlated with time in both treatments ($r = 0.55$; $p = 0.02$; $df = 14$). However, AR in the dried-rewet moss remained low over the course of the experiment, with a mean (138.5 $\text{nmol g}^{-1} \text{DW} \pm 112.2$) and maximum cumulative value (237.7 $\text{nmol g}^{-1} \text{DW}$) at 120 h after rewetting that was four times lower than the cumulative AR levels (939.8 $\text{nmol g}^{-1} \text{DW}$) in the continuously moist moss at 120 h. Interestingly, mean AR in the continuously moist moss was more than ten-fold higher after 48 hours (302.3 $\text{nmol g}^{-1} \text{DW} \pm 146.8$) than after 120 h (22.2 $\text{nmol g}^{-1} \text{DW} \pm 8.2$) (Table 2).

Table 2. N₂ fixation (μmol acetylene reduced m^{-2}) in *P. schreberi* measured at various time points (2 – 120 h) after rewetting. Given are means and standard error of acetylene reduction measured in the dried-rewet moss and in the continuously moist moss.

| Time (h) | N ₂ fixation in dried-rewet moss (μmol acetylene reduced m^{-2}) | N ₂ fixation in constant moist moss (μmol acetylene reduced m^{-2}) |
|----------|---|--|
| 2 | 0.5 ± 0.5 | 1.8 ± 1.0 |
| 4 | 0.7 ± 0.7 | 7.1 ± 2.7 |
| 8 | 1.1 ± 0.6 | 26.9 ± 4.3 |
| 16 | 2.2 ± 0.2 | 97.0 ± 89.1 |
| 24 | 4.7 ± 1.7 | 58.0 ± 32.8 |
| 32 | 7.0 ± 0.4 | 130.4 ± 10.3 |
| 48 | 7.3 ± 3.8 | 157.9 ± 77.3 |
| 120 | 83.4 ± 56.5 | 15.3 ± 5.5 |

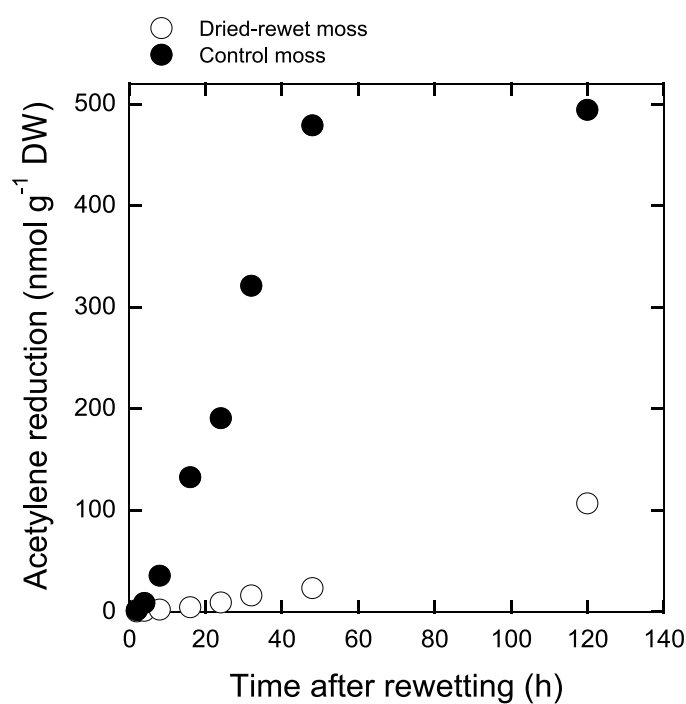


Fig. 2. Cumulative N₂ fixation (nmol acetylene reduced g^{-1} DW) in *Pleurozium schreberi* in the dried-rewet moss (open symbols) and in the control moss (filled symbols) at different time points after rewetting (2 – 120 h).

Discussion

N₂ fixation time series

Our results demonstrate that the outcome of the AR assay is highly dependent on the incubation time with acetylene. Particularly, an incubation time of 40 h or longer appeared to result in exponentially higher N₂ fixation rates than shorter incubation times. Thus, incubation times of samples that are compared in regard to their N₂ fixation rates should be as similar as possible. Alternatively, N₂ fixation could be re-calculated incorporating the specific incubation times assuming a linear relationship of N₂ fixation rates with time (incubation periods of less than 40 h). Generally, long incubation times should be avoided since the samples are exposed to unnatural conditions in the vials making extended incubations increasingly unrealistic for estimating N₂ fixation under natural conditions (Hardy et al. 1973). Regardless, acetylene reduction to ethylene is a highly sensitive and efficient means of estimating N₂ fixation (Hardy et al. 1973).

Drying and rewetting

N₂ fixation in *P. schreberi* was significantly reduced after drying and its recovery after subsequent rewetting was slow. Only after ~5 days did the levels of N₂ fixation in the dried-rewet moss reach the levels of the continuously moist moss measured at the beginning of the experiment. Fast as well as short drying and rewetting cycles have been found to be more damaging to moss than slow drying and longer cycles since protective and repair mechanisms have to be set in place (Proctor et al. 2007). Additionally, the duration of the desiccation period is crucial for recovery time of N₂ fixation by cyanobacteria, the longer the cells are drought-stressed, the longer it takes to recover (Scherer et al. 1984).

Although recovery of N₂ fixation in the dried and rewet moss was slow, it did recommence several hours after rewetting, suggesting that 3 day air-drying at 28 °C is sufficient enough to reduce N₂ fixation almost to zero and at the same time not to completely destroy cyanobacteria cells and their activity. Gundale et al. (2012) also found N₂ fixation in *P. schreberi* to recover from increased temperatures and less frequent rainfall events in the field. However, the sharp decline in AR in the continuously moist moss at the last sampling point indicates that cyanobacterial cells have greatly declined towards the end of the experiment. Although the cause of this decline is not clear, it is possibly due to the light conditions in the growth chamber or

possibly due to excessive build up of reduced N in the tissue following weeks without rain events to flush out accumulated NH_4^+ .

Recovery time of photosynthesis and respiration is dependent on the duration of the desiccation period (Proctor et al. 2007). However, N_2 fixation seems to recover slower than photosynthesis after rewetting due to the required synthesis of new proteins (Hawes et al. 1992) and newly differentiation of heterocysts responsible for N_2 fixation (Scherer et al. 1984). Further, N_2 fixation is an extremely energy demanding process (Turetsky 2003; Houlton et al. 2008), thus energy must first be generated via photosynthesis to be able to regain nitrogenase activity. This could lead to a lag phase before N_2 fixation can recommence after drying and rewetting due to the required production of energy prior to N_2 fixation. Scherer and Zhong (1991) found decreasing N_2 fixation rates after drying in free-living cyanobacteria, but stability increased after several drying cycles, suggesting that the free-living cyanobacteria developed a desiccation tolerance induced by repeated cycles of rewetting and desiccation (Scherer and Zhong 1991). Moss-cyanobacteria-associations in boreal forests could be adapted to drying and rewetting cycles since they experience large variations in their hydration status over the course of only few hours (Startsev and Lieffers 2002). Further, feather mosses are known for their ability to retain water (Turetsky 2003) and could represent moist spots in the boreal understory in contrast to other plants, facilitating colonization by cyanobacteria. When measuring the effects of desiccation on N_2 fixation rates in free-living cyanobacteria, Scherer and Zhong (1991) found that maximum nitrogenase activity in free-living cyanobacteria was achieved at water content of 71 % of fully rewetted colonies. Although not directly comparable in regard to N_2 fixation in cyanobacteria, mosses can stay active at water contents as low as 20 % (Proctor et al. 2007). Thus, free-living cyanobacteria seem to require higher moisture conditions for their activity than mosses. Even though the moisture content of the air-dried moss was very low (8.6 %, see Table 1), we could detect low activity of the cyanobacterial nitrogenase enzyme, suggesting that the moss is able to maintain a suitable environment for the cyanobacterial symbiont, leaving the moss as a more favorable habitat than the free-living status of cyanobacteria in soil.

In our experiment, recovery of N_2 fixation was fully reached between 2 – 5 days after rewetting, whereas complete recovery of photosynthesis is reached only few hours after rewetting in mosses (Proctor et al. 2007) and in cyanobacteria (Hawes et al. 1992; Satoh et al. 2002). However, we have to keep in mind that the feather moss-

cyanobacteria association is an alliance of two different organisms, making a distinction between enzymatic processes in the plant versus that in bacteria difficult. The feather moss-cyanobacteria association is assumed to be mutualistic (Turetsky 2003), in which both partners exchange resources (N, carbon (C)) with each other. But if both partners experience physiological stress through drying and rewetting, and both have to invest energy in membrane repair, photosynthesis and N₂ fixation (Glime 2007; Turetsky 2003), the question is which partner is faster in recovering to be able to exchange resources with the (symbiotic) partner. Photosynthesis seems to recover faster than N₂ fixation after rewetting (Hawes et al. 1992; Proctor et al. 2007) and mosses seem to be more desiccation tolerant than cyanobacteria (see above), suggesting that mosses could start fixing C earlier than cyanobacteria start fixing N₂ after rewetting, and could provide the cyanobacterial partner with energy and C to regain N₂ fixation ability assuring an endogenous N supply for the moss.

Our results suggest that dry summer days or weeks in the boreal forest could lead to a significant reduction of N₂ fixation performed by the feather moss-cyanobacteria association, resulting in less available N. Sun exposed patches that dry more quickly, may partially explain the high degree of spatial and temporal variability in N₂ fixation in *P. schreberi* reported previously (Zackrisson et al. 2004). Given the predicted increase in air temperatures in Northern latitudes (IPPC 2007), which will result in more frequent periods of desiccation of the moss, the N input via N₂ fixation in the feather moss-cyanobacteria association is likely to be compromised in the future. On the other hand, N₂ fixation is capable of recovery from desiccation stress upon rewetting and further, mosses are known to release large amounts of nutrients upon rewetting (Carleton and Read 1991). Thus, feather-moss cyanobacteria associations could represent a significant N source immediately upon rewetting via moss leachate and a longer-term N source via continuation of N₂ fixation by the cyanobacterial associate. However, in order to assess the impacts of natural drying-rewetting cycles on N₂ fixation in boreal forests, the degree of drying event needs to be carefully evaluated and N₂ fixation needs to be measured in recurrent drying-rewetting cycles with varying degrees of desiccation. Further, N₂ fixation and photosynthesis should be assessed in parallel during these fluctuations in hydration status in the feather moss-cyanobacteria association.

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IV.

Feather moss nitrogen acquisition across natural fertility gradients in boreal forests

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Author contributions: KA, OZ, THD designed the study and performed the fieldwork. OZ and THD performed the nitrogenase analyses. KA performed the soil nutrient analyses. JR conducted the PLFA analyses. KA and JR conducted the uptake experiment. KA analyzed the data. KA wrote the first draft, with all authors contributing to the final manuscript.

Feather moss nitrogen acquisition across natural fertility gradients in boreal forests

Kathrin Ackermann, Johannes Rousk, Davey L. Jones, Olle Zackrisson,
Thomas H. DeLuca

Abstract

Feather mosses utilize various sources of nitrogen (N): they absorb N deposited on leaf tissue, they host N₂ fixing cyanobacteria, and they are able to take up N directly from soil. In addition to their importance as primary producers in boreal ecosystems, feather mosses play a significant role in N cycling. However, estimates of their ability to take up N from soil *in situ* are scarce. Further, connecting uptake of N from soil with N₂ fixation could significantly improve our understanding of their role in ecosystem N cycling, but to date this issue has not been addressed. We report results from an uptake experiment in which we tracked ¹³C-carbon (C), ¹⁵N-alanine and ¹⁵N-ammonium chloride (NH₄Cl) into feather moss (*Pleurozium schreberi* (Brid.) Mitt.)-soil cores taken along natural fertility gradients in Northern Sweden. The varying fertility conditions coincided with a N₂ fixation gradient in the feather moss. We found that *P. schreberi* takes up C and N directly from soil. However, the moss did not show a preference for inorganic or organic N sources and only 1.4 % of the added amino acid appeared to be taken up from soil in an intact form. No differences in uptake of C or N from soil along the fertility gradients were detected. Nitrogen fixation rates in the moss were thus not correlated with C or N-uptake from soil. Nitrogen fixation in the moss as well as uptake of C and N from soil seem to be unaffected by C or N availability in the soil, suggesting that the moss can cover its nutrient demand by absorption of throughfall N and via associated N₂-fixing cyanobacteria without soil-N supplementation. We suggest further, that the moss can represent a (temporary) N-sink in the boreal forest, and that the moss' mechanism of uptake and release thereby will characterize the ecosystem N cycle.

Keywords: Acetylene reduction, bryophytes, cyanobacteria, microbial community, N deposition, N limitation, organic nitrogen

Introduction

Boreal forests are considered to be nitrogen (N)-limited (Tamm 1991) with N demand of plants and microbes satisfied by a combination of sources and pathways (Jones et al. 2005). Besides the uptake of mineralized, inorganic N (ammonium (NH_4^+) and nitrate (NO_3^-)), plants, as well as microbes, possess the ability to take up organic N in form of amino acids, urea, polyamines and small polypeptides (Kielland 1994; Schimel and Chapin 1996; Schimel and Bennett 2004; Persson and Näsholm 2008; Hill et al. 2011). Furthermore, in N-limited systems like boreal forests, organic N can be found in higher concentrations than inorganic N in soil solution (Kielland 1995; Nordin et al. 2001; Finzi and Berthrong 2005).

The N-limited conditions in boreal forests result in strong competition for N resources between plants and soil microbes. This is particularly true in the rhizosphere where plant exudation of low molecular weight amino acids, sugars and organic acids creates a zone of high microbial activity (Curl and Truelove 1986; Jones et al. 2004), resulting in rapid turnover rates of nutrients (Jones 1999; Jones and Kielland 2002; Rousk and Jones 2010). In terrestrial ecosystems, microbes are usually carbon (C)-limited (Demoling et al. 2007), however, microbial N-limitation can also occur (Sistla and Schimel 2012). Thus, organic N in form of amino acids should be readily taken up by microbes and suggests that microorganisms are better competitors for organic N than plants (e.g. Jones et al. 1996; Owen and Jones 2001).

In addition to vascular plants, mosses, including feather mosses, have been found to take up amino acids from solution and directly from soil (Ayres et al. 2006; Krab et al. 2008). Feather mosses, however, are thought to satisfy most of their N demands via absorption of N originating from atmospheric deposition and throughfall N (Li and Vitt 1997; Kotanen 2002) and via fixation of atmospheric N_2 through epiphytic cyanobacteria (DeLuca et al. 2002). There is also evidence for uptake of N from soil through rhizoids and possibly through fungal associations (Kausserud et al. 2008; Davey et al. 2009). Due to their association with cyanobacteria, which fix substantial amounts of atmospheric N_2 in pristine environments (DeLuca et al. 2002), feather moss turnover represents a major N input in nutrient poor systems, contributing approximately $2 \text{ kg N ha}^{-1}\text{yr}^{-1}$ to the N pool in mature forest ecosystems (DeLuca et al. 2002; Zackrisson et al. 2004). However, N_2 fixation by cyanobacteria is highly sensitive to inorganic N inputs, the more N is added and available, the less N_2 is fixed (DeLuca et al. 2008; Gundale et al. 2011; Ackermann et al. 2012). To date, it is unknown if there is a connection

between N₂ fixation in feather mosses and their ability to take up N from soil and with that, their role as a net N source due to their cyanobacterial associates or as a N sink due to their ability to take up N from soil.

We investigated the N acquisition of pleurocarpous feather mosses in boreal forests. To do this, we tested the ability of the ubiquitous and dominant feather moss *Pleurozium schreberi* (Brid.) Mitt. to take up N directly from soil, assessing the competition for C and N with microbes by analysing moss and microbial ¹³C and ¹⁵N enrichment after injection of ¹³C, ¹⁵N-alanine and ¹⁵N-NH₄Cl into soil. In addition, we analysed soil microbial community size and composition using PLFA-analyses and we tested mineralization rates of various amino acids in soil. To accomplish this, we collected intact cores of soil, humus and feather moss along natural fertility gradients in northern Sweden. The fertility gradients are the result of groundwater discharge (zone of high fertility) and recharge (zones of low fertility), enabling us to compare the uptake of N from soil at sites with contrasting fertility (Giesler et al. 2002). Further, we can relate uptake of N from soil to different rates of N₂ fixation in the moss-cyanobacteria association, which is expected to vary along the gradients. We hypothesize that (1) mosses are able to take up N from soil; (2) uptake of N from soil by the moss is negatively correlated with N₂ fixation since the moss presumably receives N from associated cyanobacteria (Adams and Duggan 2008); (3) mosses are poor competitors for organic forms of N in contrast to inorganic N.

Materials and Methods

Study sites

Feather moss and soil samples were collected in June 2010 along natural fertility gradients in Northern Sweden in three different forests (Varjisån, Kryddgrovan, Pite Älven). The sites are located between latitude 64 - 65° N, longitude 18 - 19° E and between 230 and 540 m above sea level. Each forest possesses three different fertility conditions (high, medium, low) due to respective inputs and losses from groundwater discharge and recharge. The low and medium fertility stands fall within a groundwater recharge zone while the high fertility stands fall on groundwater discharge zones thereby receiving subsurface nutrient inputs (Giesler et al. 2002). Mean annual temperature and precipitation are approximately 1 °C and 570 mm, respectively. The vegetation at the high fertility sites were dominated by *Paris quadrifolia*,

Gymnocarpium dryopteris, *Sorbus aucuparia*, *Geranium sylvaticum*, *Actaea spicata*, *Pinus sylvestris*, *Picea abies*, *Betula pubescens*, *Solidago virgaurea*, *Rubus spec.* and *Maianthemum bifolium*. The medium fertility sites were dominated by *Vaccinium vitis-idaea*, *V. myrtillus*, *Hylocomium splendens*, *P. schreberi*, *Empetrum nigrum* and at the low fertility sites by *V. vitis-idaea*, *V. myrtillus*, *P. sylvestris*, *P. abies*, *Cladonia spec.*, *Calluna vulgaris*, *H. splendens* and *P. schreberi*. Soils at the low and medium fertility sites were classified as Typic Haplocryods, the high fertility soils are Eutric Haplocryods (Soil Survey Staff 2010). All sites represented stand successional ages older than 120 years (estimated by tree ring counts; O. Zackrisson, unpublished).

Sampling and soil nutrient analyses

For the uptake experiment, six moss-soil-cores (5 cm diameter, 15 cm depth) with shoots from *P. schreberi* were collected at each fertility stand in each forest site (a total of 54 moss-soil-cores). Additional soil samples for analyses of total C (TC), total N (TN), extractable NH_4^+ -N, NO_3^- -N, free amino acids, amino acid mineralization and microbial community analyses (PLFA) were collected using a 2.5 cm diameter stainless steel soil core to a depth of ~ 10 cm, separating out only the O-layer, and were directly returned to the laboratory and stored at 5 °C until analysis within 5 days. Soil pH was determined in the lab on field-moist soil (1:1 w/w soil:distilled water). Soil moisture content was estimated gravimetrically by measuring the mass loss after drying for 24 h at 80 °C.

For analyses of net N mineralization and nitrification potential, we conducted 28-day aerobic incubations. In brief, a 5 g sample of fresh soil was extracted with 20 ml 0.5 M K_2SO_4 , shaken for 30 minutes, centrifuged (15 minutes at 4000 rpm) and subsequent filtered through Whatman 42 filters. The extracts were analysed by microplate-colorimetric technique using the salicylate-nitroprusside method of Mulvaney (1996) for NH_4^+ -N and the vanadium method for NO_3^- -N (Miranda et al. 2001). A second soil sample (5 g fresh weight) was incubated in 50 ml polycarbonate tubes at 20 °C for 28 days in a growth chamber with 16/8 h light/dark cycles; followed by extraction and analyses for NH_4^+ -N and NO_3^- -N as described above. Net nitrification was calculated as NO_3^- -N at day 28 minus NO_3^- -N at time zero; net ammonification was calculated as NH_4^+ -N at 28 days minus NH_4^+ -N at time zero, and net mineralization was calculated as total inorganic N (NH_4^+ -N plus NO_3^- -N) at day 28 minus total inorganic N at time zero.

Samples from the time zero extraction were used for analyses of dissolved organic N (DON) and dissolved organic carbon (DOC) using a Shimadzu TCV-TN analyser (Shimadzu Corp., Kyoto, Japan). Total C and TN in soil samples were analysed on dried soil by oxidative combustion using an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer (IRMS) (Sercon Ltd., Cheshire, UK).

To monitor throughfall N over one year we used an ion exchange system adapted from “resin lysimeters” described by Susfalk and Johnson (2002). Throughfall collectors were constructed by placing the resin capsule between 5 mm of clean, nutrient free mineral wool in open bottomed conical polycarbonate tubes, measuring 2.5 cm in diameter at the surface opening and 5 cm in depth and with a 0.8 cm bottom opening (DeLuca et al. 2008). The ionic resins in the collectors were 1.0 g of mixed bed, anion-cation exchange resins contained in a polyester mesh capsule (Unibest, Walla Walla, WA, USA). Throughfall collectors were placed at moss height with the opening exposed to the atmosphere, receiving N from wet and dry deposition, from canopy and understory throughfall and leachate from litter deposition. After 1 year in the field, the throughfall collectors were collected and the resins were extracted by placing the intact mesh capsules into 10 ml of 0.5 M K₂SO₄ in a 50 ml centrifuge tube, shaken for 30 minutes and the solution phase decanted into a fresh tube. This procedure was repeated 3 times to create an end volume of 30 ml. The extracts were then analysed for NH₄⁺-N, NO₃⁻-N, DOC and DON as described above.

N₂ fixation

N₂ fixation was estimated by measuring nitrogenase activity using a ¹⁵N₂-calibrated acetylene reduction assay (Schöllhorn and Burris 1967) as described by Zackrisson et al. (2004). In brief, 30 moss shoots of *P. schreberi* from each site were placed in a 50 ml tube, sealed and 10 % of the headspace was replaced with acetylene. Moss samples were incubated for 24 h under field conditions. Ethylene generated in the headspace was measured by gas chromatography equipped with a Porapac T column and a flame ionization detector (SRI Instruments, Torrance, California, USA). N₂ fixation data are presented as acetylene reduced to ethylene in μmol m⁻² d⁻¹.

N uptake experiment

We ran two pilot-studies prior to the uptake experiment: (1) we tested mineralization rates of acidic (glutamic acid), neutral (alanine) and basic (lysine) amino acids in soil samples (see below); (2) we tested the ability of *P. schreberi* to take up different forms of N (glutamic acid, lysine, alanine, NH₄Cl) from solution with moss shoots collected from a local Scots pine forest in Newborough, UK (53°92653 N, 4°223605 W).

Informed by the results from both pilot studies, we created the following experimental conditions for the N uptake experiment: each soil core was injected at five points with a total of 5 ml of 1 mM ¹³C, ¹⁵N dual, universally labelled alanine (U-¹³C₃ 98 at %; ¹⁵N 96-99 at %), or 5 ml of 1 mM ¹⁵N labelled NH₄Cl (¹⁵N 98 at %) or deionized water as control. Solutions were added as 1 ml injections every 1 cm diagonal downwards on the cores beginning at the moss-soil interface resulting in even distribution of the label within the soil. The solution was injected while the needle was slowly withdrawn from the moss-soil-cores. Alanine was used because it is a neutral, easily formed amino acid, which is essential for major metabolic pathways in every living organism (e.g. glycolysis, glycogenesis, citric acid cycle) (Nelson and Cox 2005) and is abundant in boreal forest soils (Nordin et al. 2001).

Prior to the injections, the moss-soil-cores were sprayed with distilled water from above and were placed in a tray containing distilled water to moisten the cores from below to prevent desiccation of moss and soil and to prevent passive transport of the solutions along the moss profile. After 24 h incubation in the greenhouse (20 °C) we harvested. The moss was cut at the moss-humus interface, rinsed with 0.5 mM CaCl₂ to remove any unincorporated label, dried (48 h; 80 °C) and ground to a fine powder using a ball mill. Soil was homogenized and was subjected to chloroform-fumigation extraction (Vance et al. 1987). In brief, 5 g of soil was immediately extracted with 20 ml of 0.05 M K₂SO₄. Another 5 g of soil was fumigated with ethanol free chloroform and extracted with 20 ml of 0.05 M K₂SO₄ after 24 h. We used a lower concentration of K₂SO₄ for the extraction to minimize the salt content of the extracts to achieve a higher sensitivity for detecting ¹³C and ¹⁵N in the samples (RG Joergensen, personal communication) (e.g. Wichern et al. 2007). Both types of extracts were freeze-dried using a lyophilizer (Edwards High Vacuum Int., West Sussex, UK). Moss, soil and freeze-dried extracts were analysed by oxidative combustion using an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer (IRMS) (Sercon Ltd., Cheshire, UK) for ¹³C and ¹⁵N content. Excess of ¹³C, ¹⁵N was calculated

by subtracting the ^{13}C , ^{15}N values (in atom %) in the control samples from the atom % values in the ^{13}C , ^{15}N treated samples.

PLFA analyses

Phospholipid fatty acids (PLFA) were determined by treating 1 g of fresh soil according to the method described by Frostegård et al. (1993) and as modified by Nilsson et al. (2007). The PLFAs chosen to indicate bacterial biomass were i15:0, a15:0, i16:0 16:1 ω 9, 16:1 ω 7t, i17:0, cy17:0, 17:0, 18:1 ω 7 and cy19:0, while 18:2 ω 6,9 and 18:1 ω 9 were used to indicate fungi (Frostegård and Bååth 1996). The quantities of the fatty acids were obtained using 19:0 as internal standard.

Total amino acids and amino acid mineralization rates

Total free amino acids in soil were analyzed on a Cary Eclipse Fluorometer (Perkin Elmer Corp., Boston, MA) using the fluorometric *o*-phthaldialdehyde- β -mercaptoethanol procedure (Jones et al. 2002). Soil solution was obtained by centrifugation-drainage of fresh soil samples at 18000 g for 2 min.

Rates of amino acid mineralization were determined using a ^{14}C -labelled amino acid incubation assay following the procedure described in Jones (1999). Briefly, 1 g of fresh soil (4 replicates from each fertility stand) was added to a polypropylene tube. Three different amino acids (glutamic acid, alanine, lysine) were then added to the soil (200 μl , 37 kBq ml^{-1}), a 1 M NaOH trap placed on the soil surface to catch evolved $^{14}\text{CO}_2$ and the tubes hermetically sealed and incubated at 20 °C. The NaOH traps were changed after 0.5, 1, 3, 6, 8, 10, 24, 48, 96, 144, 192 and 240 h. Radioactivity in the NaOH traps removed from the microcosms was then determined by liquid scintillation counting using a Wallac 1414 counter (EG&G, Milton Keynes, UK).

Statistical analyses

Differences in nutrient concentrations and pH in soil samples, N_2 fixation rates in moss, inorganic N concentrations in the throughfall collectors, as well as differences in C and N uptake from soil as well as fungal and bacterial PLFA concentrations were analysed using 2-Way Analysis of Variance (ANOVA) followed by Tukey's Post Hoc Test to detect differences along the fertility gradients using fertility as fixed factor (three levels) and forest sites as random factor (blocked). Carbon and N uptake from soil and N_2 fixation rates were subjected to linear regressions analyses. Data from the uptake

experiment are represented as excess ^{13}C , ^{15}N in the moss tissue and in the microbial biomass using ^{13}C and ^{15}N atom % values. For the microbial biomass, the values from the un-fumigated samples were subtracted from the fumigated samples and divided by the conversion factors (0.45 for C (Wu et al. 1990) and 0.54 for N (Joergensen and Mueller 1996)). The relative amounts (mol-%) of individual PLFAs were subjected to a principal component analyses (PCA) after standardising to unit variance. The resulting factor scores of the first and second principal component (PC1, PC2) were tested for correlation with soil nutrients. Further, the PC1 and PC2 scores were subjected to 2-Way ANOVAs with fixed (fertility) and random (forest sites) factors as previously described. A double exponential decay model ($Y = A_0 + B_0 e^{-k_1x} + C_0 e^{-k_2x}$) was fitted to the amino acid mineralization data (see e.g. Dempster et al. 2012) using KaleidaGraph 4.1.3. (Synergy Software). Significant differences in mineralization rates between the fertility conditions were assessed by ANOVA. All analyses were performed using R 2.14 (R Development Core Team 2011).

Results

Soil characteristics and nutrients

Soil parameters differed significantly between the three fertility conditions (Table 1). Soils were consistently more acidic in the low and medium fertility sites than in the high fertility sites ($F = 56.0$, $p < 0.0001$). Soil pH ranged between 5.8 and 7.5 in the three high fertility sites, between 4.0 and 6.2 in the medium fertility sites and between 3.7 and 5.6 in the low fertility sites, and averaged 6.4 ± 0.14 (mean \pm standard error (SE)), 5.1 ± 0.14 , and 4.8 ± 0.13 , in the high, medium and low fertility sites, respectively. DON, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$ and net mineralization in soil samples were significantly higher in the high fertility sites than in the medium and low fertility sites ($F > 11.5$, $p < 0.0001$) (Table 1). Soil C/N ratios were lower in the high fertility than in the medium and low fertility sites ($F = 4.6$, $p = 0.04$) (Table 1). Amino acid concentrations in soil solution were similar along the gradients (16.6 ± 3.2 ; 19.0 ± 2.8 ; 12.7 ± 2.6 μM , for the high, medium and low fertility sites, respectively). Throughfall $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ were significantly higher in the high fertility sites compared to the medium and low fertility sites ($F = 10.6$, $p < 0.0001$; $F = 16.6$, $p < 0.0001$) for $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$, respectively (Table 2; see also Fig. 1). C/N ratios of *P. schreberi* tissue were significantly lower in the high fertility than in the medium and low fertility sites ($F = 36.4$, $p < 0.0001$).

Further, the ratios were significantly correlated with N₂ fixation in the moss ($r = 0.62$, $p < 0.0001$, $df = 34$).

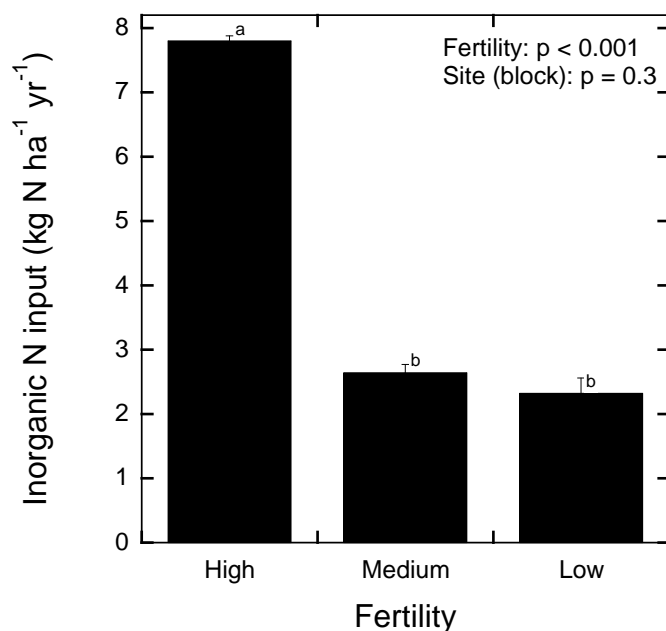


Fig. 1. Throughfall of inorganic N accumulated in resin collectors along the fertility gradients in Northern Sweden. Mean values ($n = 36$) \pm SE of inorganic N ($\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$) ($\text{kg ha}^{-1}\text{yr}^{-1}$) in the resin extracts are shown. Given are p -values for the factors fertility and site obtained from 2-Way ANOVAs. Different letters indicate significant differences tested with Tukey's Post Hoc Test.

Table 1. Selected soil characteristics along the fertility gradients (high, medium, low fertility) at Kryddgrovan, Varjisån and Pite Älven in Northern Sweden. Values are means (n = 6) \pm SE. Also mean C/N values (n = 4) \pm SE of *Pleurozium schreberi* tissue collected along the sites are given.

| | Kryddgrovan | | | Varjisån | | | Pite Älven | | |
|--|----------------|---------------------|-----------------|----------------------|---------------------|---------------------|----------------|---------------------|----------------------|
| | High | Medium | Low | High | Medium | Low | High | Medium | Low |
| NH ₄ ⁺ -N ($\mu\text{g g}^{-1}$ soil) | 1.8 \pm 0.6 | 0.08 \pm 0.04 | 0.02 \pm 0.01 | 2.7 \pm 0.7 | 0.05 \pm 0.01 | 0.12 \pm 0.06 | 2.0 \pm 0.2 | 0.2 \pm 0.04 | 0.09 \pm 0.03 |
| NO ₃ ⁻ -N ($\mu\text{g g}^{-1}$ soil) | 0.2 \pm 0.05 | 0.01 \pm 0.004 | 0 | 0.006 \pm 0.006 | 0.01 \pm 0.004 | 0.03 \pm 0.008 | 0.1 \pm 0.03 | 0.02 \pm 0.008 | 0.006 \pm 0.006 |
| DON ($\mu\text{g g}^{-1}$ soil) | 63 \pm 24.6 | 27.1 \pm 3.3 | 33 \pm 9.7 | 72 \pm 11.1 | 30 \pm 6.9 | 12 \pm 4.5 | 59 \pm 7.9 | 20 \pm 4.5 | 8.8 \pm 2.4 |
| TC (mg g ⁻¹ soil) | 410 \pm 109 | 320 \pm 6.4 | 240 \pm 176.2 | 250 \pm 52 | 311 \pm 43 | 310 \pm 91 | 320 \pm 78 | 390 \pm 109 | 370 \pm 6.0 |
| TN (mg g ⁻¹ soil) | 15 \pm 0.8 | 15 \pm 4.9 | 6 \pm 3.8 | 15 \pm 6.0 | 7 \pm 0.6 | 12 \pm 0.0 | 15 \pm 0.6 | 10 \pm 2.3 | 9 \pm 0.5 |
| C/N _{soil} | 28 \pm 8.7 | 25 \pm 8.6 | 36 \pm 6.3 | 19 \pm 4.3 | 45 \pm 2.5 | 25 \pm 7.5 | 21 \pm 6.0 | 38 \pm 2.4 | 43 \pm 1.5 |
| C/N _{Moss} | 28 \pm 1.7 | 41 \pm 0.5 | 45 \pm 0.1 | 36 \pm 6.3 | 48 \pm 4.7 | 51 \pm 9.3 | 36 \pm 0.2 | 39 \pm 2.4 | 48 \pm 1.4 |
| pH | 7.0 \pm 0.1 | 5.2 \pm 0.2 | 5.2 \pm 0.1 | 6.1 \pm 0.1 | 5.6 \pm 0.1 | 4.3 \pm 0.2 | 5.9 \pm 0.0 | 4.6 \pm 0.2 | 5.0 \pm 0.2 |

Table 2. Throughfall $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$ ($\text{kg ha}^{-1}\text{yr}^{-1}$) accumulated in resin collectors along the fertility gradients in Northern Sweden. Mean values ($n = 36$) \pm SE of the inorganic N forms collected in the resin extracts are shown.

| | High | Medium | Low |
|--|---------------|---------------|---------------|
| $\text{NH}_4^+\text{-N}$ ($\text{kg N ha}^{-1}\text{yr}^{-1}$) | 6.3 ± 0.1 | 2.6 ± 0.1 | 2.2 ± 0.1 |
| $\text{NO}_3^-\text{-N}$ ($\text{kg N ha}^{-1}\text{yr}^{-1}$) | 1.5 ± 0.1 | 0.0 ± 0.0 | 0.1 ± 0.0 |

N₂ fixation in P. schreberi

Average N_2 fixation as measured by acetylene reduction was 29.4 ± 5.8 ; 437.2 ± 52.2 and 570.4 ± 69.1 $\mu\text{mol m}^{-2} \text{d}^{-1}$ for the high, medium and low fertility sites, respectively (Fig. 2). Thus, the rates differed significantly between fertility conditions ($F = 27.8$, $p < 0.0001$).

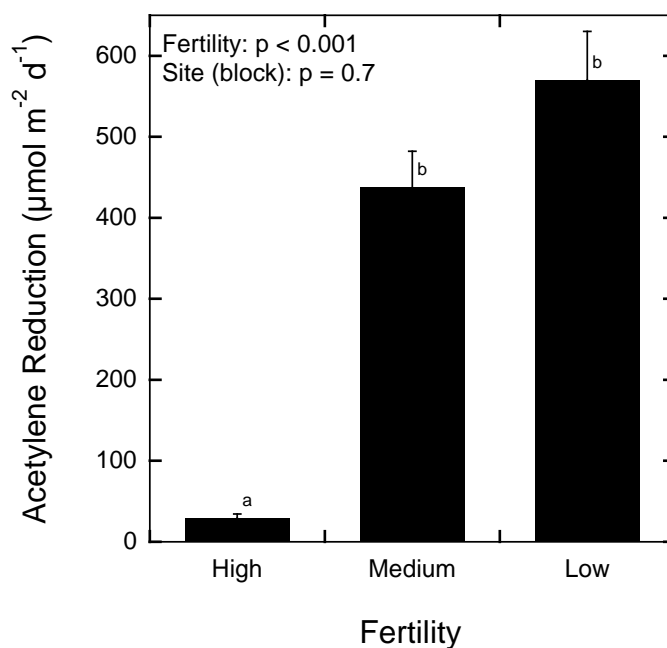


Fig. 2. N_2 fixation (acetylene reduction ($\mu\text{mol m}^{-2} \text{d}^{-1}$)) measured in the feather moss *Pleurozium schreberi* along the fertility gradients in Northern Sweden. Values are means ($n = 12$) \pm SE. Given are p values for the factors fertility and site obtained from a 2-Way ANOVA. Different letters indicate significant differences tested with Tukey's Post Hoc Test.

Uptake of C and N from soil by P. schreberi and microbes

The feather moss *P. schreberi* was found to be capable of accessing C and N directly from soil as indicated by enrichment of ^{13}C and ^{15}N in the moss tissue for all treatments (Fig. 3). That is, the ^{13}C and ^{15}N concentrations in ^{13}C , ^{15}N -alanine and ^{15}N - NH_4Cl treated mosses were higher than in the control (non- ^{13}C , ^{15}N -labelled) treatments in all fertility conditions. The ^{15}N uptake by moss was similar for the organic and inorganic N sources along the gradients (Fig. 3): mean excess ^{15}N for the ^{15}N - NH_4Cl addition was 0.052 ± 0.02 (SE) (at %), 0.054 ± 0.02 (at %) and 0.06 ± 0.01 (at %) for the high, medium and low fertility sites, respectively. Mean excess ^{15}N in the ^{13}C , ^{15}N -alanine plots was 0.016 ± 0.008 (at %), 0.018 ± 0.003 (at %) and 0.046 ± 0.02 (at %) for high, medium and low fertility conditions, respectively. Also mean excess ^{13}C in moss was not significantly different along the gradients: 0.0003 ± 0.0002 (at %), 0.0013 ± 0.0008 (at %) and 0.0024 ± 0.0005 (at %) in the high, medium and low fertility sites, respectively. On average, this yielded an excess uptake of 0.06 ± 0.01 (at %), 0.03 ± 0.007 (at %) and 0.001 ± 0.0004 (at %) for ^{15}N - NH_4Cl , ^{15}N -alanine and ^{13}C -alanine, respectively. The obtained values for excess ^{13}C , ^{15}N -alanine and ^{15}N - NH_4Cl were significant different from zero ($t = 3.2, 3.7, 4.8$; $p = 0.01, 0.006, 0.001$ for ^{13}C -alanine, ^{15}N -alanine and ^{15}N - NH_4Cl , respectively). Excess ^{13}C -alanine and ^{15}N -alanine in moss tissue were positively correlated ($r = 0.7$, $p = 0.02$; $df = 7$) (Fig. 4). The theoretical maximum slope for uptake of ^{13}C , ^{15}N -alanine in an intact form would be 3, because 1-N and all 3 C-atoms in the amino acid were labelled. Thus, a slope of 3 would indicate that the uptake of alanine was 100 % as intact amino acid. We found a slope of 0.042 in moss tissue, which suggests that 1.4 % of the alanine uptake was in an intact form (Fig. 4). We did not find significant correlations between N_2 fixation rates in the moss and ^{13}C , ^{15}N uptake by moss along the gradients. However, uptake of N from soil was correlated with C/N ratios of the moss in the medium and low fertility sites ($r = 0.96$, $p < 0.0001$; $df = 9$).

We found no detectable enrichment of ^{13}C or ^{15}N in the chloroform-fumigation microbial biomass estimates.

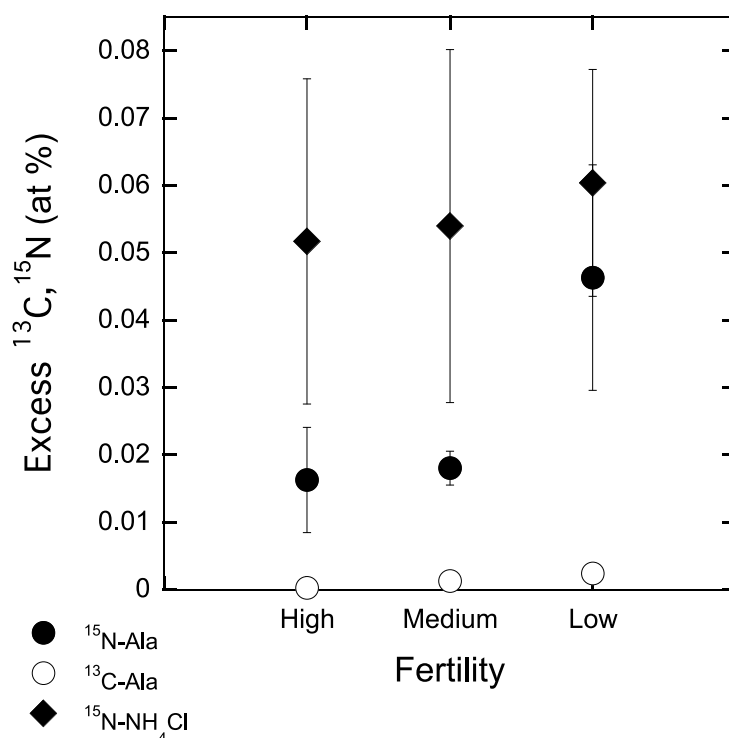


Fig. 3. Enrichment of ¹³C-alanine (white circles), ¹⁵N-alanine (black circles), and ¹⁵N-NH₄Cl (black rhombi) as excess label (at %) in moss tissue 24 h after injection of ¹³C, ¹⁵N-alanine and ¹⁵N-NH₄Cl into moss-soil cores. Moss-soil cores were collected along the three fertility gradients in Northern Sweden. Data points are means (n = 6) ±SE for the different fertility sites (high, medium, low).

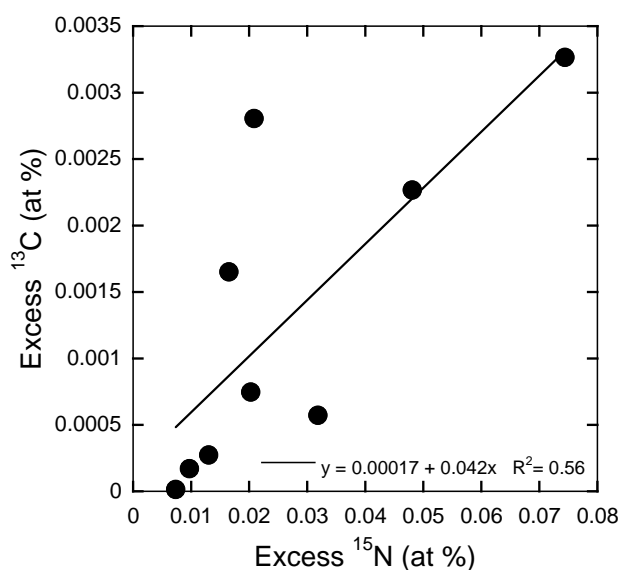


Fig. 4. Relationship between excess ¹³C-alanine and ¹⁵N-alanine in the tissue of *Pleurozium schreberi* as an indicator for intact uptake of the amino acid. A slope of 3 would imply that alanine was taken up intact (all 3 C-atoms were labelled). Regression equation, regression line and r^2 value are given.

PLFA concentrations

There were no significant differences in bacterial or fungal PLFA concentrations between the different fertility conditions (Table 3). However, the PCA analyses revealed a separation of the PLFA composition following the high, medium and low fertility conditions along the first PCA axis (PC1) ($F = 19.3$, $p < 0.001$) as well as along PC2 ($F = 3.7$, $p = 0.05$) (Fig. 5a). The high fertility sites were clustered toward higher PC1 and PC2 values and the medium and low fertility sites were clustered toward lower PC1 and PC2 values (Fig. 5a). The main fungal PLFA biomarkers (18:2 ω 6,9 and 18:1 ω 9) were found to the left in the panel (lower PC1 values), and the main bacterial PLFA biomarkers (18:1 ω 7, 16:1 ω 7c, 16:1 ω 5, 16:1 ω 9, cy19:0) were oriented toward the right of the panel (higher PC1 values), reflecting a pattern between the fungal:bacterial balance and the fertility sites (Fig. 5b). PC1 showed a negative correlation with soil-C/N ratios ($r = -0.72$, $p < 0.001$; $df = 16$).

Table 3. PLFA concentrations in nmol g⁻¹ soil fresh weight along the fertility gradients (high, medium, low fertility) in Northern Sweden. Given are means \pm SE ($n = 6$) for total, bacterial and fungal PLFA concentrations.

| | High | Medium | Low |
|------------------------------------|---------------|---------------|--------------|
| Total PLFA (nmol g ⁻¹) | 460 \pm 114 | 770 \pm 200 | 480 \pm 93 |
| Bacteria (nmol g ⁻¹) | 240 \pm 54 | 240 \pm 46 | 150 \pm 22 |
| Fungi (nmol g ⁻¹) | 40 \pm 20 | 190 \pm 78 | 110 \pm 40 |

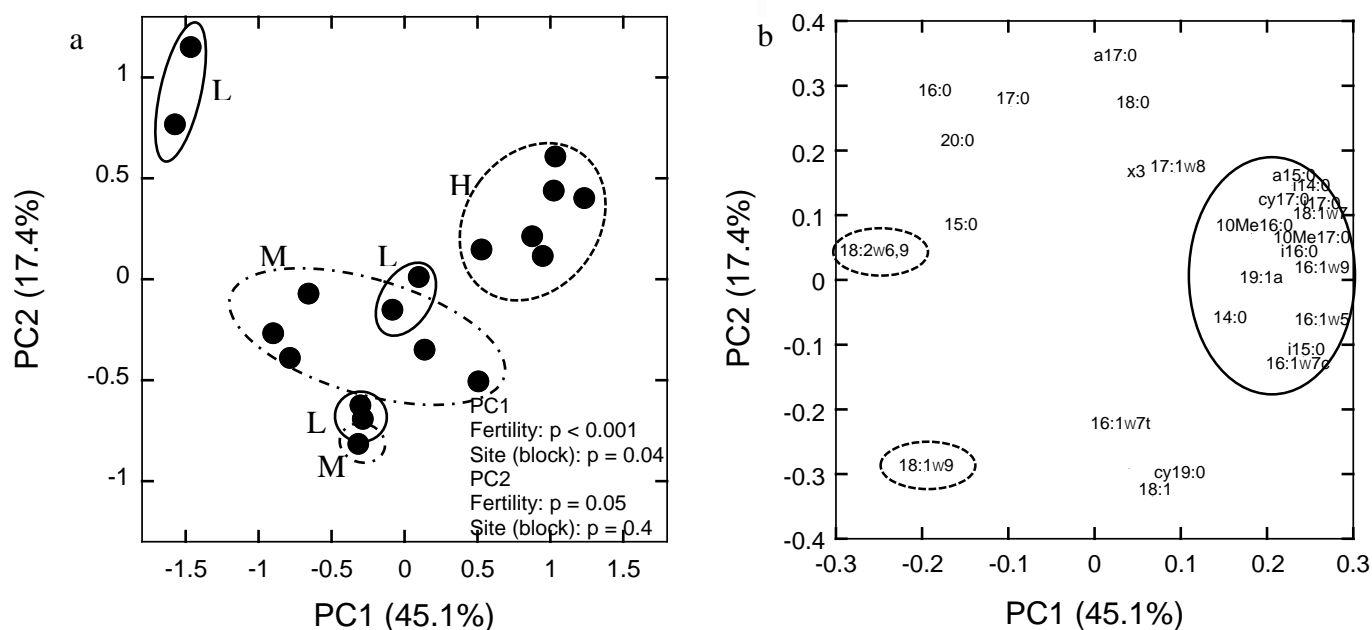


Fig. 5. Results of the PLFA analyses in soil samples collected along the fertility gradients in Northern Sweden. PLFAs were subjected to PCA. (a) PCA case scores from the high (H), medium (M) and low (L) fertility sites. Solid lines represent the L, dotted-dashed the M and dotted lines represent the H fertility sites. Given are p values for the factors fertility and site obtained from a 2-Way ANOVA for PC1 and PC2. (b) PCA variable loadings: Fungal and bacterial PLFA marker. Dashed lines indicate the main fungal biomarker; solid lines indicate the main bacterial biomarker.

Amino acid mineralization

Amino acid mineralization rates were similar in all forest sites and under all fertility regimes, thus, nutrient resource conditions had no overall effect on amino acid mineralization rates (Fig. 6).

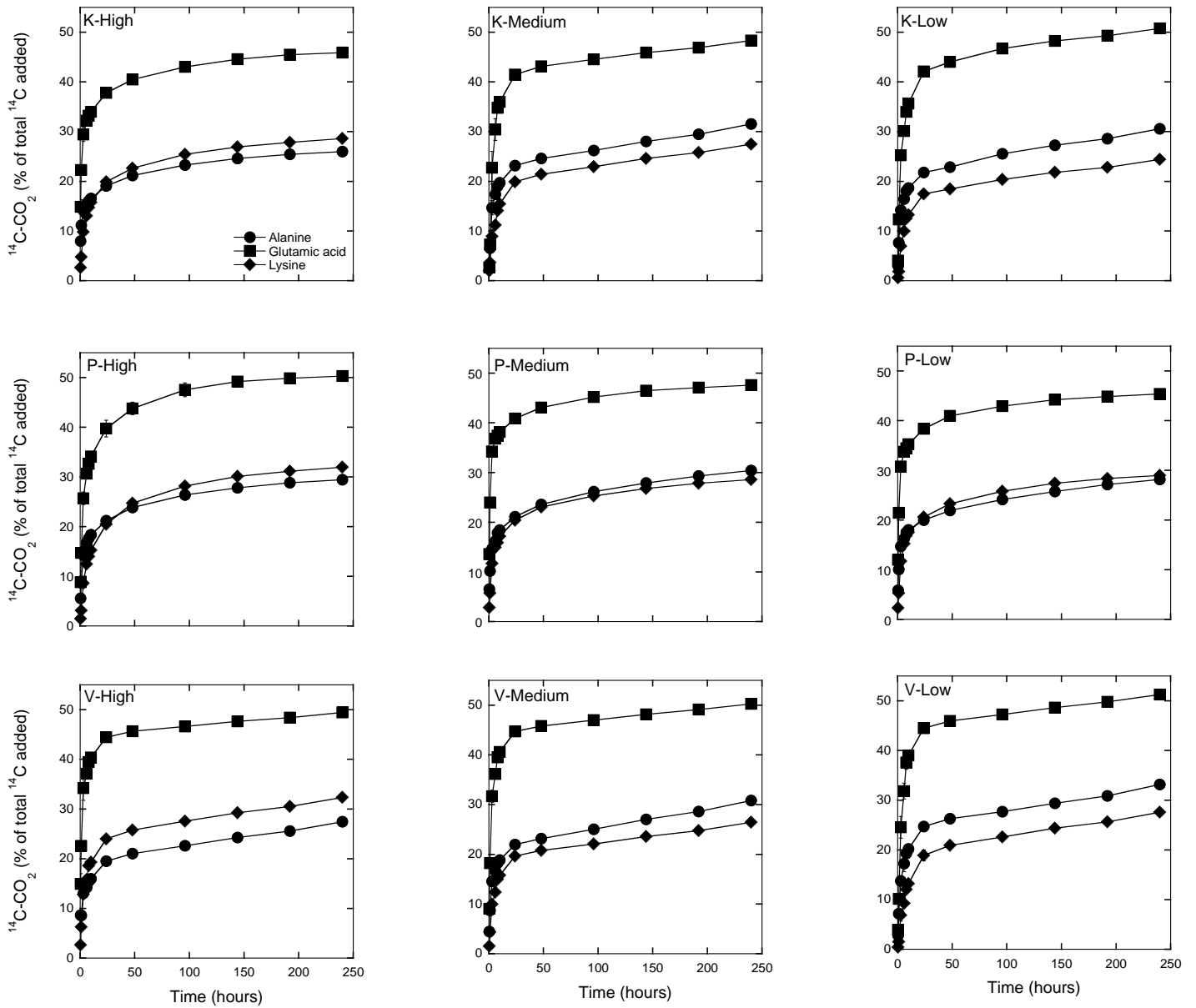


Fig. 6. Mineralization of three amino acids in soil samples collected along the fertility gradients in Northern Sweden. Shown is percentage of ^{14}C evolved as CO_2 in the three forest sites (K = Kryddgrovan; P = Pite Älven; V = Varjisån) in the three fertility stands (high, medium, low). Given are means for the CO_2 evolution after adding alanine (circles), glutamic acid (squares) and lysine (rhombi) and $\pm\text{SE}$ ($n = 4$). Error bars are hidden behind symbols. The curves represent the best fit to the kinetic model.

Discussion

Feather mosses in intact microcosms were found to be capable of C and N uptake from the soil humus layer. Although C and N uptake from soil by moss was low, the occurrence of ^{13}C and ^{15}N signal in the moss tissue demonstrates the capacity of the feather moss *P. schreberi* to take up C and N directly from soil, corroborating our first hypothesis. Since mosses possess rhizoids rather than roots, which reach only a few cm into the humus layer, their ability to use this pathway of nutrient utilization is surprising. However, uptake of N from soil is likely a minor source of N for mosses (Ayres et al. 2006). The more prominent means of N uptake is the absorption of N from throughfall and atmospheric deposition (Turetsky 2003) as well as the relocation of nutrients from dead moss tissue (Aldous 2002). The lack of detectable differences of C and N uptake from soil by moss between the fertility conditions further suggest that the moss is less affected by changes in soil-C and N concentrations than by changes in N input via throughfall from the canopy, which was significantly different along the gradients. Uptake of soil nutrients as well as N_2 fixation in the moss seem to be mainly driven by throughfall from the canopy (see below), reflecting a rather closed moss-nutrient cycle. There is no source of additional atmospheric N deposition other than the low background N deposition ($2 \text{ kg N ha}^{-1}\text{yr}^{-1}$) along the gradients, thus, the observed sensitivity of N_2 fixation in the moss is only caused by the different vegetation along the gradients that lead to differences in N deposited on the moss via throughfall. Thus, depending on throughfall N, the form in which N enters the system is very different: either as organic N via N_2 fixation by the moss-cyanobacteria association or, if N throughfall is high enough to inhibit N_2 fixation, the moss layer is bypassed and N enters the system as inorganic N, which could significantly affect soil-N cycling and the competition between plants and microbes for N.

We expected higher microbial activity in soils from the high fertility sites due to higher nutrient availability. Microbial biomass (PLFA concentrations) was higher in the more fertile sites, which also coincided with a shift toward bacteria dominated rather than fungi dominated communities, as indicated by a separation of the main fungal and bacterial PLFA biomarkers along the fertility gradients. Both higher microbial biomass (Stockdale and Brookes 2006) and a shift toward a higher relative dominance of bacteria have been associated with a more rapid turnover of nutrients (Hedlund et al. 2004; Moore et al. 2005; Neutel et al. 2007), and PLFA biomarkers linked to bacteria have been previously and repeatedly found in more fertile conditions, whereas PLFA

biomarkers linked to fungi are relatively more dominant in less fertile conditions, as shown in boreal forest soils (Nilsson et al. 2005), as well as in N-fertilization experiments in grassland soils (Rousk et al. 2011). However, since the bacterial and fungal PLFA concentrations were not significantly different between the fertility conditions, the lack of a pattern in amino acid mineralization rates along the fertility gradients is plausible (Fig. 6). Furthermore, the high fertility sites could be prone to phosphorus (P)-limitation due to increased P immobilization in aluminium- and iron-complexes (Giesler et al. 2002), which could inhibit microbial activity. Also, C compared to N is likely to limit microbial activity in the high fertility sites as indicated by the lower C/N ratios in these soils than in the low and medium fertility sites. In contrast, it is possible that the less fertile sites are limited by both C and N (Demoling et al. 2008; Sterner and Elser 2002). Thus, the soil microbial community may be exposed to alternating nutrient limitations along the fertility gradients.

Mosses are associated with a variety of fungi (Kausarud et al. 2008; Davey et al. 2009), which could reflect a mutualistic relationship, similar to mycorrhiza, or a neutral to parasitic relationship (Carleton and Read 1991; Davey and Currah 2006). We did not find differences in C or N uptake from soil by the moss along the fertility gradients, despite a relatively fungal dominated community in the medium and low fertility sites. Therefore, we cannot conclude that associations with fungi would enhance nutrient uptake from soil.

Nitrogen throughfall was fundamentally higher in the high fertility sites than in the medium and low fertility sites, which could have been sufficient to cover the moss' N requirements, since this is the main N source for mosses (Turetsky 2003) and therefore, uptake of N from soil is not necessary to cover N demand in areas with enhanced N input from throughfall. We did not account for organic N in our estimate of throughfall N along the gradients, which is likely to contribute to the total N in throughfall in boreal forests, representing another source of N for feather mosses (Forsum et al. 2006).

We found higher N₂ fixation rates in cyanobacteria associated with mosses from the medium and low fertility sites, as expected, but uptake of C and N from soil by moss was marginally higher here than in the high fertility sites. However, we did not find a correlation between uptake of C and N from soil and N₂ fixation, indicating that N₂ fixation rates are not affected by C and N uptake from soil by moss and vice versa. Throughfall N in the high fertility sites likely suppresses N₂ fixation rates, but at the

same time does not promote uptake of N from soil suggesting that N input via throughfall is the driver of the various N utilization strategies of the moss (see above). The increased N throughfall at the high fertility sites could make uptake of N from soil unnecessary. In contrast, the minimal N throughfall at the medium and low fertility sites could lead to higher N₂ fixation rates and uptake of N from soil. Also, tissue N concentrations were much lower in moss collected at the medium and low fertility sites, indicating that the mosses are N-limited. However, in the moss-cyanobacteria association, the moss likely receives N from the cyanobacteria and the cyanobacteria receives shelter and C in return, since it is assumed that cyanobacteria shut down their own photosynthesis when living in association with plant partners (Steinberg and Meeks 1991; Adams and Duggan 2008). The tendency for higher uptake of C and N-alanine in the low fertility sites could suggest that increasing N₂ fixation rates lead to higher demands of C and N by the moss either (1) as a result of higher C transfer to the cyanobacteria as an exchange for fixed N, or (2) the moss requires more nutrients from soil when N throughfall is too low to cover its N demand. This would imply that the N transfer from cyanobacteria to moss is insufficient to satisfy the moss' N demands which would explain why throughfall N concentrations up to 6 kg N ha⁻¹ yr⁻¹ (Gundale et al. 2011) and N additions in the lab of 10 kg N ha⁻¹ (Ackermann, unpublished) do not reduce N₂ fixation in the moss. In addition, the C/N ratios of the moss imply that the moss is N-limited in the low and medium fertility sites, which stands in contrast to the high N₂ fixation rates in these sites.

Although we found a correlation between alanine derived ¹³C and ¹⁵N uptake into moss tissue, only 1.4 % of the added amino acid seemed to be taken up in an intact form. Estimating preferences for inorganic or organic N uptake by moss is therefore difficult. The low uptake of intact amino acid by the moss could be a result of ¹³C-loss via moss-respiration during the 24 h incubation period. Another explanation is that mineralization of alanine could have taken place prior to uptake. Turnover rates of amino acids in soils are fast (< 1 h), thus, our 24 h incubation period left enough time for mineralization by microbes (e.g. Rousk and Jones 2010, see below). However, we did not fractionate ¹³C and ¹⁵N labels into the different moss parts (e.g. green parts and brown parts, rhizoids), which could bias our findings since alanine could have been mineralized in the rhizoids and ¹⁵N could have been transferred to the moss shoots. Warren (2012) suggests that amino acids are metabolized in plant roots before the N is transferred to the leaves via the xylem sap. However, mosses lack roots and they

possess a great capacity to absorb and scavenge N (Svensson 1995; Kotanen 2002), thus comparison with vascular plants is challenging.

Unfortunately, we are not able to quantitatively estimate microbial uptake in our experiment. We expected the microbial community to be limited in different nutrients along the fertility gradients (possibly P, C-limitation in the high fertility sites, N-limitation in the medium and low fertility sites), which could have added to the lack of detectable differences in mineralization rates of amino acids along the gradients. The mineralization curves for alanine suggest that 75 % is immobilized after 24 h (Fig. 6), thus, after 24 h of tracer additions, we should have been able to detect enrichment in the fumigated soil. However, for the detection of intact amino acid uptake, 24 h could have been too long since microbial turnover of nutrients takes place within few hours (Jones and Kielland 2002; Jones et al. 2004; Rousk and Jones 2010). The half-life of amino acids in soils can be as short as 1.6 h (Finzi and Berthrong 2005). Our pilot study (mosses with rhizoids submerged in solution) showed that N uptake from solution by *P. schreberi* was higher after 24 h than within few hours (mean excess ^{15}N (at %) after 6h: -0.0003 ± 0.0001 , after 24 h: 0.0004 ± 0.0003 ; $p = 0.05$). Also, 24 h is a commonly used incubation time for measuring uptake of nutrients by mosses (Krab et al. 2008), and incubation times of up to 7 days are used as well (Ayres et al. 2006). Nevertheless, in our pilot study, the rhizoids would have lacked competition from a microbial community, which would be absent in nutrient solution.

Conclusions

The results from this set of studies show that the feather moss *P. schreberi* is capable of N-uptake from soil in the form of $\text{NH}_4^+\text{-N}$ and N-alanine as well as of uptake of C from soil. However, our results suggest that microbial uptake was faster, which probably compromised our ability to quantify uptake rates for microbes after a 24 h incubation period. Fertility did not affect the uptake of C or N from soil by moss, suggesting that other mechanisms could have been the more crucial factors and driving forces. Throughfall N is likely the most influential factor dictating N uptake by the moss, whereas the microbial response to N availability is obscured by other limiting factors, possibly including both C and P. Furthermore, N_2 fixation in the moss was not correlated with uptake of C or N from soil, corroborating the importance of throughfall N as a driver of N_2 fixation in the moss rather than soil-N availability. Hence, the way N

enters the ecosystem is driven by throughfall N: either as inorganic N when N throughfall is high, leading to suppression of N₂ fixation in the moss layer, or as organic N when N throughfall is low enough to promote N₂ fixation in the moss. Additionally, mosses efficiently absorb and hold onto N from atmospheric deposition and throughfall, releasing only minor fractions of N into the soil. Thus, forest ecosystems that exhibit a moss-dominated ground cover are dependent on the N release from moss carpets.

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The cyanobacterial role in the resistance of feather mosses to decomposition - toward a new hypothesis

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The cyanobacterial role in the resistance of feather mosses to decomposition – toward a new hypothesis

Kathrin Ackermann, Thomas H. DeLuca, Johannes Rousk

Abstract

Cyanobacteria-plant symbioses play an important role in many ecosystems due to the fixation of atmospheric nitrogen (N) by the cyanobacterial symbiont. The ubiquitous feather moss *Pleurozium schreberi* (Brid.) Mitt. is colonized by cyanobacteria in boreal systems with low N deposition. Here, cyanobacteria fix substantial amounts of N₂ and represent a potential N source. The feather moss appears to be resistant to decomposition, which could be partly a result of toxins produced by cyanobacteria. To assess how cyanobacteria modulated the toxicity of moss, we measured inhibition of bacterial growth. Moss with varying number of cyanobacteria was added to soil bacteria to test the inhibition of their growth using the thymidine incorporation technique. Moss could universally inhibit bacterial growth, but moss toxicity did not increase with N₂ fixation rates (numbers of cyanobacteria). Instead, we see evidence for a negative relationship between moss toxicity to bacteria and N₂ fixation, which could be related to the ecological mechanisms that govern the cyanobacterial – moss relationship. We conclude that cyanobacteria associated with moss do not contribute to the resistance to decomposition of moss, and from our results emerges the question as to what type of relationship the moss and cyanobacteria share.

Keywords: Acetylene reduction, boreal forest, bryophytes, evolution, mutualism, N deposition, parasitism, symbiosis

Introduction

Symbiotic relationships between plants and microbes have been proposed to be important drivers of evolution (Wernegreen, 2004). Cyanobacteria are widespread and well-known symbionts, colonizing bryophytes, lichens and higher plants (Rai et al., 2000; Adams and Duggan, 2008; Meeks, 2009). Cyanobacteria are facultative autotrophs, generally fixing atmospheric carbon (C) and nitrogen (N), but are also known to use host-C through symbiotic relationships (Meeks, 2009). The general

assumption is that cyanobacteria-plant associations are mutualistic symbioses: the plant host receives N in the form of ammonium (NH_4^+) or amino acids and in return provides carbohydrates, shelter and protection (Meeks et al., 1985; Steinberg and Meeks, 1991), suggesting ecological specialization where the cyanobacteria down-regulate their own photosynthesis (Meeks and Elhai, 2002; Adams and Duggan, 2008).

In the northern boreal forest, atmospheric N deposition is generally low ($1 - 3 \text{ kg N ha}^{-1} \text{ yr}^{-1}$), however, mosses colonized by N_2 fixing cyanobacteria may contribute a further $2 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, thus representing a major N input pathway in this pristine environment (DeLuca et al., 2002; Gundale et al., 2011). Due to the strong N limitation of these ecosystems (Tamm, 1991), the moss-cyanobacteria association therefore characterizes the productivity of the ecosystem as well as its biogeochemical budget. One example is the relationship between one of the dominant primary producers in boreal forests, the feather moss *Pleurozium schreberi* (Brid.) Mitt. and associated cyanobacteria. Substantial amounts of atmospheric N_2 are fixed by cyanobacteria colonizing *P. schreberi* (DeLuca et al., 2002). However, this process is sensitive to N inputs: higher N input through deposition results in lower numbers of cyanobacteria in the feather mosses and consequently, in lower N_2 fixation rates (DeLuca et al., 2008; Gundale et al., 2011; Ackermann et al., 2012).

In spite of their dominant presence as a ground cover in the boreal forest, feather mosses are consumed by very few herbivores (Prins, 1982). Although other bryophytes have been found to be even more resistant to decomposition (Lang et al., 2009; Fenton et al., 2010), it has been noted that even the herbivores that graze mosses appear to specifically avoid the ubiquitous *P. schreberi* and *Hylocomium splendens* (Eskelinen, 2002). Additionally, moss litter is known to be highly resistant to microbial decomposition in high latitude ecosystems (Hobbie, 1996). However, the low quality of moss as a substrate or food source (Prins, 1982; Hobbie, 1996) appears an insufficient explanation for the lack of utilization of this ample potential resource. The cyanobacteria that associate with feather mosses are known to produce toxins (Cox et al., 2005; Adams and Duggan, 2008; Kaasalainen et al., 2012), which thus could play an important role in the resistance of mosses towards decomposition. This would be a hitherto unrecognized dimension of the relations between feather moss and their cyanobacterial colonizers.

In this experiment we assess the contribution by cyanobacteria to the resistance to decomposition characteristic for moss. More precisely, we hypothesize that moss

toxicity increases with higher levels of cyanobacterial colonization. To assess this hypothesis, we used a microbial bioassay to estimate the toxicity of *P. schreberi* by determining its propensity to inhibit soil bacterial growth (Aldén and Bååth, 2008; Rousk et al., 2011, 2012) across a gradient of cyanobacterial colonization.

Materials and Methods

Sampling, N₂ fixation analyses and cyanobacterial counts

We used shoots of the feather moss *P. schreberi* from four mid- to late succession forests (Dötternoive, Borup, Nyvall, Kuottavare) in Northern Sweden that have been shown to fix sufficient amounts of N to be used in our experiment (Ackermann et al. 2012). The sites were located between latitude 64-65°N and longitude 18-19°E and have been described previously (DeLuca et al. 2002; Zackrisson et al. 2004; Ackermann et al. 2012) (see also Table 1). Mean annual temperature and precipitation were 1 °C and 570 mm, respectively. The vegetation consists of Scots Pine; Norway Spruce; *Vaccinium vitis-idaea*; *V. myrtillus*; *Empetrum hermaphroditum*; *H. splendens* and *P. schreberi*. Soils at the sites are classified as Typic Haplocryods (FAO, Cambic Podzol). All sites are mid- to late succession forests.

Table 1. Mean values (n = 18) ±SE of the main soil properties from the moss-sampling sites in Northern Sweden. Data for soil nutrients are given in mg g⁻¹ soil dry weight.

| pH | TC | TN | C/N | DOC | DON | DIN |
|-------|------|-------|-----|--------|--------|----------|
| 4.4 | 369 | 11 | 48 | 1.46 | 0.02 | 0.002 |
| ± 0.3 | ± 23 | ± 3.6 | ± 1 | ± 0.17 | ± 0.02 | ± 0.0002 |

TC = Total Carbon; TN = Total Nitrogen; DOC = Dissolved Organic Carbon;

DON = Dissolved Organic Nitrogen; DIN = Dissolved Inorganic Nitrogen

Nitrogen fixation was measured using a ¹⁵N₂-calibrated acetylene reduction assay (Schöllhorn and Burris 1967) as described by Zackrisson et al. (2004). Briefly, the green parts of 10 moss shoots (> 800 leaves shoot⁻¹) from each site were placed in a 20 ml tube, sealed and 10 % of the headspace was replaced with acetylene. Moss samples were incubated for 24 h at room temperature. Ethylene generated in the headspace by the cyanobacterial nitrogenase enzyme was measured by gas

chromatography equipped with a Flame Ionization Detector (Varian, Santa Clara, USA).

The fresh moss shoots were ground using a ball mill (2 min., 30 s⁻¹) forming a moss slurry, which was subsequently used in the bacterial growth assay to determine toxicity (below). Until processing, samples were kept at 4 °C. Five different fronds with 10 leaves each were counted per sample. For that, moss leaves were destructively harvested from the fronds through scraping to enable microscopy counting.

Cyanobacteria cells were then counted using an ultraviolet-fluorescence micrograph (Zeiss Axiophot 2 fluorescence microscope with 20x magnification) with a green filter. Cyanobacteria possess auto-fluorescent phycobiliproteins to capture light; therefore no staining is needed to visualize them using a fluorescence microscope.

Moss toxicity assay using bacterial growth as a bioindicator

Soil bacterial growth rates were estimated using the thymidine (TdR) incorporation technique (Bååth 1992; Bååth et al. 2001) rather than the leucine incorporation technique since the latter is susceptible to confounding isotope dilution effects, which the present assay would be sensitive to (non-radioactive leucine concentrations were likely to scale with the moss-addition, causing unwanted isotope dilution effects). The determination of moss toxicity was otherwise determined analogously to salt toxicity in Rousk et al. (2011). Briefly, 2 g of wet weight (ww) of soil (a composite and homogenized sample of the soil from all sites) was mixed with 20 ml of distilled water. The soil suspensions were vortexed for 3 min and then centrifuged for 10 min at 1000 g to obtain a bacterial suspension in the supernatant. From the soil bacterial suspension, 1.35 ml was used for the thymidine incorporation measurements in 2 ml microcentrifuge tubes. Subsequently, 0.15 ml moss (see below) was added to the 1.35 ml of the bacterial suspension in the microcentrifuge tubes.

Eight different concentrations of moss was used for each sample to determine its toxicity to bacterial growth; 0, 0.14, 0.41, 1.2, 3.7, 11, 33, 100 mg moss ml⁻¹ were used in the moss toxicity determination as final concentrations in the bacterial suspensions. Distilled water was added to the control and to dilute the moss in the dilution series. Thymidine ([*Methyl*-³H]thymidine, 25 Ci mmol⁻¹, 1.0 mCi ml⁻¹, Perkin Elmer) was added to a final concentration of 130 nM in the bacterial suspensions. The samples were then incubated at approximately 20 °C for 2 h. The incorporation of TdR was terminated by the addition of 75 µL of 100% trichloroacetic acid. Non-incorporated

TdR was then removed as described in Bååth et al. (2001), and ^3H -TdR incorporation was measured in a scintillation counter (Wallac EG&G, Milton Keynes, UK).

Total phenols in moss tissue

Total phenols were measured in moss tissue that was first ground by placing 1 g dry weight equivalent of fresh moss tissue in a ball mill and then suspended in 10 ml of distilled water. Samples were centrifuged at 4000 rpm and the supernatant analyzed for phenols by using the Folin and Ciocalteu's reagent (Swain and Hillis, 1959) and absorbance measured at 725 nm using a microplate reader.

Statistics and calculations

Toxicity values were expressed as the concentration resulting in 50 % inhibition (EC_{50}) of the bacterial growth in the soil suspensions. More toxic moss inhibited the bacterial growth at lower concentrations and, therefore, has a lower value of EC_{50} than a less toxic moss. The EC_{50} -values of the bacterial communities were calculated using a logistic model, $Y = c/[1 + e^{b(X-a)}]$, where Y is the TdR incorporation rate, X is the logarithm of the moss concentration, a is the value of $\log(\text{EC}_{50})$, c is the TdR incorporation rate in the control, and b is a parameter (the slope) indicating the inhibition rate. Kaleidagraph 4.0 for Mac (Synergy software) was used to fit a logistic curve to the data using the equation. Relationships between variables of the different soils were investigated using regression analyses or using analysis of variance (ANOVA) with Tukey's HSD post hoc pair-wise comparisons using JMP 9.0 for Mac (SAS Institute, Cary, NC, USA).

Results

Moss toxicity and soil bacteria

N_2 fixation rates were found to scale linearly with the numbers of cyanobacteria in the mosses, ranging between 0 and 200 cells leaf $^{-1}$ ($p < 0.0001$; $r^2 = 0.94$) (Fig. 1), where low counts corresponded to low rates, and *vice versa*, and where a N_2 fixation rate of 0 $\mu\text{mol m}^{-2} \text{d}^{-1}$ acetylene reduced corresponded to 0 cyanobacteria cells, and a N_2 fixation rate of 91 $\mu\text{mol m}^{-2} \text{d}^{-1}$ acetylene reduced corresponded to 200 cyanobacteria cells leaf $^{-1}$ ($16 \cdot 10^4$ cells shoot $^{-1}$).

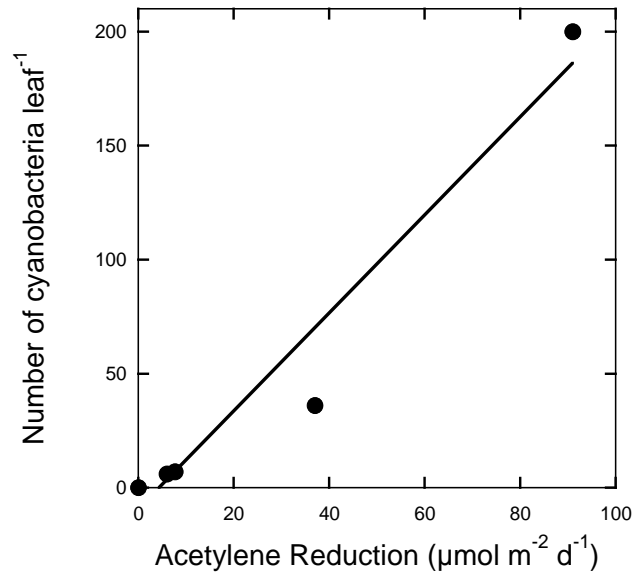


Fig. 1. Numbers of cyanobacteria cells leaf⁻¹ in relation to acetylene reduction ($\mu\text{mol m}^{-2} \text{d}^{-1}$) in the feather moss *P. schreberi*.

We were able to demonstrate clear dose-response relationships between bacterial growth and moss addition for the six sites studied here (Fig. 2), with lower bacterial growth following exposure to higher concentrations of moss. The relationship between bacterial growth and moss exposure could be modeled well using a logistic model ($r^2 = 0.99, 0.93, 0.96, 0.96, 0.95$ and 0.84 for the six sites, ordered from low to high N_2 fixation rates / cyanobacteria numbers). Higher concentrations of moss effectively inhibited bacterial growth as indicated by suppression of growth by 90% at the highest moss addition rate.

The EC_{50} -values were used as indices for moss toxicity to bacterial growth. The EC_{50} -values were related to the presence (cyanobacterial cell count; $p < 0.05$; $r^2 = 0.72$) and activity (acetylene reduction; $p = 0.01$; $r^2 = 0.81$; Fig. 3) of cyanobacteria on the mosses.

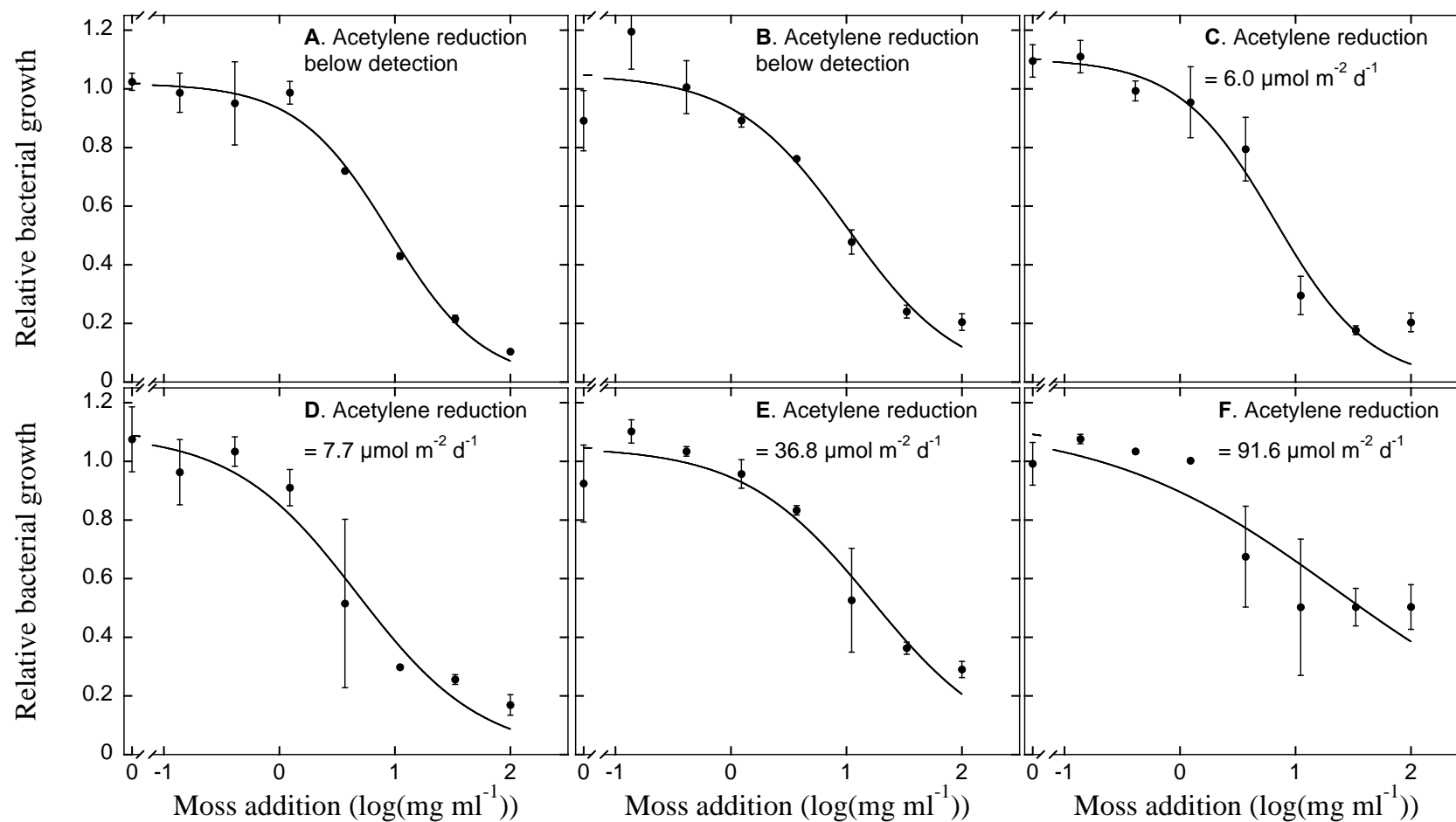


Fig. 2. Relationship between relative bacterial growth and the differently concentrated *Pleurozium schreberi* solutions. Panels A-F are different moss samples hosting different numbers of cyanobacteria and with that, different N₂ fixation rates. Data points are mean values (n = 2) \pm SE.

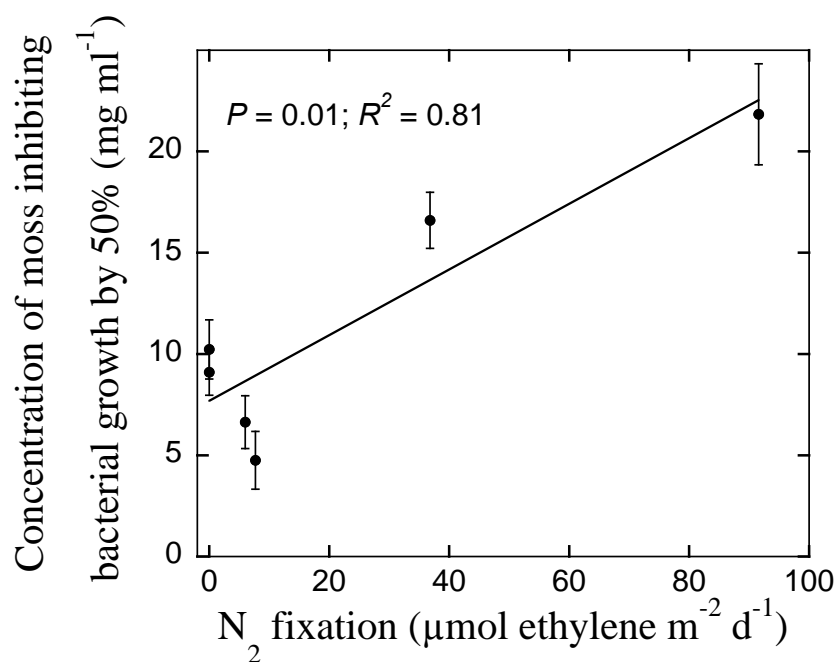


Fig. 3. Relation between EC_{50} values as a mean for moss toxicity and N_2 fixation rates in *P. schreberi*. Shown are mean values \pm SE derived from the inhibition curves.

Total phenols

Total phenols in moss tissue ranged between 5.4 and 9.0 mg phenol g^{-1} fresh weight (mean: 7.0; SE \pm 0.6) (Table 2) and were not significantly different between the moss samples. No correlation was found between phenols and N_2 fixation and between phenols and EC_{50} -values.

Table 2. Main characteristics of *Pleurozium schreberi*-tissue collected in Northern Sweden.

Shown are means \pm SE.

| pH | C/N | Phenols (mg g^{-1} fw) | Chlorophyll (SPAD-Units) |
|------------|-----------|----------------------------------|--------------------------|
| (n = 35) | (n = 18) | (n = 24) | (n = 20) |
| 4.4 | 46.3 | 7.0 | 3.7 |
| \pm 0.04 | \pm 1.1 | \pm 0.6 | \pm 0.6 |

Discussion

The use of biomarkers has been an invaluable tool to directly assess the toxic effect of substances in the environment (van Gestel and van Brummelen, 1996). While the interpretation of toxicity assessments based on biomarkers must consider the particular sensitivity or tolerance of the organisms used, it is generally held that the small size of microorganisms (and high area per volume) make them particularly exposed to toxins and thus sensitive indicators of the substances' general toxicity (Kahru et al. 2008). Moreover, while single species tests can be strongly biased by the properties of the particular strains included and must be supplemented by a battery of tests of different organisms before their effects can be generalized, assessing whole bacterial communities (as in the present study) are more robust in principle: it takes advantage of the naturally highly diverse bacterial communities to provide a continuous toxicity response that is a powerful tool to accurately establish the propensity for a substance to be toxic (Brandt et al. 2004). Indeed, previous comparative assessments have validated the use of bacterial growth to assess environmental toxicity (e.g. Ore et al 2010; Brandt et al. 2010; Aldén-Demoling et al. 2008).

Bacterial growth was universally inhibited by moss additions irrespective of cyanobacterial presence or activity, as shown by the clear dose-response relationship that could be established for all tested mosses. We hypothesized that moss toxicity, as indicated by the propensity of mosses to inhibit bacterial growth, would increase with higher numbers of cyanobacteria present. What we found was that cyanobacteria did not contribute at all to the toxicity of moss to bacteria. We cannot rule out a higher susceptibility of the other major decomposer group, fungi, to the toxic potential of mosses. However, in a thorough assessment of the abundance of fungi (ergosterol concentration) in relation to a previously noted N₂-fixation gradient across a chronosequence since last fire of boreal forests in Northern Sweden (see Zackrisson et al. 2004), we found no differences in fungal presence (J. Rousk and K. Rousk, unpublished). Moreover, rather than less, it would be reasonable to suppose that bacteria should be more susceptible than fungi to toxins, due to their high area per volume. This implicitly suggests that they are not responsible for contributing for the slow decomposition rates described for feather mosses (Hobbie, 1996).

That moss can inhibit bacterial growth is not surprising, and probably is partly related to the inhibitory nature of constitutional plant compounds, such as phenols, which have a known inhibitory effect on microorganisms (e.g. Vanderpoorten and

Goffinet, 2009). However, moss phenol content could not be used to explain any differences in the inhibition of bacterial growth with regard to presence of cyanobacteria, as phenol concentrations in moss samples were similar despite differences in cyanobacterial colonization and were not correlated with moss toxicity (EC₅₀ values). Moreover, toxins produced by cyanobacteria are not exclusively phenolic, rather the majority are alkaloids and cyclic peptides (Cox et al., 2005).

The obtained results on the variation of moss toxicity are of a negative nature, lending no support to our hypothesis stating that cyanobacteria contribute to the toxicity of moss. However, rather than no relationship (i.e. no slope in Fig. 3), we have a suggestion for a higher toxic effect by mosses that was correlated with lower cyanobacteria numbers and activity. Could these results be used to guide our understanding of the hitherto elusive ecology of moss-cyanobacteria relations? A range of observations from previous experiments and field investigations could be combined with our here reported results to develop a way forward to address this.

P. schreberi collected in Wales (UK), exposed to relatively high rates of N deposition ($> 12 \text{ kg N ha}^{-1} \text{ yr}^{-1}$; Jones et al., 2004), are not colonized by cyanobacteria and with that, do not fix N₂ (K. Ackermann and T.H. DeLuca, unpublished). When stored in a laboratory this remained true, but when N starvation was experimentally induced (rinsing with de-ionized water) cyanobacterial colonization and N₂ fixation both commenced. It has also been found that cutting off the rhizoids and dead moss associated with moss carpets (i.e. severing its N supply from the soil) and returning it to the forest floor leads to a significant increase in cyanobacterial colonizers and N₂ fixation rates (T.H. DeLuca, unpublished results). Further, the cutting of moss carpets from N rich environments and subsequent transplantation to N poor environments results in colonization of moss leaves by cyanobacteria and increased N₂ fixation (DeLuca et al., 2007). Taken together, these and other observations suggest that a regulation of cyanobacterial colonization of moss is very sensitive to the environmental growth conditions of the moss.

A possible mechanism for the regulation of cyanobacterial colonization of moss is the production of toxic secondary metabolites (Kaasalainen et al. 2012), such as oxylipins, derived from fatty acids (Croisier et al., 2010; Matsui, 2006). It is possible that such compounds could be used to control the colonization by bacteria generally, including that of cyanobacteria. The underlying ecology and causality of such a regulation would be ambiguous, however. One possibility would be that moss actively

down-regulates the production of toxins during times of N deficiency to enable colonization by cyanobacteria and with that, a cyanobacteria powered endogenous supply of the limiting resource. Another possibility would be that conditions of N deficiency compromised the moss' ability to defend against opportunistic microorganisms generally, and cyanobacteria with their independent N supply in particular. These forwarded hypotheses call into question the placement of the cyanobacterial-moss symbiosis on the mutualism – parasitism continuum, which presently is an active area of research (Kiers and Denison, 2008; Grman, 2012). The substantial work on the mycorrhizal-plant symbiosis (Smith and Read, 2008) could act as a lens through which to focus experimental work to resolve the ecological interactions between the associated moss and cyanobacteria.

To conclude, we find no support for any contribution by cyanobacteria to the ability of feather mosses to resist decomposition. Instead, our results suggest a negative relationship between moss toxicity and cyanobacterial colonization. Our findings generate novel questions regarding the type of relationship that characterizes the ecology of moss and cyanobacteria – mutualistic or parasitic symbiosis?

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VI.

Feather mosses in boreal forests: Nitrogen (N) sink or source? Insights from a ^{15}N -application-experiment

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Author contributions: KA designed the study. KA, TD set up the field sites and the experiment. KA, TD collected the samples. KA analyzed the data. KA wrote the first draft of the manuscript, with all authors contributing to the final manuscript.

Feather mosses in boreal forests: Nitrogen (N) sink or source? Insights from a ^{15}N -application-experiment

Kathrin Ackermann, Davey L. Jones, Thomas H. DeLuca

Abstract

Cyanobacteria living epiphytically on feather mosses in pristine, unpolluted areas fix substantial amounts of atmospheric nitrogen (N). This moss-cyanobacteria association likely represents a primary source of N in N-limited ecosystems like boreal forests. However, the fate of the fixed N_2 is unclear. Little knowledge exists about how and when the fixed N_2 entering the soil will become available for plant and microbial uptake. In this study, we applied ^{15}N -ammonium chloride ($^{15}\text{N-NH}_4\text{Cl}$) onto carpets of the feather moss *Pleurozium schreberi* (Brid.) Mitt. and tracked the ^{15}N label into green (living) and brown (senescent) moss and into the upper soil layer over time. Further, we separated the moss from the humus and placed filters between moss and soil to assess the role moss-associated fungi play for N-transfer to the soil. The experiment was conducted along endpoints of a N_2 fixation gradient in Northern Sweden. The feather moss efficiently retained the applied N in the green moss parts over several months, and no ^{15}N -enrichment was found in the brown moss parts or in the soil in the first months after the application. The filter treatment did not result in differences in ^{15}N -enrichment of the soil. Nitrogen retention in the moss was similar regardless of position along the endpoints of the N_2 fixation gradient. Thus, feather mosses seem to represent a short-term (< 1 year) N sink due to their effective retention of acquired N. However, due to its association with N_2 fixing cyanobacteria, it is possible that the feather moss represents a N source over the long-term (> 1 year)

Keywords Biological N_2 fixation, forest ecology, N cycle, N deposition, N limitation, stable isotopes

Introduction

Feather moss-cyanobacteria associations may represent a major nitrogen (N) source in N-limited ecosystems due to the ability of the cyanobacteria to fix atmospheric N_2 (DeLuca et al. 2002; Gundale et al. 2011). The feather moss-cyanobacteria association

contributes $> 2 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ to total N input in pristine, undisturbed boreal forests (DeLuca et al. 2002). In these environments, atmospheric N deposition is low ($1 - 2 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) resulting in low rates of total N input to these systems (Tamm 1991; Gundale et al. 2011). Given the abundance and biomass of feather mosses in boreal forests (Oechel and Van Cleve 1986), their N input could constitute a major component of the N cycle. Although there have been reports that significant amounts of nutrients can be leached from dried moss upon e.g. rewetting (Carleton and Read 1991), mosses are usually efficient at absorbing aerial deposited N and in recycling and retaining trapped N (Aldous 2002; Friedrich et al. 2011), thus the mosses create a regulating layer between atmospheric-N and soil-N.

Mosses lack roots, their connection to soil is limited to rhizoids that reach only surface layers of soil. However, frequent reports have shown that mosses grow in association with different fungi (Kausrud et al. 2008; Davey et al. 2009), which could function as to transfer N from moss to soil via fungal hyphae. In spite of numerous studies on feather mosses and the role they play in ecosystem N dynamics, there is currently little known about the amount and extent in which the N_2 fixed by cyanobacteria is transferred to the soil. Thus, the role and importance of feather mosses in the boreal N cycle, as a long-term N source, or short-term N sink remains unresolved.

Nitrogen fixation in the feather moss-cyanobacteria association is affected by numerous abiotic factors. For instance, increased N inputs may lead to a partial or even total reduction of N_2 fixation in the feather moss-cyanobacteria association (Zackrisson et al. 2004; DeLuca et al. 2008; Ackermann et al. 2012). In contrast, cutting moss shoots above the humus layer results in increased rates of N_2 fixation (T.H. DeLuca, unpublished). The purpose of the studies reported herein was to assess the fate of $^{15}\text{NH}_4^+$ within the moss layer and evaluate the role of rhizoids in this process. A field experiment was conducted that combined both observations (N_2 fixation inhibition by N input and N_2 fixation promotion by cutting the moss) with an estimation of N-transfer from moss to soil. For that, we applied ^{15}N -ammonium chloride ($^{15}\text{N-NH}_4\text{Cl}$) on carpets of the ubiquitous feather moss *Pleurozium schreberi* (Brid.) Mitt. in three forest sites in Northern Sweden and analyzed the ^{15}N content of the moss and soil below the moss at several time points after the ^{15}N addition, to identify if, and when, the moss releases plant and microbial available N into the soil. Further, we cut the moss above the humus layer and placed filters between moss and soil in the same plots to assess the importance of fungal hyphae for the transfer of fixed N_2 from moss to the soil and to estimate the

effects of disturbances on soil-N-transfer. We established the ^{15}N addition plots at 2 distances (0 m; 150 m) away from three busy roads in Northern Sweden. These sites represent the endpoints of a N_2 fixation gradient with increasing N_2 fixation in the *P. schreberi*-cyanobacteria association with increasing distance away from the roads (Ackermann et al. 2012). With this approach, we aimed to gain a deeper understanding of the role of the *P. schreberi*-cyanobacteria association for the N cycle by relating the release of N to the soil with N_2 fixation rates in the moss.

Material and Methods

Study sites

The sites are located in Northern Sweden between latitude 64-66°N, longitude 18-19°E and between 230 and 540 m above sea level and have been described previously in Ackermann et al. (2012). Mean annual temperature and precipitation are 1 °C and 570 mm, respectively. Our study included three busy, paved road segments (Borup (65.0060°N, 19.2509°E); Nyvall (65.2308°N, 19.2632°E); Strömsforsheden (65.0813°N, 18.5179°E)), on busy highways in northern Sweden. The vegetation at all sites was dominated by Scots pine (*Pinus sylvestris* L.), Norway spruce (*Picea abies* L. (Karst)), feather moss carpets composed of *Pleurozium schreberi* and *Hylocomium splendens* (Hedw.), shrubs like *Vaccinium vitis-idea* (L.), *V. myrtillus* (L.) and *Empetrum hermaphroditum* (Hagerup). The soils were classified as Typic Haplocryods (USDA, 2003). All forest stands were of mid- to late succession status.

^{15}N application experiment and sampling

The experiment was established in June 2011 at 0 m and 150 m distance from the road edge, representing the two endpoints of a N_2 fixation gradient, in which N_2 fixation increases with distance from the roads (Ackermann et al. 2012). Four treatments with three replicate plots (1 m² each) per site per distance were established. The treatments were as follows: (1) control (100 ml distilled water) with filter barrier; (2) control without filter; (3) 0.5 g N m⁻² as ^{15}N - NH_4Cl with- and (4) 0.5 g N m⁻² as ^{15}N - NH_4Cl without filter, which was equivalent to 5 kg N ha⁻¹. For the treatments with filters, the moss was cut above the humus layer prior to ^{15}N application, the filter placed between moss and humus and the moss subsequently placed back on the filter (Munktel Analytical Filter papers 3, 15 cm diameter, 90 g m⁻², pore size >10 µm, cellulose paper

with 0.06 % ash content). The solutions were evenly sprayed on the feather moss carpets using plant-spraying bottles. At different time points after the applications (2 h, 2 weeks, 2 months), moss and soil samples were collected. The moss shoots were cut off the humus and separated into brown (senescent and dead) and green (alive) parts. Soil samples were collected beneath the moss in each treatment with a 2.5 cm stainless steel soil core to depth of ~ 10 cm. Three replicate soil and moss samples were collected in each plot from each treatment. Moss and soil samples were dried at 80 °C for 24 h and ground to a fine powder using a ball mill. Total N (TN) and ^{15}N in soil and moss samples were analysed by oxidative combustion using an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer (IRMS) (Sercon Ltd., Cheshire, UK). Excess ^{15}N was calculated by subtracting the ^{15}N values (in atom %) of the control samples from the ^{15}N values (in atom %) of the treated samples.

Statistical analyses

^{15}N data are represented as excess ^{15}N (atom %). To test for significant differences in excess ^{15}N and total N between the samples (green moss, brown moss and soil), between times after application of $^{15}\text{N}\text{-NH}_4\text{Cl}$, and between the N input effects (no filter, 0 m from road; with the filter, 0 m from road; no filter, 150 m from road; with the filter, 150 m from road), we used 2-way-ANOVA approaches followed by Tukey's Post Hoc Test. Road site was used as a random factor (block). All data analyses were performed in R 2.14.0 (2011).

Results

Excess ^{15}N in moss and soil

At 2 h after the ^{15}N application, the green moss showed higher ^{15}N excess than the brown moss parts and the soil ($F = 69.7$; $p < 0.0001$) (Fig. 1). The filter treatment as well as distance from the road did not result in differences in excess ^{15}N in the moss or soil samples (Fig. 1).

The distribution of excess ^{15}N along the moss-soil-profile was similar 2 weeks after the ^{15}N addition: the green moss had higher ^{15}N excess than the brown moss and the soil ($F = 44.0$; $p < 0.0001$). However, the samples with the filter exhibited higher ^{15}N excess than the samples without filters for the green moss (1.1 ± 0.15 with filter, 0.41 ± 0.06 without filter), brown moss (0.31 ± 0.13 with filter, 0.042 ± 0.01 without

filter) and for the soil samples (0.001 ± 0.0008 with filter, 0.001 ± 0.003 without filter) ($F = 7.7$; $p = 0.0001$).

The same pattern was also seen 2 months after the ^{15}N application, in which the green moss still had the highest ^{15}N signal ($F = 27.2$; $p < 0.0001$). No differences in excess ^{15}N in moss or soil between the filter treatments and the distances were detected.

Excess ^{15}N did not change with time in moss or soil samples.

Total N in moss and soil

Total nitrogen (TN) in moss tissue was similar in the control and ^{15}N plots, but higher in soil samples collected from the ^{15}N application plots as compared to the control plots ($F = 9.0$; $p = 0.003$). The green and brown moss parts showed similar TN concentrations, and were higher than soil TN ($F = 20.7$; $p < 0.0001$) (Table 1). The filter treatments did not result in differences in TN in moss or in soil samples (Table 1). Total N in soil samples collected at 150 m away from the road was approximately $10 \mu\text{g g}^{-1}$ soil higher than collected close to the road ($F = 13.8$; $p = 0.0002$) (Table 1). No consistent differences in moss tissue-TN over time or between the two distances were found. Soil-TN values from the 2 weeks sampling point were lost and therefore excluded.

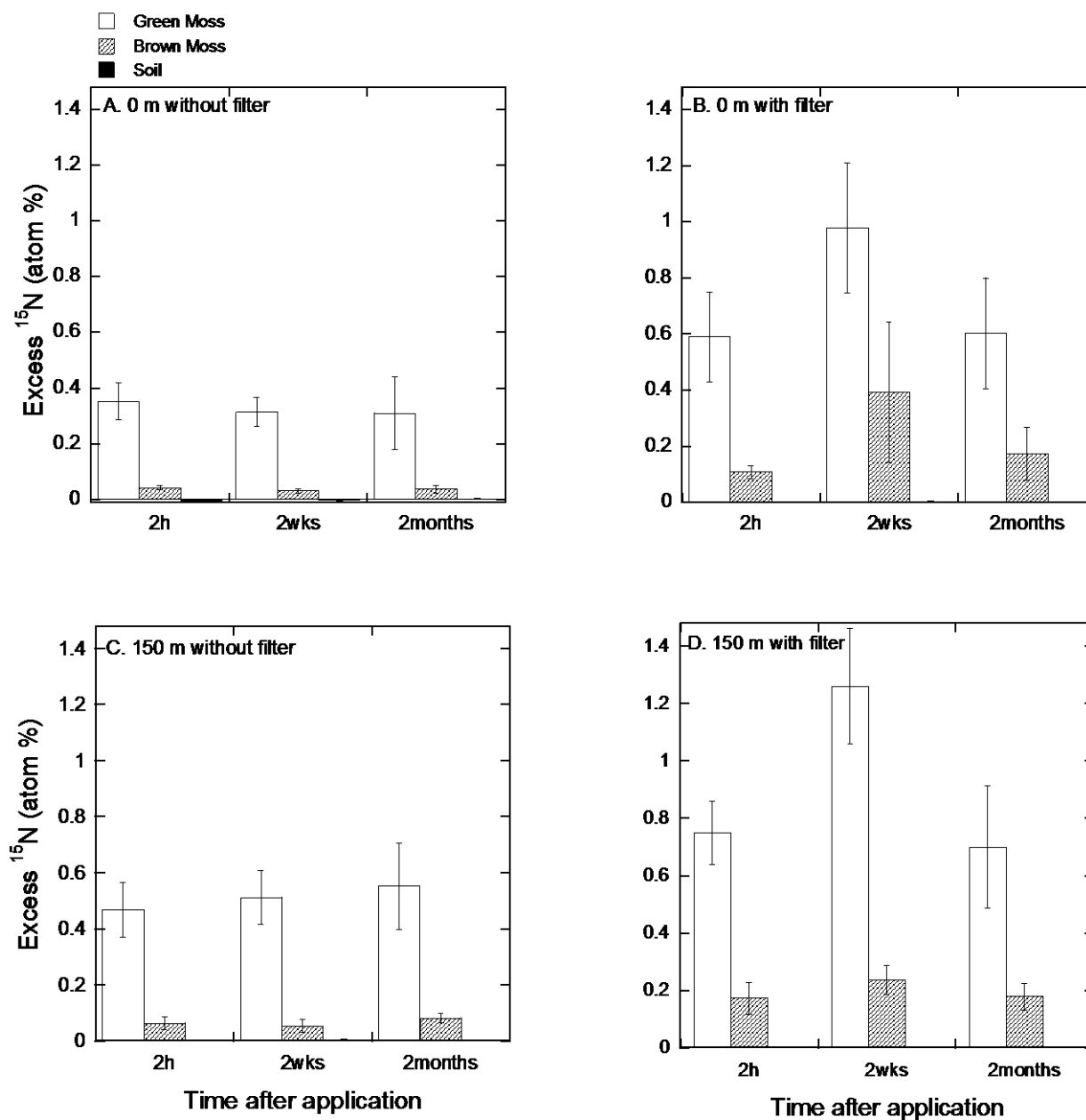


Fig. 1. Excess ^{15}N (atom %) in green moss (white bars), brown moss (striped bars) and soil (black bars) at 0 m (A, B) and 150 m (C, D) from the roads, with- (B, D) and without filter treatment (A, C). Mean ($n = 9$) and $\pm\text{SE}$ are given. Moss and soil were collected 2 h, 2 weeks and 2 months after the $^{15}\text{N-NH}_4\text{Cl}$ applications in Northern Sweden. Note that the bars for soil samples can be difficult to distinguish due to low values.

Table 1. Total nitrogen (TN) ($\mu\text{g g}^{-1}$ soil or moss) in soil, brown moss and green moss from the ^{15}N addition plots at 0 m and 150 m away from the roads at three sampling times (2 h, 2 weeks, 2 months) after the addition of $^{15}\text{N-NH}_4\text{Cl}$. Given are mean values ($n = 9$) \pm SE of the samples collected from plots without and with filters placed between the dead moss and the humus layer. n.a. = not available.

| Soil | 0 m | | 150 m | |
|-------------------|--------------|--------------|--------------|--------------|
| | - Filter | + Filter | - Filter | + Filter |
| 2 h | 37 \pm 2.6 | 35 \pm 3.6 | 50 \pm 3.0 | 51 \pm 3.6 |
| 2 weeks | n.a. | n.a. | n.a. | n.a. |
| 2 months | 36 \pm 4.5 | 36 \pm 4.3 | 46 \pm 6.6 | 46 \pm 3.7 |
| Brown Moss | 0 m | | 150 m | |
| | - Filter | + Filter | - Filter | + Filter |
| 2 h | 38 \pm 2.9 | 38 \pm 2.4 | 43 \pm 2.5 | 40 \pm 3.6 |
| 2 weeks | 36 \pm 3.5 | 34 \pm 1.9 | 34 \pm 1.5 | 36 \pm 3.5 |
| 2 months | 36 \pm 2.2 | 40 \pm 2.1 | 38 \pm 2.9 | 36 \pm 3.8 |
| Green Moss | 0 m | | 150 m | |
| | - Filter | + Filter | - Filter | + Filter |
| 2 h | 35 \pm 2.3 | 41 \pm 3.7 | 37 \pm 1.9 | 37 \pm 2.6 |
| 2 weeks | 33 \pm 2.3 | 31 \pm 1.1 | 31 \pm 1.3 | 32 \pm 3.7 |
| 2 months | 33 \pm 1.5 | 36 \pm 2.2 | 31 \pm 1.8 | 33 \pm 1.7 |

Discussion

We did not find an increase of ^{15}N -enrichment in the soil with time after the application of a simulated atmospheric pulse addition of N to moss canopies. After 2 months, no more ^{15}N was detected in the soil than found immediately (2 h) after the tracer application. The green moss still showed the highest enrichment after 2 months. Thus, we conclude that the transfer of fixed N_2 from the moss to the soil is slow, suggesting that the moss is a short-term (< 1 year) N sink in our study system. Whereas the humus layer is a long-term N sink in boreal forests, accumulating and storing N over several thousands of years, from which N is released by forest fire (Berg and Dise 2004). However, Friedrich et al. (2011) showed that in heathlands, mosses can turn from a short-term N sink to an N source. In their study, shortly after a ^{15}N tracer pulse addition,

64 % of the tracer was recovered in the *P. schreberi*-dominated moss layer, while after 1 year, only 41 % of the added tracer was recovered in the moss and the soil became the most important N sink. In an N addition experiment, Aldous (2002) showed that N retention was highest in the capitula and the upper stem parts of *Sphagnum*-moss and decreased with increasing depth along the moss profile, which is in accord with our findings where we observed higher ^{15}N enrichment in the upper (green) part of the moss. However, in the study by Aldous (2002), N retention by the moss was strongly related to moisture availability, suggesting that the hydrological status of the moss is a crucial factor for moss-N cycling and N_2 fixation in the moss (see also Smith 1984, Gundale et al. 2009, 2012a; Jackson et al. 2011). In addition, large differences in N retention between seasons were observed, with higher retention in summer (July) vs. spring (May-June) (Aldous 2002). Also, N_2 fixation in feather mosses fluctuates seasonally, with highest rates in early and late summer (June, September) and low activity in mid summer (July) (Zackrisson et al. 2004; 2009). This could indicate a shift in the N utilization pathway by mosses: from N_2 fixation in early summer to N absorption from deposition and throughfall in high summer, returning to N_2 fixation towards the end of the summer (Fig. 2). The low N_2 fixation rates in summer are likely a result of light and temperature conditions for moss and cyanobacteria (Gundale et al. 2012b), leading to a reduction of activity, and promoting passive acquisition of N. In contrast, we did not find significant differences between ^{15}N -enrichment in June (the first two sampling times) and at the end of August, which could suggest that the hydrological status of the moss at a given sampling time is the most crucial factor for N cycling within the moss.

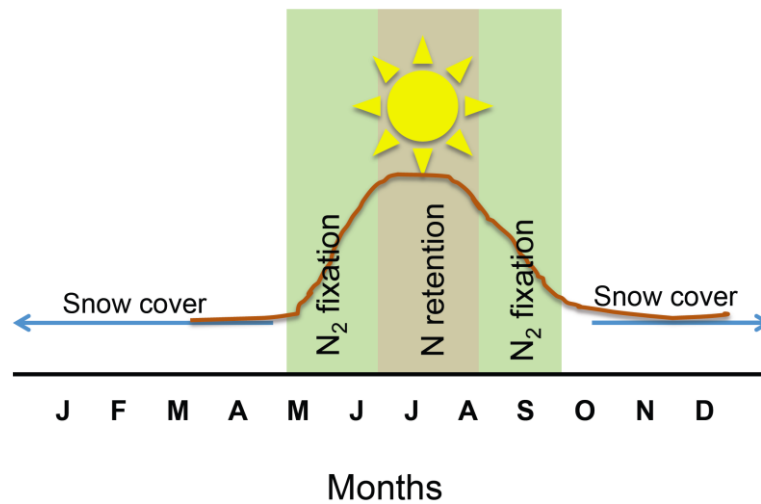


Fig. 2. Schematic representation of the hypothetical seasonal shift in the N utilization pathways by mosses. Nitrogen fixation (green bars) is highest in early summer and late summer, whereas passive N utilization (N absorption from throughfall and deposition) (brown bar) is highest in mid summer, when temperatures (red line) are highest in northern ecosystems.

The ^{15}N signal along the moss profile or in the soil did not show a consistent change over time. The highest ^{15}N enrichment was found in the green moss where the ^{15}N was directly applied. We were interested in the gradual emergence of the ^{15}N signal in the soil (via the brown moss) as an indicator for the rate of transfer of fixed N_2 from cyanobacteria to moss to soil. The ^{15}N -enrichment in the soil samples was very variable and close to background values. These large variations in excess ^{15}N indicate that the natural abundance of ^{15}N in some control soil samples was higher than in some soils of the ^{15}N treated plots, this was most apparent in the samples close to the roads and without the filter treatment. At 150 m away from the road, soil samples in the ^{15}N treated plots had higher ^{15}N signals than soil collected in the control plots.

Although N input derived from roads decreases with increasing distance from the roads (Ackermann et al. 2012), we found higher concentrations of total N in soil collected at 150 m away from the road than close to the road. This could be a result of transfer of fixed N_2 from the moss to the soil at 150 m away from the road, where N_2 fixation in the feather moss is high (Ackermann et al. 2012).

Mosses are conservative and very efficient in recycling and translocating N within the moss profile (Oechel and Van Cleve 1986; Li and Vitt 1997; Aldous 2002). In addition, mosses lack roots, thus transfer to the soil is mainly passive via leaching

after drying-rewetting events (Carleton and Read 1991) and when the cyanobacterial-N-enriched moss is slowly decomposing. Thus, mosses represent an N source that is likely to become slowly available for soil-N cycling. The ^{15}N signal did not change in the brown moss parts over time, indicating that the live moss parts efficiently retained the added N. Although the ^{15}N -sprayed moss was cut above the humus layer, the soil samples collected in these plots with the filter between moss and humus layer showed higher ^{15}N enrichment than the no-filter treatment. Thus, we cannot conclude that the transfer of fixed N_2 from moss to soil is enhanced by fungal hyphae, suggesting that fungal associations with the moss are less important for the direct transfer of N from moss to the soil. Also, with their endogenous N supply via cyanobacteria and their effective adsorption of throughfall N, feather mosses are independent of fungal associations to satisfy their N demand.

No significant differences in excess ^{15}N in moss or soil between the two distances from the roads (reflecting an N_2 fixation gradient) were detected, indicating that the moss possess similar abilities to retain N along the gradient, independently of endogenous N_2 fixation rates. This would suggest that the moss covers most of its N demands by absorption of deposited N rather than by cyanobacterial fixed N_2 . Hyodo et al. (2013) proposed that *P. schreberi* does not use the N from colonizing cyanobacteria, rather the N_2 fixed by cyanobacteria likely undergoes mineralization prior to uptake by mosses and other plants.

Mosses serve as nutrient filters; they trap and retain much of the nutrients from throughfall and deposition before they can reach the soil (Oechel and Van Cleve 1986; Glime 2007), making them a short-term nutrient sink (Startsev and Lieffers 2006; Glime 2007; Friedrich et al. 2011). In a fertilization experiment, Startsev et al. (2008) showed that 72 % of applied fertilizer (100 kg ha^{-1} of NH_4NO_3) was retained in the moss and litter layer, and only low concentrations of total N was captured in the leachate. However, when the fertilizer dose was increased (330 kg ha^{-1}), the moss was significantly damaged, which led to less retention of the fertilizer in the moss and litter layer and higher concentrations of total N in the leachate (Startsev et al. 2008). Thus, at low rates of N input, the moss acts like a sponge, but above a certain level of N input, the moss is damaged and N enters the soil bypassing the moss layer.

The slow nutrient release by mosses is also a result of their ability to move N along the moss shoot as well as to recycle captured N within the moss (Weber and Van Cleve 1984; Oechel and Van Cleve 1986). Re-absorption of leached nutrients upon

rewetting of dried moss has also been observed, confirming the notion that mosses represent short-term sinks of N (Startsev and Lieffers 2006).

Taken together, in ecosystems where the groundcover is dominated by a thick moss carpet, the moss layer acts like a filter or sponge between deposited N and soil-N cycling (Li and Vitt 1997; Glime 2007). Thus, mosses could limit primary productivity over short time scales in forests that exhibit a moss-dominated understory vegetation if the amount of N input does not exceed a level that damages the moss. Regardless of short-term N-limitation, mosses ultimately reflect a net source of N to the ecosystem, but delivery of the fixed N₂ to higher plants must follow a tortuous pathway requiring decomposition of moss detritus and eventual organic N uptake or liberation of inorganic N from moss, moss detritus or humus through disturbance and, in particular, fire.

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4. Conclusions, Commentary, Future Perspectives

The input of biologically available nitrogen (N) has increased dramatically over the past five decades (e.g. Galloway and Cowling 2002), emphasizing the need to identify the consequences of increased N loads in natural ecosystems, how various components of a system will respond, and if resilience to this disturbance exists. This is especially important in N limited ecosystems like boreal, arctic and Antarctic systems (Shaver and Chapin 1980; Vitousek et al. 1989; Tamm 1991), which are predicted to be the most vulnerable to enhanced levels of available N (Vitousek 1994). A major source of N for these N limited ecosystems is the fixation of atmospheric N₂ performed by free-living and symbiotic cyanobacteria. Nitrogen-fixing cyanobacteria have been found to colonize a range of moss species in pristine, unpolluted environments (Basilier and Granhall 1977; DeLuca et al. 2002; Sorensen et al. 2006; Ininbergs et al. 2011), where the N₂ fixation of the moss-cyanobacteria association can contribute 50 % to the total N input in these systems (DeLuca et al. 2002; Gundale et al. 2011; Sorensen and Michelsen 2011). Besides that, feather mosses represent an iconic and important feature in boreal forests due to their ability to influence soil hydrology and chemistry; they form extensive carpets and account for 60 - 90 % of the ground cover in these forests (Oechel and Van Cleve 1986). Further, the associated N₂ fixing cyanobacteria could alleviate the pronounced N limitation in boreal forest ecosystems (Gundale et al. 2011). However, N₂ fixation is affected by various abiotic factors. For instance, N deposition (DeLuca et al. 2008; Gundale et al. 2011; Ackermann et al. 2012) and moisture content of the moss (Jackson et al. 2011; Gundale et al. 2012) seems to be important drivers of N₂ fixation. Reports on the effects of these factors on N₂ fixation are scarce, however, and a more systematic assessment of the impact of these controlling agents of N₂ fixation in mosses has yet to be accomplished.

In this project, we addressed several pressing questions regarding the feather moss-cyanobacteria association to be able to facilitate an improved understanding of their role in the N cycle in boreal forests. We were able to link a fundamental ecosystem function, N₂ fixation, with various abiotic factors. For instance, we showed that N availability (Chapter I, II, IV) and the hydrological status of the moss (Chapter III) had fundamental impacts on N₂ fixation rates. Considering future scenarios predicting

changes in these particular factors, the effects on the N cycle in boreal forest could be dramatic.

We could show that N₂ fixation in the feather moss-cyanobacteria association is a sensitive indicator for N deposition in boreal forests, e.g. for road-derived N input. While other measured factors (soil-N concentration, microbial biomass, N mineralization rates etc.) along the investigated roads did not show a distance effect, N₂ fixation in the moss was inhibited close to busy roads and increased with increasing distance to those roads (Chapter I). By using laboratory experiments, we could identify a threshold of total N input above which N₂ fixation in the feather moss-cyanobacteria association is inhibited. This threshold is likely to be higher than 10 kg N ha⁻¹ (Chapter II) and falls within the range of other reported critical loads of N input for boreal ecosystems (Achermann and Bobbink 2003). Further, N inputs along artificial (Chapter I) as well as along natural N gradients (Chapter IV) have similar inhibitory effects on N₂ fixation, corroborating our first hypothesis. However, we could show that N₂ fixation in the feather moss-cyanobacteria association is able to recover from increased N loads (Chapter II) as well as from water stress (Chapter III) upon removal of these stressors, indicating its resilience to these disturbances. We hypothesized that recovery of N₂ fixation upon rewetting of dry moss is rapid (within hours), however, we found that recovery was very slow. It took five days after rewetting of dried moss for N₂ fixation to recommence and to reach levels comparable to constant moist moss (Chapter III).

We proved that the moss can take up N from soil (Chapter IV), showing that feather mosses use various pathways of assessing and utilizing N. The moss did not show a preference for organic (alanine) or inorganic (ammonium) N sources and only a small fraction of the added amino acid seemed to be taken up from soil in an intact form. The uptake of N from soil was independent of the soil-N availability. Further, N uptake from soil was not correlated with N₂ fixation rates in the moss, suggesting that the moss is independent of the soil-N condition and can cover its N demand via absorption of deposited N from the atmosphere and throughfall as well as via N₂-fixing cyanobacteria.

In accordance with our hypothesis, the moss effectively retained acquired N over several months and only slowly releases N (Chapter VI). Most of the N retained in the moss tissue becomes available after disturbances like drying-rewetting events and fire (Carleton and Read 1991), suggesting that the moss represents a short-term (< 1 year) N sink.

Overall, the project contributed strongly to improving our understanding of the ecology of the feather moss-cyanobacteria association by showing that colonizing cyanobacteria do not contribute to the moss' resistance towards decomposition by soil bacteria, rather, the un-colonized moss appeared to be more resistant to soil bacterial decomposition (Chapter V), which stands in contrast to our hypothesis. This suggests that either (1) the moss down regulates its defensive compounds when it is deprived of N to promote colonization by cyanobacteria in order to gain an endogenous N source, or (2) the cyanobacteria colonize a weakened moss and use the moss as a habitat.

To summarize, N₂ fixation in the feather moss-cyanobacteria association seems to be resilient to desiccation and N input; feather mosses are able to take up N from soil, but this is likely a minor source of N for the moss; cyanobacteria do enrich the colonized moss tissue with N, which becomes slowly available for soil N cycling. Thus, feather mosses that host N₂ fixing cyanobacteria are likely to contribute fundamentally to the N budget of boreal forest ecosystems.

The work performed in this thesis goes a long way to addressing some of the unknowns regarding the feather moss-cyanobacteria association, but more important, it has raised numerous new questions to be addressed in the future. For instance, the role mosses play in N cycling remains poorly understood. Further, a qualitative evaluation of abiotic controls on N₂ fixation in moss-cyanobacteria associations is a challenge yet to be addressed. In addition, and more pressingly, boreal, arctic and Antarctic ecosystems are assumed to be the most vulnerable ecosystems towards increased N deposition, temperature and CO₂ levels (see ACIA 2005). Thus, estimating the effects of these parameters on ecosystem functioning, e.g. N₂ fixation is crucial in order to predict possible consequences on ecosystem- and global scales.

More research is also needed to explore the relationship mosses and cyanobacteria share. Many plant-microbe-associations represent mutualistic relationships, in which the partners exchange carbon (C) and N with each other (Turetsky 2003). While at least as many plant-microbe-associations are of parasitic nature, many relationships are likely to be found along a continuum between the two extremes. Surprisingly, no attempts have been made so far to place moss-cyanobacteria associations along a mutualism-parasitism continuum (see e.g. Johnson et al. 1997). In addition, the balance of nutrient exchange between the partners seems to depend on nutrient demands of the partners (Johansson et al. 2011), as well as on nutrient supply, light and moisture conditions, which can upset the relationship between the partners in

lichen symbioses (Scott 1960). To date, it is unclear how much of the fixed N_2 by the cyanobacterial colonizer is transferred to the moss. By measuring photosynthesis rates in parallel with N_2 fixation rates in the moss and also, by analyzing the effects of N additions, recurrent drying-rewetting events and N deprivation on both biologically fundamental processes simultaneously, would provide a more complete picture of how both partners respond to these stress factors, e.g. which process recovers faster and at which rate, revealing which partner is more inhibited. This could indicate which partner is actually driving the relationship. In addition, by measuring photosynthesis and N_2 fixation rates simultaneously, we could specify if the cyanobacteria on moss leaves remain photosynthetically active and quantify the exchange of C and N between the partners. More directly, treating moss samples with antimicrobial agents to control the numbers of colonizing cyanobacteria and label the moss with the stable isotopes of C ($^{13}CO_2$) and N ($^{15}N_2$) with subsequent tracking of the label into moss and cyanobacterial biomarkers, could provide an indication of the nutrient trading between the two partners. Further, it would be of great interest and importance to identify the resource optimization in moss-cyanobacteria associations, in particular, when does it pay off to invest in N_2 fixation? Nitrogen fixation is energy expensive (Scherer and Zhong 1991; Turetsky 2003; Houlton et al. 2008), thus is not found at high-N conditions (DeLuca et al. 2008; Gundale et al. 2011). The threshold value, that is, under which (N-) conditions it pays off to invest in N_2 fixation remains unexplored to date.

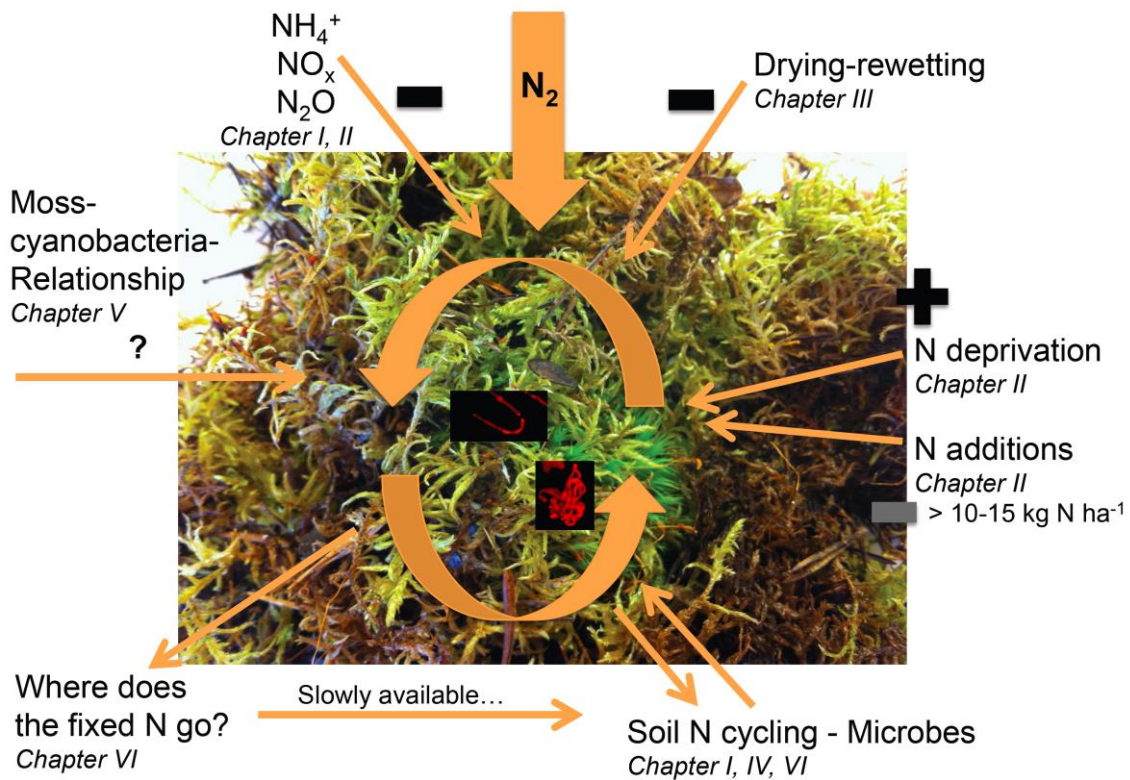


Fig. 1. Schematic diagram summarizing the work performed in my thesis with references to the chapters. Shown are the directions (positive or negative) of the effects of evaluated factors on N_2 fixation in the feather moss-cyanobacteria association; the relation with the soil-N cycle as well as the unknown relationship between the feather moss and its colonizing cyanobacteria.

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