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Sanna Leppänen

Bacterial Nitrogen Fixation in Boreal Mosses

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ABSTRACT

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Biological nitrogen (N_2) fixation in forest and wetland mosses has been shown to enhance carbon (C) fixation and growth of mosses, thus playing an important role in both N and C cycling in boreal forest and wetland habitats. However, the main N_2-fixing bacteria (diazotrophs) in mosses are considered to be *Cyanobacteria* and the N_2 fixation activity has been measured with the indirect acetylene reduction assay (ARA). In this thesis I hypothesized that N_2 fixation is a substantial N source for boreal mosses living in forests, wetlands and rivers, and that methane (CH_4)-oxidizing (methanotrophic) and heterotrophic bacteria are important participants in moss-associated N_2 fixation. ARA and the direct $^{15}N_2$ tracer method were compared in forest mosses, and the latter proved to be more sensitive than ARA when measuring low activities of N_2 fixation associated with feather mosses. At one subarctic site, N_2 fixation in feather mosses was equal to the annual bulk N deposition. Both methanotrophy and diazotrophy were detected from wetland *Sphagnum* mosses, and at some sites CH_4 oxidation enhanced N_2 fixation. Single cell analysis revealed that these two processes were linked also at the bacterial-level. Various factors regulating N_2 fixation were studied, and moss moisture content, water table level and flark-hummock variation were found to be major regulators, N_2 fixation being always higher in moist conditions. Sequencing of the *nifH* gene (encoding nitrogenase reductase) revealed that in wetlands and rivers most diazotrophs were affiliated to *Proteobacteria* instead of *Cyanobacteria*. Methanotrophic *nifH* sequences were present especially in river moss *Fontinalis dalecarlica* and in *Sphagnum* mosses from fens. Bacterial N_2 fixation was proven as an important N source for mosses and, for the first time, N_2-fixing methanotrophs were shown to be an active and abundant link in C and N cycles.

Keywords: $^{15}N_2$ tracer method; acetylene reduction assay; diazotroph; feather moss; *Fontinalis dalecarlica*; methanotroph; nanoSIMS; *Sphagnum*.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV.

Sanna M. Leppänen planned the experiment and sampling in II and III, and jointly with other co-authors in I and IV. SML conducted the sampling, incubations and laboratory work in II and III, except that in II sampling was conducted jointly with MarjaTiirola and DGGE was carried out by Antti J. Rissanen. In I SML, Maija Salemaa, Aino Smolander and Raisa Mäkipää conducted the incubation setup, SML conducted the stable isotope analysis and AS and MS conducted the gas chromatography. In IV SML, Tuula Larmola and Maija Aarva conducted the sampling and incubation setup, SML and MA did the stable isotope analysis and TL, Päivi Merilä and Eeva-Stina Tuittila the supporting analyses. SML conducted the statistical analyses in II and III, except that in II analyses considering DGGE were conducted by AJR. SML did the statistical analyses together with MS in I. SML wrote papers I, II and III. Paper IV was written jointly with TL and MT. All papers were finalised with all co-authors.

I Leppänen S.M., Salemaa M., Smolander A., Mäkipää R. & Tiirola M. 2013. Nitrogen fixation and methanotrophy in forest mosses along a N deposition gradient. *Environmental and Experimental Botany* 90: 62–69.

II Leppänen S.M., Rissanen A.J. & Tiirola M. 2013. Non-cyanobacterial diazotrophs in boreal *Sphagnum* mosses. Submitted manuscript.

III Leppänen S.M., Musat N., Saarenheimo J., Kuypers M.M.M. & Tiirola M. 2013. Localizing nitrogen fixing methanotrophs in aquatic mosses. Manuscript.

IV Larmola T., Leppänen S.M., E.-S. Tuittila, Aarva M., Merilä P., Fritze H. & Tiirola M. 2013. Methanotrophy induces nitrogen fixation during peatland development. Submitted manuscript.

1 INTRODUCTION

1.1 N_2 fixation

1.1.1 Biological N_2 fixation in boreal forests and peatlands

Nitrogen (N) is one of the key elements sustaining life, since it is required for protein and nucleic acid synthesis (Postgate 1998). Most organisms can assimilate N only in combined forms such as ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and urea. However, the largest pool of N on earth is the atmospheric molecular N_2 gas, which is unavailable to most organisms. Some prokaryotes, including *Bacteria* and *Archaea*, carry the oxygen (O_2)-sensitive nitrogenase enzyme, by which they can reduce N_2 gas to NH_3 in an energy (ATP) expensive process called biological N_2 fixation (Postgate 1998):

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \quad [1]$$

Biological N_2 fixation supplies 40 % of the world´s soil and water N (Postgate 1998), but non-biological N_2 fixation can also occur, naturally through lightning and industrially through the Haber-Bosch process and during combustion at high temperatures. Actually, anthropogenic activities have more than doubled the input of fixed N_2 to the terrestrial biosphere and this anthropogenic N input is continuously increasing (Gruber & Galloway 2008). Humans have been taking advantage of biological N_2 fixation for centuries in agriculture, and in 1888 it was discovered that legumes assimilate free N_2 with the help of microorganisms (Galloway et al. 2004). Nowadays 50–70 Tg N yr^{-1} is fixed biologically in agricultural systems (Herridge et al. 2008), but still most of the anthropogenic N is of industrial origin (Schlesinger 2008).

Boreal forests are usually N limited (Tamm 1991), due to the accumulation of recalcitrant litter and plant material with high carbon-to-nitrogen (C/N) ratios, leading to rapid immobilization of inorganic N and decreased net N mineralization rates (Scott & Binkley 1997). Most N enters boreal forests as a deposition or is biologically fixed mainly by cyanobacterial associates of feather

mosses; for example, in northern Sweden the atmospheric deposition and N_2 fixation inputs are equal (Sorensen & Michelsen 2011), being about 2 kg N ha^{-1} y^{-1} each (DeLuca et al. 2002). The boreal forest biome accounts for 17 % of the Earth´s land surface (DeLuca et al. 2008), so the ability of this ecosystem to sustain productivity is important when considering the Finnish and Scandinavian economy (Anon. 2013) as well as global biogeochemical budgets.

Peatlands cover about 350 million ha in the northern hemisphere (North America, Russia and Europe) (Strack 2008). Before large scale drainages 50 years ago, mires comprised 37 % of the Finnish land area (Solantie 2006). In Finland, peatlands can be roughly divided in 2 main types (mire complex types), aapamires and raised bogs, that locate within northern and middle boreal zones and within southern boreal zone, respectively (Solantie 2006). These types can be further divided in 4 main peatland types: spruce mires, pine mires, fens and eutrophic fens. Also combination site types occur (Eurola et al. 1995). Boreal peatlands are regarded as effective N sinks, where the main N pool resides in dead organic matter and only a minor part is stored in living organisms (Limpens et al. 2006); hence, peatlands are highly N-deficient and, as in boreal forests, net mineralization rates are low (Regina et al. 1998, Kaiser et al. 2005). The main N inputs in peatlands are N deposition, biological N_2 fixation and N inflow through upland runoff or discharge, but their amounts vary widely, and N_2 fixation rates in peatlands remain poorly quantified (Limpens et al. 2006). Pine mires mainly depend on the atmosphere as a nutrient source; hence they are considered ombrotrophic. Fens can be ombrotrophic, but usually they have also some surface water inflow, therefore being minerotrophic. Spruce mires and eutrophic fens are mostly minerotrophic (Eurola et al. 1995). In addition, peatlands form an important sink for the most important greenhouse gas carbon dioxide (CO_2), storing about one-third of the global terrestrial C (Gorham 1991). Northern peatlands have also an important role in global methane (CH_4) cycle (see below).

1.1.2 The role of methane in N_2 fixation

CH_4 is the second most important greenhouse gas (Anon. 2007), and is mainly produced by methanogenic *Archaea* in the anoxic water layer of wetlands, including boreal peatlands (Basiliko et al. 2004) that produce $10–65\times10^{12}$ g CH_4 yr^{-1} (reviewed by Strack et al. 2008). Natural wetlands are regarded as the single greatest CH_4 source, accounting for 20 % of global CH_4 emissions (Wuebbles & Hayhoe 2002), other CH_4 sources including rice paddies, soils and ruminants (reviewed by Hanson & Hanson 1996). However, CH_4-oxidizing bacteria (methanotrophs), present mainly in oxic environments, can act as a sink for CH_4 and reduce CH_4 emissions (Hanson & Hanson 1996).

Methanotrophs are a subset of Gram-negative methylotrophic bacteria that can use reduced one-carbon compounds, such as methanol or CH_4, as a C and energy source. Methanotrophs utilize CH_4 in an energy-yielding oxidation process (Kirchman 2012):

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \qquad \Delta G^0 = -814 \text{ kJ mol}^{-1} \qquad [2]$$

Aerobic methanotrophs belong to *Alpha-* or *Gammaproteobacteria* or *Verrucomicrobia* (Murrel & Jetten 2009, Op den Camp et al. 2009). Anaerobic methanotrophs, which use nitrite (NO_2^-) as an electron acceptor instead of O_2, belong to the NC10 phylum (Ettwig et al. 2010). *Alpha-* (also referred as type II methanotrophs) and *Gammaproteobacteria* (also referred as type I) are considered the most abundant methanotrophs in peatlands (Kip et al. 2010).

Steudler et al. (1989) showed that nitrogenous fertilization represses methanotrophic CH_4 consumption in forest soils. After that general agreement was that fertilizers inhibit CH_4 oxidation, although contradictory studies were also published (reviewed by Bodelier & Laanbroek 2004). Bodelier et al. (2000) discovered that ammonium-based fertilizers actually stimulated CH_4 oxidation around rice roots, while Cai & Mosier (2000) showed that initially fertilization might decrease CH_4 oxidation, but in the long run it would increase CH_4 oxidation. These studies imply that N may limit the growth and activity of methanotrophic populations and that N may actually be an essential factor on the sink side of the global CH_4 budget. In this thesis it was studied, whether N_2 fixation and CH_4 oxidation could be linked in N-deficient habitats where CH_4 is readily available, such as in boreal peatlands, forests and rivers. In these ecosystems, proteobacterial aerobic CH_4 oxidation could provide an energy source for N_2 fixation.

Genetic and biochemical analyses have provided evidence that N_2 fixation capabilities are broadly distributed among type I and II proteobacterial methanotrophs (Auman et al. 2001), verrucomicrobial methanotrophs (Khadem et al. 2010) and the deep-sea anaerobic CH_4-oxidizing *Archaea* (Dekas et al. 2009). It has also been hypothesized, that the contribution of methanotrophs to N_2 fixation may be significant in waterlogged soils (Kravchenko & Doroshenko 2003), but the coupling between the CH_4 and N cycles remains poorly understood (Murrel & Jetten 2009) and totally ignored in plant endosymbionts.

1.2 Mosses

1.2.1 Boreal mosses

Bryophytes (Bryobionta) are non-vascular plants that have no roots. They are usually small (1–10 cm) and reproduce via spores. There are 3 lineages within Bryobionta: Marchantiophyta (liverworts), Bryophyta (mosses) and Anthocerophyta (hornworts), liverworts and mosses being the most common lineages in Finland. Mosses are usually further divided in Sphagnopsida, Andeaeopsida, Polytrichopsida and Bryopsida (Goffinet et al. 2008). In this thesis mosses from Sphagnopsida (*Sphagnum* sp.) and Bryopsida (*Hylocomium splendens, Pleurozium schreberi* and *Fontinalis dalecarlica*) were studied. These mosses are all

circumpolar, except *H. splendens* which is bipolar, and common in Finland forming dense carpets where they grow (Syrjänen 2002).

Mosses are crucial in boreal forest and peatland habitats due to their traits (e.g. N_2-fixing capacity, resistance to herbivores, photosynthetic capacity etc.) that influence the biochemistry of soils and peatlands (Cornelissen et al. 2007), being important drivers for the functioning of their characteristic ecosystems (Nilsson & Wardle 2005). In boreal forests, mosses contribute fundamentally to the biomass and productivity, sometimes even exceeding the tree biomass (Turetsky 2003, Lindo & Gonzalez 2010). For example, feather moss *P. schreberi* can account for up to 80 % of the ground cover in boreal forests (DeLuca et al. 2002). *Sphagnum* mosses are successful inhabitants of peatlands, because their morphology, anatomy, physiology and composition make them effective ecosystem engineers (van Breemen 1995) and they can incorporate more C than any other plant genus (Laine et al. 2009). Mosses are as well important in stream ecosystems, where their primary production can equal or exceed that by epilithic and periphytic algae, but they often remain overlooked (Anon. 1999).

Mosses are poikilohydric, so they have no physical mechanism to prevent desiccation, and moisture and nutrients are absorbed over their entire surface (Lindo & Gonzalez 2010). However, *Sphagnum* mosses can draw water up through the capillary spaces formed by overlapping pendent branches that hang down against the stem (Fig. 1a). They are also able to store water in the porous, dead hyaline cells (Fig. 1b-c), which make up about 80 % of the plant's volume (van Breemen 1995). Mosses have a stem and small leaves, which are usually only one cell layer thick. In the leaves of *Sphagnum* mosses, thin photosynthetic cells and large hyaline cells alternate (Fig. 1b-c).

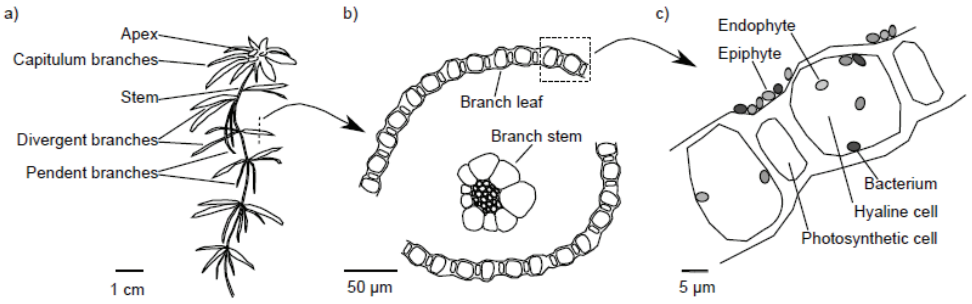

FIGURE 1 The structure of a *Sphagnum* moss. a) The moss consists of a stem and branches that can be divergent or pendent. b) A cross-section of a branch, where the branch stem is in the middle and the branch leaves are on sides. c) The leaves consist of an alternating pattern of large hyaline cells and thin photosynthetic cells. Bacteria can be found from within the hyaline cells (endophytes) or on the leaves (epiphytes). The scale bars are approximate.

1.2.2 Diazotrophs of boreal mosses

N_2-fixing *Cyanobacteria* have been found to inhabit mosses in boreal forests (*P. schreberi* and *H. splendens*; e.g. DeLuca et al. 2002, Ininbergs et al. 2011) and in

peatlands (genus *Sphagnum*; e.g. Granhall & Hofsten 1976, Basilier 1980, Markham 2009). In forest mosses, *Cyanobacteria* live as epiphytes, and the number of colonizing cyanobacterial cells and the N_2 fixation rate show a positive linear relationship (DeLuca et al. 2007). As mentioned earlier, the N input of moss-associated *Cyanobacteria* can equal the atmospheric N deposition in some boreal areas (DeLuca et al. 2002, Sorensen & Michelsen 2011). In *Sphagnum* mosses, bacteria can live as epiphytes or endophytes (Fig. 1c). The epiphytes inhabit mainly plant parts that are submerged and the endophytes are present above the water table in the hyaline cells (Basilier 1979). Dead moss parts usually contain no associative bacteria, indicating that the living moss provides bacteria with more than physical support (Basilier & Granhall 1978). Granhall & Hofsten (1976) reported that *Sphagnum* mosses also host other bacteria within their hyaline cells, and these bacteria would decrease the O_2 concentration and increase the CO_2 concentration of the hyaline cell by their cellular respiration. The high CO_2 concentration would stimulate cyanobacterial N_2 fixation and provide N for the host plant and also for the other bacteria. Berg et al. (2013) showed that N_2 fixation by *Cyanobacteria* associated with *Sphagnum* mosses had an immediate effect on the C fixation by *Sphagnum*, indicating that factors regulating N_2 fixation will have a direct effect on the role of *Sphagnum*-dominating ecosystems with respect to C cycling. These studies suggest that the relationship between diazotrophs and moss is symbiotic, i.e. a close physical association between species, in which a symbiont (diazotroph) occupies a habitat provided by a host (moss).

Sphagnum mosses can carry methanotrophic endosymbionts, which can provide up to 30 % of the C in the plant (Raghoebarsing et al. 2005, Larmola et al. 2010). Most of these methanotrophs belong to *Proteobacteria* (Raghoebarsing et al. 2005, Kip et al. 2010), which are known to possess the N_2 fixation capacity (Dedysh et al. 2004). However, the role of methanotrophs in N_2 fixation has been overlooked; since the reaction conditions for N_2 fixation and CH_4 oxidation are opposite (N_2 fixation does not tolerate O_2, which is a prerequisite for CH_4 oxidation). Yet the cultured methanotroph *Methylosinus trichosporium* (strain 41) was able to fix N_2 and oxidize CH_4 at the same time when the O_2 concentration was lowered to 5 % (DeBont & Mulder 1974). Diazotrophic methanotrophs usually occur in natural environments at sites with relatively low pO_2 values (Dedysh et al. 2004).

To date, *Cyanobacteria* are being considered the main diazotrophs of mosses (e.g. Houle et al. 2006, Gavazov et al. 2010, Sorensen & Michelsen 2011, Berg et al. 2013). Although some studies considering heterotrophic N_2 fixation have been conducted (e.g. Waughman & Bellamy 1980, Kravchenko & Doroshenko 2003), the role of heterotrophs and methanotrophs has not been taken into account in the moss-associated N_2 fixation. By knowing the identities of diazotrophic associates and what their relationship is with the moss can help in evaluating the responses that different environmental factors may cause on N_2 fixation and eventually on C sequestration in mosses.

1.2.3 Factors that affect N_2 fixation in mosses

N_2 fixation in mosses is controlled by biotic and abiotic factors. The main controllers for N_2 fixation are the nutrient level and moisture, which also determine the moss species (reviewed by Rousk et al. 2013a) and microbiota of moss (Ininbergs et al. 2011, Sorensen & Michelsen 2011). The N_2 fixation rate can then be mediated both by moss responses and by microorganism responses to environmental changes (Sorensen & Michelsen 2011). Also the presence of CH_4 may affect the composition of moss microbiota (Putkinen et al. 2012, Bragina et al. 2013).

Since N_2 fixation is an energy expensive process, bacteria only use it if it is the only way to gain N, so the nutrient level of a habitat usually determines the general potential for N_2 fixation. However, feather moss-associated N_2 fixation has been noted to increase towards late succession in boreal forests (Zackrisson et al. 2004), which contrasts with the belief that N_2 fixation is more important during the early stages of succession due to the lack of nutrients (Rastetter et al. 2001). This might be explained by the incomplete bacterial colonization of mosses at the early stages (Zackrisson et al. 2004), and with the lack of inorganic, biologically available N in late successional forests, which creates the need for N_2 fixation. There are differences in the observed N_2 fixation rates between peatlands of different fertility levels; for example, when extremely nutrient-rich fens and nutrient-poor bogs were compared to medium-nutrient fen types, both extreme types had lower N_2 fixation rates than the fen types with medium nutrient levels. Extremely rich fens had an additional N influx from groundwater and on the other hand poor bogs had a shortage of energy substrate that possibly limited the N_2 fixation rates, which were measured using the indirect acetylene reduction assay (Waughman & Bellamy 1980). However, systematic stable isotope studies focusing on N_2 fixation in *Sphagnum* mosses in successional gradients of peatlands have not been conducted before this study.

Mosses effectively absorb nutrients from atmospheric deposition, which is their main source of N, making them extremely sensitive to increased nutrient input (e.g. Bengtsson et al. 1982, Ackermann et al. 2012). Also artificial N additions have been shown to significantly decrease numbers of cyanobacterial cells on moss leaves (Gundale et al. 2011) and feather moss-associated N_2 fixation was downregulated by the anthropogenic N deposition (Zackrisson et al. 2009).

When the soil C/N ratio is 30:1 or higher, soil heterotrophic microorganisms are considered to be N-limited (Kaye & Hart 1997). Compared to that, the C/N ratio of *Sphagnum* litter is high (> 50:1; van Breemen 1995), indicating that constant N deficiency prevails in peatlands. Previously the C/N ratio and the N_2 fixation rate have been found to correlate positively (Cusack et al. 2009, Rousk et al. 2013b). However, at certain times C might be the limiting factor for N_2 fixation despite the high C/N ratio; for example in peatlands where most of the C is considered biologically inactive, glucose addition increased N_2 fixation by inducing nitrogenase synthesis or activation (Kravchenko & Doroshenko 2003). In coniferous bark litter the addition of glucose also sped up the initiation of N_2 fixation (Weber & Sundman 1986). This was explained by the

increase of microbial activity and growth, which would have enhanced the O_2 consumption reducing the O_2 to levels more suitable for N_2 fixation.

Moisture has been found to positively affect N_2 fixation in *P. schreberi*, as daily watering increased N_2 fixation, but extreme drought decreased it (Gundale et al. 2009). Also the sensitivity to moisture variation was greater in old forests than in young forests (Gundale et al. 2009). In addition, rainfall frequency has been found to positively affect N_2 fixation in boreal feather mosses (Jackson et al. 2011, Gundale et al. 2012a). However, if the dry period was intense and the mosses got too dry, the N_2 fixation capacity recovered only after 5 days of rewetting, indicating that short and recurrent cycles of intensive drying and rewetting could potentially eliminate N_2 fixation in *P. schreberi* (Ackermann 2013). In contrast, Markham (2009) did not observe any effect of precipitation on N_2 fixation in *P. schreberi* and *Sphagnum capillifolium*.

As in the above mentioned litter study (Weber & Sundman 1986), partial pressure of O_2 is an important determinant for the N_2 fixation rate, due to the sensitivity of the nitrogenase enzyme to O_2. In addition, Weber & Sundman (1986) noted that soil microbes did not fix N_2 in normal O_2 conditions (pO_2 = 0.21), but when the partial pressure of O_2 was lowered to 0.05, N_2 fixation started immediately. Kravchenko & Doroshenko (2003) observed the highest N_2 fixation rates in aerobic conditions, where N_2 fixation was almost twofold compared to N_2 fixation in anaerobic conditions. These studies indicate that the diazotrophs in these habitats were mostly microaerophiles, at least at the time when they were fixing N_2 (see also Postgate 1998).

In the forest mosses *P. schreberi* and *H. splendens,* the N_2 fixation rates have been reported to have monthly variation, peaking in early spring after snowmelt (May-June) and in late summer (September), and dropping in midsummer (July-August; Basilier & Granhall 1978, DeLuca et al. 2002, Zackrisson et al. 2004). However, in *Sphagnum* mosses such a trend has not been detected (Granhall & Hofsten 1976, Basilier 1979, Gavazov et al. 2010) and actually sometimes N_2 fixation has been observed to peak in midsummer (Basilier & Granhall 1978, Markham 2009). The drop in the N_2 fixation rates of feather mosses in midsummer has been attributed to photoinhibition (Zackrisson et al. 2004). Gentili et al. (2005) was able to measure N_2 fixation in *P. schreberi* both at low and high light intensities, although more N_2 was fixed at higher intensities. In *Sphagnum* peat the most N_2 was actually fixed in dark aerobic conditions, which indicates the participation of heterotrophic bacteria in N_2 fixation as well (Kravchenko & Doroshenko 2003). These studies indicate that the effect of light on moss-associated N_2 fixation may differ between habitats and moss species.

Temperature and N_2 fixation have been shown to correlate positively (Markham 2009), and for *Sphagnum* peat the temperature optimum was 25–30°C (Kravchenko & Doroshenko 2003) but only 16°C for *Sphagnum riparium* stems (Basilier & Granhall 1978). The minimum in both cases was 5°C, but 2°C temperature minimum for feather mosses has been reported (DeLuca et al. 2002, Gentili et al. 2005). In addition, Gentili et al. (2005) discovered different temperature optima for different cyanobacterial species associated with *P. schreberi*.

Moss species may also affect the N_2 fixation rate via the selection of microbial communities. Bragina et al. (2011) discovered that *Sphagnum* species harboured species-specific microbiomes, and Ininbergs et al. (2011) showed a correlation between the diazotroph community structure and the N_2 fixation rate in *H. splendens*. However, Basilier (1979) did not find differences between the N_2 fixation rates of *Sphagnum* species. In addition, pH might have an effect on N_2 fixation, e.g. pH 3.8 has been reported to be too low for moss-associated N_2 fixation (Granhall & Hofsten 1976). Usually N_2 fixation occurs in a wide pH range (4.3–6.8; Basilier & Granhall 1978, Kravchenko & Doroshenko 2003).

In most of the previous studies listed in this chapter, the assumption has been that *Cyanobacteria* are the main diazotrophs of mosses. Due to this, these factors and their effect on N_2 fixation need further studying, when heterotrophs and methanotrophs are considered as well active participants of moss-associated N_2 fixation.

1.3 Analysis of N_2 fixation and methane oxidation

1.3.1 Process measurements

A variety of methods are available for measuring N_2 fixation rates from cell culture analyses to ecosystem-scale measurements, including the acetylene (C_2H_2) reduction assay (ARA), stable isotope tracer ($^{15}N_2$) method, mass balance approach and stable isotope budgeting (reviewed by Zehr & Montoya 2007). In moss-associated N_2 fixation studies, ARA is the most used method, and to date only one ecological study (excluding this thesis) has applied the $^{15}N_2$ tracer method in moss research (Gavazov et al. 2010).

The nitrogenase enzyme can reduce other substrates as well as N_2, including acetylene, hydrogen cyanide (HCN) and azides (N_3^-; Hardy & Knight 1967). In ARA, the activity of nitrogenase enzyme is estimated by measuring acetylene reduction to ethylene (C_2H_4), which is then converted to represent the amount of reduced N_2 (Hardy et al. 1968). In theory, the production of 3 ethylene molecules represents 1 fixed N_2 molecule, the conversion factor then being 3:1, but usually the acetylene reaction occurs in saturating conditions, where H_2 by-production is inhibited (see reaction 1), and the ratio is closer to 4:1 (Capone 1993, Mulholland et al. 2004). ARA is an indirect way to measure N_2 fixation; therefore each sample set should be calibrated with the $^{15}N_2$ tracer method, because of the high variation in the conversion factor (Postgate 1998, Zehr & Montoya 2007). ARA is fairly straightforward and economical to use, as samples (e.g. mosses, soil or aquatic bacteria, pure cultures) are incubated in vials with 10 % acetylene, and the development of ethylene is monitored with a gas chromatograph. However, while acetylene competes with N_2 (Schöllhorn & Burris 1967), it disrupts the natural N metabolism in bacterial cells in the long term (Capone 1993) and even more importantly, it inhibits the function of methane monooxygenase in methanotrophs (DeBont & Mulder 1976). Therefore

it has been suggested that samples containing methanotrophs should be measured using the $^{15}N_2$ tracer method (Postgate 1998, Zehr & Montoya 2007).

A direct way to measure N_2 fixation is the $^{15}N_2$ tracer method ($^{15}N_2$ method from now on), which provides estimates of the net rate of incorporation of $^{15}N_2$ into biomass (Zehr & Montoya 2007). The $^{15}N_2$ method is also easy to conduct, but the need for tracer gases and the sample analysis with a stable isotope mass spectrometer (SIRMS) makes it more costly than ARA. The advantage of this method is that multiple tracers can be used at the same time, e.g. labelling the sample with $^{13}CH_4$ along $^{15}N_2$ is possible, which then reveals the methanotrophic N_2 fixation and CH_4-C sequestration simultaneously. At low N_2 fixation rates, the $^{15}N_2$ method is more sensitive than ARA (Montoya et al. 1996). The $^{15}N_2$ method is performed by incubating the sample with tracer gas additions and then calculating the tracer enrichment with the help of an un-enriched control sample. Tracers are added in such amounts that isotopic fractionation and the natural occurrence of the tracer gases (^{15}N 0.366 % and ^{13}C 1.106 % of the total N and C pool, respectively) can be neglected when comparing the difference between the added tracer and the natural N (^{14}N) or C (^{12}C) (Fry 2006).

The $^{15}N_2$ enrichment provides data at the plant level (moss stem or parts of the stem), but it can also be used to focus on single bacterial cells. When the samples are analysed with SIRMS, bulk data are obtained which contains both the microbial and moss biomasses. This is important when ecosystem-level results are needed. When bacterial metabolism and moss-bacteria associations are of interest, *in situ* single cell-level data can be obtained by conducting the sample analysis with secondary ion mass spectrometry (SIMS). Shortly, SIMS analysis starts with bombarding the sample surface with primary ions to make the surface atoms sputter. The sputtering detaches the atoms and atom groups from the sample, and the loose atoms get ionized. These ionized atoms, secondary ions, represent the atomic structure of the sample and they are analysed according to their mass, as in SIRMS. Finally, images with quantitative information for selected masses are formed (Lechene et al. 2006). The internal resolution for modern day SIMS is usually ~50 nm (nanoSIMS), which makes it possible to determine the amounts of different substances within a bacterial cell e.g. N_2 fixation and CH_4 oxidation rates in moss-associated bacteria and if the bacterium releases the end-products of these processes for its host.

The mass balance method can be used in terrestrial systems to estimate N_2 fixation by net change in total N with time, and it has also been successfully used in measuring moss-associated N_2 fixation (Berg et al. 2013). However, the problem is that all N inputs and outputs must be known (reviewed by Zehr & Montoya 2007). Stable isotope budgeting is based on the natural abundance of the ^{15}N, and it can provide a long term *in situ* measure of the impact of N_2 fixation on the local N budget (reviewed by Zehr & Montoya 2007).

ARA and the $^{15}N_2$ tracer method with SIRMS and SIMS measurements were used in this thesis.

1.3.2 Molecular microbial analyses

Culturing has been used to isolate diazotrophs for decades, but it only yields a small percentage of organisms that are present in the environment. Culture-independent molecular biology methods dominate the current research in microbial ecology, and they can be used to assay the potential for N_2 fixation and to identify the organisms involved. The most often used techniques are based on polymerase chain reaction (PCR), which amplifies the target gene from DNA or RNA extract of an environmental sample. Imaging techniques based on messenger RNA (mRNA) hybridization are also common in environmental microbiology. In addition, immunological techniques have been used in aquatic systems to identify bacteria that contain nitrogenase (Currin et al. 1990).

Various genes are used in molecular phylogenetic studies in microbial ecology, but most widely used are the genes encoding for 16S rRNA (Madigan et al. 2009). The 16S rRNA gene alone does not provide information about the physiology or metabolic features of the bacterium, so molecular methods targeting functional genes can be useful for linking functions within bacterial communities. The *nifH* gene encodes a subunit for nitrogenase (Postgate 1998), and it has been widely used for studying the presence and diversity of diazotrophs. The *nifH* gene contains conserved regions, which can be used for targets when planning binding sites for universal or broad-range PCR amplification primers (e.g. Zehr & McReynolds 1989, Poly et al. 2001a). The *nif*-gene cluster contains also other genes, *nifD* and *nifK*, but *nifH* is the most sequenced and has become a marker gene when studying the phylogeny, diversity and abundance of N_2-fixing bacteria (Gaby & Buckley 2012), thus having the largest reference database.

The most common PCR-based profiling methods are fingerprinting methods, e.g. denaturing gradient gel electrophoresis (DGGE; Muyzer et al. 1993), (cloning-)sequencing methods and gene expression analyses (quantitative PCR, reverse transcriptase PCR and their combination, RT-qPCR; Heid et al. 1996). DGGE relies on separation of PCR-amplified genes on the basis of differences in their GC-content and sequence, which then leads to differences in their melting properties as they migrate through a gel matrix containing a gradient of a denaturing chemical. The separation provides information about bacterial community structure within and between samples. In Sanger sequencing, a cloning step is usually needed, and information about bacterial species (operational taxonomic units, OTUs) and about the OTU diversity is obtained. The difference between these methods is that DGGE is based on millions of molecules, but can usually resolve bands which make at least 1 % of the total PCR product, while the cloning and sequencing approach is limited to some hundreds of random molecules (Prosser et al. 2010). The new, next generation sequencing (NGS) approaches (e.g. 454, Illumina, SOLiD, PacBio, Ion Torrent), in which cloning steps are not needed and which can provide even 0.1 to 1000 million reads per sequencing run (Glenn 2011), should also be mentioned, despite not being used in this thesis. In addition, RT-qPCR is useful in determining the amount of active diazotrophs, but it has been mainly used in

measuring marine or freshwater diazotroph activities (reviewed by Farnelid 2013).

Fluorescence *in situ* hybridization (FISH) employs the specificity of fluorescently labeled DNA sequences (probes), which then hybridize with the matching mRNA inside the bacterium. Regions that bind the probe are imaged with microscopy techniques revealing identities and amounts of bacteria in environmental samples (reviewed by Moter & Göbel 2000). Catalyzed reported deposition FISH (CARD-FISH) is a modification of the traditional FISH, where the oligonucleotide probes used have a horseradish peroxidase (HRP) label that is further amplified with fluorescent tyramide. With CARD-FISH also weak probe signals can be detected due to the extra amplification step (Kerstens et al. 1995). FISH has also been connected to microautoradiography (MAR-FISH; Lee et al. 1999), which provides information about bacterial metabolism as well, when radioactively labeled substrates are used. MAR-FISH has been used for detecting the filamentous methanotroph *Crenotrhix polyspora* from drinking water (Stoecker et al. 2006). However, diazotrophic activity cannot be studied by this technique due to the lack of a long-lived radioactive isotope for N. The coupling of the SIMS technology and halogen *in situ* hybridization (HISH-SIMS; Musat et al. 2008) led to a useful tool, which can be used to study identities, activities and locations of methanotrophic and diazotrophic microbes at a single cell resolution.

In HISH-SIMS the hybridization procedure is essentially same as that used for CARD-FISH and the same HRP probes can be used. The main difference is that the tyramides used in the amplification step are ^{19}F-labeled, and this label can further be detected with nanoSIMS. When the ^{19}F-labeling is combined with stable isotope labeling (e.g. $^{15}N_2$ and $^{13}CH_4$ tracer gases used in this thesis) the metabolically active cells with ^{15}N and ^{13}C labels can at the same time be phylogenetically identified due to the^{19}F-labeled probe (Kuypers & Jørgensen 2007, Musat et al. 2011). Workflow for HISH-SIMS is described in Fig. 2.

Sanger sequencing, DGGE and HISH-SIMS were used in this thesis.

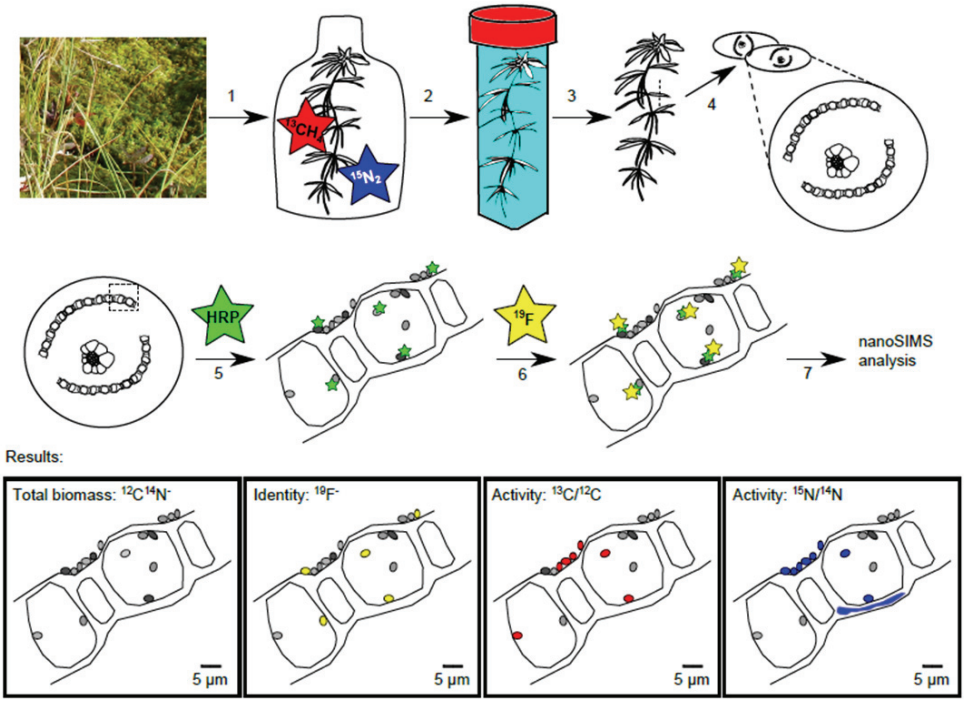

FIGURE 2 HISH-SIMS workflow. The first step is the incubation of environmental samples (e.g. mosses) with labeled substrates (e.g. $^{15}N_2$ and $^{13}CH_4$ gases) for some hours or days. In the second step the labeling experiment is ended by using chemical fixatives (e.g. paraformaldehyde). Third, the most interesting parts of the moss, based on preceding SIRMS analyses, are selected for cross-sectioning. Fourth, thin (~5 µm) cross-sections are prepared on a filter covered by conductive elements (e.g. gold-palladium) and then hybridized with an HRP probe (step 5, middle row). The probe signal is amplified with fluorescing fluorine-containing tyramide (step 6), which allows examination of the hybridization results and the quality of the sample by fluorescence microscopy. Finally, the filter is analyzed using nanoSIMS. As a result, the total biomass (shown by the $^{12}C^{14}N^-$ secondary ion image), phylogenetic identity ($^{19}F^-$ image) and metabolic activity of the analyzed cells are obtained. Using the secondary ion images (data, not shown), ratio images $^{13}C/^{12}C$ and $^{15}N/^{14}N$ can be calculated and then used to estimate the substrate uptake rates of single cells or plant material.

1.4 Aims

Boreal forest and wetland habitats are C sinks, reducing the amount of greenhouse gas CO_2 from the atmosphere. In these habitats the net mineralization rates for N are low, so biological N_2 fixation has an important role in regulating the amount of new C that is sequestered into the biomass. Especially important is to determine N_2 fixation rates at sites that lack available N in mineral form, like peatlands. So any factor that has the potential to increase N_2 fixation, like CH_4, needs further studying. In addition, N addition via N_2 fixation can be an essential factor in reducing global CH_4 budget by increasing the methanotrophic activity in boreal peatlands. Furthermore, while mosses are important drivers of the characters of their ecosystems, the environmental factors that affect the mosses, most likely affect also the activity and abundance of their diazotrophic associates. By knowing the identities of these associates can help in evaluating the responses that different environmental factors may cause on N_2 fixation. In sum, boreal moss-associated N_2 fixation can possibly have a major role in reducing the two main greenhouse gases. Due to this, the aim of this thesis was to study the rates of moss-associated N_2 fixation and the effect of CH_4 on this fixation in boreal habitats. The main study hypotheses were:

1. N_2 fixation forms a significant source of new N in boreal mosses.

2. In addition to *Cyanobacteria* and heterotrophs, methanotrophs participate in the moss-associated N_2 fixation.

These hypotheses were addressed with stable isotope incubation experiments and *nifH* gene analyses. Since acetylene inhibits the metabolism of methanotrophs, ARA was compared with the direct $^{15}N_2$ tracer method (I) and thereafter the $^{15}N_2$ tracer method was solely used in the other experiments to measure N_2 fixation (II–IV). Identities of diazotrophs were elucidated with Sanger sequencing of the *nifH* gene (I–III), and diazotroph community structures from 4 *Sphagnum* species in 2 habitats were compared (II). The share of photosynthetically driven cyanobacterial N_2 fixation was determined by comparing light and dark treatments (IV, this synthesis). The connection between methanotrophy and diazotrophy was studied at the moss stem level with SIRMS (I–IV) and at the single cell level as well with nanoSIMS (III).

2 MATERIALS AND METHODS

2.1 Study areas, sampling and experimental design

2.1.1 Forest sites (I)

The 7 study sites are located in southern (sites 4–7) and northern (sites 1–3) boreal zones (Fig. 3). The understory of all sites represents typical boreal vegetation rich in mosses and dwarf shrubs (Salemaa et al. 2008); the main moss species at all of these sites are *Hylocomium splendens* (Hedw.) Schimp. and *Pleurozium schreberi* (Willd. ex Brid.) Mitt. Five of the sites (1, 3–5 and 7) are dominated by Scots pine (*Pinus sylvestris* L.) and two (2 and 6) by Norway spruce (*Picea abies* L. (Karst.)). The forest types of the sites are given in Table 1 and more information about understory vegetation, air quality and wet deposition are presented in the national report of forest condition monitoring in Finland (Derome et al. 2007).

H. splendens and *P. schreberi* samples were collected into 500 ml containers, 15 containers from each site per moss species, in September 2010. The samples were stored in dark at 4°C until the experiment started and the samples for microbial analysis were stored at –20°C.

Mosses were acclimatized for 4 d before incubation, and then incubated for 2 d at the common incubation site (in the yard of the Finnish Forest Research Institute, Vantaa) under 4 treatments (A C_2H_2, B $^{15}N_2$, C $^{15}N_2$+$^{13}CH_4$ and D unlabelled control) in the ambient conditions. The treatments were used to reveal 1) the differences between ARA and $^{15}N_2$ tracer method (A vs. B), 2) the effect of CH_4 addition on N_2 fixation (B vs. C) and additionally 3) the effect of sampling site (= N deposition) on N_2 fixation, and 4) the effect of moss species and moss part on N_2 fixation. Site-specific coverages for the mosses were calculated as in Salemaa et al. (2013).

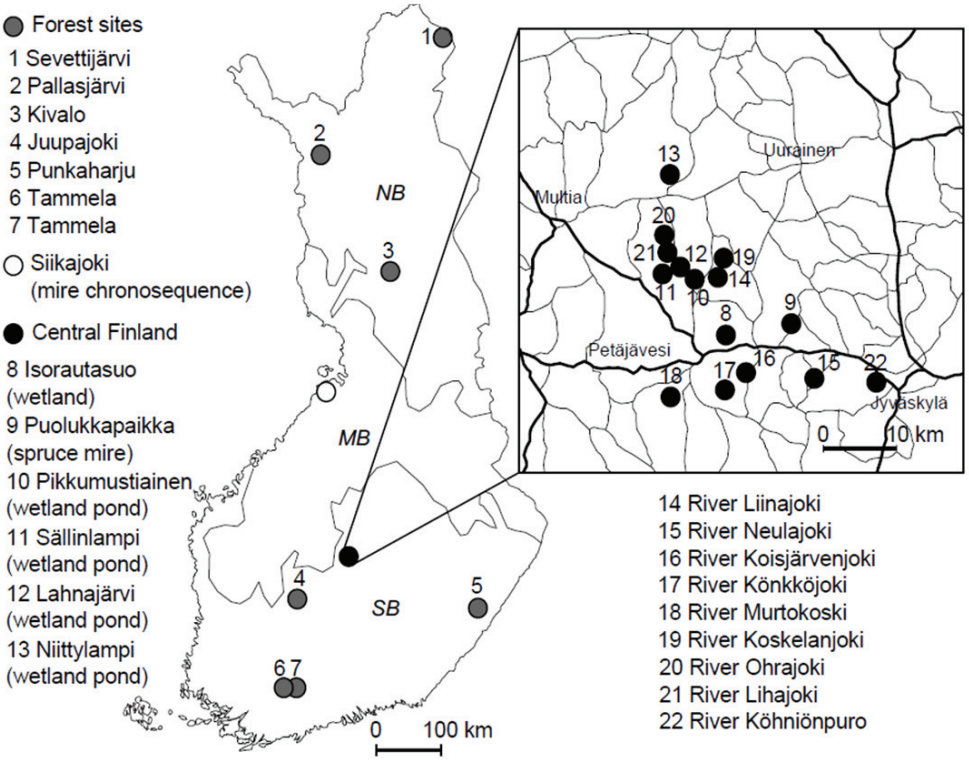

FIGURE 3 Locations of the forest sites (1–7; *Hylocomium splendens* and *Pleurozium schreberi*), wetland and wetland pond sites (8–13; *Sphagnum* mosses), rivers (14–22; *Fontinalis dalecarlica*) and mire chronosequence sites (*Sphagnum* mosses) in the climate zones of Finland (NB = northern boreal, MB = middle boreal, SB = southern boreal zone).

TABLE 1 The forest types of the sites 1–7.

Site	Forest type
1 Sevettijärvi	*Uliginosum-Vaccinium-Empetrum* Type
2 Pallasjärvi	*Hylocomium-Myrtillus* Type
3 Kivalo	*Empetrum-Myrtillus* Type
4 Juupajoki	*Vaccinium* Type
5 Punkaharju	*Vaccinium* Type
6 Tammela	*Myrtillus* Type
7 Tammela	*Vaccinium* Type

2.1.2 Wetland and wetland pond sites (II, III)

Wetland Isorautasuo (site 8; Fig. 3) includes two sub-sites, an oligotrophic *Sphagnum* bog (III), dominated by *Sphagnum papillosum* Lindb., and a pristine oligotrophic low-sedge fen (II–III) dominated by *Sphagnum fimbriatum* Wils., *Sphagnum balticum (*Russ.) C. Jens., *Sphagnum fallax* (Klinngr.) Klinngr. and *Sphagnum riparium* Ångstr. The forest site (site 9; Fig. 3) is a meso-eutrophic thin-peated *Vaccinium-Myrtillus* spruce mire, in which the field layer is dominated by *Sphagnum* mosses (*Sphagnum angustifolium* (Russ.) C. Jens., *S. girgensohnii* Russ. and *S. riparium*; II–III). The dominant moss species were collected from three random plots from the fen and forest in August 2008 (II).

The 4 wetland ponds (sites 10–13; Fig. 3) show significant *Sphagnum* establishment along the littoral zone, being at the early stage of bog development. *Sphagnum majus* (Russ.) C. Jens. is the most abundant *Sphagnum* species in all ponds (III).

The samples of the most abundant *Sphagnum* species (listed above) were not stored prior to incubations, since all the incubations started on the sampling day. Samples for microbial analyses were stored at −20°C. See Table 2 for experimental designs.

TABLE 2 The experimental designs for *Sphagnum* mosses from wetlands and wetland ponds.

Paper	Incubation	Treatments	Replicated*
II	*in situ*	A $^{15}N_2$	in air and water headspace
		B $^{15}N_2$+$^{13}CH_4$	
		C control	
II	laboratory	A–C	top, middle and bottom moss parts
III	Lake Jyväsjärvi	A–C	

*See details (e.g. moss species) from the corresponding paper.

2.1.3 River sites (III, river and season experiments)

The study rivers (sites 14–22: Fig. 3) are typical acidic Finnish streams, where *Fontinalis dalecarlica* (Bruch & Schimp.) covers 100 % of the river bottom vegetation. In August 2008, *F. dalecarlica* samples were collected from each river during one day and transported to the laboratory at 5°C (the river experiment). At Liinajoki site (site 14), samples were collected from 3 sampling sites, which were 2 m apart. At site I, the water current was weak, at site II the water current was strong and site III was in a backwater. In all other experiments, the samples were always collected from site II. Also river water was collected into separate bottles. One *F. dalecarlica* stem was placed in the incubation vial and then filled with the corresponding river water. Incubations were conducted in a similar way as in III with the following exceptions. The samples were incubated under four

treatments (A $^{15}N_2$, B $^{15}N_2$+$^{13}CH_4$, C $^{15}N_2$+glucose and D control) at a common incubation site (Köhniönpuro, site 22) in ambient conditions for 24 h. The treatments were used to reveal 1) net N_2 fixation and CH_4 oxidation in the rivers (A, B, D), 2) the effect of CH_4 addition on N_2 fixation (A vs. B) and 3) the effect of an extra-carbon source on N_2 fixation (A vs. C). Glucose (D(+)-glucose anhydrate, J.T. Baker B.V., Deventer, Holland) was added at 12.5 mg C^{-1} per sample. Incubations were terminated and the stable isotope analysis was conducted as in I.

During 2009, the River Liinajoki (site 14) was sampled once a month (the season experiment). In September 2009 additional samples were taken for the single cell experiment (III). Every month 3 treatments (A $^{15}N_2$, B $^{15}N_2$+$^{13}CH_4$ and C control) were conducted and incubated in Liinajoki for 2 d in the ambient conditions. The same treatments were replicated in the laboratory, but always at 15°C and 12 h light/dark cycle. In addition, the treatments A and B were replicated in the dark (incubation vials covered with tin foil) and for washed moss stems (*F. dalecarlica* washed 4 times in clean tap water). Otherwise the incubations, their termination and the stable isotope analysis were conducted as in III and I.

At each sampling time, samples were taken for microbial analyses as well, and these samples were stored at –20°C. Only preliminary results for the river and the season experiment are presented in Results and Discussion, as they need additional analyses.

2.1.4 Successional gradient (IV)

The Siikajoki mire chronosequence (Fig. 3) is a replicated primary successional series of mire ecosystems in the land-uplift coast of the Gulf of Bothnia of the Baltic Sea, Finland (Tuittila et al. 2013). The study sites consist of 12 mires that form a replicated successional series in 4 stages, the age of which varies from 200 to 2500 years (see Table 1 in IV). In June 2010, dominant *Sphagnum* mosses were collected from wet depressions (flarks) and hummocks of each of the study mires. The moss sampling was based on a vegetation survey conducted by Tuittila et al. (2013). Experimental incubations started immediately after sampling.

Mosses were incubated *in situ* under three treatments (A $^{15}N_2$+$^{13}CH_4$, B $^{15}N_2$ and C unlabelled control), each under prevailing light conditions (day length 20 h) and in dark for 2 d. The treatments were used to reveal 1) net N_2 fixation (A–C), 2) the effect of CH_4 addition on N_2 fixation (A vs. B) and 3) the active N_2-fixing microbial groups. Light, dark and CH_4 treatments were used to separate diazotrophs utilizing different sources of energy and C (photosynthesis for cyanobacteria, organic matter breakdown for heterotrophs and CH_4 for methanotrophs).

2.2 Stable isotope analyses (I-IV)

Stable isotopes of N and C were analysed from native (control samples) as well as tracer-incubated mosses. $^{15}N_2$ and $^{13}CH_4$ gases were used as isotopic tracers to measure N_2 fixation and CH_4-C uptake of moss-associated bacteria. The isotopic values of the tracer gases were $\delta^{15}N$ 98 ‰ and $\delta^{13}C$ 99 ‰ for $^{15}N_2$ and $^{13}CH_4$, respectively, while the expected natural values for N_2 and CH_4 gases are 0 ‰ and –43 ‰, respectively. The natural $\delta^{15}N$ and $\delta^{13}C$ values within the moss tissue are also close to these values. The final enrichment levels (atom % of the labelled gas: $at\%^{15}N_{final}$ or $at\%^{13}C_{final}$) of the incubation vials were calculated using a simple mixing model (Fry 2006)

$$at\%^{15}N_{final} = f_1 \times (at\%^{15}N_{air} - at\%^{15}N_{tracer\ gas}) + at\%^{15}N_{tracer\ gas} \quad \text{[Eq. 1]}$$

where f_1 = fractionation factor for N_2 in air, $at\%^{15}N_{air}$ = 0.36630 and $at\%^{15}N_{tracer\ gas}$ = 98. $At\%^{13}C_{final}$ was calculated accordingly ($at\%^{13}C_{air}$ = 1.05837). If the vial headspace was filled with water, the f_1 was calculated using solubility and V_m values of the corresponding gas at the incubation temperature.

N_2 fixation and CH_4 oxidation (CH_4-C uptake) in the mosses were determined by counting the difference (enrichment $\delta_{1,2}$) between the stable isotope values of the experimental treatments and the corresponding control treatment with the following equation (Fry 2006)

$$\delta_{1,2} = \frac{\delta_1 - \delta_2}{\delta_2 + 1000} \times 1000 \quad \text{[Eq. 2]}$$

where δ_1 = $\delta^{13}C$ or $\delta^{15}N$ value of a sample, δ_2 = $\delta^{13}C$ or $\delta^{15}N$ value of a corresponding control.

Details of the sample preparation and stable isotope analysis can be found from I and II.

2.3 Microbial analyses

2.3.1 Molecular analyses

Bacterial nucleic acids from moss samples (I–III) were extracted from 0.3–0.8 mg of the moss using a modified version of the bead-beating and phenol-chloroform extraction protocol of Griffiths et al. (2000) (full description in I).

The OTUs of the moss-associated diazotroph communities were studied with cloning and Sanger sequencing of the *nifH* gene (I–III). Amplification of the *nifH* sequences of divergent diazotrophs from DNA extracts was done using a nested PCR protocol according to Demba Diallo et al. (2004). The primer sets used were: FGPH19 (5´– TAC GGC AAR GGT GGN ATH G – 3´) (Simonet et al.

1991) and PolR (5´– ATS GCC ATC ATY TCR CCG – 3´) for the first round, and PolF (5´– TGC GAY CCS AAR GCB GAC TC – 3´) and AQER (5´– GAC GAT GTA GAT YTC CTG – 3´) (Poly et al. 2001b) for the second round. A full description of the cloning and sequencing can be found in I.

Spatial variations (within site and between sites) in the moss associated diazotroph communities were assessed using DGGE (II). Bacterial DNA was amplified using a nested PCR protocol for *nifH* gene as described before, except in the second PCR reaction the primer PolF included a GC-clamp to enable the DGGE analysis.

2.3.2 Single cell analysis

The locations, activities and identities of N_2-fixing methanotrophs in mosses were determined with a single cell analysis using HISH-SIMS (III). The moss samples were first incubated in the presence of $^{15}N_2$ and $^{13}CH_4$ tracer gases and when the incubation was terminated the samples were fixed in 1 % paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature, transferred to 50 % ethanol and kept at –20°C until further processing (III). The oligonucleotide probes used in CARD-FISH were chosen according to the observed *nifH* gene phylogeny in the study mosses (Fig. 2 in III), hence ALF968 (5´– GGT AAG GTT CTG CGC GTT – 3´) (Neef 1997) was selected for *S. majus* and Mα450 (5´–ATC CAG GTA CCG TCA TTA TC – 3´) (Eller et al. 2001) for *F. dalecarlica*.

2.4 Statistical analyses (I-IV)

The specific statistical analyses made in the study, and, in which of the papers the analyses are fully described, are presented in Table 3.

TABLE 3 Statistical analyses used in this study.

Analysis	Description
Linear regression	I
t-test	I, II
Analysis of variance (ANOVA) with Tukey's multiple comparisons or simple main effects analysis[1]	I, II
Mann-Whitney U-test	I, II, III
Kruskall-Wallis test with *post hoc* testing	II, III
Spearman rank correlation	II, IV
Permutational multivariate analysis of variance (PERMANOVA)[2]	II
Multi-response permutation procedure (MRPP)	II
Non-metric multidimensional scaling (NMS)	II
Mantel´s test	II
Pearson correlation analysis	III
Nested analysis of covariance (ANCOVA)	IV
Bonferroni *post hoc* test	IV
Stepwise regression	IV

[1]Quinn & Keough 2002, [2]McArdle & Anderson 2001

3 RESULTS AND DISCUSSION

3.1 Comparison of the acetylene reduction assay and the $^{15}N_2$ tracer method with forest mosses (I)

Of the 54 acetylene treated forest moss samples, only 24 showed an ethylene signal, while 50 of the 54 $^{15}N_2$ incubated samples had significantly enriched $\delta^{15}N$ values; hence the $^{15}N_2$ method was more sensitive than ARA at the studied sites. The greater sensitivity of the $^{15}N_2$ method can be explained by the differing detection limits of the measuring devices; the detection level for SIRMS was 0.03 nmol N_2 g^{-1} h^{-1} at the 20 % $^{15}N_2$ labelling level, but for ARA the detection level for the gas chromatographic procedure used was 0.2–0.5 nmol N_2 g^{-1} h^{-1}. When the N_2 fixation results from the different sites and moss parts were compared between the 2 methods, the calibrated values did not differ between the northern boreal samples, but the $^{15}N_2$ method was more sensitive than ARA for measuring the low N_2 fixation activity of southern samples. The higher sensitivity of the $^{15}N_2$ tracer method has been noted in previous studies (Montoya et al. 1996, Kayanne et al. 2005). However, both methods indicated higher N_2 fixation activity in the upper (green) part than in the lower moss part. Gavazov et al. (2010) also measured higher N_2 fixation activities from the upper part of *H. splendens*, which supports the important role of light in N_2 fixation (Meeks 1998, Gundale et al. 2012b).

The conversion factor for *P. schreberi* was 3.2, for *H. splendens* 3.4 and for pooled data it was 3.3. This means that 3.3 nmol of ethylene represents 1 nmol of fixed N_2. These values are close to the theoretical conversion factor of 4.0 (Capone 1993) as well as the values obtained from previous moss studies (3.0; DeLuca et al. 2002, 3.3; Zackrisson et al. 2009). Environmental factors (e.g. moss biomass, climate factors and the length of active period) may cause uncertainties in the quantification of N_2 fixation, reducing the importance of the second digit of the conversion factor. However, at the lowest ethylene concentrations ARA gave lower N_2 fixation values than the $^{15}N_2$ method, which decreased the conversion factor (Fig. 4). The same phenomenon has been detected by Montoya

et al. (1996) and it may be due to microbial usage of the produced ethylene (Capone 1993), to changes in the stress level of the host plant (Schwinzer & Tjepkema 1994) or to differences in the symbiotic relationship between the host and *Cyanobacteria* (Postgate 1998).

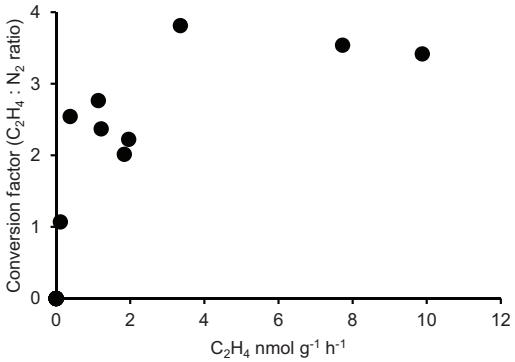

FIGURE 4 Conversion factor (C_2H_4 : N_2 ratio) counted separately for each moss batch (n = 3). At low N_2 fixation activity, measured ethylene production (C_2H_4 nmol g^{-1} h^{-1}) was low decreasing the batch specific conversion factor.

3.2 Factors that affect N_2 fixation rates in boreal mosses

3.2.1 Overall N_2 fixation rates (I-IV)

The N_2 fixation rates varied between 0.03–2.89 nmol N_2 g^{-1} h^{-1} in the forest mosses *P. schreberi* and *H. splendens* (I), with the variation due to the differences between the sampling sites (see 3.2.5). These rates are in line with previous studies conducted with the same moss species at the same latitudes (DeLuca et al. 2002, Zackrisson et al. 2004, Lagerström et al. 2007), and at the Pallasjärvi site the N_2 fixation (1.9 kg N ha^{-1} y^{-1}) rate exceeded the areal inorganic bulk deposition. On average, N_2 fixation at the northern sites was 0.68 kg N ha^{-1} y^{-1} and at the southern sites 0.04 kg N ha^{-1} y^{-1}. Our N_2 fixation rates for *P. schreberi* were 2–5 times higher than in the study of Gavazov et al. (2010), but variation may arise due to seasonal changes or from deviating sampling procedures. On the other hand, the N_2 fixation rates for air incubated *Sphagnum* mosses (II) were of the same order of magnitude (41.7–2 960 nmol N_2 g^{-1} h^{-1}) as in Gavazov et al. (2010), but in the submerged fen mosses our N_2 fixation rates (17 920 nmol N_2 g^{-1} h^{-1}) were twofold greater than in their study. *S. majus* from a wetland pond fixed N_2 on average at 27 000 nmol N_2 g^{-1} h^{-1}, the maximum rate being 81 000 nmol N_2 g^{-1} h^{-1} (III). The highest N_2 fixation rates, up to 118 000 nmol N_2 g^{-1} h^{-1}, were measured from mid-successional mesotrophic fens (IV), exceeding the current wet inorganic deposition in the region. In addition, in Siikajoki the N_2 fixation rates were 3 times higher in light than in dark, although N_2 was also fixed in

darkness, indicating that the most active diazotrophs were phototrophic organisms (IV).

These values for *Sphagnum*-associated N_2 fixation are within the wide range of the few estimates available (Granhall & Selander 1973, Basilier & Granhall 1978, Waughman & Bellamy 1980, Markham 2009, Gavazov et al. 2010), although when compared to Limpens et al. (2006), biological N_2 fixation forms more considerable input in peatlands according to our results. The previous studies has been conducted with ARA (excluding Gavazov et al. 2010), which may have caused underestimations in methanotrophic N_2 fixation rates; hence explaining the higher accumulation rates gained in this thesis. In addition, Elbert et al. (2012) have calculated that N_2 fixation in cryptogams (lichens, bryophytes and their associates) covers nearly half of the biological N_2 fixation on land and that it is also crucial for C sequestration by plants. These studies indicate that mosses, due to their high biomass and overall abundance, form an important part of N- and C-economy in boreal and subarctic forests and peatlands.

3.2.2 Methane (I-IV)

The forest mosses, *P. schreberi* and *H. splendens,* did not show significant CH_4 oxidation activity (I), which can be explained by the low concentration of CH_4 in forest habitats and the lack of suitable moisture conditions for methanotrophs (Larmola et al. 2010). However, in all other studies (II-IV) performed with *Sphagnum* mosses, CH_4 oxidation was detected at varying rates along with N_2 fixation.

N_2 fixation was confirmed in most (91 %) and CH_4 oxidation in nearly half (44 %) of the 163 double-labelled fen and forest *Sphagnum* mosses collected from Central Finland in late summer (II). When both of these processes were measured simultaneously, CH_4 oxidation did not enhance the N_2 fixation rates at either habitat, which was probably due to the low CH_4 oxidation rates (0–4 ‰ enrichment, representing up to 830 nmol C g^{-1} h^{-1}) that were of 3 orders of magnitude lower than in the previous study conducted using gas chromatographic analysis (Larmola et al. 2010). However, in early summer CH_4 addition increased N_2 fixation in *S. papillosum*, indicating methanotrophy induced N_2 fixation (II). Since in late summer the N_2 fixation rates were high, ammonium evolved from N_2 fixation could have inhibited the CH_4 oxidation pathway at the molecular level (Toukdarian & Lindström 1984, Bodelier & Laanbroek 2004) or the active diazotrophs (cyanobacteria and heterotrophs) might have outcompeted methanotrophs in nutrient competition (e.g. for phosphorus, P, resources). Regardless, the control of methanotrophy on N_2 fixation seems to vary seasonally. At the fen, CH_4 was oxidized by all *Sphagnum* species, but at the forest only *S. riparium* and *S. girgensohnii* indicated CH_4 oxidation activity. CH_4 oxidation rates may vary between moss species (Basiliko et al. 2004), but the dispersal of methanotrophs via water (Putkinen et al. 2012, Bragina et al. 2013) might have created the differences between the submerged *S. riparium*, semi-submerged *S. girgensohnii* and hummock growing *S. angustifolium*. In comparison, at the fen all mosses were submerged.

In 2 of the 4 wetland ponds, the N_2 fixation and CH_4 oxidation rates of *S. majus* correlated positively, but the dual-labelling experiment implied that CH_4 oxidation did not enhance N_2 fixation as such. River moss *F. dalecarlica* oxidized CH_4 at the highest rate, the maximum rate being 447 060 nmol C g^{-1} h^{-1} (III), but CH_4 addition did not enhance N_2 fixation in *F. dalecarlica* either. This indicates that methanotrophic N_2 fixation may be a slower process than non-methanotrophic, and longer incubation periods are needed to see significant effects in bulk measurements. However, the measured CH_4 oxidation rates in *S. majus* (average 20 000 nmol C g^{-1} h^{-1}) were considerably higher than in II. In the *S. majus* samples, analysed at the bacterial cell resolution with nanoSIMS, CH_4-C fixation was detected from 146 bacterial cells, from which 60 also indicated N_2 fixation activity at the same time. Therefore, this study is the first to show N_2 fixation and CH_4 oxidation *in situ* in plant samples at the cellular level. As calculated in III, oxidation of 1 mole of CH_4 could provide energy for the reduction of 1.6 moles of N_2, which signifies that the ability to utilize methanotrophy could be an important phenotypic trait for symbiotic diazotrophic bacteria of mosses.

At the mire chronosequence, the highest moss-associated CH_4 oxidation rates were observed in mesotrophic fens, continuing at moderate rates in the flarks of the older stages. The share of CH_4-induced N_2 fixation was over one-third of the N_2 fixation in the 3 younger mire stages but negligible in the fen-bog transitional mires (IV). 70 % of N and 40 % of C has accumulated during the first 1000 years of the 2500 year period (Tuittila et al. 2013), which is a period when N_2 fixation peaked and had the strongest response to CH_4 addition. This implies that the methanotrophic N_2 fixation contributes to the rapid N accumulation at the fen stages.

Despite the opposing reaction requirements for N_2 fixation and CH_4 oxidation, both processes were detected within same bacterial cells and the potential of CH_4 oxidation in increasing N_2 fixation was shown. In Central Finland the high N_2 fixation rates seemed to decrease CH_4 oxidation, but at Siikajoki fens the highest N_2 fixation rates were methanotrophy induced. It can be concluded that the greenhouse gas CH_4 certainly has a role in moss-associated N_2 fixation, but more studies are needed to clarify the impact of N addition via N_2 fixation on CH_4 fluxes.

3.2.3 Moss species (I, II, IV)

There was a trend for higher N_2 fixation in *H. splendens* than in *P. schreberi*, although the difference was not statistically significant (I). Among these forest mosses, N_2 fixation in *H. splendens* has been observed to be more tolerant of N fertilizer additions than N_2 fixation in *P. schreberi* (Zackrisson et al. 2009), which indicates that the N_2 fixation ability in *H. splendens* may remain in areas with higher atmospheric N deposition.

In the fen and forest habitats, the N_2 fixation rates differed significantly between the *Sphagnum* species, except between *S. balticum* and *S. fallax* in the fen (II). In the fen, N_2 fixation was most efficient in *S. riparium* and least efficient in *S.*

fimbriatum. However, the N_2 fixation rates were much lower for *S. riparium* batches that were collected from the forest, where the lowest N_2 fixation values were measured for *S. riparium* and the highest rates for *S. angustifolium*. Gavazov et al. (2010) reported differences in N_2 fixation rates between lichens, bryophytes and liverworts, although the lower-level taxonomic divergence was masked by the high variance between the individual samples. Therefore, it is important to note that the habitat had an even higher effect on N_2 fixation than the species, as *S. riparium* had the highest N_2 fixation rate of the fen species, but lowest of the forest species. Basilier (1979) detected the same pattern in *S. angustifolium* between 2 different habitats. Also submerged *S. majus,* growing in Pikkumustiainen, had different N_2 fixation rate than the same species growing in 2 other ponds, Sällinlampi and Niittylampi (X^2 = 37.528, df = 4, P < 0.001 and pairwise comparisons). Hence habitat may be the primary and moss species the secondary determinant in the moss-associated N_2 fixation rate. This is supported by the comparison of the mire chronosequence, where the most efficient N_2 fixation was observed in mid-successional mesotrophic fens (IV). Within that habitat group, *S. platyphyllum* differed significantly from other 2 flark species collected from different sites.

3.2.4 Water table level (II, IV)

At the fen, submerged *Sphagnum* mosses exhibited approximately 44 % higher N_2 fixation activity than air incubated mosses. At the forest, submergence enhanced N_2 fixation only in *S. riparium* (II). Also at the successional series, N_2 fixation was always higher in the flarks than in the hummocks (IV). This suggests that the nitrogenase activity in these mosses was mainly located in bacterial cells that were not sheltered from O_2, which implies that the active diazotrophs were not only *Cyanobacteria*, because they are able to protect their nitrogenase from O_2 (Fay 1992). Furthermore, methanotrophs were more active in flark mosses, which can be explained by the higher CH_4 concentration in the mire water table than in the atmosphere.

3.2.5 N deposition, C/N ratio of the moss and successional stage (I, II, IV)

In the forest mosses, *P. schreberi* and *H. splendens,* the N_2 fixation rates were higher at the northern than southern sites (I), which was explained by the higher atmospheric N deposition in the south. This is in line with Zackrisson et al. (2009), who showed moss-associated N_2 fixation rates increasing north of 64 °N. Gundale et al. (2011) showed that N_2 fixation was down-regulated already with N additions of 3 kg N ha^{-1} y^{-1}, which equals the N deposition at our southern study sites. Also N concentration of forest mosses is known to correlate with N deposition level (Poikolainen et al. 2009), and this was also evident in our data set as northern samples had higher C/N ratio (77:1) than southern ones (53:1). This same phenomenon was noticed in the fen and forest *Sphagnum* mosses, since N_2 fixation rates increased when the C/N ratio of mosses increased (II). Remarkably high N_2 uptake rates were detected when the C/N ratio was higher

than 45:1, consistent with the study of Cusack et al. (2009). However, at the successional series, the C/N ratio decreased when N_2 fixation increased (IV). This is in contrast with the other experiments in this thesis, but at the successional gradient mires N_2 fixation was best explained with the P availability, the N_2 fixation rates being highest at the lowest N/P ratios. Thus, diazotrophs were able to respond to N demand relative to P supply, which has also been shown previously (Houlton et al. 2008).

The highest N_2 fixation activity was measured in the mid-successional fens, not at the earlier meadow stage as was expected. The *Sphagnum* moss carpet in the meadows was not as uniform as at the older successional stages, and the colonization or moss-diazotroph symbiosis may be still developing (Zackrisson et al. 2004, DeLuca et al. 2007). There are no previous N_2 fixation studies from boreal mire primary successional gradients, but our results are in line with the studies conducted with *Sphagnum* mosses collected from different fertility levels (e.g. Granhall & Selander 1973, Basilier 1979, Waughman & Bellamy 1980), with the N_2 fixation being always higher at the minerotrophic than at the ombrotrophic sites.

3.2.6 N_2 fixation and methane oxidation in *F. dalecarlica*

F. dalecarlica associated N_2 fixation varied between the 9 studied rivers in Central Finland, although overall the rates were low (Fig. 5a). The CH_4 addition seemed to increase N_2 fixation in Könkköjoki, Murtokoski, Liinajoki I and Ohrajoki, but CH_4 oxidation was not necessarily high at these sites (Fig. 5b). Glucose addition increased N_2 fixation as well, but not more than CH_4 addition. This indicates that N_2 fixation in river habitats is dependent on an external energy source and is closely connected with CH_4 oxidation. Interestingly, the 3 closely located sampling sites at Liinajoki differed markedly in their N_2 fixation rates, the weak water current site (sampling site I) having the highest N_2 fixation rate. This indicates that sampling site I might have experienced N deficiency in the recent past and the diazotroph community was still adapted to that condition. The difference between the CH_4 oxidation rates was not that clear between these sites. Between the rivers, the CH_4 oxidation rates varied from 0‰ enrichment to 70‰ enrichment, and were affected slightly by the site-specific CH_4 concentrations (Fig. 5b).

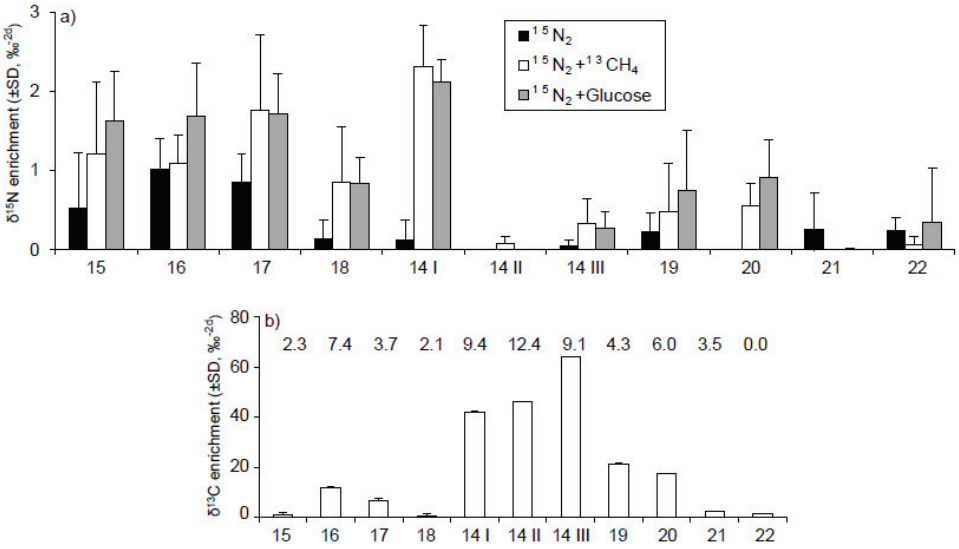

FIGURE 5 The river experiment. a) The N_2 fixation rates ($\delta^{15}N$ enrichment, n = 4) in *Fontinalis dalecarlica* with and without CH_4 or glucose. b) The CH_4 oxidation rates ($\delta^{13}C$ enrichment, n = 4) in *F. dalecarlica*. The numbers above the bars are CH_4 content (ppm) of the corresponding river water. The rivers are listed as numbers (x-axes), see Fig. 3.

Liinajoki (sampling site II) was monitored once a month during 2009, and CH_4 oxidation was detected year-round, the highest rates being measured during September (Fig. 6a). High CH_4 oxidation rates have been measured from the riverbed gravels, where the net C fixation via CH_4 oxidation was equivalent to 6% of the C fixed via net photosynthetic primary production (Trimmer et al. 2010). In the laboratory, the fluctuation in rates was stronger than in the field, which implies that the methanotroph community undergoes seasonal adaptation. Fluctuation was also evident in the laboratory N_2 fixation rates, which were higher than the corresponding N_2 fixation rates in the field (Fig. 6b). N_2 fixation remained low throughout the year, being highest in the growing season, from June to August. In addition, the effect of washing and light was tested on N_2 fixation and CH_4 oxidation every month, and washing enhanced N_2 fixation in both light and dark if CH_4 was present. This indicates that methanotrophs were responsible for N_2 fixation. CH_4 oxidation was higher in the light, and washing had a positive effect on the CH_4 oxidation rates as well. River water sometimes contains a lot of detritus, which adheres to mosses. The washing may have removed this and improved the uptake of N_2 and CH_4 from the water, suggesting that the bacteria are closely connected with the moss.

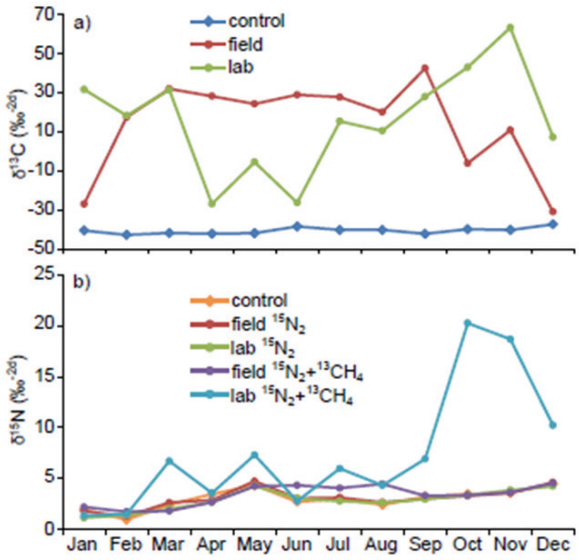

FIGURE 6 The season experiment. The a) CH_4 oxidation ($\delta^{13}C$) and b) N_2 fixation ($\delta^{15}N$) rates in *Fontinalis dalecarlica* from Liinajoki during year 2009. Field samples were incubated in the ambient conditions in the river and laboratory samples were incubated in the set conditions. N_2 fixation was measured with and without CH_4.

For the first time, N_2 fixation and CH_4 oxidation was showed to occur in river moss *F. dalecarlica*. Especially the CH_4 oxidation rates are noteworthy, being the highest measured among different mosses in this thesis. The biomass production in these habitats may mainly rely on C originated from methanotrophy and N_2 fixation aids the growth during summer seasons, but more studies are needed to resolve the higher scale implications of *F. dalecarlica* associated bacteria on the CH_4 and N cycles.

3.3 Identities and locations of diazotrophic bacteria in boreal mosses

3.3.1 *nifH* gene analysis (I-III)

The *nifH* sequences in the forest mosses *P. schreberi* and *H. splendens* were mostly affiliated (96 %) to *Cyanobacteria*, from which *Nostoc* and *Nodularia*-type sequences were equally present in all samples (I). No *nifH* genes affiliated to known methanotrophic genera were observed. This confirms the observation that *Cyanobacteria* are the prevailing diazotrophs in feather mosses (e.g. DeLuca et al. 2002, Ininbergs et al. 2011).

However, most of *Sphagnum* moss-associated diazotrophs in the fen (82 %) and the forest (65 %) belonged to the class *Alphaproteobacteria* (II), most closely

matching the heterotrophic bacterium *Bradyrhizobium elkanii* ORS391. Other *nifH* sequences were assigned to the genera *Methylocystis* and *Methylocapsa*, which belong to type II methanotrophs, and to the heterotrophic genera *Rhizobium, Azospirillum* and *Rhodopseudomonas*. Type II methanotrophs were common in the fen mosses, but less frequent in the forest mosses. Surprisingly, only a few of the *nifH* genes from *Sphagnum* mosses were affiliated to *Cyanobacteria* (*Nostoc*, fen 5 %, forest 6 %). The results are in line with Zadorina et al. (2009), in which alphaprotebacterial *nifH* genes predominated in the peat samples collected from a boreal oligotrophic *Sphagnum* bog. In addition, various culture-independent studies support the ecological importance of *Proteobacteria* in the peat accumulation of northern *Sphagnum* dominated wetlands (reviewed by Dedysh 2011) and in *Sphagnum* mosses themself (Bragina et al. 2012).

In *F. dalecarlica,* as many as 74 % of the *nifH* sequences belonged to a single OTU (MSC31), most closely matching the sequence of *Methylocystis* (AJ563952, type II methanotroph) (III). The importance of N_2-fixing methanotrophs in *F. dalecarlica* is supported also by the additional incubations (III), where CH_4 addition increased N_2 fixation and acetylene clearly inhibited CH_4-C assimilation. The bacterial composition and high CH_4 oxidation rates of *F. dalecarlica* suggest that *F. dalecarlica* has an important role in the CH_4 cycle in boreal rivers. However, no previous reports considering bacterial associates of *F. dalecarlica* are available.

The diazotroph community composition in the fen differed from that of the forest, as only 2 OTUs (HE600417 and HE600427) were shared, and *nifH*-DGGE revealed differences in the diazotroph community structures as well (II). However, no differences were found in the diazotroph communities between the sampling sites within the habitat types or between the *Sphagnum* species within the habitat. Nor did the N_2 fixation rates correlate with the diazotroph community structure in either habitat specific or pooled data. Ininbergs et al. (2011) found a connection between the *nifH*-carrying community composition and the N_2 fixation rate in forest moss *H. splendens*, but not in *P. schreberi*. Sorensen & Michelsen (2011) have proposed that responses in N_2 fixation rates can be mediated by both the moss species and the diazotrophs. Based on this, N_2 fixation in each habitat is more influenced by the moss species than by the diazotroph community structure.

3.3.2 Single cell analysis (III)

Bacteria were found from all the studied plant structures (inside hyaline cells, inside photosynthetic cells, inside plant cell walls and outside plant leaves) of *S. majus* (III). There were also bacteria inside the middle part of the branch stem, especially within the outermost, large plant cells. *Cyanobacteria* and heterotrophic bacteria have been found inside *Sphagnum* hyaline cells (e.g. Granhall & Hofsten 1976), but we also detected active bacteria within photosynthetic cells and plant cell walls, indicating a close relationship of these bacteria with the moss. Inside the plant cells bacteria were usually arranged in groups of 2–4 cells, and some of these groups were in contact with the plant cell wall. Individual bacterial cells

were also detected. In all samples the plant cells contained mucous-like material, which contained plenty of active bacteria. In some samples the mucous-like film formed thin strings, connecting bacteria or groups of bacteria together. One 24 h incubated Lahnajärvi sample also indicated that bacteria can excrete substances with high ^{15}N or with high ^{15}N and ^{13}C enrichment either inside or outside the branch leaf hyaline cell (Fig. 7a–d).

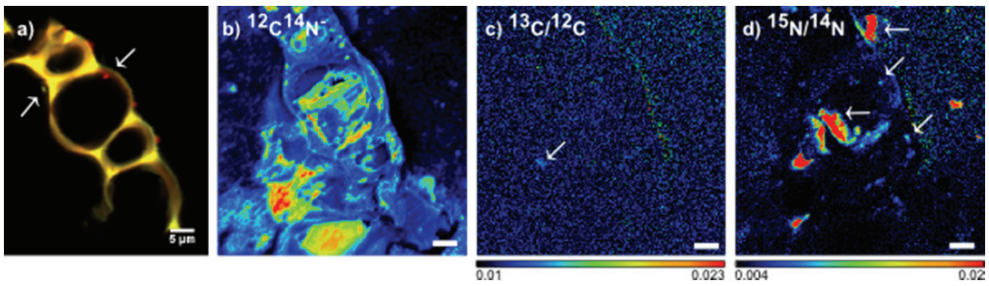

FIGURE 7 Cross section of *S. majus* branch leaf with a) *Alphaproteobacteria* (arrow pointing to a green bacterium) and other DAPI-stained bacteria (arrow pointing to a red bacterium). b) A secondary ion image of the corresponding leaf structure showing an overview of the leaf ($^{12}C^{14}N^-$ image), c) a $^{13}C/^{12}C$ ratio image showing CH_4-^{13}C enrichment (arrow, $\delta^{13}C = 45$) and d) a $^{15}N/^{14}N$ ratio image showing ^{15}N enrichment in bacterial exudates (horizontal arrows) and in bacteria attached to the plant cell wall (crooked arrows). The scale bars in b-d are 3 µm.

In *S. majus*, the bacterial cells in the leaf areas had higher N_2 fixation rates than the cells in the branch stem areas (III). Also the percentage of methanotrophs fixing N_2 was higher in the leaf (54 % of the methanotrophic cells) than in the branch stem (31 %). In the leaf and branch stem tissue, 7–11 % of active methanotroph as well as active diazotroph cells hybridized with the probe targeting *Alphaproteobacteria*, and from the N_2-fixing methanotrophs approx. 10 % were confirmed as *Alphaproteobacteria* by HISH-SIMS. The clone libraries based on the *nifH* gene sequences would have predicted higher abundance of N_2 fixing alphaproteobacterial methanotrophs; therefore it can be predicted that the number of alphaproteobacterial N_2-fixing methanotroph cells may be a serious underestimation. Permeabilization of the cells and hybridization reaction might need more optimization, or the probe match may not have been perfect, as the major part of the active bacteria was not targeted by our probe ALF968. Furthermore, although the cell size varied up to 1 µm, most of the bacteria were really small, only 0.5 µm in diameter, indicating that they belong to a group of unicellular ultramicrobacteria (Panikov 2005).

In the stream moss, *F. dalecarlica*, some ^{15}N enriched bacteria hybridized with the type II methanotroph specific probe Mα450 (III). However, only 1 bacterium with all the labels, ^{15}N and ^{13}C enrichments and type II methanotroph probe signal, was detected. Most of the microbes were located on the leaf, in the cavity between the leaf and stem, but there were also microbes inside the outer stem cells. Microbes in these locations are sheltered from the water current, which may promote a symbiotic relationship. A mucous-like material was also

detected in *F. dalecarlica* cells. The presence of mucous-like exudates may suggest a symbiotic relationship between the bacteria and moss (Solheim et al. 2004) or be a strategy for protection of the nitrogenase from O_2 (Postgate 1998). Possibly both of the explanations are valid, since the slime stabilizes the association and enables more efficient transfer of nutrients between the organisms (reviewed by Potts 1994).

3.4 Transfer of N and C from N_2 fixation and CH_4 oxidation into moss tissue (III, IV)

In *S. majus*, no signals of ^{15}N or CH_4-^{13}C tracers were ascertained within plant tissue, but fixed N_2 and oxidized CH_4 remained inside bacterial cells throughout the 24 h incubation period (III). It has been claimed that fixed N_2 is quickly used in the growth of *Sphagnum* moss instead of just being stored within the bacterial cells, as the transfer of cyanobacterially fixed N_2 into *Sphagnum* tissue was shown using ARA and the total N method (Berg et al. 2013). Even if our incubation period was relatively short, it is possible that fixed N_2 was already transferred from the vicinity of bacterial cells into the growing part of the moss when the incubation was terminated. This is a probable explanation, since in symbiotic relationships N_2-fixing *Cyanobacteria* tend to have an enhanced N_2 fixation rate (Meeks 1998), and also the inhibition of their dinitrogenase enzyme by N compounds is lowered (Sprent & Sprent 1990). In contrast, longer incubation might have increased the within-plant $^{13}CH_4$-signal. A large portion of *Sphagnum* moss C has been shown to originate from methanotrophy (Raghoebarsing et al. 2005, Kip et al. 2010, Larmola et al. 2010), but methodological differences (i.e. vastly different incubation times [1 d vs. 1–21 d] and natural isotopic labelling approaches) constrain comparisons of these works to the present study results.

Instead in the stream moss, *F. dalecarlica*, ^{15}N enrichment was not seen in the bacterial cells, but in the plant leaf (III). As the ^{15}N label was not seen in the bacterial cells, the transfer of N had probably taken place immediately after fixation. Fast transfer of fixed N_2 outside the bacterial cells has been previously shown with nanoSIMS between cyanobacteria and their diatom hosts (Foster et al. 2011) and between cyanobacteria and *P. schreberi* (Bay et al. 2013). However, no signal of $^{13}CH_4$ was detected inside the plant tissue, even if the CH_4 oxidation rates measured with SIRMS were high. The single cell analysis confirmed that the relationship between the bacteria and moss can be symbiotic, but it also indicates that the transfer of nutrients between the bacteria and its host is dependent on the environmental conditions.

At the mire chronosequence, N obtained by N_2 fixation corresponded on average to 37 % of the moss biomass N increment in flark vegetation, based on SIRMS data and on the known growth rates (Laine et al. 2011) and our measurements of the N contents of *Sphagnum* species at the study sites. This is within the same scale as in Berg et al. (2013) for *S. riparium* (35 %). The remaining

N is presumably being taken up from the moss as inorganic ions and organic N, or recycled to new growth from older parts of the moss stem (Aldous 2002). CH_4-C incorporation was faster in light than in dark, as also noted with *F. dalecarlica*, suggesting that mosses fixed additional CH_4-derived CO_2 in their photosynthesis. The contribution of CH_4-C to *Sphagnum* C in flarks was 26 % in light but 10 % in dark. These findings confirm that CH_4 can be a significant C source for submerged *Sphagnum*, as also shown in previous studies (Raghoebarsing et al. 2005, Larmola et al. 2010).

4 CONCLUSIONS

Thorough examination of biological N_2 fixation showed that bacterial N_2 fixation occurred at significant rates in boreal mosses; hence mosses form an important part of N economy in boreal and subarctic forests and peatlands due to their overall abundance and high biomass. The N_2 fixation rates varied between studied habitats, being highest in *Sphagnum* mosses at the mid-successional fens and lowest in the feather mosses at xeric forest sites, and at one forest area equalling the annual bulk N deposition. It was also shown that N obtained from N_2 fixation in flark mosses represented almost 40 % of N in the moss biomass. These high N_2 fixation rates explain the ability of rootless mosses to grow at low N deposition sites and to dominate the vegetation in nutrient-poor wetland habitats. In addition, N_2 fixation in the river moss *F. dalecarlica* was confirmed and the transfer of bacterially-fixed N_2 into plant tissue was shown, which indicate that this previously unstudied river moss might have an important role in the N dynamics of aquatic habitats. The high N_2 fixation activities measured also imply that boreal mosses sequester CO_2 to their biomass at notable rates.

The *nifH* gene sequencing together with the experimental light-dark incubations suggest that the main diazotrophs in *Sphagnum* mosses may belong to photoheterotrophic *Alphaproteobacteria*, e.g. *Rhodopseudomonas*, instead of *Cyanobacteria* as previously thought. In feather mosses *Cyanobacteria* remained the most abundant diazotrophic group, as expected. However, as the diazotroph-moss relationship is close, even symbiotic, it cannot be excluded that during daytime, the photosynthetic activity of the moss could enhance N_2 fixation activity of the other heterotrophic bacteria. In the future, quantitative PCR of messenger RNA would be a suitable way to study the identities of the active diazotrophs at different times of day and different seasons.

The N and CH_4 cycles were shown to be connected, since in *Sphagnum* mosses methanotrophic bacteria were also active participants in N_2 fixation. In flark mosses, the calculated contribution of CH_4-C was as much as one-third of the *Sphagnum* C content. The N_2-fixing methanotrophs would be especially important during the primary succession of mires, since they may be responsible for the rapid accumulation of N into the moss biomass. This also suggests that

Sphagnum moss-associated N_2 fixation should be studied with the $^{15}N_2$ tracer method instead of ARA, which was proven less sensitive at the low N_2 fixation activities in the feather moss study. The *nifH* gene analysis supported the role of methanotrophs in N_2 fixation, since several *nifH* sequences were affiliated to *Methylocystis* and *Methylocapsa* in the fen and forest *Sphagnum* mosses. In the river moss *F. dalecarlica*, the *nifH* sequences related to *Methylocystis* were clearly the most dominant (> 70 % of the sequences) group. The high CH_4 oxidation rates in *F. dalecarlica* imply that the biomass production in its habitats may mainly rely on C originated from methanotrophy, and N_2 fixation aids the growth during summer seasons. The single cell analysis confirmed that the methanotrophs were also able to fix N_2 alongside CH_4 oxidation. However, the ecological significance of methanotroph-driven N_2 fixation needs more attention, especially the temporal and spatial variation of CH_4-enhanced N_2 fixation.

The moss-associated N_2 fixation rates were affected by two main factors: the nutrient level of the site and the moisture level of the moss. N_2 fixation correlated with the P content, since it occurred rather in minerotrophic than in ombrotrophic conditions. In addition, N_2 fixation was always higher in moist conditions, supporting the hypothesis that N_2 was fixed by non-cyanobacterial diazotrophs, which can shelter their nitrogenase from O_2 only when submerged, and suggesting the importance of N_2-fixing methanotrophs. Therefore, abiotic factors characteristic to each habitat determine the prevailing moss species composition, forming the primary determinant in the moss-associated N_2 fixation rates, as shown.

In sum, moss-associated N_2 fixation forms an important N input in northern forests and wetland habitats, and is linked with C cycle via CH_4 oxidation and CO_2 sequestration.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Bakteerien typensidonta boreaalisissa sammalissa

Boreaalisen kasvillisuusvyöhykkeen metsiä ja soita voidaan pitää hiilinieluina (C), koska ne vähentävät tärkeimmän kasvihuonekaasun, hiilidioksidin (CO_2), määrää ilmakehästä. Näissä ympäristöissä kasvua rajoittava ravinne on yleensä typpi (N), joten biologisella typensidonnalla on tärkeä rooli uuden hiilen sitoutumisessa kasvibiomassaan. Biologiseksi typensidonnaksi kutsutaan bakteerien toimintaa, kun ne sitovat ilmakehän typpikaasua (N_2) ammoniumiksi (NH_4^+), joka voidaan käyttää solujen proteiini- tai nukleiinihapposynteesissä. Typensidonta kuluttaa paljon bakteerisolun energiaa, ja typpeä sitovia bakteereita kutsutaan diazotrofeiksi. Diazotrofeja on löydetty paljon erilaisista sammalista, kuten seinä- ja kerrossammalista (*Pleurozium schreberi* ja *Hylocomium splendens*) sekä rahkasammalista (*Sphagnum* sp.). Ajatellaan, että diazotrofit antavat osan sitomastaan typestä sammalelle, joka puolestaan takaa bakteereille suotuisan elinympäristön pitämällä kosteusolosuhteet ja hapen (O_2) sekä hiilidioksidin osapaineet typensidonnalle optimaalisina. Sinibakteereita (*Cyanobacteria*) pidetään sammalten yleisimpinä typensitojakumppaneina. Kuitenkin erityisesti rahkasammalvaltaisilta soilta on löydetty myös metaania (CH_4) hapettavia bakteereita (metanotrofeja), jotka elävät symbioosissa rahkasammalen kanssa luovuttaen metaaninhapetuksen lopputuotetta hiilidioksidia sammalelle yhteyttämistä varten. Jopa 30 % sammalen hiilestä voikin olla metanotrofeilta peräisin. Myös muita bakteereita, ns. heterotrofisia bakteereita, on löydetty sammalten pinnoilta ja rahkasammalten rahkasolujen sisältä. Metanotrofit ja heterotrofit kykenevät myös sitomaan typpeä, mutta niitä ei kuitenkaan ole aiemmin huomioitu tutkittaessa sammaliin liittyvää typensidontaa.

Tässä väitöskirjassa tutkittiin sammalten typensidontanopeuksia suomalaisissa typpiköyhissä ympäristöissä, kuten metsissä, soilla ja virtavesissä, vakaisiin isotooppeihin ($^{15}N_2$ ja $^{13}CH_4$) ja asetyleenin (C_2H_2) pelkistykseen perustuvin menetelmin. Myös metaanin vaikutusta typensidontanopeuksiin tarkasteltiin, koska erityisesti suot ovat tämän kasvihuonekaasun lähteitä, minkä vuoksi soilla on metaania ylimäärin. Metaanin hapetus saattaa joissakin olosuhteissa olla typpirajoitteista, joten typensidonta voisi silloin lisätä metanotrofien toimintaa ja siten olla yksi metaania ilmakehästä vähentävistä tekijöistä. Metanotrofien hapettaessa metaania vapautuu energiaa, joten tutkittiin myös, lisääkö metaaninhapetuksesta saatava energia typensidontanopeuksia. Lisäksi tutkittiin eri tekijöitä (esimerkiksi vedenpinnan taso, ympäristön ravinnepitoisuus, sammallaji), jotka vaikuttavat sammaliin ja sitä kautta diazotrofien typensidontanopeuksiin. Mittauksia tehtiin sekä sammalten että yksittäisten bakteerisolujen tasolla. Diazotrofien identiteettiä ja yhteisörakennetta tutkittiin molekyylimikrobiologian menetelmin.

Sammalten typensidontanopeudet vaihtelivat eri ympäristöjen välillä. Sukkession keskivaiheilla olevilla nevoilla rahkasammalet sitoivat typpeä eniten, kun taas metsäsammalten (seinä- ja kerrossammal) typensidontanopeudet

olivat alhaisimpia. Kuitenkin Pohjois-Suomessa eräällä tutkimusalueella metsäsammalten vuosittainen typensidonta oli samaa luokkaa alueen typpilaskeuman kanssa. Siikajoella rimmissä kasvavien rahkasammalten typestä 40 % oli peräisin biologisesta typensidonnasta, mikä myös ylitti vuosittaisen typpilaskeuman osalla soista. Virtavesisammaliin kuuluvalla virtanäkinsammalella (*Fontinalis dalecarlica*) typensidonta osoitettiin ensimmäistä kertaa ja lisäksi bakteerien sitoman typen siirto kasvin käyttöön näytettiin solutasolla. Täten virtanäkinsammalella saattaa olla tärkeä osa makean veden ympäristöjen typpitaloudessa. Yleisesti ottaen voidaan päätellä, että sammalten typensidonta on merkittävä osa boreaalisten metsien ja soiden typpitaloutta sekä näin ollen hiilidioksidin kiertoa. Lisäksi isotooppi- ja asetyleenimenetelmiä vertailtiin typensidonnan mittaamisessa, ja vertailussa selvisi, että kun typensidonta-aktiivisuus on suurta, molemmat menetelmät antavat yhtä tarkkoja tuloksia, mutta aktiivisuuden ollessa pientä isotooppimenetelmä on tarkempi.

Metanotrofit osallistuivat myös typensidontaan. Alkukesästä metaaninhapetus lisäsi typensidontaa, mutta loppukesästä ei. Metanotrofien typensidonta varmistettiin myös bakteerisolutasolla. Lisäksi Siikajoen rimpien rahkasammalten hiilestä kolmasosa oli metaaninhapetuksesta peräisin. Molekyylimikrobiologiset analyysit (*nifH*-geenin, joka koodaa typensidonnassa tarvittavaa proteiinia, sekvensointi) varmistivat metanotrofien osallisuuden sammalten typensidonnassa, sillä useat rahkasammalten diazotrofit kuuluivat *Methylocystis*- tai *Methylocapsa*-sukuihin. Virtanäkinsammalella yli 70 % diazotrofeista kuului *Methylocystis*-sukuun, ja lisäksi siltä mitattiin korkeimmat metaaninhapetusaktiivisuudet. Virtanäkinsammalen biomassan tuotanto on siis todennäköisesti suurelta osin riippuvainen metanotrofeilta saadusta hiilestä. Metanotrofien typensidontaa koskevien tulosten perusteella isotooppimenetelmä antaa luotettavamman arvion sammalten typensidonnasta kuin asetyleenimenetelmä, joka ei mittaa metanotrofien typensidonta-aktiivisuutta asetyleenin inhiboivan vaikutuksen takia. Typen- ja metaaninkierrot ovat siis yhteydessä toisiinsa, mutta metanotrofien typensidonnan ekologisen merkityksen selvittäminen vaatii vielä lisää tutkimusta.

Myös muut heterotrofiset bakteerit osallistuivat sammalten typensidontaan. *nifH*-geenin sekvensoinnin ja kokeellisten mittausten perusteella suurin osa rahkasammalten diazotrofeista kuuluu fotoheterotrofisiin alfaproteobakteereihin, esimerkiksi *Rhodopseudomonas*-sukuun eikä sinibakteereihin, kuten aikaisemmin on oletettu. Kuitenkin metsäsammalissa sinibakteerit olivat edelleen pääosin vastuussa typensidonnasta. Tieto, että heterotrofit ja metanotrofit osallistuvat typensidontaan sinibakteerien lisäksi, auttaa ymmärtämään paremmin eri ympäristötekijöiden aiheuttamia muutoksia typensidontanopeuksissa.

Tutkituista ympäristötekijöistä sammalten typensidontanopeuksiin vaikuttivat eniten alueen ravinnetaso sekä vedenpinnan taso. Typensidontanopeus korreloi positiivisesti sammalen fosforipitoisuuden (P) ja hiili-typpisuhteen (C/N) kanssa, joten typensidonta oli nopeampaa minerotrofisissa olosuhteissa kuin ombrotrofisissa. Typensidonta oli myös nopeampaa kosteissa kuin kuivissa olosuhteissa, mikä tukee heterotrofien ja metanotrofien roolia typensitojayh-

teisössä, koska sinibakteerit eivät hyödy korkeammasta kosteuspitoisuudesta samalla tavalla kuin muut bakteerit.

Yleisesti ottaen voidaan sanoa, että sammalten typensidonta muodostaa merkittävän typen lähteen boreaalisissa metsissä ja soilla. Lisäksi se on linkittynyt hiilen kiertoon metaaninhapetuksen ja sammalen hiilidioksidin yhteyttämisen kautta.

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ORIGINAL PAPERS

I

NITROGEN FIXATION AND METHANOTROPHY IN FOREST MOSSES ALONG A N DEPOSITION GRADIENT

by

Sanna M. Leppänen, Maija Salemaa, Aino Smolander, Raisa Mäkipää & Marja Tiirola 2013

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Nitrogen fixation and methanotrophy in forest mosses along a N deposition gradient

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ABSTRACT

Nitrogen deposition has decreased the plant-associated nitrogen (N_2) fixation when measured using the indirect acetylene reduction assay (ARA). However, nitrogen deposition can also lead to changes in the diversity of moss symbionts, e.g. affect methanotrophic N_2 fixation, which is not measured by ARA. To test this hypothesis we compared ARA with the direct stable isotope method ($^{15}N_2$ incorporation) and studied methanotrophy in two mosses, *Hylocomium splendens* and *Pleurozium schreberi*, collected from seven forest sites along a boreal latitudinal N deposition transect. We recognized that the two independent N_2 fixation measures gave corresponding results with the conversion factor of 3.3, but the $^{15}N_2$ method was more sensitive for finding a signal of low N_2 fixation activity. Methane carbon fixation associated with mosses was under the detection limit (<2 nmol C g^{-1} h^{-1}). N_2 fixation rates were more pronounced in the mosses with higher C/N ratio, and in the green upper parts of the shoot than in the lower brownish parts. Sequencing of *nifH* genes revealed that dominating diazotrophs were affiliated to cyanobacterial genera *Nostoc* and *Nodularia*, but methanotrophic diazotrophs were not found in the *nifH* libraries. We conclude that the suppression of N_2 fixation along the deposition gradient was consistent regardless of the measurement technique, and microbial community changes toward methanotrophic or otherwise acetylene-sensitive N_2 fixation could not explain this trend.

1. Introduction

Biological nitrogen (N_2) fixation of moss-associated microbes contributes a remarkable proportion of new nitrogen (N) used and cycled in boreal N limited forest ecosystems (DeLuca et al., 2002). Elevated N deposition can reduce the rate of N_2 fixation (Huttunen et al., 1981; van der Heijden et al., 2000; Pearce and van der Wal, 2002; Gundale et al., 2011; Ackermann et al., 2012) and the growth of mosses (Mäkipää, 1995; Salemaa et al., 2008b). The acetylene reduction assay (ARA; Hardy et al., 1968) is the most commonly used method for estimating biological N_2 fixation, and in this assay nitrogenase enzyme of the N_2-fixing bacteria (diazotrophs) reduces acetylene (C_2H_2) to ethylene (C_2H_4), analogous to the natural N_2 fixation. ARA measures the activity of nitrogenase correlating with N_2 fixation (Vessey, 1993). When nitrogenase acts in saturating concentrations of acetylene and H_2 production is inhibited, the conversion factor is 4.0 under ideal conditions (Capone, 1993; Mulholland et al., 2004). However, acetylene competes with N_2 (Schöllhorn and Burris, 1967) disrupting the N metabolism of bacterial cells in long term (Capone, 1993), and it also inhibits the function of methane (CH_4) monooxygenase in methane oxidizing bacteria (methanotrophs; DeBont and Mulder, 1976). Furthermore, some bacteria are able to use ethylene produced by diazotrophs during ARA as their growth substrate (Capone, 1993), which can cause underestimations in the ARA results. All of these mentioned factors might lead to unreliable N_2 fixation results especially in environmental samples, in which the bacterial composition is highly diverse and not fully elucidated.

A direct way to measure N_2 fixation is the $^{15}N_2$ method (also called as $^{15}N_2$ tracer method; Postgate, 1998, p. 17; Zehr and Montoya, 2007). However, most of the moss-associated N_2 fixation studies have still used ARA (e.g., Basilier, 1979; DeLuca et al., 2002; Zackrisson et al., 2004; Markham, 2009; Ininbergs et al., 2011; Ackermann et al., 2012), and to our knowledge, there is only one wider ecological study, which applied the direct $^{15}N_2$ method in forest moss research (Gavazov et al., 2010). Many N_2 fixation measurements have also been conducted for wetland-growing *Sphagnum* species (e.g., Kravchenko and Doroshenko, 2003; Markham, 2009; Sørensen and Michelsen, 2011). *Sphagnum* mosses are known to inhabit symbiotic methanotrophs, which can supply even 10–30% of the moss carbon in some mires (Raghoebarsing et al., 2005; Larmola et al., 2010). A recent study showed that methane addition stimulated N_2 fixation in soil due to type II methanotrophs (Buckley et al., 2008), but the role of

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methanotrophic diazotrophy is still an open question in most habitats. As methanotrophs are known to be especially vulnerable to acetylene inhibition (DeBont and Mulder, 1976; Urmann et al., 2008), it is important to know which bacteria are responsible for N_2 fixation before interpreting studies using ARA.

We hypothesize that if the methanotrophs are significantly fixing nitrogen in forest mosses, observed inverted correlation between nitrogen deposition and N_2 fixation might be explained by the methodological biases by ARA, when acetylene-sensitive diazotrophy has remained undetected. Our aims in this study were therefore (1) to evaluate the consistency of the indirect (ARA) and direct ($^{15}N_2$) methods in detecting N_2 fixation across moss species, shoot parts, and a nitrogen deposition gradient, and (2) to study whether N_2 fixation in forest mosses can be explained by methanotrophy, which has recently shown to be a significant process in wetland mosses. To achieve these aims parallel acetylene and dual-label ($^{15}N_2$ and $^{13}CH_4$) stable isotope incubations were performed for moss samples collected from a latitudinal boreal transect, and the results were further supported by sequencing of the *nifH* gene libraries to reveal the dominating diazotrophic microbes.

2. Materials and methods

2.1. Study sites, moss sampling, and incubation conditions

Moss samples were collected from seven intensively monitored forest sites (ICP Forests Level II) along a latitudinal transect in Finland in September 2010. Four sites were located in southern and three in northern boreal zone (Fig. 1). Five of the sites were dominated by Scots pine (*Pinus sylvestris* L.) and two by Norway spruce (*Picea abies* L. (Karst)). The understory of all stands represented typical boreal vegetation rich in mosses and dwarf shrubs (Salemaa et al., 2008a). The basic site characteristics including total N deposition are given in Table 1 and more information about understory vegetation, air quality, and wet deposition are presented in the national report of forest condition monitoring in Finland (Derome et al., 2007). A total of 15 sub-samples (a 500 ml container filled with moss shoots) were collected according to systematic sampling design from each site. *Hylocomium splendens* did not grow in the site 1, and its sampling area was extended to some 200 m apart from the monitoring site. The moss samples were stored in the dark at 4 °C for 1–2 weeks until the experiment started. Prior to incubations, mosses were cleaned from forest litter and detritus. In all seven sites, the green upper parts of *Pleurozium schreberi* and *H. splendens* shoots (4 cm from the top, representing 2–3 annual growth segments) were taken for analysis. In the two spruce sites (sites 2 and 6, Fig. 1), also the lower yellowish or brown parts of the shoots (3-6 cm that was left after cutting upper part) were studied.

The incubation samples were formed from the sub-samples so that the effect of the between-shoot spatial variation in N_2 fixation was minimized. The incubation sample of *P. schreberi* consisted of 30 shoots, so that there were always two shoots from each 15 sub-sample of the site. The incubation sample of *H. splendens* consisted of 15 shoots, one shoot from each 15 sub-samples of the site. The shoots, i.e. 15 or 30 pc, representing one incubation sample, were placed in a 125 ml glass vial. From each sampling site, there were three incubation replicates and one control sample of both moss species for both ARA and the $^{15}N_2$ method. The total number of incubation samples was 54 in both methods (upper parts: 7 (sites) × 2 (species) × 3 (replicates); lower part: 2 (sites) × 2 (species) × 3 (replicates)).

The incubation samples, called as samples hereafter, were acclimatized in the laboratory (room temperature and natural light conditions) for two days and further two days at the common incubation site (in the yard of the institute, Vantaa) in the ambient

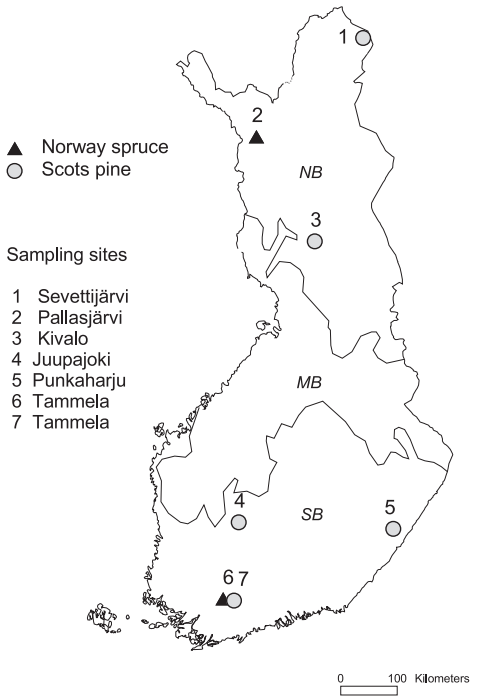

Fig. 1. Locations of the sampling sites of *Hylocomium splendens* and *Pleurozium schreberi* in the climate zones of Finland (NB = northern boreal, MB = middle boreal, SB = southern boreal climate zone). The point symbol indicates the dominant tree species.

conditions. Two days before incubations 5 ml of distilled water was added to allow optimal moisture conditions for N_2 fixation (tested with a pilot experiment). The openings of the vials were loosely covered with tin foil while the acclimatization. When the experiment started, the vials were closed with gas-tight butyl rubber caps. The experimental incubations were conducted at the common incubation site at the prevailing light and temperature conditions shaded by direct sunlight in September 2010. The intensity of photosynthetically active radiation in the early afternoon measured at the moss incubation location ranged from 120 to 160 μmol s^{-1} m^{-2} (Li-Cor LI-190SA). Mean outdoor temperature during the incubation period of 24 h (measured every 15 min with a datalogger) was 9.5 °C (min 4.5 °C, max 13.5 °C) and mean temperature in the vials with mosses was 0.5 °C higher.

2.2. Acetylene reduction assay (ARA)

After closing the incubation vials, 10% of the headspace was evacuated and replaced with acetylene. The ethylene evolved was measured after 24 h with a gas chromatograph (HP 6890), equipped with a flame ionization detector and a HP-PLOT MoleSieve5A capillary column (30.0 m × 530 μm × 50 μm) using He as a carrier gas. The temperatures of the detector, injector, and oven were 250 °C, 120 °C, and 50 °C, respectively. Column head pressure was 10 psi and column flow was 40 ml/min. Injection was manual injection

Table 1
Stand characteristics, site type, and total nitrogen (N) in bulk deposition in the study sites.

Site	Latitude	Tree species	Site type	Stand volume ($m^3\ ha^{-1}$)	N in organic layer (%)	N tot in bulk deposition ($kg\ ha^{-2}\ y^{-1}$)[a]	Stand age (years)
1	69°N	Pine	Xeric	76.4	0.71	0.83	205
2	67°N	Spruce	Mesic	72.8	0.85	1.48	145
3	64°N	Pine	Sub-xeric	167.3	0.88	1.95	60
4	61°N	Pine	Sub-xeric	210.6	1.26	2.93	85
5	61°N	Pine	Sub-xeric	358.6	0.94	2.73	85
6	60°N	Spruce	Mesic	309.4	1.18	3.72	65
7	60°N	Pine	Sub-xeric	254.5	1.07	3.85	65

[a] Year 2010 (unpublished results, A.-J. Lindroos, Finnish Forest Research Institute).

with split ratio 1:5. Endogenous ethylene release was measured from control bottles without acetylene addition.

2.3. Stable isotope analysis ($^{15}N_2$ and $^{13}CH_4$ methods)

After closing the incubation vials, 15 ml of air was removed from the vials to make space for enriched gases. Then the heavy isotope tracer gases were injected into the incubation vials, consisting of 20 ml of $^{15}N_2$ (98% enriched, Cambridge Isotope Laboratories Inc, USA) and 100 μl of $^{13}CH_4$ (99% enriched, CK Gas Products Ltd., GB). When mixed with $^{14}N_2$ and $^{12}CH_4$ present in air in the incubation vials, the final enrichment levels were 20% for $^{15}N_2$ and 82% for $^{13}CH_4$. Control samples were incubated in the ambient air. Incubation was terminated after 48 h by drying the samples at 60 °C for 60 h to constant weight. Samples were homogenized with scissors and then re-homogenized in 2 ml screw-cap plastic tubes using a bead mill (Microdismembrator U, B. Braun Bioteck Int) with one stainless steel bead (diameter 5 mm) at the rate 1500 rpm for 3 min. Then the samples were weighed (dry weight > 1.8 mg) into tin cups. The samples were combusted in an elemental analyzer (Carlo Erba Flash EA1112) coupled to a continuous flow isotope mass spectrometer (CF/IRMS, Thermo-Finnigan, Delta^{Plus} Advantage) to analyze C and N contents and stable isotope ratios of the samples. The carbon and nitrogen contents of moss samples were 45.0%(0.69%) and 0.73%(0.18%), respectively, expressed as mean (S.D.). The C/N relationship was in average 64:1, but 77:1 for northern moss samples collected from sites 1 to 3 and 53:1 for southern samples from sites 4 to 7. Stable isotope results were expressed using the standard δ notation as parts per thousand (‰) differences from the international standard (Vienna-Pee-Dee Belemnite (VPDB) for $\delta^{13}C$ and atmospheric N_2 for $\delta^{15}N$). Internal precision was always better than 0.18‰ for C and 0.12‰ for N, based on the standard deviation of replicates of the standards analyzed along the sample set. The N_2 fixation and the CH_4-carbon intake (CH_4 oxidation) was determined by calculating enrichment values, $\delta^{15}N$ and $\delta^{13}C$ (Fry, 2006, Technical supplement 6B: noisy data and data analysis with enriched samples) for the samples. The enrichment values represent the actual ^{15}N and ^{13}C accumulation in each sample at the time when the incubations were conducted (see Appendix for details of calculations).

2.4. Molecular microbiological analysis

Cloning and sequencing of the *nifH* genes was conducted for three *H. splendens* samples and two *P. schreberi* samples from the northern boreal forest sites. Samples of the upper 4 cm parts of the moss shoots were collected and pooled during original sampling as described in Section 2.1 and kept frozen until DNA extraction (−20 °C). Nucleic acids were extracted from 0.3 to 0.8 mg of the moss samples using the protocol of Griffiths et al. (2000) with the following modifications. The extractions were made in 1.5 ml steel tubes with a mixture of 0.5 mm and 0.1 mm glass beads (400 mg) and five 2.3 mm chromed steel beads (tubes and beads from BioSpec Products Inc., Bartlesville, USA). After the first centrifugation, the aqueous phase containing nucleic acids was transferred to 2 ml transparent screw cap tube and centrifuged again. After phenol–chloroform and chloroform extractions nucleic acids were precipitated from the extracted aqueous layer with 0.1 vol 7.5 M ammonium-acetate and 0.6 vol isopropanol at room temperature for 30 min, followed by centrifugation (18 000 × *g*) at room temperature for 20 min. After additional ethanol precipitation the pellets were resuspended in 75 μl of TE-buffer.

Amplification of the *nifH* sequences of divergent diazotrophs from DNA extracts was done using a nested PCR protocol according to Demba Diallo et al. (2004). The primer sets used were: FGPH19 (5′-TAC GGC AAR GGT GGN ATH G-3′) and PolR (5′-ATS GCC ATC ATY TCR CCG-3′) for the first round, and PolF (5′-TGC GAY CCS AAR GCB GAC TC-3′) and AQER (5′-GAC GAT GTA GAT YTC CTG-3′) for the second round. PCR mixtures consisted of 1× PCR buffer (DreamTaqGreen, including 4 mM $MgCl_2$; Fermentas), 0.2 mM each deoxynucleotide triphosphate, 0.3 μM each oligonucleotide primer and 1 mg ml^{-1} bovine serum albumin (Fermentas, St. Leon-Rot, Germany). In the first PCR reaction, 0.5 μl of extracted DNA was used as a template in a 20 μl PCR volume containing 1 U DreamTaq DNA polymerase. In the second PCR reaction, 0.5 μl of the PCR product from the first round was used as a template in a 25 μl PCR volume. The following cycling conditions were used in both the PCR reactions: an initial denaturation at 95 °C for 5 min and 30 cycles of amplification (94 °C for 30 s, 53 °C for 1 min, 72 °C for 3 min).

The *nifH* gene amplicons were cloned using a pCR®II-TOPO® (Invitrogen) cloning kit according to the manufacturer's instructions. Both strands were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI3100 capillary sequencing instrument (Applied Biosystems). The sequences were edited using ContigExpress (Invitrogen) and compared to *nifH* sequences in the EMBL database using BLASTN (Altschul et al., 1997) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were deposited to the EMBL database under accession numbers HE803096–HE803334, including *H. splendens* libraries from site 1, 2 and 3 (database libraries 1, 5 and 4, respectively) and *P. schreberi* libraries from sites 2 and 3 (libraries 6 and 3).

2.5. Statistical analyses

We determined the conversion factor for acetylene reduction/ethylene production values to N_2 fixation rates using the slope of the linear regression model. Regression was calculated between ARA and $^{15}N_2$ methods from the site-specific mean values ($n = 3$ per site) separately for both species and for the combined data setting the intercept as zero.

The similarity of N_2 fixation rates gained with the $^{15}N_2$ and ARA methods was compared using independent samples *t*-test for square root transformed values of subsamples (keeping three replicates per site separate). In addition, we used three-factor ANOVA for testing the effect of the method, moss species and N deposition, and their interaction on the site-specific means of N_2 fixation

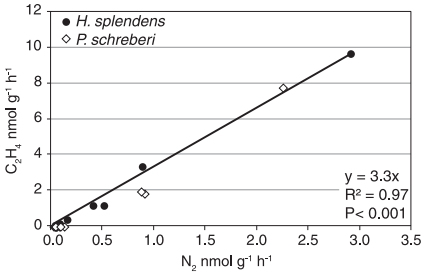

Fig. 2. Regression equation between ethylene production measured by the acetylene reduction method (C_2H_4 nmol g^{-1} h^{-1}) and N_2 fixation measured by the $^{15}N_2$ method (N_2 nmol g^{-1} h^{-1}) for site-specific mean values (n = 3 replicates for each data point). According to the slope (conversion factor), 3.3 mol of C_2H_4 equals each mol of N_2. Data points of the two moss species are indicated by different symbols.

rates. Similarly, three-factor ANOVA was used in testing the effect of method, species and shoot part (upper or lower part) on N_2 fixation rates in one northern site using data of subsamples. Mann-Whitney test was used when comparing N_2 fixation rates between the species within individual sites. PASW Statistics 18.0 (PASW Statistics 18, Release Version 18.0.0, SPSS Inc.) was used for all the analyses.

3. Results

3.1. Comparison of ARA and the $^{15}N_2$ method

The minimum amount of ethylene production the gas chromatograph reliably measured was 0.6 nmol $bottle^{-1}$ h^{-1}. In this study the lowest N_2 fixation values reliably detected and converted to N_2 ranged from 0.2 to 0.5 nmol N_2 g^{-1} h^{-1}, depending on the weight of mosses in the bottle. Of the 54 acetylene treated samples 24 showed ethylene signal. No endogenous ethylene production was observed.

The detection limit for ^{15}N enrichments in IRMS was 0.24‰ (2 × SD of the internal standard delta values), which equals the amount of 0.03 nmol N g^{-1} h^{-1} at 20% $^{15}N_2$ labeling in the incubation. Since from 54 $^{15}N_2$ treated samples 50 had enriched $\delta^{15}N$ values, the $^{15}N_2$ method found more often N_2 fixation signal than ARA in the studied sites (Table 2).

The N_2 fixation values obtained with the $^{15}N_2$ and ARA methods did not differ between the northern boreal samples (t = 0.30, df = 34, P = 0.763), but between the southern samples the two methods were inconsistent (t = 5.80, df = 46, P < 0.001), when using the joint data of both the species and all subsamples. At the lowest ethylene concentrations with relatively high uncertainties, ARA gave lower N_2 fixation values than the $^{15}N_2$ method. However the three-factor ANOVA did not find differences between the methods when analyzing the effects of the method, species and deposition level using site-specific means. The only significant effect on the N_2 fixation was observed by the deposition level (F = 6.01, P = 0.003).

The conversion factor for the pooled data of both species was gained as the slope of the regression equation between ARA and the $^{15}N_2$ method (Fig. 2):

$$C_2H_4(\text{nmol } g^{-1}\,h^{-1}) = 3.3 \times N_2(\text{nmol } g^{-1}\,h^{-1})$$

Hence 3.3 nmol of ethylene represents 1 nmol of fixed N_2. When analyzed separately for the two moss species, the conversion factor was 3.2 for *P. schreberi* and 3.4 for *H. splendens*.

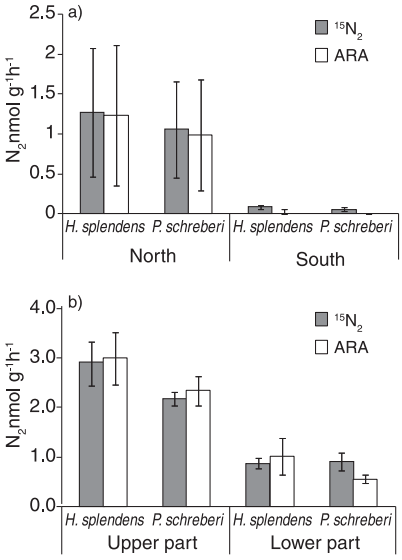

Fig. 3. The mean (±SE) N_2 fixation rate (N_2 nmol g^{-1} h^{-1}) (a) in the upper parts of *H. splendens* and *P. schreberi* shoots in North (n = 3 sites) and South Finland (n = 4 sites) measured by the $^{15}N_2$ and ARA methods, and (b) in the upper and lower parts of *H. splendens* and *P. schreberi* in the spruce forest site 2 (n = 3 replicates). Northern sites with lower N deposition levels (0.83–1.95 kg N ha^{-1} y^{-1}) showed higher N_2 fixation rates than southern ones (2.73–3.85 kg N ha^{-1} y^{-1}) and upper shoot parts showed higher N_2 fixation rates than lower parts.

3.2. The effect of latitude, C/N relationship, moss species and shoot part on the N_2 fixation activity

Both the methods indicated that N_2 fixation was higher in the northern sites with lower N deposition levels (0.83-1.95 kg N ha^{-1} y^{-1}) than in the southern sites with higher N deposition levels (2.73-3.85 kg N ha^{-1} y^{-1}) in both the species. The highest N_2 fixation rates were found in the site 2 that was an old spruce forest in the northern boreal zone (Tables 1 and 2). In overall, remarkable N_2 fixation (>0.2 nmol N g^{-1} h^{-1}) was observed in mosses with C/N ratio higher than 65:1 (Fig. 4).

Statistically significant differences between the moss species were not observed, although there was a trend for higher N_2 fixation rates in *H. splendens* than in *P. schreberi* (Table 2). When the effect of the method ($^{15}N_2$, ARA), shoot part (upper, lower) and moss species (*H. splendens* and *P. schreberi*) were studied by three-factor ANOVA using replicate samples from the site 2 (northern spruce forest), significant or almost significant effects on the N_2 fixation rate were observed by the moss part (F = 64.03, P = 0.000) and moss species (F = 4.11, P = 0.060). Although more N_2 was fixed in the upper (green) part of the mosses than in the lower part (Fig. 3b) in the site 2, the effect was not seen in the other spruce forest (site 6), located in the southern Finland, due to very low N_2 fixation in the samples in overall.

3.3. CH_4 oxidation activity

The $\delta^{13}C$ enrichment values for moss samples of all seven sites were under the detection limit (0.36‰), which equals the rate of

Table 2
N_2 fixation rates (mean and SE, $n = 3$) of the two moss species (*Hylocomium splendens* and *Pleurozium schreberi*) in seven study sites (Fig. 1) expressed as fixed N_2 in the $^{15}N_2$ method or produced ethylene in ARA, calculated for sample mass and areal activity. u.d. = under detection limit.

Site	Species	$^{15}N_2$ method				Acetylene reduction method (ARA)			
		N_2		N_2		C_2H_4		C_2H_4[a]	
		nmol g^{-1} h^{-1}	SE	μmol m^{-2} d^{-1}	SE	nmol g^{-1} h^{-1}	SE	μmol m^{-2} d^{-1}	SE
1	*H. splendens*	0.51	0.04	3.69	0.28	1.22	0.09	8.74	0.66
1	*P. schreberi*	0.88	0.02	9.57	0.25	1.96	0.04	21.31	0.39
2	*H. splendens*	2.89	0.44	23.63	3.59	9.88	1.77	80.78	14.49
2	*H. splendens*[b]	0.88	0.11	5.54	0.71	3.35	1.21	21.13	7.60
2	*P. schreberi*	2.18	0.14	20.37	1.29	7.73	0.98	72.08	9.16
2	*P. schreberi*[b]	0.91	0.18	9.19	1.81	1.84	0.29	18.51	2.89
3	*H. splendens*	0.41	0.08	1.67	0.32	1.14	0.31	4.62	1.24
3	*P. schreberi*	0.11	0.11	0.69	0.69	0.11	0.11	0.74	0.74
4	*H. splendens*	0.10	0.03	0.66	0.18	u.d.		u.d.	
4	*P. schreberi*	0.05	0.03	0.34	0.19	u.d.		u.d.	
5	*H. splendens*	0.15	0.04	1.02	0.29	0.38	0.14	2.60	0.96
5	*P. schreberi*	0.13	0.03	0.94	0.24	u.d.		u.d.	
6	*H. splendens*	0.05	0.01	0.35	0.04	u.d.		u.d.	
6	*H. splendens*[b]	0.09	0.03	0.24	0.09	u.d.		u.d.	
6	*P. schreberi*	0.04	0.02	0.24	0.13	u.d.		u.d.	
6	*P. schreberi*[b]	0.04	0.01	0.26	0.07	u.d.		u.d.	
7	*H. splendens*	0.05	0.03	0.37	0.17	u.d.		u.d.	
7	*P. schreberi*	0.03	0.01	0.19	0.06	u.d.		u.d.	

[a] The area of one shoot in different sites varied between 0.34 and 0.78 cm^2 in *P. schreberi* and 0.98 and 1.95 cm^2 in *H. splendens*.
[b] Lower (brownish) part of the shoot.

1.8 nmol C g^{-1} h^{-1}. It should be noted that the mean of measured $\delta^{13}C$ enrichments were markedly (over 20 times) lower than the mean $\delta^{15}N$ enrichments, although the labeling percentage in the incubation was four times higher for CH_4 than for N_2, which also indicates that methanotrophy was an insignificant process compared to N_2 fixation.

3.4. Microbial communities

The identity of the diazotrophs was analyzed by sequencing the *nifH* genes from the northern boreal forest samples. Of the 205 blasted sequences 96% were cyanobacterial, with closest matches to *nifH* genes of genera *Nostoc* (58%), *Nodularia* (25%), *Mastigocladus* (8%), *Tolypothrix* (4%) and *Fisherella* (2%). *Nostoc* and *Nodularia*-type sequences were equally present in all the samples, while most *Mastigocladus* and *Tolypothrix*-type sequences were obtained from the *P. schreberi* sample from site 2. No *nifH* genes affiliated to known methanotrophic genera were observed in the libraries.

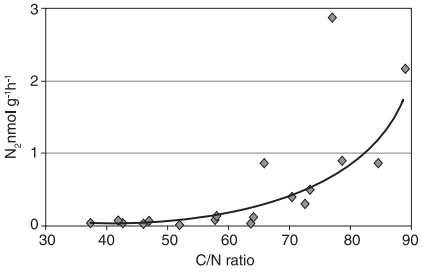

Fig. 4. Relationship between the C/N ratio of the moss sample and the N_2 fixation rate (N_2 nmol g^{-1} h^{-1}) measured by the $^{15}N_2$ method. Site-specific mean values ($n = 3$ replicates) of *H. splendens* and *P. schreberi* samples given.

4. Discussion

4.1. Methodological consistency between ARA and $^{15}N_2$ incorporation

The conversion factor (3.3) calculated for the pooled data of both species is in the same order of magnitude as in the previous moss studies. DeLuca et al. (2002) obtained the conversion factor of 3.0 for *P. schreberi*, and that value has been widely applied in other studies. Lagerström et al. (2007) determined the same factor for *H. splendens*. The obtained factors are close to the theoretical value of 4.0 (Capone, 1993). Zackrisson et al. (2009) gained conversion factor 3.3 for *H. splendens*, but instead of using that they decided to keep the same factor that was determined for *P. schreberi* (DeLuca et al., 2002) based on the similar cyanobacterial microbiota. When the overall N_2 fixation activity was low and the ethylene production was <2.0 nmol g^{-1} h^{-1}, as was the case in our moss samples from southern boreal forests, sample-specific conversion factors decreased and the variation increased. The same phenomenon was noticed in the methodological comparison by Montoya et al. (1996), when studying cyanobacterial samples of the Baltic Sea. The smaller and varying conversion factor might reflect e.g. microbial usage of the produced ethylene (Capone, 1993), changes in the host plant stress (Schwinzer and Tjepkema, 1994) or differences in the symbiotic relationship between the host and the cyanobacteria (Postgate, 1998, pp. 42–43). However, the differences in the second digit of the conversion factor may have only limited importance compared to the other uncertainties in the quantification of N_2 fixation, since other factors such as moss biomass (Sørensen and Michelsen, 2011; Gundale et al., 2011; Stewart et al., 2011), climate factors such as temperature, water, light and N-deposition (e.g. Gentili et al., 2005; Gundale et al., 2009; Sørensen et al., 2012; Gundale et al., 2012a,b), and length of the active period may have even more remarkable effects in wider ecological calculations. Thus, beside conversion from ethylene production to the actual amount of fixed N_2 also other factors involved in scaling to areal and yearly N_2 fixation values should be evaluated.

It is recommended to determine the conversion factor with the $^{15}N_2$ method each time when ARA is used (Zehr and Montoya, 2007;

Gavazov et al., 2010). However, most of the studies on mosses (e.g., Markham, 2009; Gundale et al., 2010; Jackson et al., 2010; Ininbergs et al., 2011) have earlier used the conversion factor of 3, which was obtained by measuring few replicate samples of *P. schreberi* (DeLuca et al., 2002) or *H. splendens* (Lagerström et al., 2007). Several studies have shown that acetylene reduction activity, that is used as an indicator of N_2 fixation, is affected by temperature, moisture, light and N deposition (Gentili et al., 2005; Gundale et al., 2011, 2012a; Sørensen & Michelsen, 2011). Some methodological comparisons for ARA and the $^{15}N_2$ method have been made with N_2-fixing aquatic cyanobacteria (Moisander et al., 1996; Montoya et al., 1996; Kayanne et al., 2005). Thus, further testing whether the conversion factor applies with different moss species across contrasting environmental conditions was needed for mosses and their associated bacteria.

4.2. Comparison of ARA and the $^{15}N_2$ method

The comparison of the two methods is challenging, although both measure the functioning of the same enzyme, nitrogenase. ARA measures ethylene production in the bottle headspace, while the $^{15}N_2$ method measures actual N-incorporation. In this comparison the $^{15}N_2$ method proved to be more sensitive than ARA at low N_2 fixation levels, which has also been noted in the previous studies (Montoya et al., 1996; Kayanne et al., 2005). Despite the presumably lower sensitivity, the advantage of ARA over the $^{15}N_2$ method is its cheapness and suitability for large number of samples. We therefore agree that ARA would best suit for screening noteworthy ability to fix N_2 (Rennie and Rennie, 1983; Vessey, 1993), for real-time measurements, and for collection of time series data (Montoya et al., 1996). ARA is a fast and cost efficient technique when screening large number of samples, while the $^{15}N_2$ method may be more accurate with low activities and if the microbiology is not known. The development of the multiple collector IRMS (Wieser and Schwieters, 2004) and an addition of an automated continuous-flow peripheral to IRMS has also greatly reduced the laboriousness and costs of the $^{15}N_2$ method. However, the need of $^{15}N_2$ tracer gas raises the price of the $^{15}N_2$ method. Both methods (ARA and the $^{15}N_2$ method) require in vitro incubation, so the data gained represents N_2 fixation activity within few hours or days, while the long-term data is usually more difficult to acquire. ARA has the advantage that the ethylene production of the same sample can be measured at different time points, but the inhibitory effects of acetylene exposure and blocking of methanotrophic organisms can last several weeks (DeBont and Mulder, 1976; Urmann et al., 2008).

In moss related N_2 fixation studies, the calibration of ARA with the $^{15}N_2$ method has usually been conducted for already once acetylene incubated samples (DeLuca et al., 2002; Zackrisson et al., 2004; Lagerström et al., 2007). However, repeated acetylene incubations may affect the conversion factor. In our preliminary testing, long and repetitive acetylene incubations (3 times 48 h within 2 weeks) affected the sample microbiota and blocked the actual N uptake of diazotrophs, which was detected by the increase of the conversion factor to over 8 (data not shown). Therefore we think that for avoiding the disturbance of nitrogenase enzymes of diazotrophs, calibration of ARA with parallel $^{15}N_2$ samples would be a more reliable way than performing serial measurements of individual samples with ARA and then with the $^{15}N_2$ method. With a good sampling practice and randomization parallel samples should provide both accurate and precise data.

Both methods showed that the upper part of the shoot fixed N_2 more than the lower part in both tested moss species, which supports the important role of light in N_2 fixation (Meeks, 1998; Gundale et al., 2012a). Differential N_2 fixation rates for different parts of *H. splendens* were also measured by Gavazov et al. (2010). As the activity varies in different parts of the plants, whole stem incubations in their natural spatial orientation would be essential for the most accurate upscaling and analyses of areal N_2 fixation.

4.3. N_2 fixation rates

N_2 fixation activity was higher in the mosses from the northern sites than those obtained from the southern sites, and one reason for this might be the atmospheric deposition of N, which is lower in the north. N concentration of forests mosses is known to correlate with N deposition level (Poikolainen et al., 2009), which was also evident in our data set as northern samples had higher C/N ratio (77:1) than the southern ones (53:1). Our results are also in line with the study of Zackrisson et al. (2009), where moss-associated cyanobacterial N_2 fixation rates increased north of 64°N in *P. schreberi* and *H. splendens*. Gundale et al. (2011) showed that N_2 fixation in mosses was downregulated already with N additions of 3 kg N ha^{-1} y^{-1}, which corresponds to the nitrogen deposition in our southern sites. Huttunen et al. (1981) demonstrated in a moss transplant experiment that N_2 fixation in forest mosses decreased significantly in polluted areas in Finland. Also Ackermann et al. (2012) found that N deposition inhibited N_2 fixation in mosses close to busy roads in northern Sweden and suggested N_2 fixation to be highly sensitive indicator for increased N loads to ecosystems.

Although not statistically significant, *H. splendens* had a trend to fix N_2 more actively than *P. schreberi* in all sites, except in site 1, where moss samples of different species were collected from different locations (some hundreds of meters apart from each other). Zackrisson et al. (2009) found out that *H. splendens* is tolerant to low amounts of N fertilizer (5 kg N ha^{-1} y^{-1}), whereas *P. schreberi* was already negatively affected by such N supply (Zackrisson et al., 2004). This indicates that *H. splendens* might be able to fix N_2 more effectively in areas of atmospheric N deposition. In the studies of Zackrisson et al. (2009) and Ininbergs et al. (2011), *P. schreberi* was more active N_2 fixer than *H. splendens*, but in the studies of Huttunen et al. (1981), Lagerström et al. (2007), and Gavazov et al. (2010) *H. splendens* proved to be more active. In our case site 1 was the most northern site studied with the lowest N deposition, which could explain the higher activity of *P. schreberi*. In the high latitude forests with low canopy cover, the N_2 fixation in mosses is also favored by light and moisture availability (e.g. Gundale et al., 2009, 2012a; Sørensen and Michelsen, 2011).

The N_2 fixation rates calculated in this study are in line with the previous studies conducted at the same northern latitudes. Our acetylene reduction rates of *P. schreberi* in sites 1 and 2 are in the same order of magnitude as measured in northern Sweden (DeLuca et al., 2002; Zackrisson et al., 2004; Lagerström et al., 2007). When compared to the $^{15}N_2$ labeling study also conducted in northern Sweden (Gavazov et al., 2010), our N_2 fixation rates are 2–5 times higher for *P. schreberi*, but similar for *H. splendens*. Another N_2 fixation study (Huttunen et al., 1981) conducted in coastal parts of Finland gained markedly higher variation in the acetylene reduction rates between *P. schreberi* and *H. splendens* than detected in this study. It seems that overall N_2 fixation rates associated to these two forest mosses in northern latitudes remain about the same level. The slight variation noticed between the studies might arise due to seasonal changes or from deviating sampling procedures as different parts of moss shoots harbor varying N_2 fixation activities.

4.4. Nitrogen fixation by methanotrophic or other acetylene-sensitive bacteria

The stable isotope analysis indicated that $^{13}CH_4$-carbon assimilation in this set of samples was always relatively low (<2 nmol C g^{-1} h^{-1}), hence the lower sensitivity obtained with ARA is not explained by ceased methanotrophic N_2 fixation. When

potential methane oxidation was measured in a wide peatland study of 41 *Sphagnum* species (Larmola et al., 2010), methane oxidation varied between 0 and 2.6 μmol C g^{-1} h^{-1}, which is in a different scale. Furthermore, the *nifH* sequences obtained in our study revealed that most of the forest-moss associated diazotrophs belonged to the phylum *Cyanobacteria*. Together with the high correlation between the two measurement techniques this indicated that N_2 fixation has, indeed, a geographical gradient, which was not due to methodological constraints. When considering the previously reported positive correlation of N concentration of forest mosses and N deposition level (Poikolainen et al., 2009) and the finding that higher N_2 fixation values (>0.2 nmol N g^{-1} h^{-1}) were only detected in moss samples with C/N relationship over 65:1, the results indicate that N deposition in southern boreal sites had satisfied the need of nitrogen by cyanobacterial symbionts.

5. Conclusions

We measured N_2 fixation using ARA and the $^{15}N_2$ method in two forest mosses collected from a boreal latitudinal transect. The methods gave consistent results with the conversion factor of 3.3, revealing that N_2 fixation was higher in the samples from northern latitudes with low N deposition and higher C/N ratio. Sequencing of the moss-associated *nifH* genes indicated that cyanobacteria were the primary N_2 fixers. No methanotrophic diazotrophs were found by *nifH* sequencing, and methanotrophic activity, which would be inhibited by acetylene (in ARA), could not explain N_2 fixation activity. We conclude that both the $^{15}N_2$ and ARA methods provided similar evidence of the lower N_2 fixation in sites with higher deposition, and the low activity was therefore not explained by methodological biases. The results also suggest that C/N ratio of the plant can indicate the stoichiometrical level, in which N_2 fixation will be downregulated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2012.12.006.

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Appendix: Stable isotope calculations

Calculation of enrichment values (Fry 2006)

$$\boldsymbol{\delta^{E}} = [(\delta_{sample} - \delta_{control}) / (\delta_{control} + 1000)] * 1000 \quad [1]$$

Calculation of N_2 fixation turnover rates

$$\textbf{N turnover \%} = atom\%^{E} * (100 / atom\%_{headspace}) \quad [2]$$

where $\textbf{atom\%}^{\textbf{E}} = 100 * (\delta^{E} + 1000) / [\delta^{E} + 1000 + (1000 / 0.0036765)]$ [3]

$$\textbf{atom\%}_{\textbf{headspace}} = f_{N2} * (atom\%_{N2} - atom\%_{15N2}) + atom\%_{15N2} \text{ (Fry 2006)} \quad [4]$$

where f_{N2} is the fractionation factor for N_2 in air or water, $atom\%_{N2} = 0.36630$, and $atom\%_{15N2} = 98$

$$\mathbf{f_{N2}} = V_{N2} / (V_{N2} + V_{15N2}) \quad [5]$$

where V is the volume of the corresponding gas

Calculation of moss nitrogen uptake:

$$\textbf{N uptake (µmol g}^{\textbf{-1}}\textbf{ DW)} = (1 / 100) * (\%N / 100) * [(atom\%_{sample} - atom\%_{control}) / MW(N_2)] * 10^{6} * (100 / atom\%_{headspace}) \quad [6]$$

where %N is the N percent of the dried sample, and $MW(N_2)$ is molecular weight for N_2 (28.0134462)