Symbioses Between Cyanobacterial Communities and Feather Mosses in Boreal Forests and Consequences for Dinitrogen Fixation

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Cover: Feather mosses in a boreal forest of Northern Sweden (upper left), *Nostoc* filaments on *Pleurozium schreberi* (upper right), *Nostoc* filaments on *Pleurozium schreberi* under fluorescent light (lower left), *Nostoc* filaments under white light (lower right).
(photos: G. Bay)

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Abstract

Feather moss-cyanobacteria associations, although still poorly understood, are recognised as essential for the regulation of nitrogen (N) input into N-limited ecosystems such as boreal forests due to their ability to carry out N_2-fixation. This thesis aimed to investigate the diversity and composition of feather moss-associated cyanobacterial communities, the mechanisms by which cyanobacterial colonisation on mosses occurs and the fate of the N that they fix, and how various combinations of biotic and abiotic conditions influence cyanobacterial N_2-fixation activity. The results demonstrated that the cyanobacterial communities associated with these mosses are highly host-specific and diverse, with moss species identity rather than environmental factors being the primary driver of their composition and diversity. Furthermore, cyanobacterial colonisation on feather mosses appears to be possible only if the plants are able to produce a chemo-attractant (to attract and guide cyanobacteria towards colonisation sites), the plant eventually gaining N from the cyanobacteria according to its needs. Moreover, the results reveal that both abiotic (light intensity, temperature) and biotic (host plant presence, cyanobacterial community composition, cyanobacterial density) factors must be considered when studying what causes variation in feather moss-cyanobacteria N_2-fixation rates over time and space. Overall, the results of this thesis help in developing our understanding of the drivers of cyanobacterial communities on boreal feather mosses, and highlight the important role that these mosses play in the N cycle in boreal forest ecosystems, by hosting cyanobacteria and acquiring the N_2 that they fix. In addition, this work also contributes to a better understanding of the complex interactions regulating biological N_2-fixation, a major driver of ecosystem processes.

Keywords: Boreal feather mosses, cyanobacteria, dinitrogen fixation, symbiosis, chemoattraction, species diversity, *nifH* gene.

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In loving memory of my granddad

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Ininbergs, K.*, **Bay, G.***, Rasmussen, U., Wardle, D. A. & Nilsson, M.-C. (2011). Composition and diversity of *nifH* genes of nitrogen-fixing cyanobacteria associated with boreal forest feather mosses. *New Phytologist* vol 192(2), 507-517. DOI: 10.1111/j.1469-8137.2011.03809.x

II **Bay, G.**, Nahar, N., Oubre, M., Whitehouse, M. J., Wardle, D. A., Zackrisson, O., Nilsson, M.-C. & Rasmussen, U. (2013). Boreal feather mosses secrete chemical signals to gain nitrogen. *New Phytologist* (Rapid Report) vol 200(1), 54-60. DOI: 10.1111/nph.12403

III **Bay, G**. N_2-fixation by cyanobacterial communities associated with *Pleurozium schreberi*: effect of the interaction between light intensity, temperature and cyanobacterial species (manuscript).

*Equal contributors

My contribution to the papers included in this thesis was as follows:

I Major participation in planning, field sampling, experimentation (except cloning), data analysis and writing.

II Major participation in experimentation (except nitrogen transfer), data analysis and writing.

III Planning, sampling, experimentation, data analysis and writing.

Abbreviations

µg	Microgram (or 10^{-6} gram)
µm	Micrometre (or 10^{-6} metre)
µmol	Micromole (or 10^{-6} mol)
^{14}N	Nitrogen-14 (stable isotope)
^{15}N	Nitrogen-15 (stable isotope)
A/T/C/G	Adenine/Thymine/Cytosine/Guanine
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ARA	Acetylene reduction assay
ATP	Adenosine Triphosphate
BNF	Biological (di-)nitrogen fixation
C	Carbon
ca.	*Circa* (about)
cm	Centimetre (or 10^{-2} metre)
CO_2	Carbon dioxide
cpc	Phycocyanin operon
d	Day
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
e^-	Electron
e.g.	*Exempli gratia* (for example)
EA-IRMS	Elemental analyser isotope ratio mass spectrophotometer
et al.	*Et alii* (and others)
Fe	Iron
FW	Fresh weight
g	Gram
GFP	Green fluorescent protein

H^+	Hydron
H_2	Dihydrogen
ha	Hectare
HIF	Hormogonia inducing factor
HSD	Honest significant difference
i.e.	*Id est* (that is)
ITS	Internal transcribed spacer
kg	Kilogram (or 10^3 grams)
kJ	Kilojoule (or 10^3 joules)
km	Kilometre (or 10^3 metres)
LMER	Linear mixed-effect model
log	Logarithm
m	Metre
mg	Milligram (or 10^{-3} gram)
min	Minute
ml	Millilitre (or 10^{-3} litre)
mm	Millimetre (or 10^{-3} metre)
Mo	Molybdenum
mol	Mole
N	Nitrogen
N_2	Dinitrogen (gaseous form of nitrogen)
$NaNO_3$	Sodium nitrate
ng	Nanogram (or 10^{-9} gram)
NH_3	Ammonia
NH_4^+	Ammonium
NH_4NO_3	Ammonium nitrate
nm	Nanometre (or 10^{-9} metre)
NO_2^-	Nitrite
NO_3^-	Nitrate
O_2	Dioxygen
ºC	Degree Celsius
OD	Optical density
P	Phosphorus
PCA	Principal component analysis
PCR	Polymerase chain reaction
P_i	Inorganic phosphate
qPCR	Quantitative PCR
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RuBisCO	Ribulose-1,5-bisphosphate carboxylase oxygenase

s	Second
SDS	Sodium dodecyl sulphate
SE	Standard error
SIMS	Secondary ion mass spectrometry
sp.	Species (singular)
spp.	Species (plural)
Tg	Teragram (or 10^{12} grams)
yr	Year

1 Introduction

1.1 Boreal forests and nitrogen limitation

The boreal forest forms a continuous belt across North America and Eurasia and constitutes one of the largest biomes on Earth, covering 11% of the Earth's terrestrial surface (Bonan and Shugart, 1989). These ecosystems play an important role in global climate control as collectively they store larger quantities of carbon (C) than any other terrestrial biome (Anderson, 1991). Soil represents the main sink of C, especially in the absence of wildfires and when plant species promoting below-ground C accumulation dominate (Jonsson and Wardle, 2010). Thus, these forests contribute to reducing global levels of atmospheric carbon dioxide (CO_2), particularly when in their maximum growth phase (Chen *et al.*, 2006). However, the net primary productivity of a major proportion of terrestrial ecosystems, including many boreal forest ecosystems, is constrained by the availability of nutrients, especially nitrogen (N) (Grime, 1979; Aerts and Chapin, 2000, Le Bauer and Treseder, 2008). The reduction in physiological processes which occurs when levels of biologically accessible N are low is a direct demonstration of the crucial biological role of N in such ecosystems (Box 1).

In terrestrial ecosystems, N is prone to fast turnover and/or may be lost in gaseous forms into the atmosphere (Lambers *et al.*, 2006). In boreal forest ecosystems, anthropogenic N-deposition is often very low (Gundale *et al.*, 2011), and mineralisation rates are slow due to low temperatures combined with acidic soils (Swift *et al.*, 1979; Van Cleve and Alexander, 1981). In addition, once N is incorporated into biological material such as living plant biomass, plant litter, and microbial biomass, it becomes less accessible to plants and microbes (Tamm, 1991). Further, even though N is the dominant element in the atmosphere, this atmospheric N is inaccessible for most life forms due to its non-reactive nature (Schlesinger, 1997). A few plants and fungi are known to form symbioses with micro-organisms that can fix

dinitrogen (N_2) in boreal forests (e.g. *Sphagnum* and feather mosses, cyanolichens, *Alnus*; Henriksson and Simu, 1971; Basilier, 1979; Rosén and Lindberg, 1980; Mitchell and Ruess, 2009; Pawlowski and Demchenko, 2012; Henskens *et al.*, 2013). In spite of their wide distribution in boreal forests, these species are restricted to specific habitats. Therefore, their overall contribution to the N cycle is considered insufficient to sustain the N demand of boreal forest ecosystems. For the various reasons mentioned above, even though they have a rather substantial pool of N, boreal forest ecosystems are considered to be generally N-limited because much of this N is immobilised.

Box 1. Biological role of nitrogen

Nitrogen is the main constituent of Earth's atmosphere (i.e. 78.08% by volume of dry air). Nitrogen is found in all living organisms, as it is an essential structural component of amino acids, proteins, DNA and RNA molecules (e.g. nitrogenous bases such as adenine or guanine). It is therefore crucial for all physiological processes. In plants, N is needed for two essential physiological mechanisms, i.e. photosynthesis and C assimilation, as it enters into the structure of chlorophyll and RuBisCO (see Fleming, 1967; Spreitzer and Salvucci, 2002).

1.2 Feather mosses and their impact on nitrogen availability in boreal forest ecosystems

Pleurocarpous feather mosses, i.e. bryophytes with feather-like foliage and a female archegonium on a side branch rather than on the main stem, occur widely across boreal zones of the world and often dominate the ground vegetation (Zackrisson *et al.*, 2009), where they form dense and continuous carpets, covering up to 80% of the soil surface (Mälkönen, 1974; Foster, 1985; Alexander and Billington, 1986; Oechel and Van Cleve, 1986; DeLuca *et al.*, 2002a; Zackrisson *et al.*, 2009). Although feather mosses represent a relatively small fraction of the total standing biomass of boreal forest ecosystems, their annual foliage production can potentially exceed that of trees in such environments (Weetman, 1969; Oechel and Van Cleve, 1986; Bisbee *et al.*, 2001; Bond-Lamberty and Gower, 2007; Wardle *et al.*, 2012). Consequently, feather mosses represent an important part of the ground vegetation biomass of boreal forest ecosystems and have a significant control over C and N fluxes.

There is general agreement that the moss layer is an important factor in the dynamics of boreal forests (see Bonan and Shugart, 1989; Lindo and Gonzalez,

2010), and a major driver of soil properties and decomposers, and therefore nutrient availability (Nilsson and Wardle, 2005). For instance, they regulate soil temperatures, which impact on microbial activity and nutrient mineralisation rates (Oechel and Van Cleve, 1986; Tietema *et al.*, 1992). Feather mosses are also known for their capacity to rapidly immobilise nutrients, notably N originating from tree and shrub litterfall (Weber and Van Cleve, 1984; Bonan and Shugart, 1989; Brown and Bates, 1990). This is due to their physiology (i.e. poikilohydry), which makes them act like "sponges" for nutrients and water (Turetsky, 2003). Moreover, the decomposition rate of dead feather mosses is slow due to the poor quality litter they generate, which also impacts on nutrient turnover in boreal forest ecosystems (Oechel and Van Cleve, 1986; Lang *et al.*, 2009). While feather mosses can have a negative impact on nutrient mineralisation, the moss layer can also promote higher decomposition rates of leaf litter under moisture-limited conditions (Jackson *et al.*, 2011). Furthermore, because bryophytes have poorly developed water conduction systems and lack stomata and an effective cuticle, feather mosses may be subjected to frequent hydration-desiccation cycles, causing nutrient leakage when re-hydrated; this source of nutrients can potentially be substantial (Gupta, 1977; Carleton and Read, 1991; Coxson, 1991; Startsev and Lieffers, 2006). Finally, feather mosses species have been shown to host cyanobacteria which possess the ability to fix atmospheric N_2 (DeLuca *et al.*, 2002b; Solheim *et al.*, 2002). Therefore, feather mosses play a predominant role with regards to nutrient cycling in boreal forest ecosystems.

Dinitrogen-fixation is acknowledged as a major driver of ecosystem processes, and feather moss-cyanobacteria associations are fundamental to the regulation of N input into boreal forest ecosystems (Chapin *et al.*, 1986; DeLuca *et al.*, 2002b). Recent studies have demonstrated the role of ecosystem successional stage as a driver of the N_2-fixation activity of these associations (Zackrisson *et al.*, 2004; Lagerström *et al.*, 2007). As such, moss cover increases with time since major disturbances (e.g. wildfire), in turn supporting greater populations of N_2-fixing cyanobacteria, and thereby biological N_2-fixation (BNF) (Zackrisson *et al.*, 2004; Lagerström *et al.*, 2007). However, despite growing interest in recent years, moss-cyanobacteria associations are still poorly understood. As a result, deeper understanding of the mechanisms controlling these associations (i.e. physiological, ecological, environmental) is needed in order to understand the primary source of biological N in these ecosystems.

1.3 Cyanobacteria: a key element in environmental processes

1.3.1 Life history, classification and characteristics

Cyanobacteria, prokaryotic organisms also known as blue-green algae due to their pigment composition, appeared approximately 3.5 billion years ago according to fossil records (Golubic and Seong-Joo, 1999). They are one of the most widespread groups of bacteria on Earth, found in almost all aquatic and terrestrial ecosystems (e.g. oceans, fresh waters, soil crusts, boreal forests; Whitton, 2012).

As a remnant of their endosymbiotic life (Box 2), cyanobacteria form symbioses with a wide range of organisms such as fungi (bipartite cyanolichens), both fungi and algae (tripartite cyanolichens), sponges, bryophytes, and vascular plants (see Rai *et al.*, 2002), but are mostly found as free-living in, for instance, aquatic or terrestrial ecosystems. Cyanobacteria are currently classified into five groups, based on phenotypic and developmental features (Rippka *et al.*, 1979; Rippka, 1988; Castenholz, 1992), summarised

Box 2. From cyanobacteria to chloroplasts

According to the endosymbiotic theory (i.e. the incorporation of bacterial symbionts into cells, eventually becoming organelles; Kutschera and Niklas, 2005; Falcón *et al.*, 2010; Figure 1), chloroplasts in plants originated from a common cyanobacterial ancestor (see Cavalier-Smith, 2000). This process has had a considerable influence on the evolution of life on Earth, as it enabled the emergence of land plants (e.g. gymnosperms, angiosperms, bryophytes), and by extension, of the entire terrestrial ecosystem (see Kenrick and Crane, 1997).

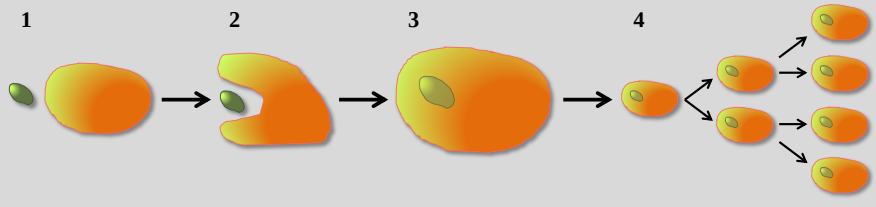

Figure 1. Schematic representation of an endosymbiosis (modified after University of California Museum of Paleontology, 2008). The process starts with two independent bacteria (1). Over time, one bacterium (the "ancestral" cell) enslaves the other via endocytosis, through invagination of its plasma membrane (2). One bacterium (the "endosymbiont") now lives inside the other. The endosymbiont eventually becomes an organelle through genetic evolution (i.e. genome shrinkage by loss of genes or gene transfer to the ancestral cell's nuclear genome, see Bock and Timmis, 2008); both partners benefiting from this arrangement (3). The organelle is then passed on from generation to generation (4).

in Table 1. However, the taxonomy of cyanobacteria remains unsatisfactory as molecular phylogenies do not always support the monophyly of all the groups (Turner *et al.*, 1999; Zehr *et al.*, 2000; Henson *et al.*, 2004). In his review, Komárek (2006) points out the complexities in cyanobacterial taxonomy, which sometimes results in misleading classifications (e.g. erroneous strain designation, based on out-of-date literature). His suggestion is therefore that cyanobacterial classifications (and especially the diagnostic phenotypic characters used to differentiate their taxa) need to be revised by combining morphological, molecular (i.e. phylogeny), and ecophysiological data. This last criterion is at least as important as the other two, because the habitat where cyanobacteria live (such as the culture medium) may influence their metabolism or morphology (Garcia-Pichel *et al.*, 1998; Komárek, 2006). A similar proposal has been made to further define what a "species" really is (Lindström and Gyllenberg, 2006), but with the support of social science and

Table 1. *Cyanobacterial taxonomy modified after Rippka* et al. *(1979).*

Group	Cell organisation	Reproduction	Representative genera	Dinitrogen fixation
I	Unicellular	Binary fission or budding	*Cyanothece* spp.	Yes[1]
			Synechococcus spp.	No[1, 4]
			Synechocystis spp.	Yes[1]
II	Unicellular	Multiple fissions	*Chroococcidiopsis* spp.	Yes[1]
			Cyanocystis spp.	No[2]
			Dermocarpa spp.	No[3]
III	Filamentous No heterocysts	Random breakage of filament and division in only one plane	*Oscillatoria* spp.	Yes[1]
			Pseudoanabaena spp.	Yes[1]
			Spirulina spp.	No[3]
IV[5]	Filamentous Heterocysts	Random breakage of filament and division in only one plane	*Calothrix* spp.	Yes[3]
			Cylindrospermum spp.	Yes[1]
			Nostoc spp.	Yes[3]
V[5]	Filamentous Heterocysts	Random breakage of filament and division in more than one plane	*Chlorogloeopsis* spp.	Yes[3]
			Fischerella spp.	Yes[3]
			Stigonema spp.	Yes[1]

[1]Bergman *et al.*, 1997; [2]Palińska *et al.*, 2012; [3]Young, 1992.
[4]One strain is able to fix N_2: *Synechococcus* PCC7335.
[5]These two groups (heterocystous cyanobacteria) have been identified as a uniform cluster (Nostocineae; Gugger and Hoffman, 2004).

bioinformatics. Even though such a system of classification may be difficult to implement because it is time-consuming and requires access to expertise in very different fields (e.g. taxonomy, molecular biology, ecology, genetics, bioinformatics), it may help in solving the recurrent problems in the taxonomic classification of cyanobacteria (Komárek, 2006).

One of the distinctive characteristics of cyanobacteria is their ability to perform oxygenic photosynthesis, i.e. the conversion of light energy into chemical energy that eventually becomes available to sustain physiological needs. During the process, CO_2 and water are used as raw materials, and dioxygen (O_2) is a resulting waste product. Photosynthesis in cyanobacteria occurs in vegetative cells, which are only formed when environmental conditions are favourable. Cyanobacteria account for 20 to 30% of the Earth's photosynthetic productivity (Pisciotta *et al.*, 2010), and are thought to be responsible for the Great Oxygenation Event, i.e. the appearance of O_2 in the Earth's atmosphere ca. 2.4 billion years ago (Hayes, 1983; Dismukes *et al.*, 2001; Canfield, 2005; Holland, 2006). Cyanobacteria also have a strong capacity to fix atmospheric N_2, a process known as diazotrophy; this ability is restricted to organisms belonging to the Bacteria and Archea domains (e.g. the bacteria *Frankia* and *Rhizobia*, methanogenic Archea). Whereas most cyanobacteria genera from the taxonomic groups I to III are capable to perform N_2-fixation, this ability extends to all genera from groups IV and V (Table 1) (see Bergman *et al.*, 1997).

1.3.2 Dinitrogen fixation by cyanobacteria

Cyanobacteria preferentially take up easily accessible forms of N present in the environment (e.g. amino acids, urea, nitrate, ammonia; Fay, 1992). When these sources are no longer available, cyanobacteria use atmospheric N_2 as their main source of N, through N_2-fixation. In the atmosphere, atoms of N are tightly associated by a triple chemical bond; its breakdown requires the expenditure of a large amount of energy, obtained through the hydrolysis of molecules of ATP (the energy of $N \equiv N$ bond is 940 kJ mol^{-1}; Turetsky, 2003). Dinitrogen-fixation in cyanobacteria is catalysed by a multimeric enzyme complex, named nitrogenase. The reaction it performs is as follows:

$$N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i$$

The nitrogenase complex is composed of two highly conserved metalloproteins (Haselkorn, 1986). The first protein is dinitrogenase (also called molybdenum-iron protein or MoFe-protein), a heterotetramer encoded by *nifD* and *nifK* genes (see Burgess and Lowe, 1996; Gaby and Buckley, 2012); it is the active site where N_2 is reduced. The second protein is

dinitrogenase reductase (also called iron protein or Fe-protein), a homodimer encoded by *nifH* gene (see Howard and Rees, 1996); it corresponds to the site where ATP is hydrolysed and electrons are transferred between proteins. About 20 *nif* genes are involved in N_2-fixation (Johnston *et al.*, 2005), some of them encoding for the nitrogenase enzyme and others for regulatory proteins of the process. In the diazotroph *Klebsiella pneumoniae* for instance, low level of fixed N triggers the production of nifA protein, which regulates the initiation of the transcription of the *nif* genes. Conversely, when there is a sufficient amount of reduced N or presence of O_2, the nifL protein is produced, which inhibits the activity of *nifA* gene (Arnold *et al.*, 1988; Fay, 1992; Martinez-Argudo *et al.*, 2004; Johnston *et al.*, 2005). The impairment of the *nifA* function in the presence of O_2 is one of the strategies that diazotrophs have evolved to protect the nitrogenase enzyme from the oxidative properties of O_2.

Cyanobacteria utilise a variety of strategies for performing N_2-fixation, while at the same time, protecting nitrogenase from the O_2 produced during photosynthesis. As mentioned above, exposure to O_2 would irreversibly inactivate nitrogenase because of its high sensitivity to oxidation, and would also repress the synthesis of its structural proteins (Robson and Postgate, 1980; Gallon, 1981). A few examples of these strategies are the use of detoxifying enzymes (which remove O_2-derived reactive oxygen species), the consumption of O_2 via increased respiration, and the temporal and/or spatial separation between photosynthesis and N_2-fixation (Bergman and Carpenter, 1991; Fay, 1992; Reddy *et al.*, 1993; Postgate, 1998). Temporal separation can be obtained by the partitioning in time of photosynthesis and N_2-fixation, with N_2-fixation only occurring when no O_2 is released through photosynthetic activity, i.e. in darkness. Spatial separation can be achieved via the formation of special thick-walled cells known as heterocysts to which N_2-fixation is restricted.

Only filamentous cyanobacteria belonging to groups IV and V (Table1) have the capacity to induce the cell differentiation needed to produce N_2-fixing-specialised cells or “heterocysts”. They are found either in the terminal position or semi-regularly distributed along the cyanobacterial filament (Figure 2; see Fay, 1992). Heterocyst frequency is species-specific and heterocysts generally make up 5 to 10% of the total number of cells when free-living (Adams and Duggan, 1999; Adams, 2000; Golden and Yoon, 2003). The induction of cell differentiation is dependent on environmental conditions (e.g. depletion of fixed N; see Wolk, 1996). Moreover, it seems that only certain cells in the filament possess the ability to differentiate, such as those with a low or zero concentration of the membrane protein PatN, whose role is to repress the initiation of heterocyst differentiation (Risser *et al.*, 2012). The nitrogenase enzyme is only expressed in mature heterocysts between 18 and 24 hours after

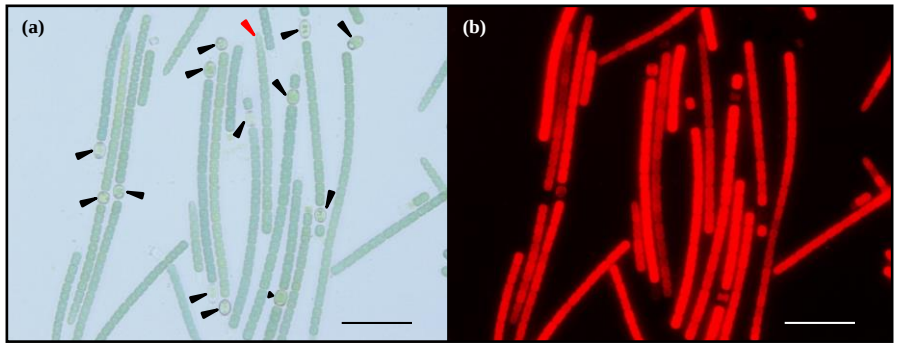

Figure 2. Photomicrographs of *Nostoc* sp. filaments observed under white light (a) and ultraviolet fluorescence with a green filter (b). Black arrowheads indicate heterocysts; red arrowhead indicates undifferentiated filament entirely composed of vegetative cells. Note that mature heterocysts are not visible in fluorescence due to the degradation of photosystem II and loss of phycobilisomes. Bars, 40 µm.

N deprivation (Elhai and Wolk, 1990; Golden *et al.*, 1991), where it is in a central location for reducing the possibility of degradation by the O_2 which might enter into the cells (Braun-Howland *et al.*, 1988; Fay, 1992). In heterocysts, other structural and physiological features exist to maintain strict anaerobic conditions in the vicinity of nitrogenase and thus prevent its inactivation. These include an O_2-impermeable barrier made of glycolipids and polysaccharides which decreases the diffusion of gases, the loss of photosystem II activity (though photosystem I is still active to generate ATP), the formation of polar nodules, and increased respiration to remove remaining traces of O_2 (see Wolk, 1996; Adams and Duggan, 1999; Meeks and Elhai, 2002; Mullineaux *et al.*, 2008). Because heterocysts do not have RuBisCO, they rely on adjacent vegetative cells for provision of C, with this C supporting N_2-fixation activity (Cumino *et al.*, 2007). Another possible exchange of metabolites could happen via the periplasm (i.e. the space between the cytoplasm and the outer membranes) which seems to be continuous (Flores *et al.*, 2006). Conversely, fixed N is transferred from the heterocysts to vegetative cells in the form of amino acids (Adams and Duggan, 1999; Meeks and Elhai, 2002), and eventually to other organisms in their vicinity in the form of ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-). This release of mineral N can in turn support the N economy of the ecosystem.

1.4 Factors regulating biological dinitrogen-fixation in boreal forests

Ecosystem properties and vegetation dynamics are known to influence cyanobacterial N_2-fixation rates (Chapin *et al.*, 1991; Rastetter *et al.*, 2001;

Vitousek *et al.*, 2002; Gruber and Galloway, 2008). As a result, several abiotic conditions regulate BNF rates, such as nutrient availability (notably of phosphorus [P] and N). Cyanobacteria require high quantities of ATP and other P-containing molecules to support N_2-fixation; BNF is therefore often considered as P-limited (see Chapin *et al.*, 2002), even though P fertilisation does not always enhance N_2-fixation activity in boreal forests (Zackrisson *et al.*, 2004; 2009). On the other hand, BNF can be reduced or even entirely inhibited when N is externally supplied (e.g. through fertilisation, pollution due to human activity). Thus, biomass of bacteria capable of BNF is often reduced by N fertilisation (Wallenstein *et al.*, 2006; Demoling *et al.*, 2008), and N_2-fixation can be inhibited by N deposition due to the N-induced modification of a nitrogenase reductase sub-unit (Gotto and Yoch, 1982; Reich and Böger, 1989; Gundale *et al.*, 2011). Further, because N_2-fixation is an energetically expensive process and cyanobacterial growth rates and abundance are light-dependent, one of the main constraints of BNF in boreal forests is the availability of or access to light. As such, forest canopies tend to close during succession which reduces the amount of light that is available to N_2-fixing organisms (Gerard *et al.*, 1990; Staal *et al.*, 2002; Rabouille *et al.*, 2006). Another factor with a strong influence on BNF rates (as well as on cyanobacterial development) is temperature, as the optimal activity of the nitrogenase enzyme is temperature-dependent, and with this optimum being species-specific (Kashyap *et al.*, 1991; Zielke *et al.*, 2002; Gentili *et al.*, 2005). Moreover, moisture conditions and BNF have been shown to be strongly correlated, i.e. N_2-fixation activity tends to be reduced when water availability declines (Kurina and Vitousek, 1999; Turetsky, 2003; Zielke *et al.*, 2005; Gundale *et al.*, 2009, Jackson *et al.*, 2011).

Although less studied than abiotic conditions, biotic factors may also play an important role in regulating BNF rates in boreal forests. For instance, BNF depends on the life form of cyanobacteria (i.e. symbiotic or free-living). Cyanobacteria in symbiosis tend to have an enhanced N_2-fixation activity (Meeks, 1998), most probably because the host facilitates the acquisition of nutrients and water, as well as provides a favourable habitat for cyanobacteria (Meeks, 1998; Rai *et al.*, 2000). Another explanation as to why greater rates of BNF rates are observed when cyanobacteria are in symbiosis is through increased heterocyst frequency, as a result of host-secreted signals (Haselkorn, 1978; Wang *et al.*, 2004). Finally, cyanobacterial community composition may also have an important effect on BNF rates because, as addressed above, the N_2-fixation response of distinct species of cyanobacteria may vary depending on the environmental conditions (e.g. temperature, light intensity).

Despite recognition of the importance of biotic and abiotic factors for BNF described above, much remains to be understood about how they control the N_2-fixation activity of feather moss-cyanobacteria associations. For instance, the interactive effect of light intensity and temperature on BNF (when moisture requirements are met) remains little investigated. In addition, nothing is known about how N_2-fixation activity differs when cyanobacteria are associated with a feather moss (as compared to in their free-living state), or how the cyanobacterial community composition may impact on N_2-fixation rates (e.g. via competition between different strains, thus reducing BNF; complementarity of different strains, thus enhancing BNF; or neutrality, with no effect on BNF).

1.5 Feather moss-cyanobacteria associations in boreal forest ecosystems

1.5.1 Contribution to the nitrogen cycle

Global N_2-fixation has been estimated to ca. 240 Tg N yr^{-1}, with the contributions of marine and terrestrial ecosystems being 40-80% and 20-60%, respectively (Berman-Frank *et al.*, 2003). Dinitrogen-fixation in boreal forest ecosystems has long been underestimated due to feather moss-cyanobacteria associations being overlooked; previous estimations for N_2-fixation in such environments were mainly based on *Sphagnum* mosses and N_2-fixing woody plants (Rosén and Lindberg, 1980; Mitchell and Ruess, 2009). Feather moss-cyanobacteria associations are now recognised to be important agents of N_2-fixation (Rousk *et al.*, 2013). As such, according to acetylene reduction assay measurements (ARA; Box 3), they fix between 1.5 and 2 kg N ha^{-1} yr^{-1}

Box 3. The acetylene reduction assay (Hardy *et al.*, 1968)

This indirect measure is one of the most common methods used to assess cyanobacterial N_2-fixation activity (DeLuca *et al.*, 2002b; Zackrisson *et al.*, 2004; Lagerström *et al.*, 2007; Ininbergs *et al.*, 2011), mainly due to its sensitivity and reproducibility. It is based on the ability of the nitrogenase enzyme to reduce triple bounded molecules such as acetylene, without inhibiting the binding of N_2. The quantity of acetylene converted to ethylene by each sample tested after a period of incubation is then quantified by gas chromatography. Finally, the amount of N_2 fixed by cyanobacteria can be calculated using a ratio of 3 moles of ethylene reduced per mole of N_2 fixed; this ratio is appropriate for feather moss-cyanobacteria associations (DeLuca *et al.*, 2002b; Zackrisson *et al.*, 2004; Lagerström *et al.*, 2007).

(Cleveland *et al.*, 1999; DeLuca *et al.*, 2002b; Lagerström *et al.*, 2007); this rate of fixation is close to 50% of the average N_2-fixation rate found on Earth (i.e. 4.7 kg N ha^{-1} yr^{-1}; calculation based on Berman-Frank *et al.*, 2003). Moreover, this input of N is greater than atmospheric N_2 deposition in several northern habitats, and may therefore contribute to enhanced N stocks in the soil (Lagerström *et al.*, 2007; DeLuca *et al.*, 2008; Gundale *et al.*, 2010, 2011).

1.5.2 Establishment of the association, role of the partners and cyanobacterial diversity on feather mosses

Feather mosses have been shown to host cyanobacteria epiphytically on or between their leaves (Figure 3) (DeLuca *et al.*, 2002b; Solheim *et al.*, 2002). However, the intrinsic factors regulating the capacity of a feather moss to become a host for cyanobacteria have yet to be determined. Until now, most of our knowledge concerning the establishment of symbioses between bryophytes and cyanobacteria has been related to liverworts (e.g. *Blasia pusilla*; Knight and Adams, 1996), hornworts (e.g. *Anthoceros punctatus*; Campbell and Meeks, 1989), and *Sphagnum* mosses (Granhall and Selander, 1973; Granhall and Hofsten, 1976). Infection of liverworts and hornworts occurs through chemical signal exchanges (see Adams, 2002). Thus, these plants secrete exudates which trigger the differentiation of cyanobacteria into hormogonia (Box 4), and eventually release a chemoattractant to guide hormogonia towards

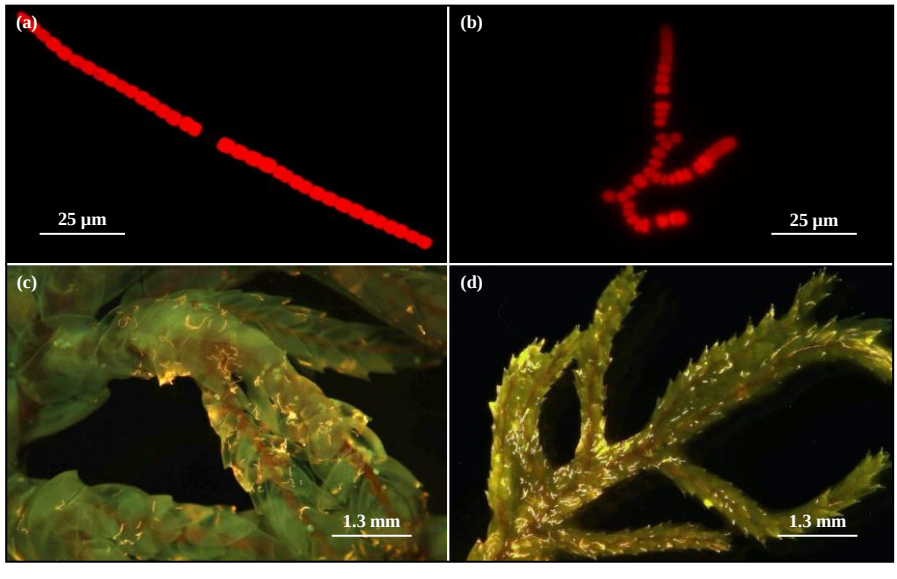

Figure 3. Examples of cyanobacteria commonly associated with boreal feather mosses (**a**, *Nostoc* sp.; **b**, *Stigonema* sp.) and *Nostoc* sp. filaments, seen in bright orange, on leaves of *Pleurozium schreberi* and *Hylocomium splendens* (**c** and **d**, respectively).

Box 4. Hormogonia formation and functional role

Hormogonia are transient, non-growing motile cyanobacterial filaments serving as dispersal units (Tandeau de Marsac, 1994; Meeks, 1998), formed by cyanobacteria from the orders Nostocales and Stigonematales. Unlike their N_2-fixing counterparts, they do not fix N_2 as they do not form heterocysts, and generally possess smaller, different-shaped cells than the vegetative trichomes (Meeks *et al.*, 2002; Figure 4). Hormogonia formation is induced by different stimuli such as nutrient and salt concentration (Herdman and Rippka, 1988), light quality (Damerval *et al.*, 1991; Tandeau de Marsac, 1994), transfer to a fresh medium (Meeks, 1998), and the secretion of hormogonia inducing factors (HIF) by plants (Campbell and Meeks, 1989; Rasmussen *et al.*, 1994). Hormogonia are subsequently attracted by chemotaxis towards their symbiotic partner (Meeks, 1998). The secretion of a HIF and a chemo-attractant is necessary for the establishment of a symbiosis between cyanobionts and various plants (e.g. *Nostoc* with *Gunnera* and *Anthoceros*; see Rai *et al.*, 2002). Even though the mechanisms by which cyanobacteria, and therefore hormogonia, move have not been fully elucidated, Adams (2001) suggests that hormogonia glide via pili produced during the early stages of hormogonia differentiation.

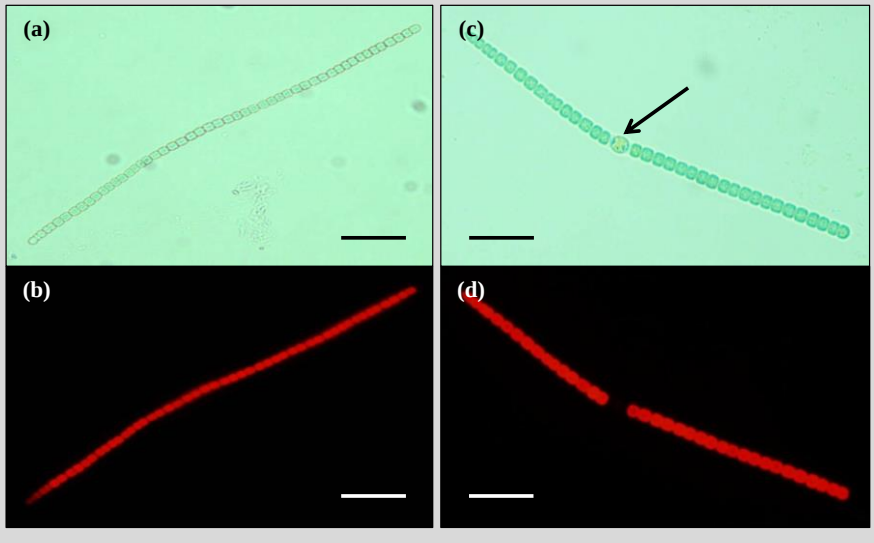

Figure 4. Photomicrographs of a *Nostoc* sp. hormogonium (**a**, **b**) and N_2-fixing filament (**c**, **d**) observed under white light and ultraviolet fluorescence with a green filter, respectively. The arrow indicates a heterocyst. Bars, 25 µm.

symbiotic structures (i.e. spherical structures, also known as auricles, for *Blasia*; slime cavities in the thallus for *Anthoceros*). In the case of *Sphagnum* mosses, cyanobacteria are found epiphytically as well as intracellularly in water-filled hyaline cells (Solheim and Zielke, 2002). As for *Sphagnum*, cyanobacteria colonising feather mosses have been thought to be transported passively and randomly, e.g. via wind or water (Solheim and Zielke, 2002). Additionally, feather mosses do not produce symbiotic structures. For these reasons, the relationship between feather mosses and cyanobacteria has been considered as accidental and therefore an association rather than as a symbiosis.

The potential benefit that feather mosses and/or cyanobacteria obtain from the association is still unknown. However, in other symbioses between plants and cyanobacteria, it is recognised that the cyanobacteria transfer the excess of N_2-derived ammonium that they produce into easily assimilated compounds such as amino acids, to sustain their host's physiological needs (e.g. cyanobacteria in symbiosis with *Azolla* or *Gunnera*; Rai *et al.*, 2002). In the case of *Nostoc* spp., this transfer may reach 90% of the N-derived molecules produced (Meeks *et al.*, 1983; Berg *et al.*, 2013). In return, the plant partner may provide the energy necessary to support N_2-fixation (Cumino *et al.*, 2007), and in some cases also provide an O_2-protection system (Mylona *et al.*, 1995). In the symbiosis between cyanobacteria and the bryophyte *Calliergon richardsonii*, these processes can be facilitated due to a moss-secreted matrix made of extra-cellular polysaccharides in which cyanobacteria are "contained" (Potts, 1994; Solheim and Zielke, 2002). Similar processes are likely to occur in feather moss-cyanobacteria associations and, therefore merit further investigation.

In boreal forest ecosystems, cyanobacteria reported to live in association with feather mosses belong to the *Nostocaceae* (e.g. *Nostoc* sp., *Nodularia* sp., *Cylindrospermum* sp.), *Rivulariaceae* (e.g. *Calothrix* sp.), *Stigonemataceae* (e.g. *Stigonema* sp.), and *Fischerellaceae* (e.g. *Fischerella* sp.) families (DeLuca *et al.*, 2002b; Gentili *et al.*, 2005; Houle *et al.*, 2006; Zackrisson *et al.*, 2009; Ininbergs *et al.*, 2011; Leppänen *et al.*, 2013). However, knowledge of the composition and diversity of N_2-fixing cyanobacterial communities associated with two of the most common feather mosses (i.e. *Pleurozium schreberi* and *Hylocomium splendens*) is still limited. Several N_2-fixing cyanobacteria other than those mentioned above are also able to form symbioses with bryophytes. For instance, *Anabaena* sp. and *Oscillatoria* sp. have been found on leaves of arctic mosses (Jordan *et al.*, 1978). However, *Nostoc* was with one exception the only genus that was found in symbiosis with the hornwort *Phaeoceros*, which demonstrates a high host-specificity at

least for some bryophytes (West and Adams, 1997). Although the host specificity between plants and symbiotic diazotrophs has been studied for some years (e.g. Bagwell *et al.*, 2001; Andronov *et al.*, 2003), the degree of host specificity between bryophytes and the cyanobacteria that they host remains little explored. Understanding this issue could give valuable insights about which factors drive cyanobacterial community composition in boreal feather mosses.

1.6 Objectives

The overall aims of this thesis are to expand our understanding of the underlying mechanisms of cyanobacterial colonisation on feather mosses, to explore the cyanobacterial community composition and diversity associated with these mosses, and to determine how cyanobacterial N_2-fixation activity varies under different combinations of biotic and abiotic conditions. Thus, Paper I focuses on the characterisation of the cyanobacterial community composition and diversity on the feather mosses *P. schreberi* and *H. splendens*, as well as on how environmental conditions impact on N_2-fixation activity by these cyanobacteria. Meanwhile, Paper II examines the mechanisms by which feather mosses are colonised by cyanobacteria, the intrinsic conditions necessary for this colonisation to occur, and the fate of the N_2 fixed by cyanobacteria. Finally, Paper III explores the role played by combinations of biotic and abiotic factors on the N_2-fixation activity of cyanobacteria associated with feather mosses.

The central questions addressed in this thesis are:

1. Based on the *nifH* gene, what is the genetic diversity and community composition of the N_2-fixing cyanobacterial communities associated with *P. schreberi* and *H. splendens* from contrasting environments? (Paper I)
2. Is cyanobacterial colonisation on feather mosses the result of chemical communication between the two partners, and does it occur only under certain physiological conditions of the host? (Paper II)
3. Is the N_2 fixed by cyanobacteria transferred to feather mosses? (Paper II)
4. What are the effects of abiotic conditions (i.e. temperature and light intensity) and biotic factors (i.e. host moss presence and cyanobacterial species identity), as well as their interactive effect, on cyanobacterial N_2-fixation activity? (Paper III)

2 Materials and Methods

2.1 Study systems: island and forest reserve chronosequences

The study area for Paper I consisted of 30 forested islands on two adjacent lakes (Lake Hornavan and Lake Uddjaure; 66º24'N - 17º54'E and 66º00'N - 17º83'E, respectively), while for Papers II and III it consisted of a natural forest reserve in Reivo (i.e. Ruttjeheden; 65º80'N - 19º10'E); both study areas are located in the northern boreal zone of Sweden (Figure 5). Mean annual precipitation for this region ranges from 600 to 750 mm, and mean temperatures from +13ºC in July to -14ºC in January (SMHI - Swedish Meteorological and Hydrological Institute). In addition, N deposition in this region is very low (< 1 kg ha^{-1} yr^{-1}; Gundale *et al.*, 2009), and therefore has a negligible impact on cyanobacterial N_2-fixation activity.

The island system has been shown to form a chronosequence (i.e. a series of sites only varying in stage of succession) driven by time since the most recent fire, which varies greatly among islands. With increasing time since fire, the islands enter a state of ecosystem retrogression as nutrients become converted to increasingly unavailable forms (Wardle *et al.*, 2003, 2004; Zackrisson *et al.*, 2004). An ecosystem in a retrogressive phase is characterised by a reduction in plant biomass, soil fertility and ecosystem productivity; these effects increase with time since disturbance (Walker *et al.*, 1983; Wardle *et al.*, 2004; Peltzer *et al.*, 2010). Lightning-ignited wildfires are more frequent on larger islands than smaller islands (because they have a higher chance of being struck by lightning), and the smaller islands have therefore entered a state of retrogression (Wardle *et al.*, 1997, 2003). In this thesis, the 30 islands were divided into size classes with 10 islands per class, i.e. small (< 0.1 ha), medium (0.1 – 1.0 ha), and large (> 1.0 ha), with a mean time since last major wildfire of 3260, 2190 and 595 years, respectively (Wardle *et al.*, 2003).

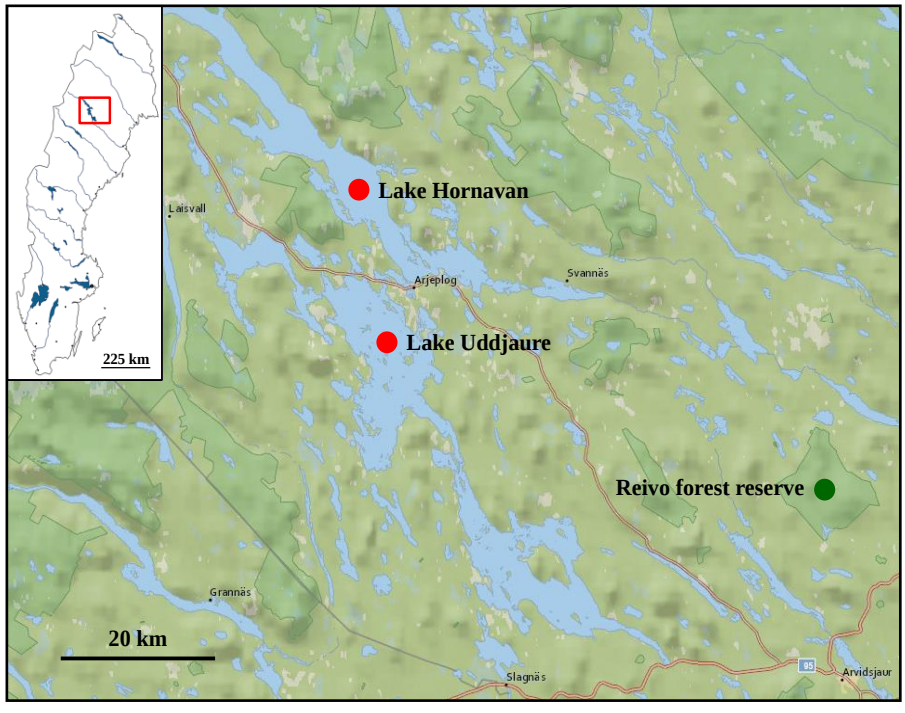

Figure 5. Sampling sites (northern Sweden). The red dots represent the lakes in which the island chronosequence (sampled in Paper I) is based, and the green dot represents the natural forest reserve (sampled in Papers II and III). (Map designed with MapMaker Interactive©, National Geographic Society, 1996-2013).

This is in accordance with previous studies performed on this island system (Wardle *et al.*, 2003; Wardle and Zackrisson, 2005; Lagerström *et al.*, 2007). Each island represents an independent replicate ecosystem with its own process rates and properties (e.g. microbial activity, productivity; Wardle *et al.*, 1997, 2003; Wardle, 2002). Considerable differences in vegetation composition and soil fertility occur between the different island classes (summarised in Table 2; Wardle *et al.*, 1997, 2003). Further, the rates of cyanobacterial N_2-fixation have previously been shown to be highly responsive to island size for both dominant moss species (i.e. *P. schreberi* and *H. splendens*); fixation was on average greater on the smaller islands that had entered a state of retrogression (Lagerström *et al.*, 2007). However, nothing was known about the molecular identity or community composition of cyanobacteria contributing to this pattern of N_2-fixation, either in this island system or for feather moss-cyanobacteria associations more generally, and was therefore investigated in this thesis.

The site in the Reivo forest reserve is a late post-fire successional forest stand. The date since last fire disturbance (i.e. 366 years ago) was determined through the study of tree ring scars by Zackrisson (1980). This site was dominated by *Pinus sylvestris* (and to a lesser extent by *Picea abies*), and the same ericaceous shrub and feather moss species as found on the island system (DeLuca *et al.*, 2002a; Zackrisson *et al.*, 2004). Similarly to the island system, feather mosses sampled from late fire successional stands (age > 290 years) in the vicinity of Reivo exhibit enhanced N_2-fixation rates, compared with nearby sites at earlier successional stages (DeLuca *et al.*, 2002b; Gundale *et al.*, 2009). For this reason, moss gametophytes originating from this sampling area were particularly suitable for the experiment in Papers II and III (see below).

Table 2. *Vegetation composition and soil fertility found on the three island classes after Wardle* et al. *(1997, 2003).*

Island class	Dominating vegetation			Soil fertility
	Trees	Dwarf shrubs (Ericaceae)	Ground level	
Small	*Picea abies*	*Empetrum hermaphroditum*	*Pleurozium schreberi* and *Hylocomium splendens*	Low
Medium	*Betula pubescens*	*Vaccinium vitis-idaea*		Medium
Large	*Pinus sylvestris*	*Vaccinium myrtillus*		High

2.2 Sample collection

Paper I is based on laboratory studies of field-collected feather moss samples originating from the island chronosequence. Collection of feather mosses[1] for N_2-fixation measurements, as well as for the study of cyanobacterial composition and diversity on these mosses, was done in June 2009. This period corresponds to the first main N_2-fixation seasonal peak of cyanobacteria-moss associations (DeLuca *et al.*, 2002b). For each of the 30 above-mentioned islands, 30 gametophytes of *P. schreberi* and 15 gametophytes of *H. splendens* (as they are generally larger than *P. schreberi* gametophytes) were sampled in a grid pattern from a 20 × 20 m study area. In each grid, 10 sampling points were selected for collecting *P. schreberi* gametophytes and five for collecting *H. splendens* gametophytes. In the vicinity of each point, three individual gametophytes of each of the moss species were randomly collected. Sampling points located < 2 m from overstory trees or in dense shrub vegetation were

1. Only the upper 3-5 cm green part of the gametophytes (ca. 10 mg dry weight) was used as this is where most of the N_2-fixation activity occurs on feather mosses (Solheim *et al.*, 2004).

avoided. Each sampling area was placed at equal distance from the shore to avoid differences between samples due to edge or microclimatic effects (Wardle *et al.*, 1997, 2003).

Papers II and III are based on climate chamber controlled experiments of feather moss samples collected from Ruttjeheden in the Reivo forest reserve. In Paper II, moss gametophytes were collected to study (i) the possible chemical communication with cyanobacteria, (ii) the N transfer between cyanobacteria and mosses, (iii) the effect of N-amendment on chemo-attraction of cyanobacteria, and (iv) the cost of producing sporophytes on chemo-attraction of cyanobacteria. Therefore, 13 gametophytes of each of four different moss species[2] were sampled to investigate their efficiency in attracting cyanobacteria. These were two of the most common boreal feather moss species (i.e. *P. schreberi* and *H. splendens*) and two acrocarpous mosses (i.e. *Dicranum polysetum* and *Polytrichum commune*) found in the study area. *Pleurozium schreberi* and *H. splendens* were used as host mosses (Ininbergs *et al.*, 2011), whereas *D. polysetum* and *P. commune* were used as non-host species because no or few cyanobacteria occur in association with them (Scheirer and Dolan, 1983). These mosses, as well as those used to study N transfer (i.e. 60 *P. schreberi* gametophytes), were randomly sampled in June 2010. In addition, five *P. schreberi* gametophytes[2] from a 1 m × 1 m N-amended plot (5 kg N ha^{-1} yr^{-1} as NH_4NO_3 dissolved in water, from June to September 2007-2010) and five gametophytes[2] from a 1 m × 1 m unfertilised control plot (to which only water was added) were randomly sampled from five sub-plots in September 2010 to assess the effect of N amendment. The N addition rate used in the N-fertilised plot is consistent with rates used in previous experiments in boreal forests (Gundale *et al.*, 2011). Finally, 36 *P. schreberi* gametophytes[2] were used for testing the cost of sporophyte production on cyanobacterial chemo-attraction. From these 36 gametophytes, 24 had well-developed sporophytes whereas the remaining 12 had no sporophytes.

For Paper III, 120 *P. schreberi* gametophytes[2] were randomly collected from the same area as for paper II (i.e. Ruttjeheden) in June 2010. They were used to study the role played by biotic and abiotic factors in determining the N_2-fixation activity of different cyanobacterial strains isolated from these mosses.

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2. Only the upper 3-5 cm green part of the gametophytes (ca. 10 mg dry weight) was used as this is where most of the N_2-fixation activity occurs on feather mosses (Solheim *et al.*, 2004).

2.3 Experimental methods and design of climate chamber experiments

2.3.1 Cyanobacteria isolation and cultures

In Papers I-III, the cyanobacteria present on the leaves of feather moss samples were isolated via agar plate culture. Mosses were placed singly on solidified BG-11_0 medium containing 0.9% Difco Bacto agar. BG-11 medium with or without $NaNO_3$ as a source of combined N (BG-11_N or BG-11_0, respectively; Stanier *et al.*, 1971; Rippka *et al.*, 1979) are standard growth media for many cyanobacteria genera and species (Stanier *et al.*, 1971; Rippka *et al.*, 1979, Lindblad *et al.*, 1989; West and Adams, 1997; Berrendero *et al.*, 2011). Due to the absence of N in its composition, BG-11_0 medium does not affect the N_2-fixation ability of heterocystous cyanobacteria and reduces the growth of other heterotrophic bacteria in the medium.

After one month under constant illumination (i.e. 18 µmol photons m^{-2} s^{-1}) at 24ºC, single cyanobacterial colonies were transferred to liquid BG-11_0 media. Purification of the different cyanobacterial cultures was achieved through multiple alternate growths on solidified and liquid BG-11_0 media. Isolated strains were subsequently observed by microscopy[3] to ensure that no other cyanobacteria (i.e. those of other genera) were contaminating the cultures. Further purifying treatments were not considered as the experiments in this thesis did not require the obtaining of axenic cyanobacterial cultures but rather monocultures. The cyanobacterial monocultures were then grown under the same conditions as above until further experimentation.

The purpose of Paper I was to obtain a "snapshot" of the cyanobacterial communities associated with both moss species at a given time. Therefore, moss gametophytes (i.e. 10 from each of the three island classes for both *P. schreberi* and *H. splendens*) were randomly selected for cyanobacterial isolation. In contrast, the preliminary aim in Papers II and III was to obtain specific strains of a heterocyst-forming cyanobacterial genus naturally associated with feather mosses (i.e. *Nostoc* spp.). This is because *Nostoc* has been suggested to be one of the most commonly occurring genera associated with *P. schreberi* and *H. splendens* (DeLuca *et al.*, 2002b; 2007; Houle *et al.*, 2006; Zackrisson *et al.*, 2009), and is an easily cultivable cyanobacterium which forms hormogonia; these were crucial requirements for the experiments in both papers. Therefore, at least 10 gametophytes of each moss species for

3. This was performed with an Olympus BX60 F5 epifluorescence microscope (Olympus Optical Co., Ltd, Tokyo, Japan) equipped with a cyanobacteria-specific green excitation filter (510-560 nm).

each of Papers II and III were observed by microscopy (using the same equipment as above) prior to cyanobacterial isolation to allow the selection of gametophytes with the highest cyanobacterial coverage, thus maximising the odds of obtaining the desired strains in culture.

In addition, cyanobacteria belonging to the genus *Stigonema* were commonly observed on leaves of the feather mosses sampled for Paper I but, in spite of several attempts, could not be isolated with the aforementioned technique (a problem also reported in other publications; Gentili *et al.*, 2005; Zackrisson *et al.*, 2009). Other isolation techniques (i.e. manual isolation from moss leaves, culture in liquid versus solidified $BG\text{-}11_0$ medium) also turned out to be inefficient. To circumvent this problem (which would otherwise have had a negative impact on the experiment), a non-axenic culture of *Stigonema ocellatum* NIES-2131 obtained from the Microbial Culture Collection (National Institute for Environmental Studies [NIES], Japan) was used as a reference strain in Paper I. It was grown in the general freshwater algae AF-6 medium (Provasoli and Pintner, 1959; Kato, 1982; Watanabe *et al.*, 2000; Kasai *et al.*, 2009), at 20ºC and with light intensity varying between 13 and 18 µmol photons m^{-2} s^{-1} (as suggested by the supplier) until further experimentation.

2.3.2 Cyanobacterial identification and diversity investigation

In Papers I-III, the identity of the cyanobacterial isolates obtained from moss leaves was first investigated through microscopic observations (see previous section for details about the microscope); this enabled identification of the different isolates in culture at the genus level. This is a fast and inexpensive method to obtain information about the identity of organisms when defining a cyanobacterium to the species level is not of crucial importance (as in Paper II).

To obtain as complete a picture of the cyanobacterial community on mosses as possible in Paper I, a method to discriminate between the different cyanobacterial species associated with boreal feather mosses was needed. Thus, the diversity (i.e. phylotype richness) and identity of these cyanobacteria were determined not only by morphological pre-identification using light microscopy, but also by molecular identification using denaturing gradient gel electrophoresis (DGGE) of the gene selected for polymerase chain reaction (PCR) amplification. This commonly used molecular technique has been proven to be efficient and sensitive enough to characterise microbial (and therefore cyanobacterial) population structures and dynamics (von Wintzingerode *et al.*, 1997; Muyzer, 1999; Maarit Niemi *et al.*, 2001; Morris *et al.*, 2002; Komárek, 2006; Wartiainen *et al.*, 2008). In brief, DGGE allows the

separation of PCR-generated DNA products based upon the sequence differences of the target gene, providing the molecular "fingerprint" of a given community where each band observed theoretically represents a different strain/phylotype. Because DGGE is based on the analysis of DNA, it consequently required DNA extraction and PCR amplification of the relevant samples. In Paper III, DNA extractions and PCR amplifications (followed by sequence analysis) were also required to confirm the identity of the isolated cyanobacterial strains.

In Paper I, DNA extractions were performed on intact moss-cyanobacteria associations because no method to separate cyanobacteria from their host moss appeared efficient or reliable enough. DNA was also extracted from the cyanobacterial cultures used as references for the phylogenetic study in Paper I (i.e. *Stigonema ocellatum* NIES-2131, *Nostoc punctiforme* PCC73102 and *Anabaena* PCC7120), as well as from the cyanobacterial isolates used in Paper III. Extractions of DNA from the different samples were achieved by Phenol:Chloroform:Isoamylalcohol 25:24:1 treatments (modified after Tillett and Neilan, 2000; Bauer *et al.*, 2008). This followed mechanical and chemical cell lysis (i.e. bead beating and xanthogenate buffer combined with sodium dodecyl sulphate [SDS], respectively; Jungblut and Neilan, 2006). Even though this method is slightly more time-consuming than the use of commercially available DNA extraction kits, its advantage is to avoid possible DNA yield loss, or even inefficiency, such as reported for microbial mats (Bauer *et al.*, 2008). The purity and concentration of DNA samples were assessed by spectrophotometry (NanoDrop Technologies, Inc., Wilmington, DE, USA), to make sure that the requirements for PCR amplification were met.

To be able to perform subsequent DGGE analyses in Paper I, the different cyanobacterial DNAs retrieved after extraction were amplified by PCR, using cyanobacteria-specific primers to ensure an amplification of the desired organisms. When studying the composition of cyanobacterial communities or their phylogeny, the highly conserved 16S rRNA gene (which codes for the small sub-unit of ribosomal RNA) is generally the preferred PCR-targeted gene (Nübel *et al.*, 1997; Redfield *et al.*, 2002; Gugger and Hoffman, 2004; Svenning *et al.*, 2005; Tomitani *et al.*, 2006). However, even though the use of this marker gene was considered for Paper I (Table 3), it was swiftly abandoned. Due to the lack of a technique to separate cyanobacteria from mosses, the PCR products obtained were mainly composed of 16S rRNA sequences originating from moss chloroplasts (due to the moss biomass being greatly in excess), preventing the retrieval of only cyanobacterial DNA by DGGE. As a consequence, cyanobacterial DNA in Paper I was PCR-amplified using the cyanobacteria-specific primer pair CNF/CNR; this primer pair targets

Table 3. *Oligonucleotide primers tested for amplification of cyanobacterial DNA in the papers presented in this thesis.*

Paper	Gene	Primer	Sequence (5'→ 3')	Reference
I, III	16S rRNA	CYA106F*	CGGACGGGTGAGTAACGCGTGA	Nübel *et al.*, 1997
I, III	16S rRNA	CYA781Ra	GACTACTGGGGTATCTAATCCCATT	Nübel *et al.*, 1997
I, III	16S rRNA	CYA781Rb	GACTACAGGGGTATCTAATCCCTTT	Nübel *et al.*, 1997
I, III	*nifH*	CNF*	CGTAGGTTGCGACCCTAAGGCTGA	Olson *et al.*, 1998
I, III	*nifH*	CNR	GCATACATCGCCATCATTTCACC	Olson *et al.*, 1998
I	*nifH*	PolF	TGCGAYCCSAARGCBGACTC	Poly *et al.*, 2001
I	*nifH*	PolR	ATSGCCATCATYTCRCCGGA	Poly *et al.*, 2001
I	*nifH*	nifH1F	TGYGAYCCNAARGCNGA	Zehr and McReynolds, 1989
I	*nifH*	nifH2R	ADNGCCATCATYTCNCC	Zehr and McReynolds, 1989
III	*hetR*	hetR1	AAGTGTGCAATATACATGAC	Rasmussen and Svenning, 2001
III	*hetR*	hetR2	TCAATTTGTCTTTTTTCTTC	Rasmussen and Svenning, 2001
III	*cpc*	PCβF	GGCTGCTTGTTTACGCGACA	Neilan *et al.*, 1995
III	*cpc*	PCαR	CCAGTACCACCAGCAACTAA	Neilan *et al.*, 1995
III	ITS	16S27F	AGAGTTTGATCCTGGCTCAG	Wilmotte *et al.*, 1993
III	ITS	23S30R	CTTCGCCTCTGTGTGCCTAGGT	Lepère *et al.*, 2000

* Used for DGGE analysis with an additional GC-clamp, as follows: CGC CCG CCG CGC CCC GCG CCG GTC CCG CCG CCC CCG CCC G.
The reverse primers CYA781Ra and CYA781Rb were used as an equimolar mixture.
Modified bases: Y = T/C, S = C/G, R = A/G, D = A/T/G, B = T/C/G, N = A/T/C/G.

the *nifH* gene, the most conserved of the genes involved in coding for the nitrogenase enzymatic complex (Postgate, 1982; Table 3). The *nifH* gene has been shown to be suitable for genetic identification of diazotrophic communities at intra-species level (and encodes the enzyme dinitrogenase reductase, see 1.3.2; Ben-Porath *et al.*, 1993; Mårtensson *et al.*, 2009). Several *nifH* primers were tested in Paper I (Table 3), but only the CNF/CNR pair successfully amplified all samples. Consequently, all other primer pairs were excluded from further analyses. After DGGE analysis, eluted DNA from excised bands was re-amplified using the same primers (i.e. *nifH*, without the GC-clamp), and the samples from the first analysis were subjected to a three-cycle PCR amplification to obtain heteroduplex-free samples (Thompson *et al.*, 2002). For Paper III, five different families of primers were tested to distinguish between the three *Nostoc* strains isolated (i.e. 16S rRNA, *nifH*, *hetR*, *cpc* [phycocyanin operon], and 16S-23S rRNA internal transcribed

spacer [ITS]; Table 3). *Cpc*, *hetR* and ITS genes have been shown to be appropriate for characterising cyanobacterial populations at species level (Neilan *et al.*, 1995; Janson *et al.*, 1999; Boyer *et al.*, 2001; respectively). However, only the cyanobacteria-specific hetR1/hetR2 primer pair (coding for heterocyst and akinete differentiation in filamentous cyanobacteria; Buikema and Haselkorn, 1991) could amplify all three *Nostoc* strains and distinguish between them; the other primers were therefore disregarded. In all cases (i.e. in Papers I and III), PCR amplifications were performed three times and PCR products were pooled to reduce possible PCR-induced biases, the eluted PCR products (from DGGEs) were purified using a commercially available PCR purification kit (Qiagen GmbH, Germany), and purities and concentrations were assessed by spectrophotometry before sequencing (see above for equipment details).

In Paper I, DGGE analysis was carried out to separate the different partially amplified *nifH* genes obtained by PCR, giving an overview of the cyanobacterial diversity of each of the samples (i.e. a "fingerprint" of the cyanobacterial community). A second analysis (performed on re-amplified DNA of the excised bands from previous DGGEs) was used to check before sequencing that the observable bands of the first analysis were not artefacts. Owing to the number of samples investigated, each of the two analyses could not be achieved at once (i.e. with only one gel for each analysis). To normalise the different DGGE gels and then enable their comparison, a ladder made of four *nifH* fragments covering the whole dispersion of expected bands was designed. Given the complexity of the patterns obtained and to avoid any sort of analytical bias, the similarity between the samples was assessed using Fingerprinting II 3.0 (Bio-Rad Laboratories, USA). Reproducibility of the DGGE patterns was confirmed by performing each analysis two to three times.

To identify accurately the *nifH* sequences retrieved from DGGE analysis, comparisons with the sequences available in GenBank (using blastn megablast; Altschul *et al.*, 1990) and Integrated Microbial Genomes (http://img.jgi.doe.gov/) were performed. The phylogenetic relationship between the different cyanobacterial species associated with feather mosses, based on the cyanobacterial sequences previously identified, was scrutinised using FastTree (Price *et al.*, 2010). Additionally, the *nifH* sequence of *Stigonema ocellatum* NIES-2131, of isolated cultures from the mosses (*Nostoc* sp. and *Cylindrospermum* sp.) and of reference strains (*Nostoc punctiforme* PCC73102 and *Anabaena* PCC7120) were incorporated into the phylogenetic analysis. This was to ascertain the retrieval of tangible products. In Paper III, the DNA sequences obtained by PCR were compared with the nucleotide collection database available in GenBank using blastn megablast (Altschul *et*

al., 1990). Sequence similarity assessment was performed using Clustal X 2.0.10 (Larkin *et al.*, 2007).

Although DGGE is recognised as a useful method for providing an accurate and rapid overview of microbial community composition (von Wintzingerode *et al.*, 1997; Muyzer, 1999; Maarit Niemi *et al.*, 2001; Morris *et al.*, 2002; Komárek, 2006; Wartiainen *et al.*, 2008), it also has several limitations which need to be taken into consideration. Potential biases, which would influence the interpretation of the results, are linked with the DNA extraction method (e.g. inefficiency in retrieving the DNA of all the organisms of interest), the PCR technique (e.g. formation of heteroduplexes), and limited sensitivity of the DGGE technique itself (e.g. detection of dominant species only, problems when separating small DNA fragments, co-migration of DNA fragments with different sequences, or differing migration of DNA fragments with similar sequences) (Muyzer and Smalla; 1998; Jackson *et al.*, 2000). Great care was undertaken in the study reported in Paper I to prevent such problems, which included the use of a reliable DNA extraction method, optimisation of the PCR/DGGE conditions, "re-conditioning PCR" (thus eliminating heteroduplexes; Thompson *et al.*, 2002), and sequencing of the PCR products. Issues regarding the migration of DNA fragments are discussed in section 3.1.

2.3.3 Hormogonia induction assessment

Several plants with the ability to form symbioses with cyanobacteria are known to secrete HIFs (Campbell and Meeks, 1989; Rasmussen *et al.*, 1994). In Paper II, a simple method was developed to assess whether different moss species could induce the formation of hormogonia in a *Nostoc* sp. culture. The concept of the experiment was to have a moss in the close vicinity of the *Nostoc* culture (isolated from *H. splendens*), with the interactive partners physically separated but not isolated from each other. Such an experimental set-up (i.e. a moss-containing cell culture insert[4] with a 1 µm-pore size membrane fitted to a well filled with 1 ml of *Nostoc* sp. culture [100 mg ml^{-1}]; Figure 6) allowed the diffusion of putative moss-secreted signals in the cyanobacterial medium, but also prevented the movement of cyanobacteria (hormogonia) towards the moss. Testing for the moss-induced hormogonia formation over time was therefore achieved through simple counting of hormogonia in the medium using a hemocytometer. This was expressed as the ratio between the number of hormogonia and the total number of cyanobacterial filaments present in the

4. BD Falcon™ cell culture inserts and plates, BD Biosciences, Erembodegem, Belgium.

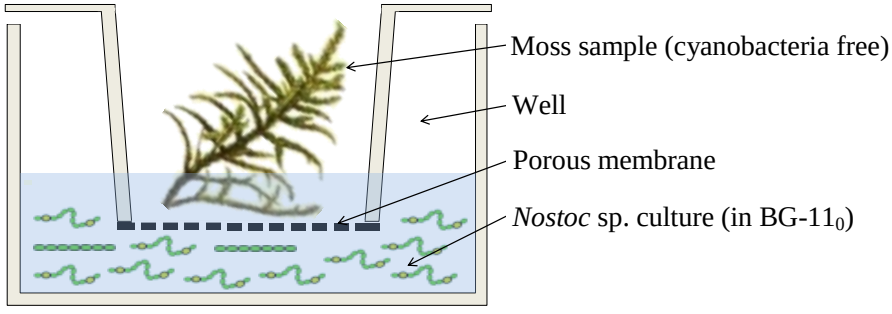

Figure 6. Schematic representation of the experimental set-up for the assessment of hormogonia induction by mosses. Note the presence of both vegetative filaments and hormogonia in the culture medium.

medium. This experiment was performed in a culture chamber with a constant light intensity of 18 µmol photons m^{-2} s^{-1} and a temperature of 24ºC, and set up with four independent replicated blocks; each block consisted of five treatments with each treatment consisting of one of the four moss species and a control (i.e. no moss). Prior to placing mosses in the cell culture inserts, they were surface-sterilised to remove all epiphytic cyanobacteria using 0.15% sodium hypochlorite for 1 min, followed by seven rinses with sterile water to remove any trace of sodium hypochlorite (modified after Misaghi and Donndelinger, 1990). Although this is the only reliable method to get rid of cyanobacteria on mosses, it is a rather rough treatment for mosses and must therefore be well handled to avoid permanent damage to the moss samples. Confirmation of cyanobacterial removal occurred through microscopic observation of the moss samples (see 2.3.1 for details about the microscope).

2.3.4 Chemoattraction assessment and measurement of cyanobacterial density on mosses

Many plants forming symbioses with cyanobacteria attract their symbiotic partners through chemotaxis (Meeks, 1998). In Paper II, the ability of mosses to attract *Nostoc* hormogonia from the culture medium was assessed after eight days of incubation (i.e. the minimum time required to allow chemotactic responses and physiological processes to occur; Meeks and Elhai, 2002; Kumar *et al.,* 2010) based on the method described above (Figure 6). The main difference with the previous experimental set-up was the use of an 8 µm-pore size membrane (instead of 1 µm). This allowed hormogonia to pass through the membrane (as their diameter does not exceed 4-5 µm) and eventually colonise the mosses. This experiment was set up with five independent replicated

blocks; each block consisted of four treatments with each treatment consisting of one of the four moss species. Colonisation of the moss was determined following incubation. In addition, this experiment was repeated in darkness to ensure that the movement of hormogonia towards the moss was not related to light attraction. Further, the effect of N-amendment on chemo-attraction was assessed using five independent replicated cell culture blocks (as described above), where each block consisted of two treatments, i.e. a N-amended *P. schreberi* gametophyte and a control gametophyte. Finally, the impact of sporophyte production on cyanobacterial chemo-attraction was assessed in an experiment with 12 independent replicated blocks. Each block consisted of three treatments with these treatments consisting of different gametophyte types, i.e. gametophytes with sporophytes, those with sporophytes removed (to test if sporophyte removal would induce a change in N_2-fixation rates), and gametophytes without sporophytes (controls).

Cyanobacterial density on mosses (a measure of the potential of the mosses to attract cyanobacteria in Paper II) was quantified using ImageJ software V.1.45s (Rasband, 1997-2012), and expressed as the proportion ratio of the total surface area occupied by cyanobacteria on mosses. The same approach was used in Paper III to determine the survival rates of cyanobacteria on mosses following different biotic and abiotic treatments (see 2.3.5 for details on the treatments). The density of cyanobacteria was expressed as the ratio between the total surface area occupied by the cyanobacteria on the moss and the weight of the moss. In Papers II and III, pictures of the moss-cyanobacteria associations were recorded using a SteREO Lumar V.12 microscope (Carl Zeiss AG, Germany) with the filter sets 31 Cy3.5 (cyanobacteria-specific; excitation 550/580 nm, emission 590/650 nm) and GFP (excitation 460/480 nm, emission 505/530 nm), and AxioVision software V.4.8.2.0 (Carl Zeiss MicroImaging GmbH, Germany).

2.3.5 Effect of abiotic and biotic treatments on cyanobacterial dinitrogen-fixation

Several factors, both abiotic and biotic, are known to influence cyanobacterial N_2-fixation rates (Gerard *et al.*, 1990; Kashyap *et al.*, 1991; Meeks, 1998; Rai *et al.*, 2000; Zielke *et al.*, 2002; Zackrisson *et al.*, 2004; Gentili *et al.*, 2005). However, the interactive effect of these factors on N_2-fixation has never been assessed, nor have the N_2-fixation rates of individual cyanobacterial species or combinations of species ever been studied. Therefore, the effects of two abiotic factors (i.e. light intensity and temperature), two biotic factors (i.e. host moss presence and cyanobacterial species identity), and their interactions on cyanobacterial N_2-fixation activity were tested under controlled conditions in

Paper III. For this experiment, four cyanobacterial species treatments (i.e. each of the three *Nostoc* species previously isolated alone or a mixture of all species, in order to investigate their response to environmental conditions) were exposed to each of three light intensities, two temperatures, and the presence and absence of host moss, in all possible combinations (i.e. 48 treatments). The experiment was set up with four independent replicate blocks for each possible treatment combination, except for the treatments involving presence of moss which were replicated five times. For this experiment, N_2-fixation activity was measured for all experimental units after eight days of treatment.

To test for the specific effect of light on N_2-fixation rates, two intensities occurring in boreal forests during the vegetation period giving "extreme" responses in terms of N_2-fixation activity were selected. The light intensities (high light = 5000 lux ≈ 70 µmol photons m^{-2} s^{-1}, low light = 1000 lux ≈ 13 µmol photons m^{-2} s^{-1}) correspond to when average N_2-fixation measurements recorded in June and September 2010 on a feather moss-cyanobacteria association in northern Sweden were the highest and lowest, respectively (DeLuca *et al.*, 2002b; Figure 7). To confirm the strong effect that we assumed light would have on N_2-fixation activity, the effect of darkness on N_2-fixation was also tested, giving three different light intensity treatments.

The effect of temperature on rates of N_2-fixation was tested with two temperatures (i.e. high temperature = 17ºC and low temperature = 10ºC). They were chosen because they correspond to the normal mean temperatures for June and September from 1960 to 1990 near the town of Arvidsjaur, 25 km from the sampling site (SMHI - Swedish Meteorological and Hydrological Institute). This is because June and September are the months in which the N_2-fixation activity of cyanobacteria associated with feather mosses has been shown to be greatest (DeLuca *et al.*, 2002b).

The experiment was performed both in the presence and absence of a *P. schreberi* gametophyte. This moss species was selected because of its widespread occurrence in boreal forests (DeLuca *et al.*, 2002b) and because the data which were used to determine the selection of the three light intensities (see above) originated from *P. schreberi*-cyanobacteria associations (Figure 7).

Finally, the N_2-fixation response of distinct species of cyanobacteria to environmental conditions was tested on each of the three *Nostoc* species previously isolated alone or on a mixture of all species. To ensure an equal amount of cyanobacteria in each experimental unit, the chlorophyll *a* of the cyanobacterial cultures was extracted in cold absolute methanol. The optical density (OD) was then measured following Nagarkar and Williams (1997), using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Cambridge,

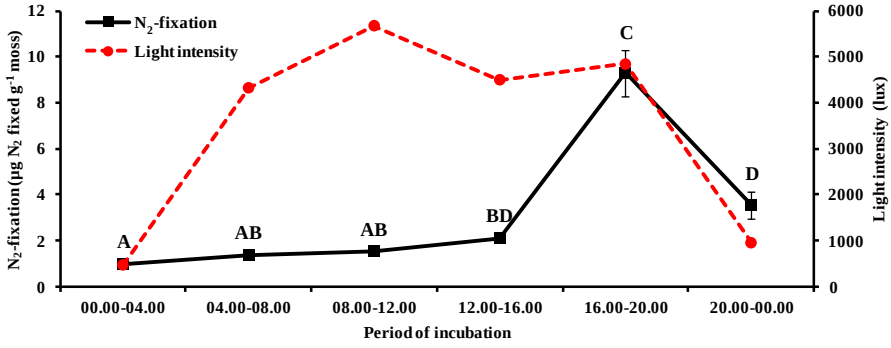

Figure 7. Fluctuations of light intensity and N_2-fixation for *Pleurozium schreberi* gametophytes over a 24-hour (one day) period. Consecutive measurements of N_2-fixation rates were each performed after a 4-hour incubation (corresponding to each data point on the figure) at a natural forest site near Umeå, northern Sweden, in June 2010[5] (Bay *et al.*, unpublished). Light intensity measurements performed at the same site, prior to each N_2-fixation measurements. Significant differences for N_2-fixation measurements are indicated by different letters above each data point (A, B, C or D; ANOVA followed by Tukey's HSD test, $p \leq 0.001$). Statistical analysis performed on log-transformed data. Error bars, ± SE ($n = 3$ for moss samples and $n = 4$ for light intensity).

England). Following the OD measurement, each culture was diluted with BG-11_0 medium to obtain a chlorophyll *a* concentration of 1.6 µg ml^{-1} before use.

Prior to N_2-fixation measurements (i.e. the main experiment), the moss-cyanobacteria associations and *Nostoc* cultures were maintained for eight days in a culture chamber with a constant light intensity of 18 µmol photons m^{-2} s^{-1} and a temperature of 24ºC, to allow cyanobacteria to develop on the moss and acclimatise to the environmental conditions.

2.3.6 Dinitrogen-fixation and nitrogen transfer

Dinitrogen fixation rates of all samples collected for Papers I-III were measured by ARA (see Box 3 for its principle), as described by DeLuca *et al.* (2002b). Samples were processed within days after collection, thus reducing possible shifts in the cyanobacterial populations associated with the feather mosses (e.g. due to death). The mosses were also regularly misted with deionised water to keep both cyanobacteria and mosses healthy until further experimentation. Rates of N_2-fixation activity were expressed as µg of N_2 fixed g^{-1} moss d^{-1} (Papers I-III) or as ng of N_2 fixed experimental $unit^{-1}$ d^{-1} (Paper III), using a ratio of 3 moles of ethylene reduced per mole of N fixed (DeLuca

5. The light intensities corresponding to the highest and lowest N_2-fixation activities in September were similar to those observed in June (data not shown).

et al., 2002b; Zackrisson *et al.*, 2004; Lagerström *et al.*, 2007; Ininbergs *et al.*, 2011).

The transfer of $^{15}N_2$ from cyanobacteria to *P. schreberi* samples was monitored and quantified (as a ratio between ^{15}N and ^{14}N measured in the samples) through secondary ion mass spectrometry (SIMS) analysis in Paper II. SIMS is a qualitative and quantitative technique used to determine the isotopic, elemental or molecular composition of the surface of samples. To verify the incorporation of $^{15}N_2$ into the cyanobacteria (associated with *P. schreberi* mosses), the samples were analysed for ^{15}N content prior to SIMS analysis with an automated elemental analyser isotope ratio mass spectrophotometer (EA-IRMS), following the procedure described by Ohlsson and Wallmark (1999).

2.4 Statistical evaluation of the data

In Paper I, principal components analysis (PCA) was applied to the *nifH* gene compositional data for the 60 samples (2 moss species × 30 islands), to summarise the data into a smaller number of variables; we used primary and secondary ordination axis (PC1 and PC2) scores for subsequent analysis. Split-plot analysis of variance (ANOVA) was used to estimate the effects of island size, moss species and their interaction on each response variable (i.e. N_2-fixation, number of bands obtained by DGGE, and the two principal axis scores from PCA). Island size was the main plot factor whereas moss species was the subplot factor. Pearson's correlation coefficient (r) was used to determine the relationship for each moss species between N_2-fixation and the number of DGGE bands obtained and ordination scores for the band data. All data were transformed as necessary to satisfy the assumptions of parametric data analysis.

In Paper II, the ability of the different moss species to induce hormogonia was tested in a linear mixed-effect model (LMER), using a Gaussian error. The fixed effect was the moss treatment (i.e. each of the four moss species and the moss-free control) and the random effect was the time of exposure of the *Nostoc* culture to the different mosses. The effect of time on hormogonia formation was tested by ANOVA for each time point, on Box-Cox transformed data (Box and Cox, 1964; Osborne, 2010). The choice of a LMER test was because of the repeated nature of the measurements (i.e. measurement of hormogonia formation on the same samples over time) and non-normality of the data. Several transformations of the data were tested (i.e. square root, log, $\log^2$, 1/x) but none gave better results in terms of normality than the Box-Cox transformation (Box & Cox, 1964); it was consequently used when necessary.

The effects of moss species, N amendment and sporophyte presence on cyanobacterial colonisation and N_2-fixation were each assessed by ANOVA, on Box-Cox transformed data (Box & Cox, 1964; Osborne, 2010). Tukey's honest significant difference (HSD) post-hoc tests at $P \leq 0.05$ were subsequently performed to investigate significant differences between moss species, and between treatments (i.e. N amendment and sporophyte presence). The effect of $^{15}N_2$ exposure on $^{15}N_2$ enrichment in gametophytes was assessed by a non-parametric Wilcoxon test.

In Paper III, the effects of light intensity, temperature, host moss presence, and cyanobacterial species identity on N_2-fixation rates was compared with ANOVA. The data set was previously tested for normality and subsequently Box-Cox transformed (Box & Cox, 1964; Osborne, 2010). Tukey's HSD post-hoc test at $P \leq 0.05$ was performed to investigate significant differences between means. Similar procedures were followed to compare the effect of light intensity, temperature and host moss presence on cyanobacterial density on mosses.

All statistical analyses were carried out using R software v.2.13.1. (R Development Core Team, 2007). Data transformation in Papers II and III was performed using statistical software XLSTAT 2012.5.02 (Addinsoft, 1995-2012).

3 Results and Discussion

This thesis investigates how cyanobacterial communities and their associated N_2-fixation activities were influenced by (i) the type of moss hosting these cyanobacteria, and (ii) contrasting environments in the boreal forest (Paper I). It further investigates the factors driving the colonisation of these mosses by cyanobacteria and the fate of the N fixed by cyanobacteria (Paper II), as well as how cyanobacterial N_2-fixation activity is influenced by various biotic and abiotic factors (Paper III). The main results are now presented and discussed.

3.1 Community composition and diversity of cyanobacteria associated with boreal feather mosses are driven by the host mosses

One of the main goals of Paper I was to characterise the cyanobacterial community associated with two common boreal feather mosses collected from environments differing in their productivity and N availability. The species of moss hosting the cyanobacterial communities and the type of environment from which these mosses were sampled represented the main factors that were tested. Specifically, this work involved the characterisation of *nifH* gene sequences retrieved from cyanobacterial communities found on *P. schreberi* and *H. splendens* samples from contrasting environments.

The results from the DGGE fingerprinting and sequence analyses demonstrated that the cyanobacterial community composition and diversity were both responsive to moss species. Thus, *P. schreberi* and *H. splendens* harboured different cyanobacterial communities and certain strains of cyanobacteria proved to be host-specific as they were only found on one of the moss species. This translated into the detection by DGGE of six specific *nifH* phylotypes (i.e. bands) that were specific to *P. schreberi* and 17 that were specific to *H. splendens* (out of 35 unique *nifH* phylotypes in total). This result

was also supported by dendrogram analysis of the sequences obtained after DGGE which revealed five clusters; two of these clusters were composed only of *nifH* phylotypes from cyanobacteria restricted to *H. splendens*. Further, the dendrogram based on the presence versus absence of bands in the DGGE profiles showed that all samples from each of the moss species clustered together. This result demonstrates that the cyanobacterial communities found on the different *P. schreberi* samples were similar, but also somewhat distinct from the communities found on the different *H. splendens* samples, and vice-versa. Moreover, the number of *nifH* phylotypes per sample, reflecting the diversity of the cyanobacterial community, was statistically significantly responsive to moss species; the number of *nifH* phylotypes was on average larger on *H. splendens* samples than on *P. schreberi* samples. Therefore, these findings provide evidence for the predominant role moss species play in selecting their cyanobacterial partners. However, great within-species variations of the number of *nifH* phylotypes (ranging from 1 to 14) were also found for both moss species. Despite the possible omission of weak bands during the analysis of the DGGE profiles, this variability in the number of *nifH* phylotypes per sample indicates that the selection of cyanobacteria by mosses may also be dependent on other factors (e.g. variable diversity in the pool of cyanobacteria available where the moss grows; see Garbeva *et al.*, 2004), despite the overriding influence of moss species identity.

Surprisingly, neither the DGGE nor dendrogram analysis revealed any effect of island size (and therefore local environmental conditions; Wardle *et al.*, 1997; Lagerström *et al.*, 2007; Gundale *et al.*, 2010) on cyanobacterial community or diversity for either moss species. This is indicated in the DGGE analysis by the absence of a clear arising pattern in the distribution of *nifH* phylotypes among samples for both moss species. Moreover, the effect of island size on the number of *nifH* phylotypes was not statistically significant, contrary to what was observed for moss species. In addition, no clear-cut pattern could be inferred from the dendrogram analysis of sequences, as the five gene clusters each contained representative *nifH* phylotypes from all three island classes. Therefore, cyanobacterial community composition and diversity on *P. schreberi* and *H. splendens* could not be explained by the parameters known to influence moss performance on these islands (e.g. soil properties, nutrient availability, moisture conditions, light; Wardle *et al.*, 1997). Consequently, moss identity appears to be the primary factor in driving cyanobacterial community composition and diversity on feather mosses. This is in accordance with findings on other bryophyte-bacteria associations showing that the bacterial community is specific to individual moss species, irrespective of their geographical location (Opelt *et al.*, 2007).

Isolation of the associative cyanobacterial populations from both *P. schreberi* and *H. splendens* resulted in the morphological identification of four different heterocystous strains belonging to two genera (three of *Nostoc* and one of *Cylindrospermum*), although these were not identified to the species level. In the case of the *Nostoc* isolates, three morphotypes could be distinguished which differed in cell size and shape. Another heterocystous genus, *Stigonema*, was observed on leaves of both moss species but could not be isolated. Notwithstanding the obvious limitations of such an approach for identifying organisms (e.g. possible exclusion of uncultivable or rare strains, confusion during identification), microscopic observations enabled the detection of *Cylindrospermum*, a genus of N_2-fixing cyanobacteria not previously reported to live in association with feather mosses. The dearth of sample sequence clustering with the *Cylindrospermum* isolate (in the phylogenetic analysis) suggests that this strain was not one of the dominant species of cyanobacteria colonising these feather mosses, at least when sampled. Consequently, this emphasises the usefulness of using both a culture-based and a DNA-based technique for identification, as this genus would not have been detected in the absence of both approaches. Additionally, strain isolation confirmed that several different cyanobacterial genera and species co-exist on each of the feather moss species tested (though with a relative predominance of *Nostoc* species, as a majority of phylotypes are related to *Nostoc*), providing evidence that mosses "recruit" cyanobacteria with diverse genetic backgrounds.

Previous work on other ecosystems such as stromatolites, seagrasses and marine microbial mats showed the associated diazotrophic communities to be highly diverse (Steppe *et al.*, 2001; Yannarell *et al.*, 2006; Bauer *et al.*, 2008; Severin and Stal, 2010). In this study, 35 *nifH* phylotypes were retrieved from the cyanobacterial populations of *P. schreberi* and *H. splendens*. Although each single band found by DGGE is supposed to represent a specific organism (which would mean that 35 different cyanobacterial strains were found on the mosses), caution in interpreting the data is needed. Based on the similarity with *nifH* sequences located outside the *nif* operon (from complete genomes of heterocystous cyanobacteria), the *nifH2* cluster appears to contain sequences known to be copies of *nifH*. Thus, we cannot exclude the possibility of an overestimation of cyanobacterial diversity in this study, as it is likely that other *nifH* phylotypes detected by DGGE may also be gene copies rather than true genotypes. For instance, *Nostoc punctiforme* is known to possess three copies of the *nifH* gene (Meeks *et al.*, 2001); a duplication which could take place in other cyanobacteria found in this work. Moreover, co-migration of DNA fragments with different sequences and variable migration of DNA fragments

that possess the same sequence are well-known issues (Jackson *et al.*, 1998; Muyzer and Smalla, 1998; Jackson *et al.*, 2000). Since only a selection of bands retrieved by DGGE was sequenced (i.e. for each of the 35 phylotypes, two to three bands were selected for sequencing), such migration issues may also have occurred in this analysis. As a consequence, this suggests that cyanobacterial diversity on feather mosses might not be as high as the results suggested. The results further suggest that the diversity of cyanobacteria on *P. schreberi* and *H. splendens* is lower than in other ecosystems (Steppe *et al.*, 2001; Yannarell *et al.*, 2006; Bauer *et al.*, 2008; Severin and Stal, 2010).

Overall, these findings stress that the diazotrophic communities associated with *P. schreberi* and *H. splendens* are highly host-specific and diverse, with their composition and diversity being under the control of the host moss rather than extrinsic environmental factors. Therefore, these findings help in developing our understanding of the dynamics of cyanobacterial communities on boreal feather mosses, and point towards a more finely tuned relationship between the two partners than previously thought (see Marshall and Chalmers, 1997). The host specificity observed could therefore indicate a certain degree of co-evolution between feather mosses and particular cyanobacterial strains, as previously shown for *Azolla*-cyanobacteria symbioses (Papaefthimiou *et al.*, 2008). Although the exact reason as to why these two moss species associate with different strains of cyanobacteria remains unclear, it is tempting to speculate about the close relationship they might maintain with their N_2-fixing partners. The host moss could, for instance, provide a micro-environment adapted for certain groups of cyanobacteria only (e.g. groups sharing similar physiological requirements), which would explain why some strains seem to develop better on one moss species than the other. In addition, it is plausible that the cyanobacterial strains shared by both *P. schreberi* and *H. splendens* (corresponding to ca. a third of the *nifH* phylotypes) consist of the most represented cyanobacterial strains on the island system, at least at the time of sampling, or that they can tolerate a wider range of conditions (making them less selective towards their host moss).

3.2 Colonisation of boreal feather mosses by cyanobacteria is dependent on moss-secreted chemicals

One of the aims of Paper II was to explore the mechanisms as well as the physiological conditions under which feather mosses are colonised by cyanobacteria. It is known that other plants forming symbioses with cyanobacteria secrete chemical compounds (HIFs and chemo-attractants; Campbell and Meeks, 1989; Rasmussen *et al.*, 1994; Knight and Adams, 1996;

Ow *et al.*, 1999), which in turn leads to the establishment of cyanobacteria on the host plant. Based on this knowledge, the ability of common boreal feather mosses that host cyanobacteria (*P. schreberi* and *H. splendens*) and of other boreal bryophytes that do not (*D. polysetum* and *P. commune*) to produce molecules that might attract cyanobacteria was assessed under controlled conditions. Additionally, to elucidate how N limitation or the need for N may impact on cyanobacterial colonisation, the effects of N-amendment and moss reproductive cost were studied on gametophytes of *P. schreberi*, the most abundant feather moss in boreal forests (DeLuca *et al.*, 2002b).

3.2.1 Different mosses induce the formation of cyanobacterial hormogonia

The results from hormogonia formation assessments showed that after only 24 hours of incubation, all mosses (i.e. the host mosses *P. schreberi* and *H. splendens* and the non-host mosses *D. polysetum* and *P. commune*) were able to induce differentiation of *Nostoc* sp. filaments into hormogonia at significantly higher levels than in the controls (i.e. *Nostoc* sp. cultures without moss). Further, the promotion of hormogonia formation peaked after 48 hours of incubation, representing ca. 45% and 60% of the total number of cyanobacterial filaments in the media for the host and non-host mosses, respectively. As only 10% of hormogonia formed naturally during the experiment in the controls, this result demonstrates that the four moss species tested promote hormogonia differentiation, most likely through the release of a molecule in the media (presumably a HIF) which has similar effects to what was previously observed with *Gunnera* and *Anthoceros* (Campbell and Meeks, 1989; Rasmussen *et al.*, 1994). Additionally, the slight acidification of the cyanobacterial medium observed over time did not have any significant effect on hormogonia differentiation. Therefore, the HIFs secreted by the mosses are strong enough to override the possible negative effect of the pH on hormogonia formation and gliding ability (Hirose, 1987; Rasmussen *et al.*, 1994).

Interestingly, the same pattern of hormogonial induction was observed for the two host mosses, as well as for the two non-host mosses. This similarity in potency suggests that the HIFs secreted by *P. schreberi* and *H. splendens* (and therefore *D. polysetum* and *P. commune*) may be chemically close (if not identical). Moreover, the non-host mosses promoted a faster and more efficient induction of hormogonia than did the host mosses during the first 48 hours of the experiment. There are two plausible explanations for this pattern: either *D. polysetum* and *P. commune* secrete higher quantities of HIFs or they secrete more effective HIFs. The latter, if confirmed, would imply that the non-host mosses produce HIFs with a different molecular composition than those of the host mosses. Nevertheless, none of the HIFs produced by these four mosses is

as efficient as the HIF produced by *Anthoceros punctatus* or *Gunnera*, which can induce hormogonia formation in *Nostoc* cultures of over 90% (Campbell and Meeks, 1989; Rasmussen *et al.*, 1994). This indicates that mosses may secrete HIFs which are chemically different from these two plants, and therefore may also suggest that the composition of each HIF is species-specific.

After 72 hours of incubation, the ratio of hormogonia (to the total number of cyanobacterial filaments) induced in the media by non-host and host mosses decreased or remained constant respectively. These patterns are in accordance with previous results showing that hormogonia turn back to their vegetative state after a period of 48 hours (Meeks and Elhai, 2002). This variation in hormogonia induction raises an interesting question with regards to the frequency of HIF secretion. A constant increase in hormogonia differentiation would be expected if the secretion of HIF was continual, even if some cyanobacteria were then to revert to their vegetative state and were then unable to form hormogonia for a period of time (Meeks and Elhai, 2002). Since the ratio of hormogonia in the media does not increase constantly, it is therefore possible that the mosses do not continuously produce HIFs. Although much work remains to be done to understand how HIF secretion in mosses varies (if indeed it does), a certain form of regulation would not be surprising given that HIF production is likely to be an energy-demanding process. If this is indeed the case, this would also mean that mosses are able to "sense" cyanobacteria in their vicinity, which could potentially trigger them to produce HIFs, maybe as a consequence of cyanobacteria-secreted compounds.

Even if the putative chemicals produced by the four moss species tested have neither been isolated nor characterised (which remains to be achieved in order to fully understand how mosses and cyanobacteria communicate), the production of HIF appears as the most likely explanation for the significant levels of hormogonia differentiated throughout the experiment. However, one aspect of these results is surprising, as it is known that *D. polysetum* and *P. commune* (both of which were found to produce HIFs) do not host cyanobacteria (Scheirer and Dolan, 1983). Therefore, the reason why feather mosses are colonised by cyanobacteria cannot be explained entirely by the production of HIFs. Consequently, these findings suggested the intervention of another undefined process such as the production of another molecule that would be specific only to feather mosses.

3.2.2 Feather mosses attract cyanobacteria via the secretion of a chemo-attractant when in need of nitrogen

The results from assessments of *Nostoc* hormogonia colonisation on the four moss species revealed that hormogonia were exclusively attracted towards *P. schreberi* and *H. splendens* (ca. 5 to 15% of the moss surface was colonised); *D. polysetum* and *P. commune* remained free of cyanobacteria. Similar colonisation rates were found in darkness for *P. schreberi* and *H. splendens*, demonstrating that cyanobacterial attraction was not light-induced. A probable explanation is that, besides a HIF, the feather mosses also produced another molecule, presumably a compound inducing a chemotactic response (hereafter referred to as chemo-attractant) of the cyanobacteria. The reason why the other two mosses were not colonised (i.e. *D. polysetum* and *P. commune*), which is also the case in natural conditions, may then either be due to their incapacity to secrete such a type of compound or to the secretion of a repellent. Moreover, the cyanobacteria which colonised both feather mosses were able to fix N_2. This suggests that a functional association was formed with both *P. schreberi* and *H. splendens* after colonisation by the *Nostoc* strain used in this experiment. However, the quantities of N_2 fixed were higher than those observed in natural feather moss-cyanobacteria associations (Lagerström *et al.*, 2007), most likely because cyanobacterial density on the experiment mosses was greater than in the wild. As a conclusion, these findings add to our understanding of feather moss-cyanobacteria chemical communication, and demonstrate that although hormogonia differentiation is a crucial step for host plants to be colonised, this cannot be achieved without the cooperation of a chemo-attractant to attract and guide hormogonia towards suitable colonisation sites (i.e. leaves in the case of feather mosses).

To test if chemo-attractant production is influenced by the N status of the moss, cyanobacterial colonisation was assessed on *P. schreberi* gametophytes amended with N. What was observed was that the gametophytes remained uncolonised, and no subsequent N_2-fixation activity was detected. This suggests that high levels of N inhibit chemo-attractant secretion by mosses. This is plausible because under N-repleted conditions, a moss would probably spare the cost of producing chemo-attractants as it may not need to form associations with cyanobacteria. Therefore, it appears that when N is freely available (i.e. neither immobilised nor in its gaseous form), both cyanobacteria and feather mosses switch their physiology towards the most economical strategy with regards to energy (i.e. interruption of N_2-fixation or chemo-attractant secretion, respectively; Wallenstein *et al.*, 2006; Demoling *et al.*, 2008; Gundale *et al.*, 2011).

To understand if the release of chemo-attractants by feather mosses would be affected by a N-demanding process such as sporophyte production (Rydgren and Økland, 2002), *P. schreberi* gametophytes with and without sporophytes were assessed for their ability to attract cyanobacterial hormogonia. While gametophytes without sporophytes attracted hormogonia at comparable rates to those previously observed (i.e. with 5 to 10% of the moss surface colonised), a four-fold greater cyanobacterial colonisation was observed on gametophytes bearing sporophytes. Given that sporophytes serve as N sinks (Renault *et al.*, 1989), it is therefore likely that *P. schreberi* gametophytes allocate more resources to actively produce chemo-attractants when in their reproductive phase. In accordance with this theory, gametophytes from which sporophytes were removed prior to experimentation attracted hormogonia at intermediate rates, which is consistent with a reduced N demand. Therefore, the need for N turns out to be one of the main drivers of chemo-attractant secretion in feather mosses. Moreover, given that sporophyte production is seasonal (Longton and Greene, 1969), this may explain the seasonality of N_2-fixation patterns generally observed in nature (DeLuca *et al.*, 2002b). Similar source-sink relationships between flowering and N_2-fixation have been reported for legumes (Sprent, 2009).

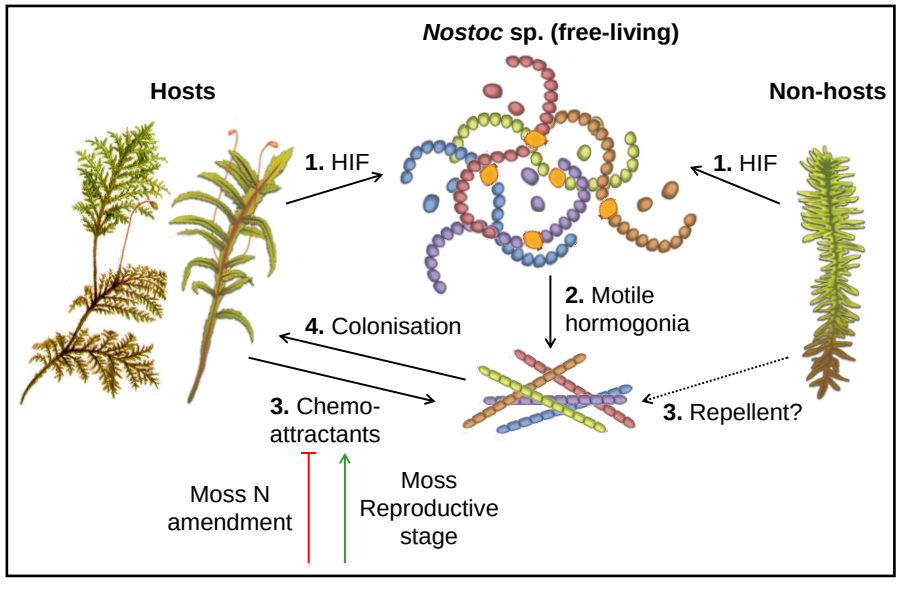

Figure 8. Summary of the sequence of events occurring during chemical communication between boreal bryophytes and hormogonia-forming cyanobacteria. Regulation of chemo-attractants secretion is indicated by a green arrow (positive interaction) and a red flat-headed arrow (inhibitory pathway).

The presence of cyanobacteria on feather mosses is traditionally believed to be a random phenomenon resulting from mechanical means of dispersal such as, for example, wind or water (Marshall and Chalmers, 1997; Solheim and Zielke, 2002). Without understating the occurrence of these means of propagation (e.g. for cyanobacterial strains which are not able to form hormogonia), these findings demonstrate that feather mosses are the main initiators and directors of their association with cyanobacteria. The factors regulating their adequacy to host cyanobacteria rest upon their ability to secrete at least a HIF and a chemo-attractant (Figure 8). Furthermore, they also seem to control the degree of cyanobacterial colonisation in accordance with their need for N. Therefore, these results suggest that the capacity of feather mosses to live in N-depleted environments may be related to their aptitude to communicate chemically with cyanobacteria. As such, associations between feather mosses and cyanobacteria appear to be well-tuned and much more complex than first assumed, and may share some similarities with other plants forming true symbioses with diazotrophs (Campbell and Meeks, 1989; Rasmussen *et al.*, 1994). Identifying the molecular structure of putative chemo-attractants secreted by those mosses (which would be an undisputable proof of the existence of these chemicals), would unveil which physiological mechanisms are triggered in cyanobacteria during moss colonisation. Furthermore, determining a possible benefit for feather mosses in hosting cyanobacteria would mean that the interaction may qualify as a true symbiosis rather than an association.

3.3 Dinitrogen fixed by associative cyanobacteria is transferred to their host moss

Cyanobacteria are recognised as the main agent of biological N input into mature forests (DeLuca *et al.*, 2002b), and are known to transfer N to their host plant when in symbiosis with cyanolichens, hornworts or liverworts (Rai *et al.*, 2002; Turetsky, 2003; Adams and Duggan, 2012). However, no experiment has yet been performed to understand the means by which N enters into the host mosses and thus boreal forest ecosystems after its fixation by cyanobacteria. Therefore, the second goal of Paper II was to determine if the cyanobacteria transfer part of the N that they fix to the moss they are associated with, and was achieved by SIMS analysis.

After just one week of exposure to $^{15}N_2$, the *P. schreberi-Nostoc* associations tested showed a significantly higher $^{15}N/^{14}N$ ratio in the moss tissues than those of non-exposed gametophytes (i.e. controls), corresponding to a 4.1% ^{15}N enrichment. After four weeks of exposure, this enrichment

increased to 34.4%. Given that all moss samples were surface sterilised prior to further experimentation (which ensured that the only diazotrophs present on moss leaves belonged to the *Nostoc* strain used in this experiment), and that no moss has been reported of being able to take up N (and therefore $^{15}N_2$) in its gaseous form, these results establish that a transfer of ^{15}N from cyanobacteria to their host moss occurred. This transfer is also supported by EA-IRMS analysis which showed that $^{15}N_2$-exposed cyanobacteria living on mosses incorporated high levels of ^{15}N (compared to non $^{15}N_2$-exposed cyanobacteria), with this ^{15}N being eventually transmitted to their host moss. Moreover, ^{15}N accumulated within the moss tissues over time, revealing that once the cyanobacteria are established on the moss, the transfer of N to the moss is a continuous process, at least during the time-course of the experiment. Although SIMS analyses are usually performed on the surface of samples, ^{15}N measurements in this experiment were implemented on moss leaf cross-sections. Therefore, this ensured that the measurements corresponded to actual levels of ^{15}N transferred from the cyanobacteria into the moss leaves.

Surprisingly, the enrichment of ^{15}N in cells of cyanobacteria associated with *P. schreberi* gametophytes did not significantly increase with time of exposure to $^{15}N_2$. Although no clear explanation for why the ^{15}N concentration in cyanobacteria does not increase, part of the answer might be that the *Nostoc* strain that was used continually transfers a large quantity of the N_2 it fixes to its host moss. Similar findings from other *Nostoc* spp-plant symbioses suggest that only a minor portion (< 10%) of the N_2 that the cyanobacteria fix is used for their own needs (Meeks *et al.*, 1983; Berg *et al.*, 2013). Nonetheless, experimental conditions (e.g. suboptimal light levels or temperature) may also have limited the accumulation of ^{15}N in cyanobacteria through affecting cyanobacterial growth and subsequent N use.

These results suggest that feather mosses benefit from their association with cyanobacteria as their N_2-fixing partners support the N economy of the plant through the supply of a readily assimilable N source, in a similar manner to that shown for other plant-cyanobacteria symbioses (Rai *et al.*, 2002; Turetsky, 2003; Adams and Duggan, 2012). Therefore, these results demonstrate that the relationship between feather moss and cyanobacteria is closer than merely a simple association, and may represent a true symbiosis. In conclusion, this work improves our understanding of how feather mosses impact on the N cycle in boreal forests; N_2 is first fixed by cyanobacteria, and is then transferred to the mosses hosting these cyanobacteria. Over time, this N may be released from a moss to other organisms in its vicinity when it decomposes, is consumed by herbivores, or leaches.

3.4 Cyanobacterial dinitrogen-fixation activity is influenced by various abiotic and biotic factors

While Paper I explored how the type of moss hosting cyanobacteria (i.e. *P. schreberi* or *H. splendens*) and island characteristics influenced cyanobacterial BNF rates, Paper III investigated more specifically how abiotic (i.e. light intensity and temperature) and biotic (i.e. symbiotic status and cyanobacterial community composition) factors, as well as their interaction, impact on the N_2-fixation process.

3.4.1 Influence of the environment and moss species on dinitrogen-fixation

In Paper I, the N_2-fixation rates among islands and within the two moss types tested were highly variable, ranging from 0.3 to 34.4 µg N g FW moss^{-1} d^{-1} for *P. schreberi* and from 0.5 to 18.4 µg N g FW moss^{-1} d^{-1} for *H. splendens*. Although variations in BNF were expected due to local environmental conditions that vary with successional stage (e.g. soil and vegetation properties, nutrient availability, moisture conditions; Wardle *et al.*, 1997; Zackrisson *et al.*, 2004, DeLuca *et al.*, 2007, 2008; Lagerström *et al.*, 2007), island size did not have a statistically significant effect on the quantity of N_2 fixed by cyanobacteria. As BNF rates in this study cannot be explained by the environment where the mosses grow, these results indicate that the factors regulating N_2-fixation activity may be different from those known to influence moss performance. Furthermore, moss species did not have a statistically significant effect on N_2-fixation rates in this study either. This suggests that while the type of feather moss hosting the cyanobacteria strongly affects cyanobacterial diversity and community composition, it is not a strong determinant of BNF. These results are somewhat surprising as they are inconsistent with several previous studies (e.g. Zackrisson *et al.*, 2004, 2009; Lagerström *et al.*, 2007; Gundale *et al.*, 2010), and may be reflective of specific conditions present during sampling for this study. Despite this, a statistically significant negative correlation was found between N_2-fixation and the number of phylotypes for *H. splendens*, and a marginally non-significant correlation was found for *P. schreberi*. Such relationships occur when dominating or high performing taxa (in this case, strains of cyanobacteria capable of high N_2-fixation) are also highly competitive and result in the exclusion of other species (Grime, 1974; Grace, 1998; Creed *et al.*, 2009). Consequently, even though no quantification of the cyanobacterial populations on these mosses was performed, these results suggest the existence of a linkage between cyanobacterial community characteristics (depicted by the number of phylotypes) and the rates of N_2-fixation (at least for *H. splendens*), which

indicates that mosses may indirectly control N_2-fixation rates via the cyanobacteria with which they form symbioses.

3.4.2 Impact of abiotic factors (light intensity and temperature) on dinitrogen-fixation

Dinitrogen-fixation has been shown to vary seasonally (DeLuca *et al.*, 2002b; Lagerström *et al.*, 2007), with higher quantities of N_2 being fixed in June and September, which helped to refine our knowledge with regards to N_2-fixation patterns. However, the time frame used in these studies (i.e. monthly measurements) did not demonstrate if more recurrent fluctuations (e.g. diurnal) in N_2-fixation occur, an issue which has been very little investigated since. A recent study did not find any diurnal variation in N_2-fixation rates in feather moss-cyanobacteria associations (Zackrisson *et al.*, 2009). This is surprising as the process is acknowledged to be dependent upon environmental conditions which vary on a daily basis, more particularly light intensity and temperature (Staal *et al.*, 2002; Rabouille *et al.*, 2006; Gentili *et al.*, 2005; Houlton *et al.*, 2008). Therefore, in addition to the three main papers included in this thesis, a study of N_2-fixation patterns over a 24h-cycle was performed in June and September 2010 on samples of *P. schreberi* and *H. splendens* to further investigate (i) if diurnal N_2-fixation variations occur and (ii) if abiotic factors such as light intensity and temperature could explain the diurnal variation.

Fluctuations of N_2-fixation activity over the 24 hours for both feather mosses were similar, irrespective of the time of the year, with maximum N_2-fixation rates observed when light intensity and temperature were among the greatest (Figure 9). Conversely, minimum N_2-fixation rates were observed when light intensity and temperature were lowest (Figure 9). As such, it is difficult to draw clear conclusions from these observations as to whether light intensity or temperature (or the interaction of these two factors) is the most important factor of the two in controlling BNF rates. However, these correlations demonstrate that N_2-fixation rates of feather moss-cyanobacteria vary and follow a diurnal pattern, matching daily light intensity and temperature variations (assuming moisture is not limiting).

To further understand how light intensity and temperature may govern BNF rates of feather moss-cyanobacteria symbioses, the impact of these two factors on N_2-fixation were tested in Paper III. Cyanobacterial N_2-fixation rates were highly responsive to both the abiotic factors investigated, with maxima reached under specific combinations of light intensity and temperature, depending on the cyanobacterial strain considered. The optimal set of abiotic conditions for enhanced N_2-fixation activity appears to be dependent on cyanobacterial strain. However, light intensity had a much more pronounced effect than temperature

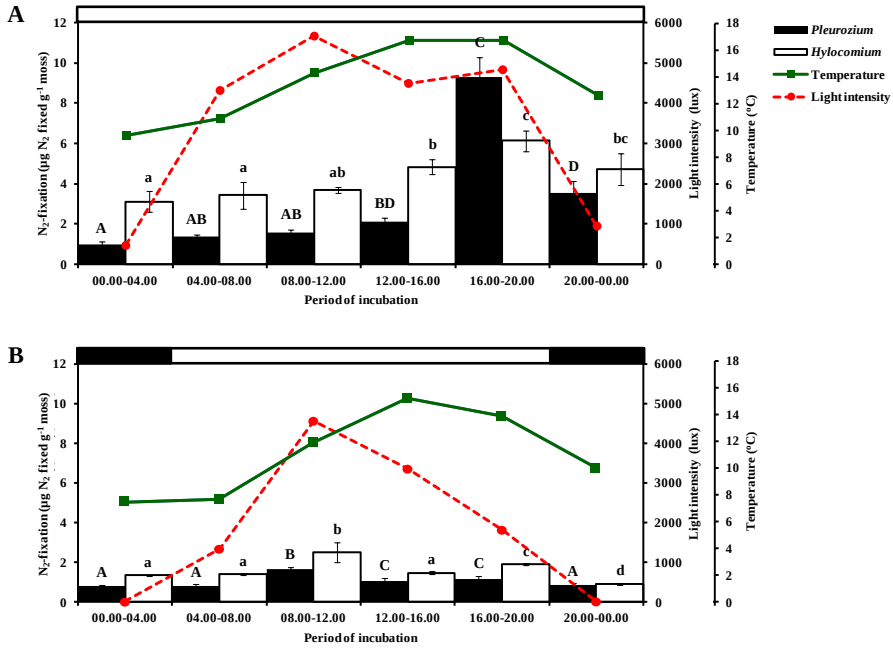

Figure 9. Variation of N_2-fixation rates of *Pleurozium schreberi* and *Hylocomium splendens*, light intensity and temperature over a 24-hour period. Mosses sampled in the Reivo forest reserve in 2010 (see 2.1); consecutive acetylene reduction assays measurements performed in June (A) and September (B) after a 4-hour incubation (corresponding to each data point on the figures) under natural conditions in Umeå (Bay *et al.*, unpublished). Significant differences for N_2-fixation measurements within species are indicated by different letters above each data point (A, B, C or D for *P. schreberi* and a, b, c or d for *H. splendens*; ANOVAs followed by Tukey's HSD test, $p \leq 0.001$). Statistical analysis performed on log-transformed data. Error bars, ± SE (for each point, $n = 3$ for moss samples, $n = 4$ for light intensity and $n = 4$ for temperature). The horizontal bar at the top of each chart represents the natural light (values > 1 µmol photons m^{-2} s^{-1}; white bar) and darkness (values < 1 µmol photons m^{-2} s^{-1}; black bar) periods of the 24-hour cycle.

on BNF rates, although temperature is also considered to be one of the main drivers of BNF (the activity of nitrogenase being temperature-specific; Kashyap *et al.*, 1991, Zielke *et al.*, 2002, Gentili *et al.*, 2005). This conspicuous impact on BNF of light intensity was due to the maintenance of a merely basal N_2-fixation activity by the cyanobacterial monocultures (i.e. without mosses) when grown in darkness, irrespective of temperature conditions, giving light intensity a statistically greater influence on N_2-fixation. Consequently, light intensity emerges as the factor with most impact on BNF, at least for the cyanobacterial strains used in this experiment. This is in accordance with several studies which have reported that N_2-fixation is an energetically expensive process (Staal *et al.*, 2002; Rabouille *et al.*, 2006), and which generally decreases during periods of darkness (Davey, 1983; Zielke *et*

al., 2002). In addition, these results demonstrate that although temperature impacts on N_2-fixation, it becomes one of the main drivers of BNF only if cyanobacteria already possess the energy necessary to fix N_2 (which is obtained through photosynthesis).

The reason for why more N_2 was fixed in June than in September (Figure 9) is still unclear, especially as no further analyses have been performed on the samples used in this experiment such as a characterisation of their cyanobacterial populations and abundances. Nevertheless, this may be explained by either (i) more suitable environmental conditions for the cyanobacterial populations in June and/or (ii) a modification in cyanobacterial populations on both moss species between June and September (the "June population" being more efficient in fixing N_2). Indeed, work remains to be done in order to fully understand natural variations of N_2-fixation rates (e.g. seasonal and diurnal patterns, effect of host plant's physiological stage, spatial variations within sites). In this respect, studying cyanobacterial communities colonising boreal feather mosses on a regular basis (e.g. a multiple year study with sampling in June and September, or more frequently if necessary) may help improve our understanding of the process.

3.4.3 Impact of biotic factors (symbiotic status and cyanobacterial community composition) on dinitrogen-fixation

Studies examining how host plants might impact BNF rates showed enhanced N_2-fixation activity of cyanobacteria when associated with their host (Meeks, 1998; Rai *et al.*, 2000). The hypotheses put forward to explain this observation were that the host may provide a favourable micro-environment for the cyanobacteria (Meeks, 1998) or trigger heterocyst formation, leading to higher N_2-fixation rates (Haselkorn, 1978; Meeks, 1998; Rai *et al.*, 2000; Wang *et al.*, 2004). As no similar study has yet been performed on feather moss-cyanobacteria symbioses, the effect of *P. schreberi* on BNF rates of different cyanobacterial strains was also explored in Paper III. The presence of *P. schreberi* had various effects on cyanobacterial N_2-fixation rates; depending on context the host induced a positive, neutral or negative N_2-fixation response when compared to the free-living *Nostoc* monocultures. While no noticeable increase in the number of heterocysts per cyanobacterium was observed in the presence of the host moss, a three-fold increase in N_2-fixation activity was sometimes found for two *Nostoc* strains when they were growing on a moss (i.e. at low light intensity/low temperature for one and high light intensity/high temperature for the other). However, N_2-fixation was impaired by mosses for one *Nostoc* strain (at low temperature), and no significant difference was observed in all other cases. Contrary to what is commonly believed, these

results show that the host is not always beneficial in terms of BNF rates, although it can be in some contexts. Additionally, the cyanobacterial response to moss presence seems specific to cyanobacterial strain and their relationships with particular environmental factors (as demonstrated by significant 3-way interactive effects on N_2-fixation of cyanobacterial identity × light intensity × host presence and of cyanobacterial identity × temperature × host presence).

Several explanations for the above observations are possible. Strong irradiances are known to induce over-excitation of the photosynthetic apparatus, leading to the formation of reactive oxygen species with detrimental effect on cell functioning (and eventually N_2-fixation; Niyogi, 1999; Tamary *et al.*, 2012). Enhanced BNF rates could therefore be explained by a reduction in the amount of light transmitted to the cyanobacteria through moss leaves serving as filters. Moreover, light-harvesting antennas in chloroplasts of higher plants are able to dissipate excess light energy as heat (van Grondelle *et al.*, 1994; Horton *et al.*, 1996; Niyogi, 1999; Ruban *et al.*, 2007). Comparable processes are likely to occur in mosses; the micro-environment within moss leaves may therefore undergo a slight increase in temperature, resulting in increased rates of BNF if it contributes to approaching nitrogenase's requirements (given that activity of the nitrogenase enzyme is temperature-dependent; Kashyap *et al.*, 1991; Zielke *et al.*, 2002; Gentili *et al.*, 2005). Feather mosses are also known for intercepting nutrients and taking them up from throughfall (Weber and Van Cleve, 1984; Bonan and Shugart, 1989; Brown and Bates, 1990). Through this uptake they could enhance cyanobacterial access to essential nutrients (P or trace elements, for instance), thus enabling them to perform higher rates of N_2-fixation. On the other hand and for similar reasons as described above (i.e. excessive light interception or temperature in the micro-environment formed by moss leaves), presence of the feather moss could also conceivably impede cyanobacterial N_2-fixation and would therefore explain the negative impact observed on BNF. Finally, the absence of effect due to the presence of a moss may be explained by the abiotic conditions being already too unfavourable for the cyanobacteria, with the moss being unable to override these negative effects. An alternative explanation could also be that cyanobacteria operate independently of the moss in some conditions, and in those conditions, the moss does not provide anything that influences the capacity of the cyanobacteria to fix N.

Another factor with the potential to impact on BNF rates in feather moss-cyanobacteria symbioses, and which remains unexplored, concerns the role of interactions among coexisting strains of cyanobacteria in the same environment. Thus, Paper III also investigated how the N_2-fixation rates of a multiple-species population of cyanobacteria differed from the fixation rates by

the component species growing alone, under different combinations of biotic and abiotic factors. When in BG-11_0 medium, N_2-fixation rates of the multi-species community were either equal or lower than those of cyanobacteria grown in monocultures, regardless of the abiotic conditions. Therefore, competition between the different *Nostoc* strains appears to be a common phenomenon during this experiment and may be explained by at least partial overlap of their respective niches (given that they belong to the same genus). Similar results were obtained with cyanobacteria grown on *P. schreberi*, except at high light/low temperature. Under this set of conditions, the amount of N_2 fixed by the multi-species cyanobacterial community exceeded the sum of the N_2-fixation rates of the corresponding monocultures. This finding shows that under specific combinations of abiotic factors (i.e. light intensity and temperature), cyanobacteria from a multi-species community in symbiosis with a moss can demonstrate synergistic activity, leading to higher rates of N_2 fixed (and possibly quantities of N transferred to the moss). As distinct strains of cyanobacteria possess species-specific requirements in terms of resources (Gerard *et al.*, 1990; Spencer *et al.*, 2011), the provision of sufficient resources to meet cyanobacterial needs enabled them to occupy different niches. Thus, competition among the different cyanobacterial species diminished, leading to a complementarity in resource use. Consequently, a moss-associated cyanobacterial community composed of diverse species can fix more N_2, but this seems to be under the control of abiotic factors. The reason for why feather mosses harbour cyanobacteria of different genetic origin may therefore be an evolutionary strategy to obtain greater amounts of N fixed from their cyanobionts at least under particular conditions.

3.4.4 Relationship between cyanobacterial density on feather mosses and dinitrogen-fixation

To understand more thoroughly how BNF rates of cyanobacteria-feather moss symbioses depend on the density of the cyanobionts, the impact of light intensity, temperature and cyanobacterial identity on total cyanobacterial density was also assessed in Paper III. Being photosynthetic organisms, the growth rate and abundance of cyanobacteria are under the direct influence of light intensity (Gerard *et al.*, 1990), as well as temperature (Spencer *et al.*, 2011). Additionally, the effects of temperature seem to be species-dependent (Nicklisch *et al.*, 2008; Giordanino *et al.*, 2011). As expected, these two factors significantly impacted on cyanobacterial density on *P. schreberi* (Paper III). However, strain identity appeared to be a much stronger driver than light intensity and temperature, indicating that cyanobacterial density is above all directed by species-specific responses to environmental conditions. This, for

instance, explains the relatively low performance of one *Nostoc* strain whose density was significantly lower than that of the other strains, possibly due to its inability to adapt to the conditions provided.

In addition, the relationship of cyanobacterial density on mosses with their rates of N_2-fixation was explored in Paper III. Depending on the cyanobacterial combination and the abiotic conditions considered, significant positive correlations (for all *Nostoc* species combinations except one) and negative correlations (for the mixture of cyanobacteria) were observed between density and N_2-fixation. As no increase in the number of heterocysts has been observed during the experiment, these positive relationships between N_2-fixation and cyanobacterial density demonstrate that when conditions are favourable for the cyanobionts, increasing densities of cyanobacteria on the moss will lead to greater N_2-fixation (and potentially, greater transfer of N to the plant). This finding is in accordance with DeLuca *et al.* (2007), where the relationship between N_2-fixation and the number of cyanobacterial cells on mosses was found to be linear. However, excessive cyanobacterial density may translate into competition for resources, causing less N_2 to be fixed. This is illustrated by the significant negative relationship observed between total density and N_2-fixation for the mixed cyanobacterial combination (which was the combination that included both the highest BNF rates and density recorded during the experiment). In conclusion, BNF can also be driven by the density of the cyanobacterial community growing on a moss, although the effect of density will be overruled by abiotic conditions in some contexts (as demonstrated by the absence of correlation between N_2-fixation and cyanobacterial density in darkness, despite the high number of cyanobacteria on mosses).

4 Conclusions and future perspectives

4.1 Summary and conclusions

Over the last decade, there has been rapidly increasing interest in the role that feather mosses play in regulating several key processes in the boreal forest, especially the input of N into this ecosystem. For this reason, the purpose of this thesis was twofold: (i) to develop our basic knowledge about feather moss-cyanobacteria associations and (ii) to understand how the N_2 that they fix enters into boreal forest ecosystems and what the factors regulating the process of N_2-fixation are.

The results presented show that, unlike what is commonly assumed, the relationship between feather moss and cyanobacteria is not a simple association but may in fact be a true symbiosis. This was demonstrated by the high degree of host specificity of various cyanobacterial strains, and high levels of diversity of the cyanobacteria living in symbiosis with both *P. schreberi* and *H. splendens*. None of these features seemed to be driven by the local environmental conditions, which underlines the predominant role played by these feather mosses in selecting their cyanobionts. The closeness of their relationship was further emphasised by highlighting the chemical communication between feather mosses and cyanobacteria, through the putative secretion by the moss of two types of compounds, i.e. a HIF and a chemo-attractant. Furthermore, the ability to release both chemical signals have been shown to be required to form functional symbioses with cyanobacteria; this is most likely an ability lacking in types of boreal mosses that remain free of cyanobacteria. Additionally, the production of a chemo-attractant appears to be directed by the N need of the moss, which in turn regulates the degree of cyanobacteria colonising the mosses. Although already explored in other distinct plant-cyanobacteria symbioses, this work is the first to reveal this phenomenon for feather mosses. It therefore represents an advance in our

understanding of how boreal feather mosses form symbioses with cyanobacteria, through unraveling the underlying driving mechanisms of this process.

The results of this thesis also provide the first evidence that feather mosses actually gain N from the cyanobacteria that they host. Indeed, this constitutes an important improvement in our understanding of how feather mosses govern N input into extensive regions of the world including throughout the boreal forests. In contrast with other studies (e.g. Zackrisson *et al.*, 2004, 2009; Lagerström *et al.*, 2007), these results also suggest that cyanobacterial N_2-fixation is influenced neither by the local environmental conditions (which would therefore only have an impact on the moss itself) nor by the type of moss hosting the cyanobacteria (its impact being only indirect through the cyanobacterial community that it hosts). In this respect, further investigation is needed as both light intensity and temperature were confirmed as strong drivers of BNF rates and appeared to be species-specific, with light intensity being of greater importance than temperature. However, these two factors are not the only factors that have a potential effect on the quantity of N_2 fixed by cyanobacteria. Given favourable abiotic conditions, the N_2-fixation activity of specific cyanobacterial species will be enhanced when in symbiosis with a feather moss, or when they co-occur in multiple-species combinations. Furthermore, an increased density of cyanobacteria on the moss will translate into higher rates of N_2-fixation in some contexts, and eventually greater amounts of N transferred to the moss. None of these biotic parameters (i.e. presence of a host moss, cyanobacterial species identity, cyanobacterial density) have previously been explored as possible factors explaining N_2-fixation variations in boreal forest ecosystems. Therefore, the results indicate that N_2-fixation is a process driven by much more complex interactions between multiple biotic and abiotic factors than has been previously thought.

Overall, the results of this thesis confirm the important role that feather mosses play in the N cycle in boreal forest ecosystems, through hosting cyanobacteria and then acquiring and potentially accumulating the N_2 that they fix. They also suggest that their capacity to live in N-depleted environments may be related to their aptitude to communicate chemically with cyanobacteria, and this strategy may help explain why feather mosses are so abundant in boreal forest ecosystems. These findings show how an ecological approach can be incorporated into physiological studies, and how this may in turn have implications that range from the molecular level to ecosystem scale (e.g. biotic interactions, plant nutrition, N cycling, forest processes and the functioning of terrestrial ecosystems).

4.2 Future perspectives

Although the work included in this thesis improves our understanding of the relationship between boreal feather mosses and cyanobacteria, as well as how these symbioses contribute to the N cycle of boreal forest ecosystems, it also raises new questions and issues. Investigating the following points would therefore certainly help to shed light on some of the remaining enigmas, further expanding our understanding of these symbioses:

- *Calothrix*, is known to live in symbiosis with boreal feather mosses (Gentili *et al.*, 2005). However, in the study of cyanobacterial community composition on *P. schreberi* and *H. splendens* in Paper I this particular strain was not retrieved. This raises the question of whether the failure to find it was the result of a lack of precision in the technique used (i.e. DGGE), bad luck during sampling (i.e. the mosses sampled did not host this cyanobacterial strain whereas it was present in the environment), or if this cyanobacterium was simply not present on the islands. One means of dealing with this issue would therefore be the use of another technique, such as 454 pyrosequencing (see Lentendu *et al.*, 2011); this may yield a more complete understanding of the full diversity of cyanobacteria associated with feather mosses (assuming that databases will be able to provide information with sufficient precision). In addition, it would also allow a precise quantification of individual cyanobacterial species on mosses, a crucial aspect that needs to be considered in further research on similar symbioses.
- The exchange of signals between feather mosses and cyanobacteria has just been discovered (Paper II) and many questions remain. What needs to be investigated to fully elucidate the process involves characterising the molecular identity of the compounds, their frequency of secretion by feather mosses and their potential specificity (since feather mosses "select" their cyanobionts as shown in Paper I). Different types of sugars are believed to impact on the establishment of *Gunnera*- and *Anthoceros-Nostoc* symbioses (Black *et al.*, 2002; Khamar *et al.*, 2010; Ekman *et al.*, 2013). This may serve as a starting point for the study of hormogonia-inducing moss-secreted compounds. Another interesting aspect of the symbiosis would be to understand if chemicals other than those secreted by mosses are exchanged (e.g. if compounds released by cyanobacteria are involved in their recognition by feather mosses).
- As demonstrated in Paper II, a transfer of N occurs between cyanobacteria and mosses. In light of this result, quantifying the proportion of N transferred from cyanobacteria to their host remains an obvious but crucial challenge. Indeed, species-specific N transfer rates may exist (based on the efficacy of the

cyanobacterial species to fix N_2?) and would also require careful scrutiny. Through such research, a more accurate estimation of the input of N into boreal forest ecosystems could be achieved.

- Besides providing their cyanobionts with a supposedly more favourable environment (demonstrated by the impossibility to artificially grow *Stigonema* strains while it is found on both feather moss species in Paper I), do these mosses transfer part of their C and/or P to the cyanobacteria (which would perhaps help understanding more precisely how cyanobacterial colonisation is regulated by the mosses)?
- Feather mosses demonstrated a strong control over cyanobacteria in Paper II, as cyanobacterial colonisation was determined by moss-released chemical compounds. To avoid being outgrown by their cyanobacterial partners (and eventually compete for the same resources; Meeks and Elhai, 2002), symbiotic plants such as *Anthoceros* control the growth of its cyanobionts. Does a similar mechanism occur in feather moss-cyanobacteria symbioses?
- Snow generally covers extensive areas of boreal forests for months in winter. Thus, ground vegetation is subjected to increased darkness as a result of the snow cover, in addition to the shortened daily irradiance at this latitude. Therefore, if cyanobacteria manage to withstand this, are feather mosses involved in their survival, and if so, how? Or do cyanobacteria colonise feather mosses every year? In this case, where do they come from (i.e. from stock in overwintering in soil, or airborne dispersal)?
- Could the low levels of N_2-fixation activity during July-August (DeLuca *et al.*, 2002b) be explained by the high cost for mosses to keep their cyanobionts (in terms of C and P for instance, if indeed they provide them with these elements), or only by environmental factors such as drought?
- It would be interesting to test if strictly marine cyanobacteria (e.g. *Richelia*) could form symbioses with feather mosses, following the same procedure as in Paper II. This would give valuable information about the degree of specificity between the cyanobacteria already acknowledged as cyanobionts and feather mosses, as well as how this symbiosis co-evolved (i.e. coincidence or real co-evolution?).
- Following N_2-fixation and cyanobacterial density of mosses over a longer period than that described in this thesis may help to understand host-cyanobiont long-term interactions.
- The use of qPCR could be valuable for generating quantitative data (e.g. *nifH* gene expression of cyanobacterial communities under various environmental conditions or during a diurnal study) and examining in more detail various physiological processes.

- Last but not least, efforts to develop a method to separate cyanobacteria from feather mosses would be highly desirable (as this would make the study of cyanobacteria colonising these plants much easier), as would the development of a sampling technique to ensure that the selected mosses do host cyanobacteria.

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