LE BACTÉRIOME DES MOUSSES ET SA FIXATION D'AZOTE EN FORÊT BORÉALE : CARACTÉRISATION ET INFLUENCE DE L'ENVIRONNEMENT

par

Marie Renaudin

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le jury a accepté la thèse de Madame Marie Renaudin
dans sa version finale.

Membres du jury

Professeur Jean-Philippe Bellenger
Directeur de recherche
Département de Chimie

Professeur Robert Bradley
Co-directeur de recherche
Département de Biologie

Professeure Nicole Fenton
Évaluatrice externe
Université du Québec en Abitibi-Témiscamingue

Professeur Philippe Dauphin-Ducharme
Évaluateur interne
Département de Chimie

Professeure Pascale Beauregard
Évaluatrice interne
Département de Biologie

Professeur Jérôme Claverie
Président-rapporteur
Département de Chimie

A mes parents,

SOMMAIRE

Dans la forêt boréale, les mousses sont des végétaux abondants ayant des fonctions écologiques et biogéochimiques majeures, telles que la régulation de la température et de l'humidité du sol et le stockage du carbone atmosphérique. Les mousses abritent diverses bactéries jouant des rôles clés dans le cycle des nutriments, comme l'oxydation du méthane et la décomposition de la matière organique. Certaines bactéries, notamment les cyanobactéries, sont également capables d'établir des associations avec les mousses et de réaliser la fixation biologique de l'azote (FBN). La FBN par le bactériome (i.e., ensemble des communautés bactériennes) des mousses est particulièrement importante car elle représente une des principales sources de nouvel azote en forêt boréale et contribue ainsi à soutenir la productivité primaire de la mousse et des plantes supérieures. Ainsi, étudier comment l'environnement (e.g., climat et nutriments) affectent le bactériome des mousses et sa FBN est essentiel pour mieux comprendre et prédire l'influence du changement climatique sur le cycle global de l'azote en forêt boréale.

La FBN étant directement liée à la quantité de cyanobactéries colonisant les mousses, le premier objectif de cette thèse (Chapitre 2) était de développer une méthode rapide et robuste pour déterminer la biomasse cyanobactérienne associée aux mousses. En adaptant une technique employée pour les cyanobactéries aquatiques, nous avons montré que l'extraction et la quantification de la phycocyanine (i.e., pigment photosynthétique cyanobactérien) par spectrofluorimétrie permet d'estimer rapidement et à moindre coût la biomasse de cyanobactéries vivant sur les mousses. Cette méthode s'est révélée être répétable, efficace avec un haut taux de recouvrement, et ne présente pas d'effets de matrice détectables.

Le deuxième objectif de cette thèse (Chapitre 3) était de déterminer les principaux facteurs environnementaux contrôlant *in situ* la biomasse cyanobactérienne et la FBN de deux espèces de mousses dominantes dans la forêt boréale de l'est Canadien. En réalisant un échantillonnage au Québec, sur un transect latitudinal de 1000 km constituant un gradient climatique et de dépôts atmosphériques en nutriments (e.g., azote, métaux), nous avons démontré que la température, les précipitations et le phosphore contrôlaient la biomasse de cyanobactéries associées aux mousses,

alors que la température, le molybdène et le vanadium contrôlaient la FBN. Nous avons aussi montré que l'espèce de mousse et son rapport carbone/azote avaient des effets importants sur la biomasse de cyanobactéries et la fixation d'azote.

Le troisième objectif de cette thèse (Chapitre 4) était de caractériser le bactériome actif global et diazotrophique (i.e., bactéries capables de réaliser la FBN) des mousses et d'évaluer comment ce bactériome est affecté par les conditions environnementales. Grâce au séquençage d'amplicons des gènes 16S et *nifH* à partir d'ARN provenant d'échantillons de mousses similaires à l'objectif 2, nous avons montré que les cyanobactéries dominaient le bactériome des mousses et représentaient 65 % des communautés de diazotrophes. Nous avons également pu déterminer que, en plus des cyanobactéries, certains méthanotrophes (i.e., bactéries oxydant le méthane) étaient présents sur les mousses et contribuaient activement à la fixation d'azote. D'autre part, en réalisant des sections le long du brin de mousse, nous avons observé que les bactéries occupaient des niches écologiques liées à leurs fonctions : les bactéries favorisant la croissance de la mousse étant localisées sur la partie verte apicale du brin et les bactéries aidant à la décomposition étant localisées sur la partie basale et sénescente du brin. Enfin, nous avons mis en évidence que le climat, les nutriments (i.e., azote, phosphore, molybdène, vanadium et fer), la densité d'arbres et l'espèce de mousse influençaient la structure et la composition du bactériome des mousses, affectant potentiellement ses fonctions microbiennes associées.

En conclusion, les résultats de cette thèse permettent d'identifier les genres de cyanobactéries et de méthanotrophes qui contribuent activement à la FBN et qui constituent donc des acteurs clés du cycle de l'azote en forêt boréale. Ces résultats montrent aussi que le bactériome des mousses et sa FBN sont fortement contrôlés par leur environnement (i.e., espèce de mousse, climat, macro- et micro-nutriments) et seront donc certainement affectés positivement ou négativement par le changement global, particulièrement intense dans les écosystèmes boréaux. Ces résultats invitent donc à effectuer des études complémentaires pour estimer plus précisément les effets du changement climatique et atmosphérique sur la structure du bactériome des mousses et la FBN.

Mots-clés : Environnement, Fixation biologique de l'azote, Forêt boréale, Mousses, Bactériome, Diazotrophes, Cyanobactéries.

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TABLE DES MATIÈRES

SOMMAIRE .... **i**

REMERCIEMENTS .... **iii**

TABLE DES MATIÈRES .... **iv**

LISTE DES ABRÉVIATIONS .... **x**

LISTE DES TABLEAUX .... **xii**

LISTE DES FIGURES .... **xiv**

CHAPITRE 1 : INTRODUCTION **1**

1.1. L'écosystème forêt boréale **1**

1.1.1. Localisation, végétation et conditions climatiques 1

1.1.2. Rôles de la forêt boréale 2

1.1.3. Cycle biogéochimique de l'azote et limitation de la productivité primaire 2

1.2. Importance des mousses en forêt boréale **4**

1.2.1. Caractéristiques des mousses 4

1.2.2. Rôles écologiques et biogéochimiques des mousses 5

1.3. Le bactériome des mousses et sa fixation d'azote **6**

1.3.1. Associations symbiotiques mousses-cyanobactéries 6

1.3.2. Composition et diversité du bactériome des mousses boréales 9

1.3.3. Méthodes d'étude du bactériome des mousses 10

1.4. Facteurs biologiques et environnementaux affectant le bactériome des mousses et sa fixation d'azote **12**

1.4.1. Effets espèces 12

1.4.2. Facteurs climatiques et environnementaux 12

1.4.3. Conséquences du changement climatique 15

1.5. Objectifs de recherche **15**

CHAPITRE 2 : QUANTIFICATION DE LA BIOMASSE CYANOBACTÉRIENNE ASSOCIÉE AUX MOUSSES GRÂCE À LA PHYCOCYANINE 18

2.1. Informations préliminaires 18

2.1.1. Auteurs et affiliations 18

2.1.2. Participation de chaque auteur 18

2.2. Article « Quantification of moss-associated cyanobacteria using phycocyanin pigment extraction » 19

2.2.1. Abstract 19

2.2.2. Introduction 20

2.2.3. Materials and methods 24

2.2.3.1. Cyanobacterial strains selection and culture conditions 24

2.2.3.2. Moss sampling 25

2.2.3.2. Phycocyanin extraction and quantification procedure 25

2.2.3.3. Analytical performance parameters assessment 27

2.2.3.3.1. Analytical calibration of the method 27

2.2.3.3.2. Accuracy of the method 28

2.2.3.3.3. Nitrogen fixation measurements 29

2.2.3.3.4. Statistical analysis 29

2.2.4. Results and discussion 30

2.2.4.1. Phycocyanin extraction method characterization and validation 30

2.2.4.2. Advantages and limits of the method 34

2.2.4.3. Phycocyanin concentration and nitrogen fixation in boreal feather moss 35

2.2.5. Data availability statement 37

2.2.6. Author contributions 37

2.2.7. Funding 38

2.2.8. Conflict of interest 38

2.2.9. Acknowledgments 38

2.2.10. Supplementary material 38

2.2.11. References 38

CHAPITRE 3 : FACTEURS ENVIRONNEMENTAUX ET CLIMATIQUES CONTRÔLANT LA BIOMASSE CYANOBACTÉRIENNE ET LA FIXATION D'AZOTE ASSOCIÉES AUX MOUSSES EN FORÊT BORÉALE 50

3.1. Informations préliminaires 50

3.1.1. Auteurs et affiliations 50

3.1.2. Participation de chaque auteur 50

3.2. Article « New insights into the drivers of moss-associated nitrogen fixation and cyanobacterial biomass in the eastern Canadian boreal forest » 51

3.2.1. Abstract 51

3.2.2. Introduction 52

3.2.3. Materials and methods 57

3.2.3.1. Moss sampling 57

3.2.3.2. Sampling site environmental parameters 58

3.2.3.3. Nitrogen fixation and cyanobacterial biomass assessment 59

3.2.3.4. Moss nutrient concentration analyses 59

3.2.3.5. Statistical analyses 60

3.2.4. Results 61

3.2.4.1. Latitudinal transect environmental conditions and moss nutrient concentrations 61

3.2.4.2. Determination of the environmental drivers of moss-associated nitrogen fixation and cyanobacterial biomass 63

3.2.5. Discussion 70

3.2.5.1. Moss identity is a major driver of cyanobacterial biomass and nitrogen fixation 70

3.2.5.2. Climatic and site-specific predictors of cyanobacterial biomass and nitrogen fixation 71

3.2.5.3. The nutrients driving moss-associated cyanobacterial biomass and nitrogen fixation 73

3.2.6. Acknowledgements 78

3.2.7. Conflict of interest 78

3.2.8. Authors' contributions 78

3.2.9. Data availability statement 79

3.2.10. Supporting information 79

3.2.11. References 79

CHAPITRE 4 : CARACTÉRISATION ET INFLUENCE DE L'ENVIRONNEMENT SUR LE BACTÉRIOME GLOBAL ET DIAZOTROPHIQUE DES MOUSSES EN FORÊT BORÉALE 95

4.1. Informations préliminaires 95

4.1.1. Auteurs et affiliations 95

4.1.2. Participation de chaque auteur 95

4.2. Article « Unraveling global and diazotrophic bacteriomes of boreal forest floor feather mosses and their environmental drivers at the ecosystem and at the plant scale in North America » 96

4.2.1. Highlights 96

4.2.2. Abstract 96

4.2.3. Introduction 97

4.2.4. Material and methods 100

4.2.4.1. Moss sampling 100

4.2.4.2. Moss-associated nitrogen fixation, cyanobacterial biomass, and environmental parameters 101

4.2.4.3. RNA extraction and 16S rRNA and *nifH* amplicon sequencing 103

4.2.4.4. Statistical analyses 104

4.2.5. Results 105

4.2.5.1. Feather moss bacterial communities' structure across a latitudinal transect 105
4.2.5.2. Bacterial community distribution along the moss shoot 110
4.2.5.3. Relationship between bacterial genera and nitrogen fixation 113
4.2.5.4. Effects of environmental variables on the feather moss bacteriome.......................117
4.2.6. Discussion 118
4.2.6.1. New insights into feather moss global bacterial community structure and functions 118
4.2.6.1.1. Cyanobacteria dominate the feather moss bacteriome..118
4.2.6.1.2. Potential roles of moss-associated bacteria..119
4.2.6.1.3. Bacteriome shift along the moss senescence gradient 119
4.2.6.2. Bacteria contributing to nitrogen fixation 120
4.2.6.2.1. Cyanobacteria are the main contributors to nitrogen fixation.............................120
4.2.6.2.2. Methanotrophs contribute to nitrogen fixation...121
4.2.6.2.3. Not all cyanobacteria do their part 121
4.2.6.3. Environmental drivers of the global and diazotrophic feather moss bacteriomes ..122
4.2.6.3.1. Effects of moss species identity 122
4.2.6.3.2. Effects of temperature and precipitation 123
4.2.6.3.3. Effects of the tree density..124
4.2.6.3.4. Effects of nutrients 124
4.2.6.4. Implications for the boreal forest ecosystem..126
4.2.7. Conclusions 126
4.2.8. Data availability 127
4.2.9. Declaration of competing interests...127
4.2.10. Acknowledgements 127
4.2.11. Supplementary data 128
4.2.12. References 128

CONCLUSION GÉNÉRALE 142
BIBLIOGRAPHIE..144
ANNEXE 1. INFORMATIONS SUPPLÉMENTAIRES DU CHAPITRE 2........................158
ANNEXE 2. INFORMATIONS SUPPLÉMENTAIRES DU CHAPITRE 3........................159
ANNEXE 3. INFORMATIONS SUPPLÉMENTAIRES DU CHAPITRE 4........................161

LISTE DES ABRÉVIATIONS

ADN, DNA	Acide Désoxyribonucléique, Deoxyribonucleic Acid
ANOVA	Analyse de variance, Analysis of Variance
ARA	Test de réduction d'acétylène, Acetylene Reduction Assay
ARN, RNA	Acide Ribonucléique, Ribonucleic Acid
ASV	Variant de séquence d'amplicon, Amplicon Sequence Variant
C	Carbone, Carbon
cDNA	ADN codant, Coding DNA
FBN	Fixation Biologique de l'Azote
Fe	Fer, Iron
GC-FID	Chromatographie en phase gazeuse couplée à un détecteur à ionisation de flamme, Gas Chromatography with Flame Ionization Detection
HIF	Facteur d'induction d'hormogonies, Hormogonia Inducing Factor
HNO_3	Acide nitrique, Nitric acid
H_2O_2	Peroxyde d'hydrogène, Hydrogen peroxide
ICP-MS	Spectrométrie de masse à plasma à couplage inductif, Inductive Coupled Plasma Mass Spectrometry
LOD	Limite de detection, Limit of Detection
LOOCV	Validation croisée d'un contre tous, Leave-One-Out Cross Validation
LOQ	Limite de quantification, Limit of Quantification
LRT	Test du rapport de vraisemblance, Likelihood Ratio Test
Mo	Molybdène, Molybdenum
N	Azote, Nitrogen
N_2	Diazote, Dinitrogen
Nase	Nitrogénase, Nitrogenase
OTU	Unité taxonomique opérationnelle, Operational Taxonomic Unit
P	Phosphore, Phosphorus
PC	Phycocyanine, Phycocyanin
PCA	Analyse en composante principale, Principal Component Analysis

PCR	Réaction en chaîne par polymérase, Polymerase Chain Reaction
PERMANOVA	Analyse multivariée permutationnelle de la variance, Permutational Multivariate Analysis of Variance
qPCR	Réaction en chaîne quantitative par polymérase, Quantitative Polymerase Chain Reaction
S	Soufre, Sulphur
sp.	Espèce, Species
spp.	Plusieurs espèces, Multiple species
V	Vanadium, Vanadium

LISTE DES TABLEAUX

Tableau 1. Cyanobacterial strains used in this study 24

Tableau 2. Analytical performance parameters tested in this study...33

Tableau 3. Nutrient concentrations (*n* = 33) consisting of molybdenum (Mo), vanadium (V), phosphorus (P), carbon (C), nitrogen (N) and C:N ratio; acetylene reduction (AR; used to estimate nitrogen fixation; *n* = 33) and phycocyanin concentrations (PC; used to estimate moss-associated cyanobacterial biomass; *n* = 15) that were measured in the feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis,* collected along a latitudinal transect in Quebec, Canada in 2017, 2018 and 2019. The moss species effect on the eight variables was assessed using the Kruskal-Wallis test. 67

Tableau 4. Linear mixed-effects models using nitrogenase cofactors molybdenum (Mo) and vanadium (V) as predictors of log-transformed (a) nitrogen fixation and (b) moss-associated cyanobacterial biomass. 69

Tableau 5. PERMANOVA analyses on Bray-Curtis dissimilarities showing the moss-associated global and diazotrophic bacterial community structure variations explained by moss species, month of collection, monthly mean temperature and precipitation, tree density, total N deposition, and phosphorus (P), molybdenum (Mo), vanadium (V) and iron (Fe) concentrations measured in feather mosses collected on a latitudinal transect (a) and along moss shoots (b). 107

Tableau S1. Phycocyanin concentrations (µg.g^{-1} moss DW ± SD) measured in *Ptilium crista-castrensis* and *Pleurozium schreberi* collected in June and September 2019 in Quebec, Canada (n = 3). 158

Tableau S2. Table summarizing the characteristics of all the moss samples collected (species, date, and location of sampling), the site-specific parameters (mean monthly temperature, precipitation, location of the meteorological stations used for temperature and precipitation estimations, tree density and total nitrogen deposition) and the moss related variables (acetylene reduction, phycocyanin, nutrient concentrations) measured and included in this study. 159

Tableau S3. Linear mixed-effects model using carbon (C) and nitrogen (N) concentrations as predictors of log-transformed nitrogen fixation. 160

Tableau S4. Characteristics of all the moss samples collected (species, date, and location of sampling), site parameters (mean monthly temperature, precipitation, location of the meteorological stations, tree density and total nitrogen deposition) and moss-related variables (nitrogen fixation, cyanobacterial biomass, nutrient concentrations) measured and included in this study. 169

LISTE DES FIGURES

Figure 1. Zones bioclimatiques de végétation au Québec (adapté de Saucier *et al*., 2009).[4] La forêt boréale inclut les zones de la sapinière à bouleaux blancs, la pessière à mousses et la pessière à lichens..........1

Figure 2. Principales sources d'azote contribuant à la croissance de la forêt boréale (adapté de Sponseller *et al*., 2016).[16]..........3

Figure 3. Tapis des mousses pleurocarpes *Pleurozium schreberi* et *Ptilium crista-castrensis* (photos par Marie Renaudin et Pauline Le Monier avec permission)..........5

Figure 4. Cyanobactéries du genre *Nostoc* ayant colonisé *Ptilium crista-castrensis* (à gauche, microscope à épifluorescence) et cultivées en milieu liquide (à droite, microscope optique) (échelle similaire pour les deux photos, photos par Marie Renaudin)..........7

Figure 5. Représentation schématique des objectifs réalisés au cours de cette thèse de doctorat. 17

Figure 6. Filamentous cyanobacteria colonies (phycocyanin fluorescence in red) within *Ptilium crista-castrensis* leaves, observed under an epifluorescence microscope (Zeiss Axio Observer Z1 equipped with a Zeiss Axiocam 506 mono, objective 40X/0.95NA). 22

Figure 7. Phycocyanin (PC) extraction and quantification procedure for cyanobacteria in cultures and moss samples (left) and method validation process (right). 26

Figure 8. Phycocyanin mass mean ± SD (n = 3) linearity in cyanobacteria *Nostoc* sp. pure cultures. 30

Figure 9. Phycocyanin mass mean ± SD (n = 3) linearity in *P. crista-castrensis* (gray squares) and *Pleurozium schreberi* (black circles) feather moss. 31

Figure 10. Linear regression between acetylene reduction mean ± SD (n = 4) and phycocyanin mass mean ± SD (n = 3) of feather moss *P. crista-castrensis* and *P. schreberi* collected in Quebec, Canada in June and September 2019..........37

Figure 11. Map of the sampling sites that are located along a 1000-km latitudinal transect in Quebec, Canada. Feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis*, two

dominant species in the eastern Canadian boreal forest, were collected in June, September, and October of 2017, 2018 and 2019. 58

Figure 12. Climatic parameters (© Gouvernement du Québec, ministère de l'Environnement et de la Lutte contre les changements climatiques, 2019), total nitrogen atmospheric deposition and mean nutrient concentrations (*n* = 3) measured in the feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis* along a latitudinal transect in Quebec, Canada. 62

Figure 13. Spearman correlation between log-transformed mean nitrogen fixation (*n* = 4) and log-transformed mean cyanobacterial biomass (*n* = 3) that was measured in feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis* that were collected along a latitudinal transect in Quebec, Canada. 63

Figure 14. Predictive variable importance plots of log-transformed nitrogen fixation (a) and log-transformed total moss-associated cyanobacterial biomass (b) measured in *Ptilium crista-castrensis* and *Pleurozium schreberi* collected on a latitudinal transect in Quebec, Canada. 64

Figure 15. Spearman correlations between log-transformed mean nitrogen fixation (*n* = 4), measured in feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis*, and temperature (a), molybdenum:vanadium (Mo:V) ratio (b), carbon:nitrogen (C:N) ratio (c); and log-transformed mean cyanobacterial biomass (*n* = 3) associated with the same feather mosses and temperature (d), precipitation (e), carbon:nitrogen (C:N) ratio (f) and phosphorus (P) (g). 66

Figure 16. Summary of the main environmental and ecological drivers of moss-associated cyanobacterial biomass and their nitrogen fixation, determined using feather moss samples collected on a latitudinal transect across the eastern Canadian boreal forest...............................77

Figure 17. Location of the sampling sites along a latitudinal gradient in Quebec, Canada. 101

Figure 18. Relative abundance of the dominant bacterial genera detected in 16S rRNA and *nifH* sequences from feather mosses *Ptilium crista-castrensis* (a, c) and *Pleurozium schreberi* (b, d) collected in June and September 2017 (*n* = 6). 108

Figure 19. Alpha-diversity (estimated with Shannon index) detected in 16S rRNA and *nifH* sequences from feather mosses *Ptilium crista-castrensis* and *Pleurozium schreberi* collected along a latitudinal transect (S1-S5) in June and September 2017 (a, d) (*n* = 12) and from both species collected in June, September, and October 2017 (b, c, e, f) (*n* = 39). 110

Figure 20. Relative abundance (a, c) of the dominant bacterial genera and alpha-diversity (estimated with Shannon index; b, d) measured in 16S rRNA and *nifH* sequences of 0.5 cm-sections of *Ptilium crista-castrensis* shoots collected on S3 in September 2019 (n = 3). 112

Figure 21. Nitrogen fixation, total cyanobacterial biomass, and phosphorus (P), molybdenum (Mo), vanadium (V) and iron (Fe) concentrations measured along *Ptilium crista-castrensis* shoots collected at S3 in September 2019 (n = 3). 113

Figure 22. Random forest models and Spearman's correlations between log-transformed nitrogen fixation and log-transformed relative abundances of the dominant cyanobacteria genera identified in 16S rRNA (a) and *nifH* (b) sequences from feather mosses collected in June, September, and October 2017. 115

Figure 23. Principal Component Analysis (PCA) biplot showing correlations between environmental variables and relative abundance of the dominant bacteria genera identified in 16S rRNA (a) and *nifH* (b) sequences from feather mosses collected in June and September 2017..116

Figure S1. Phycocyanin mass mean ± SD (n=3) linearity in *Anabaena variabilis* culture.........158

Figure S2. Spearman correlations between log-transformed mean nitrogen fixation (n = 4) and log-transformed mean molybdenum (Mo) and vanadium (V) concentrations (n =3) measured in feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis* collected on a latitudinal transect in Quebec, Canada. 159

Figure S3. Relative abundance of the dominant bacterial genera detected in 16S rRNA (a) and *nifH* (b) sequences from feather mosses *Ptilium crista-castrensis* and *Pleurozium schreberi* collected on sites S1, S3, S4 in June, September, and October 2017 (n = 18). 161

Figure S4. Alpha-diversity (estimated with Chao1 index) detected in 16S rRNA and *nifH* sequences from feather mosses *Ptilium crista-castrensis* and *Pleurozium schreberi* collected along a latitudinal transect (S1-S5) in June and September 2017 (a, d) (n = 12) and from both species collected in June, September, and October 2017 (b, c, e, f) (n = 39). 162

Figure S5. Spearman's correlation between log-transformed nitrogen fixation and log-transformed alpha-diversity (estimated with Shannon index) measured in 16S rRNA (a) and *nifH* (b) sequences from feather mosses *Ptilium crista-castrensis* and *Pleurozium schreberi* collected along a latitudinal transect (S1-S5) in June, September, and October 2017. 163

Figure S6. Alpha-diversity (estimated with Chao1 index) measured in 16S rRNA (a) and *nifH* (b) sequences of 0.5 cm-sections of *Ptilium crista-castrensis* shoots collected on S3 in September 2019 (*n* = 3). 164

Figure S7. 16S rRNA- (a) and *nifH*-assigned (b) ASV differential abundance analysis between the two moss *Ptilium crista-castrensis* and *Pleurozium schreberi* collected on a latitudinal transect in June, September, and October 2017. 165

Figure S8. 16S rRNA- (a) and *nifH*-assigned (b) ASV differential abundance analysis between feather mosses collected on the sampling sites S1 and S5, in June, September, and October 2017. 166

Figure S9. 16S rRNA- and nifH-assigned ASV differential abundance analysis between feather mosses collected on a latitudinal transect in June and September (a, c) or October (b, d) 2017. 167

Figure S10. 16S rRNA- (a) and *nifH*-assigned (b) ASV differential abundance analysis between *Ptilium crista-castrensis* shoot sections of 1 cm and 6 cm from the apex. Samples were collected on S3 in September 2019. 168

CHAPITRE 1 : INTRODUCTION

1.1. L'écosystème forêt boréale

1.1.1. Localisation, végétation et conditions climatiques

La forêt boréale est un écosystème nordique s'étendant entre les latitudes 50° et 70° N. La forêt boréale est principalement située en Amérique du Nord, Scandinavie et Russie, et recouvre 17% de la surface terrestre.[1] Au Canada, on compte 270 millions d'hectares de forêt boréale[2] ce qui représente environ 27 % du territoire. Au Québec, la forêt boréale occupe environ un tiers de la superficie de la province.[3]

La forêt boréale Québécoise se divise en zones bioclimatiques variants avec la latitude (Figure 1).[4]

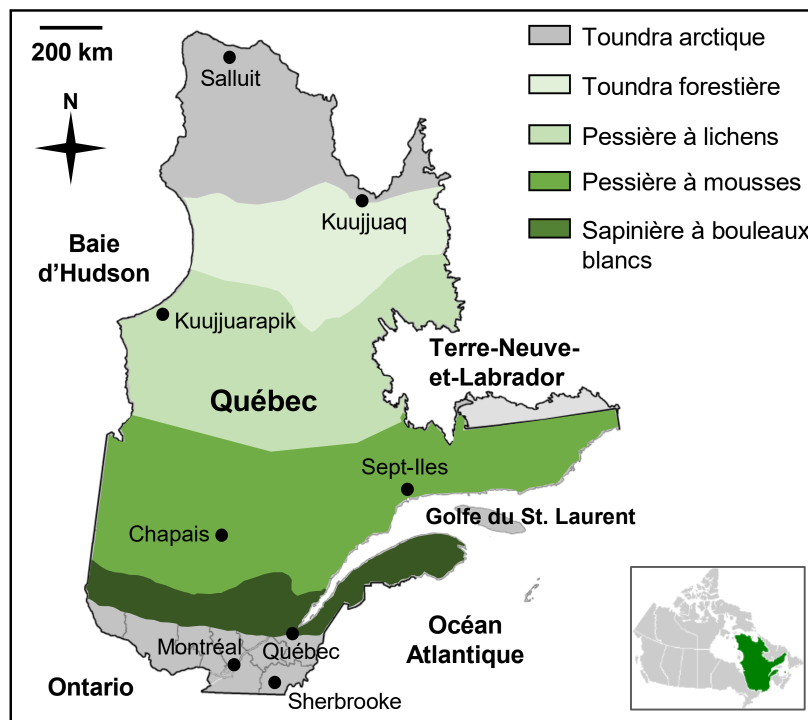

Figure 1. Zones bioclimatiques de végétation au Québec (adapté de Saucier *et al.*, 2009).[4] La forêt boréale inclut les zones de la sapinière à bouleaux blancs, la pessière à mousses et la pessière à lichens.

Au Sud, la forêt est mixte et comporte des feuillus tels que les bouleaux (*Betula* spp.) et des résineux tel que le sapin baumier (*Abies balsamea*). Cependant, plus au Nord, la forêt est majoritairement composée de conifères comme les épinettes (*Picea* spp.) et le pin gris (*Pinus banksiana*). La végétation du sous-bois est composée d'arbustes de la famille des *Ericaceas* (e.g., *Kalmia* spp., *Vaccinium* spp.) et de plantes cryptogames comme les lichens et les mousses.[5,6] Les mousses sont particulièrement abondantes, notamment dans la zone bioclimatique de la pessière à mousse (Figure 1), où elles peuvent recouvrir jusqu'à 80-100 % du sol forestier.[7]

La forêt boréale Québécoise est soumise à un climat continental caractérisé par des hivers longs et une saison de croissance relativement courte de juin à septembre. Les températures et précipitations moyennes varient selon les zones bioclimatiques et diminuent et augmentent respectivement selon un gradient latitudinal. Les températures annuelles moyennes sont basses et généralement entre -5 °C et 1 °C et les précipitations annuelles moyennes sont de 750-1000 mm.[8]

1.1.2. Rôles de la forêt boréale

La forêt boréale est un puit important de carbone (C) stocké majoritairement dans ses sols.[9] La forêt boréale représente environ 20 % des puits de carbone forestiers mondiaux et peut contribuer à réguler la concentration atmosphérique en CO_2.[10]

La forêt boréale abrite deux tiers des espèces fauniques et floristiques du Canada et constitue l'habitat privilégié de certaines espèces menacées (e.g., caribou forestier, rainette faux-grillon boréale).[11] De plus, la forêt boréale a une importance culturelle pour les communautés autochtones qui y résident (environ 70 %).[12] Cet écosystème représente aussi un intérêt économique en raison des activités récréatives qui y sont associées, du tourisme et du commerce de produits issus de l'exploitation forestière (e.g., bois, papier), qui représentent environ 3 % du PIB du Québec.[13]

1.1.3. Cycle biogéochimique de l'azote et limitation de la productivité primaire

La productivité de la forêt boréale est limitée par l'azote (N).[14,15] Dans la forêt boréale, les principales voies d'entrée de N biodisponible pour les plantes sont (i) la minéralisation de la matière organique par les micro-organismes du sol, (ii) les dépôts atmosphériques, sous forme

humides (pluie, neige, brouillard) ou sèches (particules), et (iii) la fixation biologique de l'azote (FBN) (Figure 2). Une grande partie du N provenant de la litière (e.g., feuilles, aiguilles) et des parties végétales sénescentes est également réutilisée (i.e., recyclage interne) et permet de soutenir les besoins en N de la forêt boréale.[16]

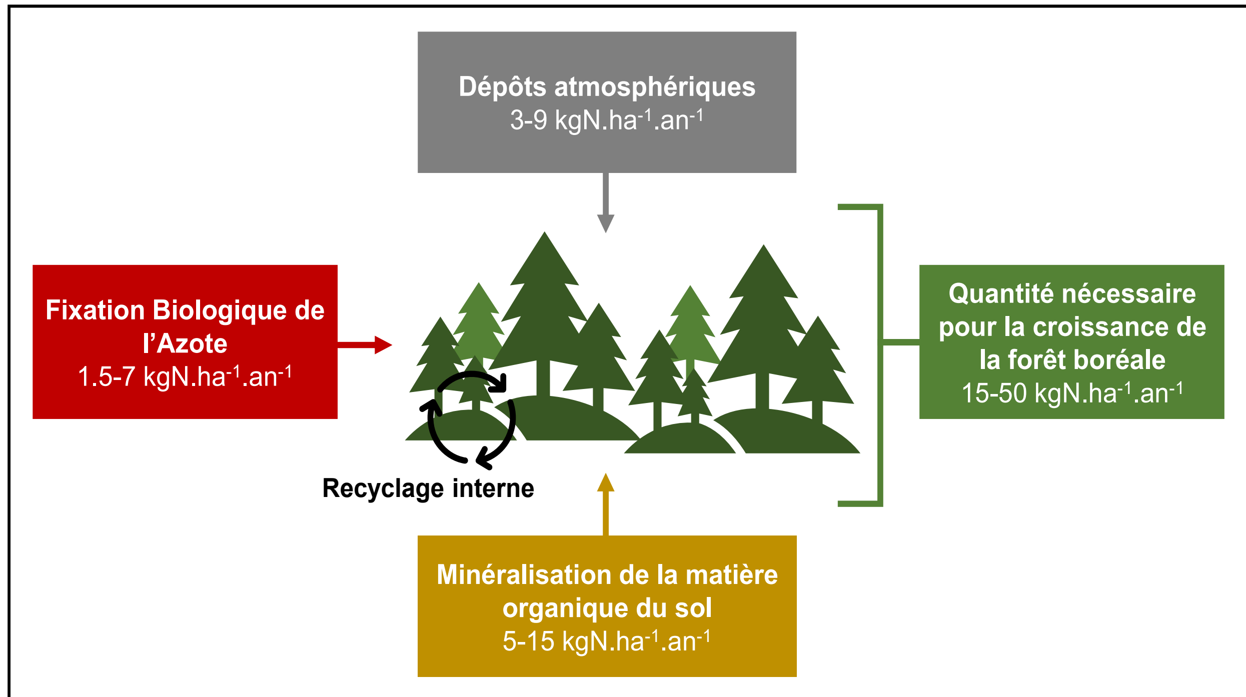

Figure 2. Principales sources d'azote contribuant à la croissance de la forêt boréale (adapté de Sponseller *et al.*, 2016).[16]

La minéralisation du N organique en ammonium représente la source majoritaire de N dans cet écosystème (5-15 kg.ha^{-1}.an^{-1}).[17,18] Cependant, bien que les sols de la forêt boréale soient riches en N (1-8 t.ha^{-1}),[19] il est peu biodisponible car lié à de la matière organique ou séquestré par des acides humiques et des composés phénoliques.[20,21] De plus, la minéralisation de la matière organique est lente, en raison des températures basses et des sols acides,[22] et le N biodisponible est rapidement immobilisé par les micro-organismes,[23] ce qui amplifie ce phénomène de limitation en N. De plus, les dépôts atmosphériques de N, surtout liés aux activités humaines (e.g., agriculture, industrie), sont généralement faibles en forêt boréale. Au Québec, les dépôts atmosphériques sont estimés entre 9 kg.ha^{-1}.an^{-1} au Sud et 3 kg.ha^{-1}.an^{-1} au Nord.[24]

La FBN est une réaction microbienne constituant la réduction du diazote atmosphérique en ammonium, catalysée par les enzymes nitrogénases (Nases) comprenant trois isoformes catégorisées selon le type de métal cofacteur de la réaction : la molybdène (Mo)-Nase, la vanadium (V)-Nase et la fer (Fe)-Nase.[25] La Mo-Nase est contrôlée par le gène *nif*, retrouvé dans tous les génomes des diazotrophes (i.e., bactéries pouvant réaliser la FBN), alors que la V-Nase et la Fe-Nase sont considérées comme des enzymes alternatives ou complémentaires, pouvant soutenir la FBN dans les milieux pauvres en Mo.[26] La FBN correspond à un apport d'environ 1.5-7 $kgN.ha^{-1}.an^{-1}$ dans la forêt boréale et constitue donc une source importante de N pour cet écosystème fortement limité.[27,28] Dans la forêt boréale, la FBN est réalisée par des bactéries établissant des associations avec certains arbres (e.g., *Alnus* spp.)[29] et lichens,[30] mais la majorité de la FBN provient du bactériome (i.e., ensemble des communautés bactériennes) des mousses.[27] La FBN du bactériome des mousses est donc essentielle pour soutenir les besoins en N et la croissance de la forêt boréale.

1.2. Importance des mousses en forêt boréale

1.2.1. Caractéristiques des mousses

Les mousses font parties du groupe des bryophytes, comprenant également les hépatiques et les anthocérotes. Avec les sphaignes, les mousses constituent plus précisément l'embranchement des *Bryophyta* et regroupent environ 13 000 espèces connues.[31] Les mousses sont des végétaux présents dans de nombreux écosystèmes terrestres et sont particulièrement abondantes en forêt boréale grâce à leur tolérance élevée à la dessication et leur habilité à alterner les périodes d'activité et de repos métabolique.[32] Au niveau morphologique, les mousses sont constituées d'un ensemble de brins (i.e., gamétophores), chacun composé d'une tige centrale, sur laquelle sont situés des rameaux recouverts de feuilles. Les mousses se développent généralement en colonies, ce qui favorise la capture et la rétention des eaux de pluie ou pluviolessivats.[33] Le brin de mousse constitue un gradient de sénescence puisqu'on peut distinguer la partie apicale verte du brin de mousse, photosynthétique et métaboliquement active, et la partie basale brune, sénescente et localisée à proximité du sol. Au contraire des plantes vasculaires comme les arbres, les mousses sont des plantes cryptogames sans systèmes vasculaire et racinaire.[34] Une grande partie de leur nutrition repose donc sur l'absorption des éléments contenus dans les dépôts atmosphériques par le système

aérien, facilité par l'absence de cuticule sur les feuilles,[35,36] ainsi que le recyclage interne de certains éléments, transférés des parties sénescentes aux parties métaboliquement actives.[37]

Les espèces de mousses dominantes en forêt boréale sont *Pleurozium schreberi*, *Ptilium crista-castrensis* (espèces étudiées dans cette thèse, Figure 3), *Hylocomium splendens* et *Sphagnum* spp.[6] *P. schreberi* et *P. crista-castrensis* sont des mousses pleurocarpes (i.e., dont le sporophyte, partie reproductrice de la plante, se développe parallèlement au brin) pouvant coloniser des substrats similaires (sol, roche, bois mort) et vivant en mélange avec d'autres espèces de mousse ou en tapis monospécifique.

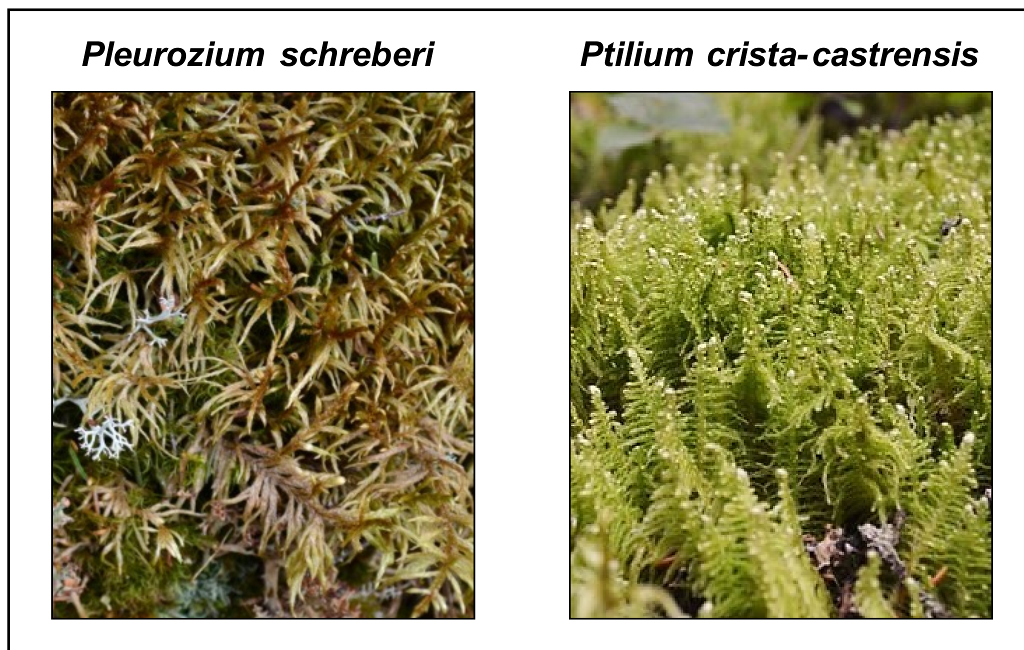

Figure 3. Tapis des mousses pleurocarpes *Pleurozium schreberi* et *Ptilium crista-castrensis* (photos par Marie Renaudin et Pauline Le Monier avec permission).

1.2.2. Rôles écologiques et biogéochimiques des mousses

Les mousses exercent un fort contrôle sur leur environnement et peuvent entrer en compétition avec les plantes vasculaires pour les nutriments et la lumière.[38] Ainsi, les mousses peuvent empêcher ou faciliter la germination des graines et le développement des jeunes conifères, et influencent la régénération et la composition des peuplements des forêts boréales.[39,40] Les mousses forment également un écosystème à part entière (la « bryosphère »)[41] en hébergeant une faune et

une flore spécifiques. Elles constituent un habitat ou une source de nourriture pour divers arthropodes (e.g., collemboles, acariens) et micro-organismes (e.g., algues, champignons, bactéries) supportant ainsi la chaîne alimentaire détritique de la forêt boréale.

Les mousses, par leurs caractéristiques morphologiques, physiologiques et fonctionnelles, jouent également un rôle majeur dans les cycles des macro-nutriments (C, N) de la forêt boréale. En effet, elles réalisent environ 13-20 % de la productivité primaire de cet écosystème[42,43] et, avec la végétation du sous-bois, peuvent avoir une productivité primaire supérieure à celle des arbres.[44] Les mousses affectent aussi la minéralisation de la matière organique par les micro-organismes en régulant la température et l'humidité du sol[45–47] et facilitent l'accumulation de C dans le sol forestier en produisant de la litière récalcitrante se décomposant lentement.[48] De plus, les mousses influencent la chimie du sol en séquestrant ou en relâchant certains nutriments. La mousse recouvrant le sol forestier peut agir comme un filtre en capturant les dépôts atmosphériques et en retenant certains éléments (e.g., N) par bioaccumulation ou recyclage interne,[37] empêchant ainsi les plantes vasculaires ou les micro-organismes d'y avoir accès.[49] Au contraire, lors des épisodes successifs de sécheresse-précipitations, les mousses peuvent libérer certains composés carbonés ou phosphorés par lessivage.[50,51] D'autre part, grâce à la FBN réalisée par son bactériome, les mousses accumulent d'importantes quantités de N relâchées lentement dans les sols lors de leur décomposition.[52]

1.3. Le bactériome des mousses et sa fixation d'azote

1.3.1. Associations symbiotiques mousses-cyanobactéries

Les mousses sont associées avec une large diversité de bactéries parmi lesquelles les cyanobactéries sont les plus étudiées en raison de leurs rôles majeurs dans les écosystèmes boréaux. Les cyanobactéries sont des procaryotes photosynthétiques et diazotrophes pouvant vivre dans une grande variété d'écosystèmes aquatiques (lacs, océans) et terrestres (sol, phyllosphère). Les cyanobactéries sont unicellulaires ou filamenteuses et peuvent être libres ou former des symbioses avec certains champignons et algues (cyanolichens, diatomées), animaux (éponges marines) et végétaux (angiospermes, gymnospermes, bryophytes).[53] Les principales plantes hôtes de ces

symbioses sont les membres des genres *Azolla* et *Gunnera*, les cycadophytes, et certains lichens et mousses.[54]

Les associations mousses-cyanobactéries ont été mises en évidence dans divers habitats autour du globe (e.g., forêts, prairies, déserts), notamment en zone polaire,[55,56] australe,[57] tropicale,[58] tempérée[59,60] et boréale (Alaska, Canada, Scandinavie).[27,61,62] Ces associations ont particulièrement soulevé l'intérêt scientifique en milieu boréal et arctique puisque les cyanobactéries, en particulier du genre *Nostoc*, représentent la majorité de la biomasse microbienne dans ces écosystèmes.[63] Parmi ces associations, les mousses sont les plantes hôtes colonisées par une plus grande diversité de cyanobactéries.[64] En forêt boréale, on retrouve majoritairement des cyanobactéries hétérocystiques filamenteuses appartenant à l'ordre des *Nostocale*s (Figure 4) colonisant les espèces de mousses pleurocarpes dominantes (i.e., *P. schreberi*, *P. crista-castrensis*, *H. splendens*).[7,61,65]

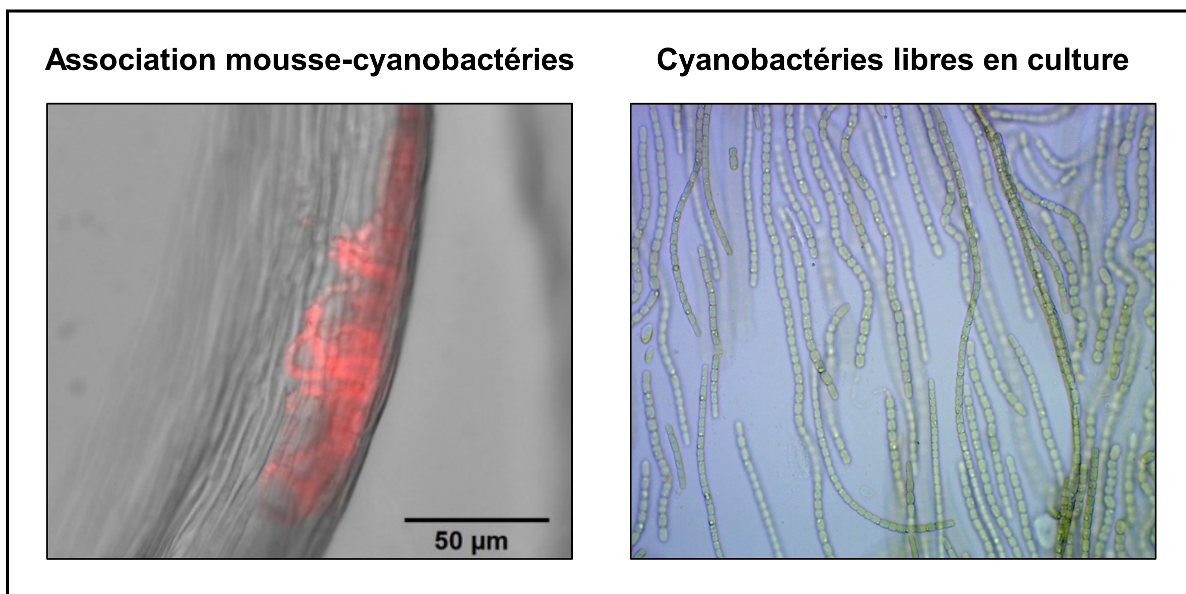

Figure 4. Cyanobactéries du genre *Nostoc* ayant colonisé *Ptilium crista-castrensis* (à gauche, microscope à épifluorescence) et cultivées en milieu liquide (à droite, microscope optique) (échelle similaire pour les deux photos, photos par Marie Renaudin).

Les connaissances liées à la mise en place des associations plantes-cyanobactéries, aussi considérées comme des symbioses, proviennent surtout d'études de cas sur certaines espèces

d'hépatiques, d'anthocérotes et de sphaignes.[54,66] Cependant, si l'établissement des symbioses entre cyanobactéries et mousses pleurocarpes semble similaire aux autres bryophytes,[67] les mécanismes exacts de la symbiose sont encore mal connus. De manière générale, chez les bryophytes, la symbiose passe par deux étapes successives, la chimiotaxie et la colonisation physique de l'hôte. Dans un premier temps, les bryophytes sécrètent des molécules chemo-attractrices appelées Hormogonia Inducing Factors (HIF) pour attirer les cyanobactéries.[67,68] Sous l'effet des HIF, les cyanobactéries passent au stade motile d'hormogonies et peuvent alors coloniser les mousses. Au contraire des autres associations plantes-cyanobactéries connues et des sphaignes, les cyanobactéries, une fois au contact de la mousse, forment des colonies uniquement épiphytiques (i.e., à la surface et dans l'incurvation des feuilles, Figure 4).

La mise en place de la symbiose passe également par un changement d'expression de certains gènes de la mousse hôte et des cyanobactéries. La mousse semble notamment diminuer la production de molécules antimicrobiennes pour faciliter sa colonisation.[69] Chez les cyanobactéries en symbiose, la différentiation en hormogonie est réprimée, la photosynthèse est réduite et la FBN est favorisée.[70] Cependant, une étude plus récente suggère que, dans le cas des symbioses avec les mousses pleurocarpes, les cyanobactéries restent motiles et la photosynthèse et la FBN ne sont pas régulées par l'hôte.[71]

Les associations mousse-cyanobactéries sont considérées comme des symbioses car elles sont avantageuses pour les deux partenaires. En effet, la mousse offre des conditions favorables à la croissance cyanobactérienne en fournissant du soufre (S) et du C[72] ainsi qu'une isolation thermale et une protection contre la dessiccation au cyanobionte. Les cyanobactéries, via la FBN, transfèrent de l'ammonium aux mousses contribuant ainsi à soutenir leurs besoins en N. La mise en place de la symbiose semble d'ailleurs dépendre des besoins en N des mousses qui attireraient les cyanobactéries en période de limitation en N[67] mais inhiberaient la colonisation lorsque N devient abondant, en arrêtant la production de HIF et en sécrétant des composés antimicrobiens de type phénol.[28,73] La quantité de N transférée à la mousse reste inconnue mais, de manière générale dans les associations plantes-cyanobactéries, 80 % du N fixé est fourni à l'hôte.[74] Cependant, les cyanobactéries ne contribuent pas toutes de manière égale à la FBN et certains genres, bien qu'abondants sur les mousses, semblent avoir une très faible FBN et ne transfèreraient donc que

très peu de N à leur hôte.[65] Par la suite, le N fixé et transféré à la mousse peut être relâché dans l'environnement lors de perturbations (feux ou périodes de sécheresse), par l'intermédiaire des champignons mycorhiziens ou par minéralisation de la matière organique de la mousse en décomposition.[52,75]

La FBN par les cyanobactéries associées aux mousses constitue une source cruciale de N pour les mousses et les écosystèmes boréaux, fortement limités en N biodisponible, puisqu'elle représente un flux de 1.5-7 $kgN.ha^{-1}.an^{-1}$ (section 1.1.3.),[27,28] soit jusqu'à 50 % des apports en N dans la forêt boréale.[76,77] La FBN est liée à la biomasse de cyanobactéries colonisant la mousse[28,57] mais aussi, plus largement, à la nature et à l'abondance des communautés de diazotrophes associés à la mousse. Pour étudier et mieux comprendre les effets de l'environnement sur la FBN, il convient donc d'examiner également la composition et la structure du bactériome des mousses.

1.3.2. Composition et diversité du bactériome des mousses boréales

Le bactériome des mousses comporte des bactéries ayant diverses fonctions écologiques et biogéochimiques particulièrement importantes pour la mousse mais aussi à l'échelle de la forêt boréale. Certaines bactéries (e.g., *Burkholderia* spp.) peuvent être bénéfiques pour la mousse en favorisant sa croissance, par la production de phytohormones, ou en réduisant les pathogènes, par la production de molécules antifongiques ou antibactériennes.[78,79] Les mousses abritent également des bactéries impliquées dans le cycle des nutriments de la forêt boréale. En plus des diazotrophes réalisant la FBN, les mousses sont colonisées par des bactéries méthanotrophes (e.g., *Methylocapsa* sp., *Methyloferula* sp., *Methylobacterium* sp.)[80] capables d'oxyder le méthane atmosphérique et de fournir du C à la mousse hôte via des transferts de lipides.[81] Les mousses hébergent aussi des bactéries photosynthétiques (e.g., cyanobactéries, voir section 1.3.1.), qui contribuent à la productivité primaire globale de la forêt boréale, et des bactéries qui participent à la décomposition de la partie sénescente de la mousse.[41]

Le bactériome des mousses en milieu boréal et toundra arctique est majoritairement composé de protéobactéries (40-50 %) et acidobactéries (11-29 %). Les phylums *Actinobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Candidatus Eremiobacterota* (WPS-2), *Planctomycetes* et *Cyanobacteria* sont aussi présents mais constituent chacun moins de 10 % du bactériome.[62,82,83] Les études concernant

plus spécifiquement le bactériome diazotrophique des mousses sont peu nombreuses et se sont surtout focalisées sur les cyanobactéries car elles sont considérées comme les principales contributrices à la FBN des mousses.[84] Les cyanobactéries colonisant les mousses en forêt boréale Canadienne ou Nord-Européenne appartiennent aux genres *Calothrix*, *Cylindrospermum*, *Fischerella*, *Stigonema*, *Tolypothrix*, *Mastigocladus*, *Nodularia* et *Nostoc*,[61,64,84,85] qui semble être le genre dominant.[86]

Avec les cyanobactéries, d'autres taxons bactériens peuvent potentiellement contribuer à la FBN des mousses. Des alphaprotéobactéries membres des familles *Beijerinckiaceae* et *Bradyrhizobiaceae*, capables de réaliser la FBN, ont été retrouvées dans le bactériome de plusieurs espèces de mousses du genre *Sphagnum*.[80,87] Plusieurs études suggèrent également l'importance des alphaprotéobactéries pour la FBN des mousses pleurocarpes en forêt boréale.[62,86] Cependant, malgré l'avancée des techniques d'exploration du bactériome et le nombre croissant d'études sur le microbiome des mousses, la diversité des bactéries associées aux mousses et leurs fonctions exactes sont encore largement inconnues. Étudier la composition du bactériome global et diazotrophique des mousses est donc crucial pour améliorer notre compréhension des processus microbiens et des cycles des nutriments (en particulier C et N) en forêt boréale, pour ainsi déterminer comment ces processus seront affectés par le changement climatique et atmosphérique.

1.3.3. Méthodes d'étude du bactériome des mousses

Différentes techniques, plus ou moins laborieuses et coûteuses, permettent de quantifier ou de caractériser les bactéries des mousses. L'utilisation du microscope à épifluorescence est la méthode la plus largement utilisée pour compter les cyanobactéries associées aux mousses. Sous l'effet d'un filtre d'excitation appliqué à l'échantillon, la phycocyanine (i.e., pigment photosynthétique synthétisé par les cyanobactéries) émet de la fluorescence rouge et les cyanobactéries peuvent être facilement détectées et dénombrées (Figure 4). Les observations peuvent être faites directement sur les brins de mousse[27] ou après extraction préalable des cyanobactéries par agitation manuelle ou sonication.[88,89] La biomasse cyanobactérienne des mousses peut également être estimée en quantifiant certains pigments spécifiques aux cyanobactéries (e.g., échinénone, phycocyanine) par chromatographie en phase liquide ou spectrofluorométrie.[57]

L'identification des cyanobactéries est également majoritairement faite par observation microscopique (fluorescence ou lumière blanche) de la mousse ou après isolation des cyanobactéries dans un milieu de culture.[85] Cependant, ces méthodes sont fortement biaisées car le comptage microscopique est affecté par la disposition des cyanobactéries regroupées en colonies relativement denses, et l'isolation permet seulement l'identification des cyanobactéries cultivables. Des techniques de biologie moléculaire plus récemment développées fournissent des informations étant davantage précises et robustes sur le bactériome des mousses grâce à des amorces moléculaires ciblant des portions spécifiques de certains gènes. Le gène le plus fréquemment ciblé pour l'identification bactérienne est le gène de l'ARNr 16S (i.e., codant pour l'ARN de la sous-unité du ribosome 16S) car il est présent dans le génome de l'ensemble des bactéries et est très conservé.[90] Pour étudier spécifiquement le bactériome diazotrophique, le gène majoritairement ciblé est *nifH*, codant pour une sous unité de la Mo-Nase et présent dans le génome de tous les fixateurs de N_2. Les portions des gènes ciblées sont amplifiées par PCR (Polymerase Chain Reaction) et quantifiées (qPCR) pour estimer la biomasse totale de bactéries ou de diazotrophes.[65,83] Les régions de gènes amplifiées (i.e., amplicons) peuvent aussi être séquencées. Le séquençage d'amplicons est une méthode relativement robuste permettant d'identifier et de déterminer l'abondance relative des bactéries et, plus spécifiquement, des diazotrophes dans un échantillon. À la suite du séquençage, les séquences d'ADN obtenues, appelées OTUs (Operational Taxonomic Unit) ou ASVs (Amplicon Sequence Variant), sont assignées à des taxons bactériens à l'aide de bases de données.

Les techniques d'étude du bactériome utilisant l'ADN permettent de dresser un portrait général des bactéries colonisant la mousse mais incluent les bactéries mortes ou dormantes.[91,92] Au contraire, l'ARN (i.e., produit de transcription de l'ADN) est lié à l'expression génétique et représente exclusivement les bactéries métaboliquement actives. L'utilisation de l'ARN au lieu de l'ADN apporte donc une vision du bactériome centrée sur ses activités métaboliques et permet de mieux lier la composition du bactériome à ses fonctions écologiques et biogéochimiques.[93] En particulier, l'ARN permet d'identifier et de quantifier les diazotrophes des mousses qui sont actifs et qui contribuent réellement à la FBN.

1.4. Facteurs biologiques et environnementaux affectant le bactériome des mousses et sa fixation d'azote

1.4.1. Effets espèces

L'identité de la mousse est un facteur contrôlant la composition du bactériome et la FBN. De nombreuses études rapportent que, même collectées sur des sites exposés à des conditions similaires, différentes espèces ou familles de mousses ont des taux de FBN contrastés.[84,94,95] Ces différences de FBN pourraient être expliquées par la structure du bactériome qui semble également être spécifique à l'espèce de mousse.[62,64,65] En effet, les cyanobactéries associées aux mousses ont des taux de FBN variables.[85] La FBN pourrait donc dépendre de la nature des genres et espèces de cyanobactéries colonisant la mousse, mais aussi de l'abondance relative de chaque genre et de la biomasse cyanobactérienne totale de la mousse.

Les mousses ont également des caractéristiques morphologiques, physiologiques et fonctionnelles (e.g., disposition et densité des feuilles, surface spécifique, capacité de rétention hydrique et d'échange cationique)[96–98] qui constituent un micro-habitat propre à chaque espèce et qui serait potentiellement plus ou moins favorable à la croissance et à l'activité des bactéries. En particulier, le taux d'humidité de la mousse ainsi que sa concentration en phénols et le nombre et la taille de ses feuilles sont des particularités influençant l'abondance des cyanobactéries et la FBN.[99]

1.4.2. Facteurs climatiques et environnementaux

La structure du bactériome des mousses et sa FBN présentent une importante variabilité spatio-temporelle et sont fortement contraintes par leur environnement. Étant donné son importance écologique, les facteurs contrôlant la FBN sont relativement bien connus. Cependant, les effets de l'environnement sur la colonisation et la croissance des bactéries associées aux mousses sont encore incertains. La température et l'humidité sont parmi les variables influençant le bactériome des mousses les plus étudiées. La température favorise la FBN jusqu'à un certain optimum, spécifique à chaque diazotrophe, mais estimé à environ 25 °C.[100] Cependant, des conditions extrêmes de température ainsi que des épisodes de sécheresse peuvent entraîner un stress cellulaire chez les diazotrophes et affecter négativement leur croissance et leur FBN.[101–103] La température

modifie également la composition du bactériome des mousses mais son effet dépend de l'intensité et de la durée du traitement. Récemment, une étude Islandaise a montré qu'une élévation des températures estivales de 1-2 °C sur 20 ans entraînait une augmentation de l'abondance relative des protéobactéries mais une diminution de l'abondance relative des acidobactéries et des cyanobactéries (en particulier *Nostoc* spp.).[83] D'autre part, une hausse des températures similaire mais à plus court terme (2 ans) pourrait favoriser la croissance de *Nostoc* spp. colonisant les mousses.[80] Les effets du climat sur le bactériome des mousses sont aussi visibles au cours de la saison de croissance pendant laquelle les conditions de température et d'humidité varient, modifiant ainsi les genres de cyanobactéries contribuant activement à la FBN.[65]

La FBN est aussi régulée par la dynamique de la forêt boréale puisqu'elle varie avec l'âge et le type des peuplements.[84,86] La végétation, via la canopée, exerce un fort contrôle sur la lumière et les nutriments disponibles. Une grande partie des diazotrophes connus étant aussi photosynthétiques, la lumière peut influencer la composition du bactériome des mousses[62] et être un facteur limitant de la FBN, surtout lorsque la canopée est dense. Ainsi, la litière des arbres feuillus peut diminuer la lumière disponible et réduire l'abondance des *Nostocaceae* et la FBN des mousses.[86] Cependant, l'effet de la lumière est corrélé avec celui de la température : à 16-22 °C, la lumière favorise la FBN mais elle a un effet négatif lorsqu'elle est combinée à des températures supérieures à 30 °C.[104]

La disponibilité des nutriments est un autre facteur affectant la biomasse cyanobactérienne des mousses et la FBN. Les cyanobactéries peuvent obtenir leurs nutriments par les dépôts atmosphériques ou par l'intermédiaire de la mousse. En effet, les mousses accumulent d'importantes quantités de nutriments sur leurs parois cellulaires, qui sont alors potentiellement disponibles pour les cyanobactéries. De plus, comme vu précédemment (sections 1.2.2. et 1.3.1.), les mousses peuvent aussi relâcher des nutriments par lessivage ou transférer activement du C ou du S au cyanobionte. La FBN étant un processus coûteux en énergie, les apports de N par les dépôts atmosphériques ou par ajouts artificiels (souvent sous forme de N inorganique comme le nitrate d'ammonium) peuvent réduire ou supprimer l'activité des diazotrophes.[105,106] Cependant, le seuil d'inhibition de la FBN est variable puisque des ajouts de 3 $kgN.ha^{-1}.an^{-1}$ sur des mousses pleurocarpes collectées en forêt boréale ont montré une réduction de la FBN, alors que des

amendements entre 5 et 50 $kgN.ha^{-1}.an^{-1}$ ont montré peu d'effets ou une augmentation de la FBN.[73,107,108] La disponibilité en N semble également être le facteur principal contrôlant la biomasse cyanobactérienne des mousses puisque le nombre de cyanobactéries colonisatrices augmente quand N est limitant mais diminue fortement quand des ajouts de N sont effectués (voir section 1.3.1.).[17,109] Cet effet négatif peut être dû à un changement métabolique de la mousse qui produirait plus de molécules antimicrobiennes pour empêcher sa colonisation par les cyanobactéries ou à la diminution du pH de la mousse à la suite d'ajouts de N inorganique (e.g., nitrate ou chlorure d'ammonium).[109] Le micro-habitat de la mousse deviendrait alors moins favorable pour la colonisation et la croissance des cyanobactéries. D'autre part, des expériences de fertilisation avec du Mo et du phosphore (P) ont montré que ces éléments favorisaient la FBN et l'abondance des cyanobactéries sur les mousses.[110] Mo et P sont deux éléments essentiels au fonctionnement de la FBN et peuvent potentiellement être limitants mais les ajouts de P sur les mousses montrent des réponses contrastées en forêt boréale. [7,111,112] Cependant, de récentes méta-analyses suggèrent une absence de limitation de la FBN par P dans les mousses en zone boréale.[113,114] Au contraire, la limitation de la FBN par Mo semble être un phénomène commun dans les forêt boréales[26,113] car ce sont des écosystèmes souvent reculés et recevant de faibles dépôts atmosphériques,[115] mais aussi car la concentration en Mo dans les sols est faible.[116] Pour pallier à la limitation en Mo, certains diazotrophes comme *Nostoc* spp. peuvent synthétiser la V-Nase, une Nase alternative ou complémentaire utilisant un cofacteur métallique plus abondant dans l'environnement (voir section 1.1.3.).[117–119] En forêt boréale, la V-Nase peut contribuer jusqu'à 15-50 % de la FBN des cyanolichens[26] et joue donc un rôle important pour soutenir les apports de N dans cet écosystème.

A ce jour, l'influence des cofacteurs des Nases (i.e., Mo, V et Fe) sur le bactériome des mousses et leur FBN reste non élucidée. Plus généralement, les effets de l'environnement, en particulier des nutriments, sur le bactériome des mousses ont jusqu'à présent majoritairement été estimés grâce à des études manipulatoires (e.g., fertilisation, incubation) réalisées en Europe du Nord. Cependant, les forêts boréales Européenne et Canadienne sont soumises à des climats (e.g., températures hivernales, précipitations)[120] et à des techniques de management (e.g., coupes, drainage des sols)[121] différents, qui influencent la lumière et les nutriments disponibles pour les mousses. Les effets de

paramètres environnementaux majeurs (e.g., climat, nutriment, canopée) sur la FBN et le bactériome des mousses restent donc à être caractérisé *in situ* en forêt boréale Canadienne.

1.4.3. Conséquences du changement climatique

Le changement climatique est particulièrement intense dans les écosystèmes boréaux[122] et est associé à une élévation des températures (+ 2 °C d'ici 2050)[8] et une augmentation de la concentration en CO_2 atmosphérique (+ 75-955 ppm d'ici 2100).[123] Le changement climatique entraîne également l'allongement de la saison de croissance[124] ce qui pourrait favoriser la FBN et la croissance bactérienne. En revanche, les épisodes de sécheresse devraient être plus fréquents dans l'est du Canada et pourraient contrer les effets positifs du réchauffement sur la FBN.[13] La fréquence des perturbations de la forêt boréale (i.e., feux, maladies) devrait également augmenter,[125,126] modifiant la structure de la canopée et affectant potentiellement la FBN des mousses. Les dépôts atmosphériques de certains nutriments, notamment Mo, V et Fe, cofacteurs des nitrogénases, augmentent continuellement depuis la révolution industrielle du début du 19e siècle.[115,127,128] L'augmentation des dépôts atmosphériques liée au développement des activités humaines dans la forêt boréale (e.g., exploitation forestière et minière), notamment via le « Plan d'action nordique 2020-2023 » mis en place par le gouvernement du Québec, pourrait diminuer la limitation en certains nutriments et favoriser la croissance bactérienne et la FBN. Au contraire, de fortes émissions de N pourraient réprimer la FBN, mais les prévisions ne semblent pas tendre vers une hausse importante au Québec au cours des prochaines décennies.[129,130]

Les conséquences du changement climatique et des perturbations de la forêt boréale sur la composition du bactériome des mousses restent à éclaircir mais plusieurs études suggèrent que certains taxons bactériens, diazotrophes ou non (e.g., protéobactéries, cyanobactéries), pourraient être affectés par la hausse des températures sur le long terme,[80,83] influençant indirectement la FBN et le N disponible pour les mousses.

1.5. Objectifs de recherche

Les mousses et leur bactériome jouent des rôles écologiques et biogéochimiques clés dans la forêt boréale, tels que la photosynthèse et la FBN (voir sections 1.2.2. et 1.3.3.). La FBN réalisée par les

diazotrophes colonisant les mousses représente une entrée importante de N pour cet écosystème. De plus, les cyanobactéries associées aux mousses sont considérées comme les principales responsables de la FBN. Ainsi, mieux connaître les effets de paramètres environnementaux majeurs sur la biomasse et l'activité des cyanobactéries colonisant les mousses contribue à mieux prédire les conséquences du changement climatique sur la FBN et sur les apports de N en forêt boréale (voir section 1.4.3). Cependant, les méthodes actuellement utilisées pour estimer la biomasse cyanobactérienne des mousses sont laborieuses, coûteuses et parfois peu précises (e.g., comptage au microscope, voir section 1.3.1.). **Le premier objectif de cette thèse (Chapitre 2) est donc de développer une méthode rapide et robuste pour déterminer la biomasse cyanobactérienne associée aux mousses.** Cette méthode pourra par la suite être utilisée pour réaliser les autres objectifs de cette thèse.

La quantité de cyanobactéries colonisant les mousses est peu souvent mesurée, malgré leur rôle majeur dans le cycle du N de la forêt boréale. De plus, les connaissances actuelles sur les associations mousse-cyanobactéries et leur FBN proviennent majoritairement d'études localisées dans la forêt boréale d'Europe du Nord (Suède, Finlande) et utilisant des expériences manipulatoires (e.g., réchauffement et ajouts artificiels de nutriments, voir section 1.4.2.). **Le deuxième objectif de cette thèse (Chapitre 3) est de déterminer les facteurs environnementaux principaux contrôlant *in situ* la biomasse cyanobactérienne des mousses et leur FBN dans la forêt boréale de l'est Canadien.**

Malgré l'intérêt croissant de la communauté scientifique pour le bactériome des mousses et ses fonctions, la nature et la diversité des communautés bactériennes métaboliquement actives colonisant les mousses boréales sont peu connues. En effet, jusqu'à présent, le bactériome des mousses en forêt boréale a été étudié via des analyses d'ADN qui, au contraire de l'ARN, incluent les bactéries mortes ou dormantes (voir section 1.3.1.). De plus, le bactériome diazotrophique des mousses reste en grande partie inconnu et certains travaux suggèrent que, mis à part les cyanobactéries, d'autres phylums bactériens contribueraient significativement à la FBN (voir section 1.3.3.). **Le troisième objectif de cette thèse (Chapitre 4) est de caractériser le bactériome actif global et diazotrophique des mousses et d'évaluer comment ce bactériome est affecté par les conditions environnementales.**

Les trois objectifs de cette thèse (Figure 5) se concentrent sur les deux espèces de mousses dominantes en forêt boréale Canadienne, *Pleurozium schreberi* et *Ptilium crista-castrensis* (Figure 3, section 1.2.1.), exclusivement collectées au Québec dans les zones bioclimatiques de la sapinière à bouleaux blancs, la pessière à mousses et la pessière à lichens (voir section 1.1.1.).

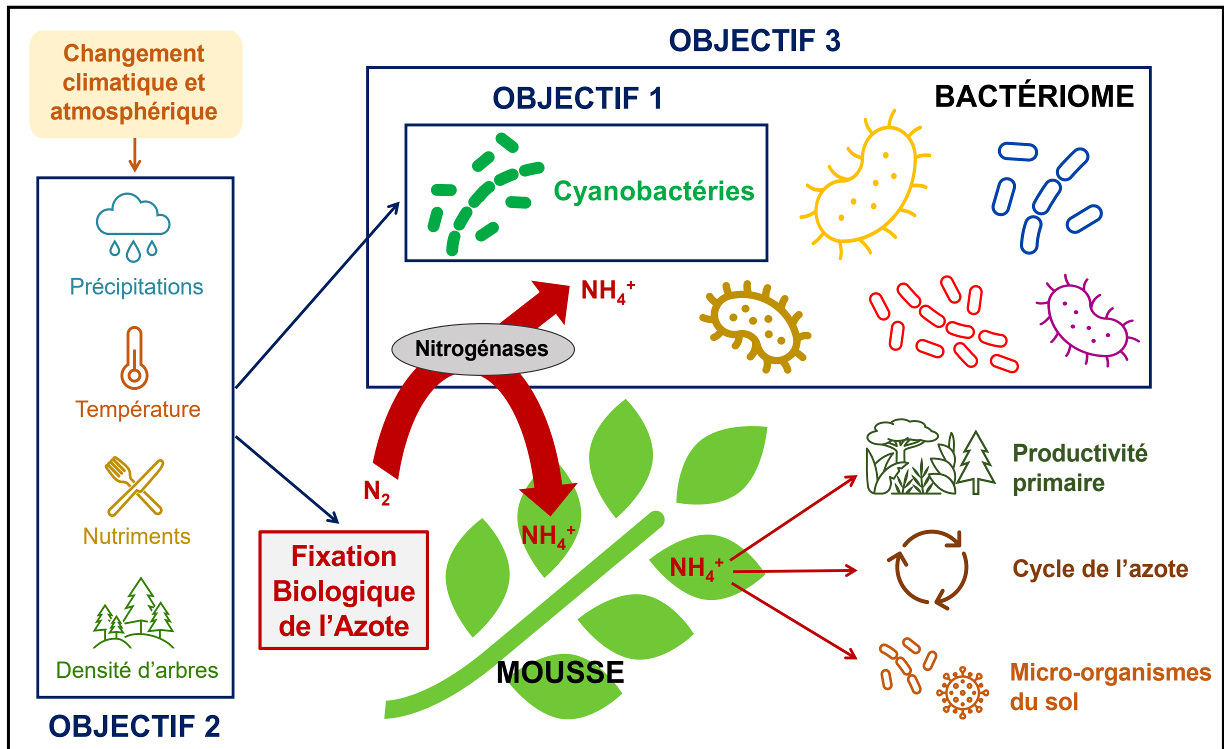

Figure 5. Représentation schématique des objectifs réalisés au cours de cette thèse de doctorat.

CHAPITRE 2 : QUANTIFICATION DE LA BIOMASSE CYANOBACTÉRIENNE ASSOCIÉE AUX MOUSSES GRÂCE À LA PHYCOCYANINE

2.1. Informations préliminaires

L'article présenté dans ce chapitre a été accepté et publié dans le journal *Frontiers in Microbiology* le 5 janvier 2021. Voir la référence : Renaudin, M; Darnajoux, R.; Bellenger, J. P. Quantification of Moss-Associated Cyanobacteria Using Phycocyanin Pigment Extraction. *Frontiers in Microbiology*. **2021**, *11*, 611792. https://doi.org/10.3389/fmicb.2020.611792.

Les modifications effectuées dans ce chapitre par rapport à la version publiée sont de natures typographiques. Seule la responsabilité de l'étudiante est engagée dans les modifications effectuées.

2.1.1. Auteurs et affiliations

Marie Renaudin[1], Romain Darnajoux[2], Jean-Philippe Bellenger[1*]

[1] Centre Sève, Département de Chimie, Université de Sherbrooke, Sherbrooke, QC, Canada

[2] Department of Geosciences, Princeton University, Princeton, NJ, United States

[*] Corresponding author: Jean-Philippe Bellenger; jean-philippe.bellenger@usherbrooke.ca.

2.1.2. Participation de chaque auteur

M.R., R.D. et J-P.B. ont conçu cette étude et les expériences associées. M.R. a réalisé toutes les expériences et analyses de données, et a rédigé le manuscrit. R.D. et J-P.B. ont révisé le manuscrit. Tous les auteurs ont contribué à cet article et ont approuvé la version finale.

2.2. Article « Quantification of moss-associated cyanobacteria using phycocyanin pigment extraction »

2.2.1. Abstract

In the boreal forest, cyanobacteria can establish associations with feather moss and catalyze the biological nitrogen fixation (BNF) reaction, consisting in the reduction of atmospheric dinitrogen into bioavailable ammonium. In this ecosystem, moss-associated cyanobacteria are the main contributors to BNF by providing up to 50% of new N input. Current environmental changes driven by anthropogenic activities will likely affect cyanobacteria activity (i.e., BNF) and populations inhabiting mosses, leading to potential important consequences for the boreal forest. Several methods are available to efficiently measure BNF activity, but quantifying cyanobacteria biomass associated with moss is challenging because of the difficulty to separate bacteria colonies from the host plant. Attempts to separate cyanobacteria by shaking or sonicating in water were shown to be inefficient and non-repeatable. The techniques commonly used, microscopic counting and quantitative PCR (qPCR), are laborious and time-consuming. In aquatic and marine ecosystems, phycocyanin (PC), a photosynthetic pigment produced by cyanobacteria, is commonly used to monitor cyanobacterial biomass. In this study, we tested if PC extraction and quantification can be used to estimate cyanobacterial quantity inhabiting moss. We report that phycocyanin can be easily extracted from moss by freeze/thaw disturbance of cyanobacteria cells and can be quickly and efficiently measured by spectrofluorometry. We also report that phycocyanin extraction is efficient (high recovery), repeatable (relative SD < 13%) and that no significant matrix effects were observed. As for aquatic systems, the main limitation of cyanobacteria quantification using phycocyanin is the difference of cellular phycocyanin content between cyanobacterial strains, suggesting that quantification can be impacted by cyanobacterial community composition. Nonetheless, we conclude that phycocyanin extraction and quantification is an easy, rapid, and efficient tool to estimate moss-associated cyanobacteria number.

Keywords: phycocyanin, cyanobacteria, nitrogen fixation, feather moss, boreal forest.

2.2.2. Introduction

Mosses are cryptogamic plants found in a very large range of terrestrial and aquatic ecosystems around the globe (Fogg, 1998). Mosses are particularly abundant in the boreal forest, the largest terrestrial biome on Earth (DeLuca and Boisvenue, 2012), where they can cover up to 70–100% of the ground (Oechel and Van Cleve, 1986). Mosses affect microbial activity in soil by regulating soil temperature and moisture (Luthin and Guymon, 1974; Gornall et al., 2007) and by releasing nutrients, such as dissolved organic carbon and potassium (Wilson and Coxson, 1999). In the boreal forest, feather mosses also play an important role in the carbon (C) and nitrogen (N) cycles and contribute up to a third of the total forest primary productivity (DeLuca et al., 2002; Turetsky, 2003; Turetsky et al., 2010; Wardle et al., 2011; Liu et al., 2020). In addition, the reaction of biological nitrogen fixation (BNF), catalyzed by diazotrophic bacteria associated with feather moss, can contribute up to 50% of new N inputs (Turetsky et al., 2012; Rousk and Michelsen, 2017) on par with atmospheric deposition. Several cyanobacterial genera (e.g., *Calothrix*, *Cylindrospermum*, *Fischerella*, *Nostoc*, and *Stigonema*) were found living epiphytically on boreal feather mosses (DeLuca et al., 2002; Gentili et al., 2005; Houle et al., 2006; Zackrisson et al., 2009; Ininbergs et al., 2011) and are considered the main contributors to moss BNF in the boreal forest (Leppänen et al., 2013).

Several studies reported a positive linear relationship between cyanobacterial abundance and BNF activity in boreal mosses and suggest that moss can regulate cyanobacteria colonization according to their N needs (DeLuca et al., 2007; Gundale et al., 2011; Rousk et al., 2013, 2017). Besides moss N demand, other environmental parameters can affect moss-cyanobacteria associations (cyanobacteria abundance and BNF), such as moisture, temperature, heavy metals, and phosphorus deposition (Gundale et al., 2012; Rousk et al., 2017; Jean et al., 2018; Scott et al., 2018). With global warming and the development of human activities at northern latitudes, boreal forests and feather mosses will undergo important changes in climatic conditions (i.e., average temperature and water regime) and atmospheric deposition (nutrients). Indeed, it has been predicted that the average annual temperature in the boreal forest will increase by 2°C by 2050 (Price et al., 2013). This will extend the growth season length (Ouranos, 2015) and, combined with increasing CO_2 atmospheric concentration, will impact boreal forest primary productivity and N demand (Lloyd and Bunn, 2007; Sigurdsson et al., 2013; Tagesson et al., 2020). Evaluating how these

environmental changes will affect moss-associated N-fixing bacteria is essential to help better predict the response of the boreal forest to global change. Thus, consistent and rigorous methods are needed to characterize how the moss-associated cyanobacteria biomass and BNF are affected by environmental factors.

Cyanobacterial BNF activity in moss can be easily assessed, indirectly, using the reaction of acetylene reduction into ethylene as a proxy (i.e., Acetylene Reduction Assay (ARA) method, Hardy et al., 1968) or directly, by the incorporation of ^{15}N tracer (Leppänen et al., 2013; Jean et al., 2018). Accurately quantifying cyanobacteria quantity, on the other hand, remains challenging. Three approaches have been used to estimate cyanobacteria quantity associated with moss. In the first approach, cyanobacteria are directly counted on whole moss shoots or leaves under an epifluorescence microscope (DeLuca et al., 2007; Gundale et al., 2011; Rousk et al., 2013). This approach is laborious, time consuming and, because cyanobacteria are often grouped into multilayer colonies located within leaf incurves (DeLuca et al., 2002), accurately counting individual cells is complicated. Moreover, counting is only performed on a relatively small number of moss shoots and leaves, which makes it difficult to extrapolate to a cyanobacteria quantity per surface *in situ*. In the second approach, cyanobacteria colonies are extracted from moss prior to being counted under a fluorescence microscope, as in the first approach. This technique allows estimating cyanobacteria number on a larger amount of moss stems with reducing errors due to variation in cyanobacteria density between stems. Different cyanobacteria separation techniques have been explored. Sonication was used to isolate cyanobacteria from the moss (Lindo and Whiteley, 2011) but it has been reported to lead to bacteria cell lysis (Reksten, 2014), whereas shaking or vortexing moss shoots immersed in distilled water (Jean et al., 2012; Rousk et al., 2017) result in variable extraction efficiencies that can only be overcome by performing a very large number of replicates. Moreover, as for the first approach, counting colonies after extraction only provides rough estimates of cyanobacteria quantity and is probably biased by differences of extraction efficiencies between cyanobacteria genera (Whiteley and Gonzalez, 2016). The last approach, more rarely used, relies on molecular biology techniques, such as quantitative PCR (qPCR), to estimate global cyanobacteria quantity or genera/species relative abundance (Warshan et al., 2016). Primers targeting the cyanobacterial 16S rRNA gene CYA 359F and CYA 781Ra/Rb (Nübel et al., 1997) are usually used. The qPCR approach is relatively sensitive but time

consuming, costly and is based on primers selectivity, which can bias cyanobacteria quantification in moss samples. Moreover, variation in 16S rRNA gene copy number has been demonstrated for several cyanobacteria genera (Engene et al., 2010; Engene and Gerwick, 2011) and could affect qPCR results when studying mixed-genera cyanobacteria communities present in moss. More recently, Arróniz-Crespo et al. (2014) proposed an alternative for the quantification of cyanobacteria number living on moss based on the extraction and quantification of the echinenone pigment by HPLC separation coupled with a photodiode array detector.

The aim of this study was to develop and test an easy, quick and affordable method based on the extraction and quantification of another pigment, the phycocyanin (PC), to estimate moss-associated cyanobacteria quantity. This approach is inspired by a method commonly used to monitor cyanobacteria blooms in lakes for the last 20 years (Seppälä et al., 2007; McQuaid et al., 2011). PC is a photosynthesis pigment produced by cyanobacteria and located in phycobilisome structures in the thylakoid membrane (MacColl, 1998). This pigment is already commonly used to observe and count moss-associated cyanobacteria by epifluorescence microscopy (Figure 6).

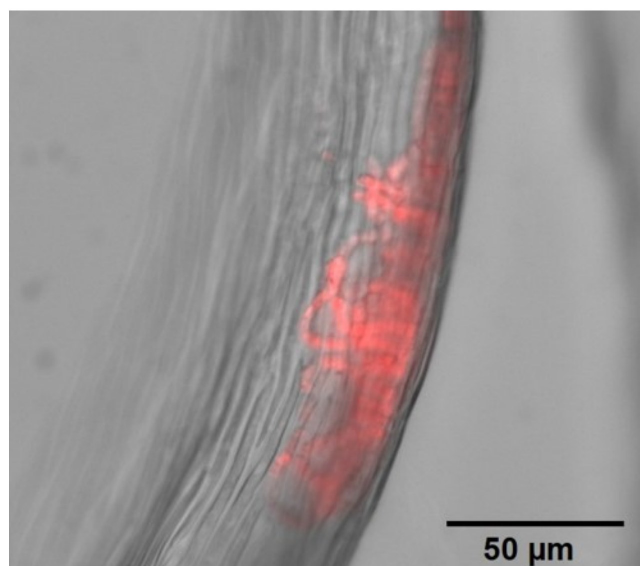

Figure 6. Filamentous cyanobacteria colonies (phycocyanin fluorescence in red) within *Ptilium crista-castrensis* leaves, observed under an epifluorescence microscope (Zeiss Axio Observer Z1 equipped with a Zeiss Axiocam 506 mono, objective 40X/0.95NA).

For widefield fluorescence, an excitation filter of 533-558 nm and an emission filter of 570-640 nm were used.

Phycocyanin has been reported as the most abundant pigment among phycobiliproteins, the major light-harvesting pigments, and can account for 20% of the total proteins in the cyanobacteria dry mass (de Marsac, 1977; Stanier and Cohen-Bazire, 1977). Choosing phycocyanin as a quantitative marker has several advantages over other cyanobacterial pigments. First, phycocyanin is produced only by cyanobacteria and two groups of algae (the cryptophytes and the rhodophytes; Kirk, 1994) and is not found in moss, unlike some chlorophylls and carotenoids (such as echinenone; Czygan, 1981). Then, it is water-soluble and can be easily measured by fluorimetry. Fluorescence spectroscopy is a very sensitive technique and is less laborious than HPLC based methods, which require chromatographic separation, that are commonly used to quantify other cyanobacteria marker pigments like chlorophyll a or echinenone (Schalles and Yacobi, 2000; Poza-Carrión et al., 2001; Schlüter et al., 2004; Seppälä et al., 2007; Arróniz-Crespo et al., 2014). Phycocyanin measurements have been extensively used to quantify cyanobacteria in pure cultures (Herrera et al., 1989; Lee et al., 2017; Basheva et al., 2018; Piron et al., 2019) and water samples (Izydorczyk et al., 2005; Cotterill et al., 2019; Cegłowska et al., 2020) but, to our knowledge, it has never been applied to moss samples.

We proposed that phycocyanin quantification could be a reliable proxy to estimate moss-associated cyanobacteria quantity. To evaluate if this new approach can be extensively used, we tested four important analytical performance parameters. We first performed the analytical calibration of the method by evaluating (i) cellular phycocyanin linearity in pure cultures of five cyanobacterial strains, (ii) the range of applicability of the method applied to moss samples, and (iii) the detection and quantification limits of phycocyanin. Then, we studied (iv) the accuracy of the method by measuring three performance parameters: phycocyanin apparent recovery assessed using both phycocyanin standard and cyanobacteria culture spikes on moss, matrix effects and repeatability. Then, we applied the method directly on two feather moss species that are dominating in the eastern Canadian boreal forest, *Pleurozium schreberi* (Brid.) Mitt. and *Ptilium crista-castrensis* (Hedw.) De Not. (Harper et al., 2003). Finally, because the quantity of cyanobacteria associated with feather moss has been reported to be correlated with BNF activity in *P. schreberi*, using microscopic counting (DeLuca et al., 2007) and qPCR (Warshan et al., 2016), we also assessed the relationship between phycocyanin and BNF in moss samples to evaluate if the relationship observed with other quantification methods was similar when using phycocyanin measurements.

2.2.3. Materials and methods

2.2.3.1. Cyanobacterial strains selection and culture conditions

To evaluate phycocyanin linearity and phycocyanin apparent recovery (see analytical performance parameters assessment section), we cultivated cyanobacteria in defined laboratory conditions.
To test phycocyanin linearity in cyanobacteria cultures, we used five cyanobacterial strains belonging to the *Nostoc* genus, isolated from the feather moss *P. crista-castrensis* and *Peltigera* cyanolichens collected in Quebec, Canada, and Iceland (Tableau 1).

Tableau 1. Cyanobacterial strains used in this study

Species	Strain	Isolation	Country of Origin
***Nostoc* sp.**	210A	*Peltigera membranacea*	Iceland
***Nostoc* sp.**	213	*Peltigera membranacea*	Iceland
***Nostoc* sp.**	232	*Peltigera membranacea*	Iceland
***Nostoc* sp.**	MR100	*Ptilium crista-castrensis*	Canada
***Nostoc* sp.**	MR101	*Peltigera* sp.	Canada
Anabaena variabilis	ATCC 29413	Freshwater	USA

We selected *Nostoc* sp. strains because they were found to be commonly associated with boreal feather moss (DeLuca et al., 2002; Ininbergs et al., 2011). To determine phycocyanin apparent recovery in moss samples, we spiked moss with *Anabaena variabilis* (ATCC 29413) cells. *Anabaena variabilis* is an aquatic cyanobacteria often assessed during bloom monitoring (Li et al., 2016). We used *A. variabilis* for the phycocyanin apparent recovery experiments because of its high phycocyanin cell content (10 times higher than *Nostoc* sp. in average), which allows to have a higher phycocyanin signal using less cyanobacteria cells. Moreover, in cultures, *A. variabilis* produced significantly less biofilm than *Nostoc* sp. strains, allowing for a more accurate cell harvesting by pipetting. All cyanobacteria strains were grown on a liquid N-free $BG11_0$ medium (Rippka et al., 1979) at 22°C, under continuous white fluorescent light tubes (T8 bulb, Sylvania

Gro-Lux) at 30 μmol.$m^{-2}.s^{-1}$ and without agitation. Cyanobacteria cells were harvested at the beginning of the stationary growth phase (20 days).

2.2.3.2. Moss sampling

Feather moss samples were collected for both the evaluation of analytical performance parameters (see section below) and to test the relationship between BNF and phycocyanin quantity. *Pleurozium schreberi* and *Ptilium crista-castrensis* were collected on four boreal forest sites along a 500-km latitudinal transect in Quebec, Canada in June and September 2019. All sites are located between N47° and N51°, in the black spruce forest bioclimatic zone. As environmental parameters are known for affecting cyanobacteria BNF, sampling sites are spread along a latitudinal gradient of temperature, moisture, and atmospheric deposition to examine the relationship between phycocyanin and BNF for moss samples displaying potentially contrasting BNF activities. Samples were collected in plastic bags and kept at 4°C in the dark until being processed, within 2 weeks after collection.

2.2.3.2. Phycocyanin extraction and quantification procedure

The phycocyanin extraction method is divided in four consecutive important steps: (i) sample dilution in sodium phosphate buffer, (ii) disruption of cyanobacteria cell membranes, (iii) phycocyanin solubilization in sodium phosphate buffer, and (iv) quantification by fluorescence of the solubilized phycocyanin. These steps are described in detail in the following section and in Figure 7.

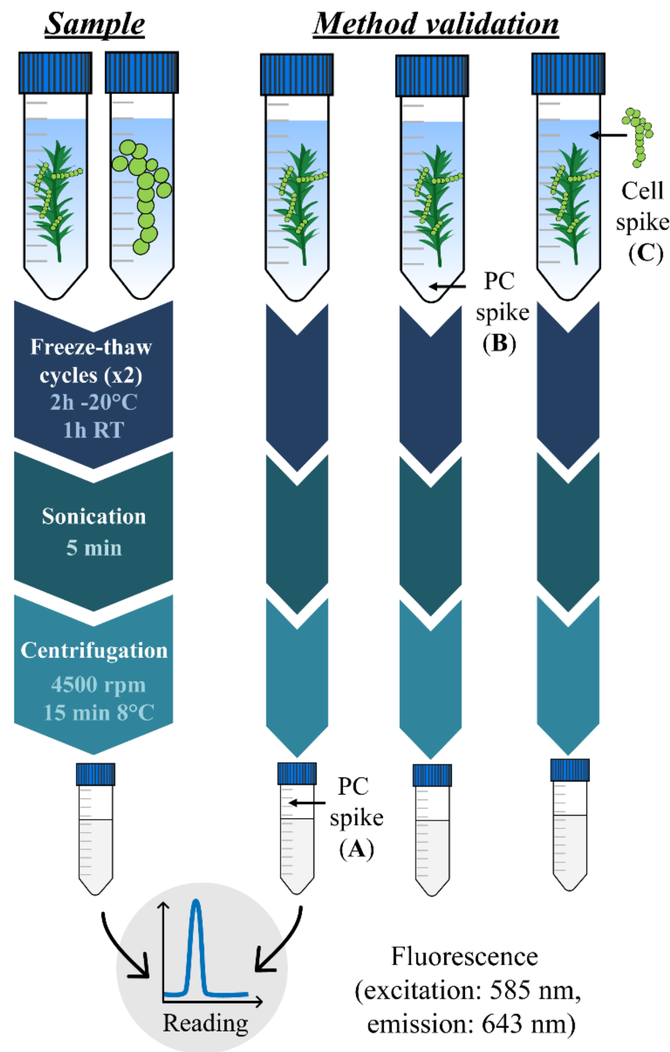

Figure 7. Phycocyanin (PC) extraction and quantification procedure for cyanobacteria in cultures and moss samples (left) and method validation process (right).

Capital letters in brackets refer to three analytical performance parameters evaluated for the method validation. (A) corresponds to matrix effects, (B) corresponds to standard phycocyanin apparent recovery, and (C) corresponds to cellular phycocyanin apparent recovery.

For phycocyanin extraction of cyanobacteria in cultures, we performed a series of dilution of cell suspensions for each strain. Between 2.5 and 12.5 ml of culture were harvested and diluted in sterilized sodium phosphate buffer (0.025 M, pH 7) to reach a final volume of 15 ml. Cyanobacteria cell concentrations, corresponding to the number of individual cells per ml of culture, are presented in Figure 8. For phycocyanin extraction of cyanobacteria living on feather moss, samples were

oven-dried at 35°C for 4 h and milled with a blender. Then, 0.15–0.20 g of moss sample were placed in 50 ml sterile tubes with 15 ml of sterilized sodium phosphate buffer (0.025 M, pH 7; Sarada et al., 1999; Furuki et al., 2003; Horváth et al., 2013) prepared by mixing 2.99 g of $Na_2HPO_4 \cdot 2H_2O$ and 1.91 g of $NaH_2PO_4 \cdot 2H_2O$ in 1 L of Milli-Q water. Phycocyanin extraction procedure was then performed similarly for cyanobacteria cells and moss samples. All samples (i.e., cyanobacteria cultures or moss in sodium phosphate buffer) were homogenized by vortexing and shaking manually for 10 s. To achieve cyanobacteria cell membrane disruption, samples were subjected to two freeze-thaw cycles (2 h at −20°C followed by 1 h at room temperature), mixed by vortexing for 10 s between cycles (Lawrenz et al., 2011; Horváth et al., 2013) and sonicated for 5 min in an ultrasound bath. Then, samples were centrifuged at 3,400 × *g* at 8°C for 15 min. Supernatants were transferred to 15 ml tubes and stored at −80°C until analysis. In these conditions, extracted phycocyanin can be stored for up to 6 months without noticeable degradation (Lawrenz et al., 2011). Phycocyanin was quantified by spectrofluorometry (excitation at 585 nm and emission at 643 nm; Seppälä et al., 2007) on a QuantaMaster 400 Phosphorimeter (PTI) using a commercial standard (C-Phycocyanin, Sigma-Aldrich). All tubes were covered by aluminum foil during the entire procedure to limit photodegradation. After phycocyanin extraction, moss samples were oven-dried at 50°C and weighted. Results were expressed in µg of phycocyanin (mass) or in µg of phycocyanin per g of moss (concentration). All phycocyanin concentrations measured were above detection and quantification limits.

2.2.3.3. Analytical performance parameters assessment

To assess the reliability of the method and evaluate if it can be applied to moss, we tested different analytical performance parameters.

2.2.3.3.1. Analytical calibration of the method

We first tested if phycocyanin mass was linear within a large range of cyanobacteria cell quantity by measuring phycocyanin in cyanobacteria pure cultures. For each cyanobacteria strain, cultivated cells were harvested and diluted in triplicates with sodium phosphate buffer, prior to phycocyanin extraction. Both procedures are described in the section above. The number of vegetative cells in cyanobacteria cultures was measured using a counting chamber (Petroff-Hausser counting

chamber, Hausser Scientific). Briefly, 2 µl of each culture were placed in the counting chamber and individual cells were counted using a bright field microscope (Motic BA210). The counting procedure was repeated five times per strain. Cell number per ml of culture was calculated according to the formula provided by the manufacturer and final cyanobacteria cell concentrations were attributed to each culture dilution. All the following analytical performance parameters were measured for both moss species *P.-castrensis* and *P. schreberi*.

We determined the range of applicability of the method (i.e., the range of mass of moss within which phycocyanin quantity is linear) by extracting phycocyanin from six different mass of moss, comprised between 0.25 and 1.25 g. The exact same moss sample was used for this experiment and three technical replicates per mass of moss were performed.
Limit of detection (LOD) and limit of quantification (LOQ) of phycocyanin were respectively calculated by adding 3 × SD and 10 × SD to the average signal measured in analytical blanks.

2.2.3.3.2. Accuracy of the method

Then, we determined the accuracy of the method (i.e., estimation of the total error impacting the method; Raposo and Ibelli-Bianco, 2020) composed of two elements, the trueness (i.e., evaluation of the systematic error), and the precision (i.e., evaluation of the random error). Trueness was examined by measuring phycocyanin apparent recovery and matrix effects. Precision was evaluated through testing the repeatability. Phycocyanin apparent recovery is defined as the ratio between observed values (i.e., measured phycocyanin) and reference values (i.e., estimated added phycocyanin). We determined (i) the standard phycocyanin apparent recovery by adding a C-phycocyanin commercial standard (Sigma-Aldrich) on moss and (ii) the cyanobacteria cellular phycocyanin apparent recovery by adding *A. variabilis* cells on moss. Cellular phycocyanin linearity in *A. variabilis* culture was checked prior to this experiment (Supplementary Figure S1, Annexe 1) and both standard and cellular phycocyanin apparent recoveries were determined using four replicates of the same moss sample. Standard phycocyanin (Figure 7B) and cellular phycocyanin apparent recoveries (Figure 7C) were respectively determined by spiking phycocyanin standard at 0.5×, 2×, and 15× the average phycocyanin content in moss and by spiking between 2 and 12 ± 1.5 × 10^6 *A. variabilis* cells, which corresponds to additions of 14–84 µg of phycocyanin (Supplementary Figure S1, Annexe 1), on 0.60 ± 0.1 g of moss.

Matrix effects (i.e., components present in samples potentially affecting phycocyanin quantification; Raposo and Barceló, 2020) were evaluated by adding a C-phycocyanin commercial standard to samples of phycocyanin extracted from moss (Figure 7A). Standard additions were performed on five replicates at 0.5×, 2×, and 15× the average phycocyanin content in moss.

Finally, the repeatability of the method (i.e., the closeness of phycocyanin results obtained by analyzing the same sample using the same procedure and under similar conditions; Thompson and Wood, 1993) was assessed by extracting phycocyanin from six replicates of the same moss sample for 3 consecutive days. Analytical blanks were performed and analyzed for each set of experiment.

2.2.3.3.3. Nitrogen fixation measurements

Green parts (i.e., photosynthetic parts of the moss shoots containing the cyanobacteria colonies) of *P. schreberi* and *P.-castrensis* shoots were hydrated with deionized water to homogenize their hydration state and placed into 250 ml glass jars. This experiment was performed in four replicates for all moss species, site, and date of collection. Samples were acclimated for 5 days in a growth chamber (18°C; 16 h light, 8 h dark) and moss-associated bacteria BNF was assessed using ARA (Hardy et al., 1968). Acetylene gas was produced by adding 25 ml of H_2O to 5 g of CaC_2 (Acros Organics) in Tedlar® gas sampling bags (Sigma-Aldrich). Moss samples were incubated with 20% of acetylene for 24 h at 18°C under continuous light. Ethylene production was measured on a gas chromatograph (Shimadzu 8A with an FID detector and a Supelco column 01282011). Following ARA, moss samples were processed for phycocyanin extraction, therefore moss dry mass could not be directly determined to standardize ARA. Moss dry mass was calculated by measuring the average moss water content by surface unit on four replicates per species and per sampling site. The estimated moss dry mass used for the ARA was comprised between 1.8 and 3 g per replicate.

2.2.3.3.4. Statistical analysis

The test of Kruskal and Wallis (1952) followed by the Dunn's (1965) *post hoc* test were used to examine the effects of moss species and month of collection on phycocyanin concentrations. Normality was tested with the Shapiro-Wilk test (Royston, 1995) and linear regression outliers were checked using the test of Grubbs (1969). Linear regressions and statistical tests were

respectively performed using GraphPad Prism (version 8.0.2) and R (version 3.4.3, R Core Team, 2017) with the Stats package. Statistically significant differences were accepted for value of $p < 0.05$.

2.2.4. Results and discussion

2.2.4.1. Phycocyanin extraction method characterization and validation

All the analytical performance parameters measured in this study and used to validate the method are presented in Tableau 2. We first tested phycocyanin linearity for a wide range of cyanobacteria cell densities in pure liquid cultures of five strains of *Nostoc* sp. extracted from the boreal moss species *P.-castrensis*, and *Peltigera* cyanolichens (Tableau 1). For all strains, phycocyanin mass was strongly correlated with cell density ($R^2 > 0.93$, Figure 8), showing that phycocyanin is a reliable proxy for quantifying cyanobacteria cells, even over a large range of cell counts.

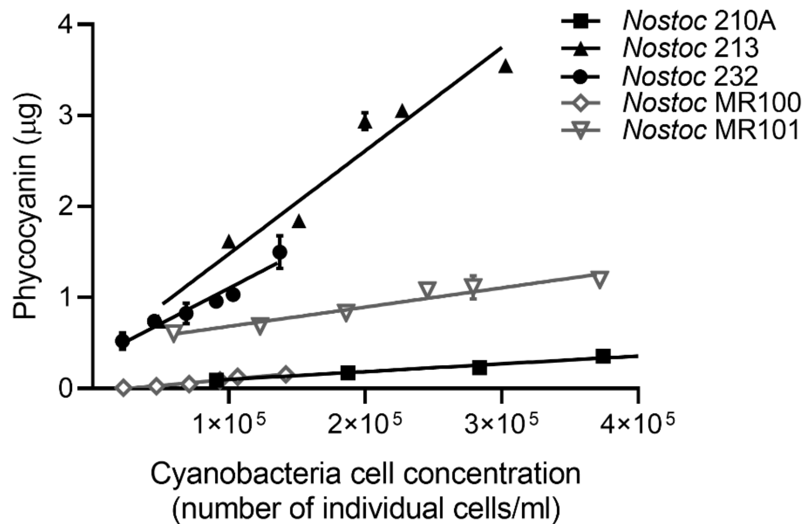

Figure 8. Phycocyanin mass mean ± SD (n = 3) linearity in cyanobacteria *Nostoc* sp. pure cultures.

Straight lines represent the linear regression between phycocyanin mass and the number of individual cyanobacteria cell per ml of culture, for each cyanobacterial strain. For all strains, $R^2 > 0.93$ and $p < 0.002$.

However, significant differences in phycocyanin cellular concentration were observed between strains. Using data from Figure 8, we calculated phycocyanin cellular concentration for each cyanobacteria strain. *Nostoc* 232 achieved the highest phycocyanin cellular concentration with an average of $1.18 \pm 0.39 \times 10^{-6}$ µg.cell^{-1}, followed by *Nostoc* 213 ($9.24 \pm 1.1 \times 10^{-7}$ µg.cell^{-1}), *Nostoc* MR101 ($3.58 \pm 1.6 \times 10^{-7}$ µg.cell^{-1}), *Nostoc* MR100 ($8.4 \pm 1.5 \times 10^{-8}$ µg.cell^{-1}), and *Nostoc* 210A ($6.73 \pm 9.6 \times 10^{-9}$ µg.cell^{-1}). Then, we tested the range of applicability of the method for moss samples by examining phycocyanin linearity for a large range of moss masses. For both moss species, phycocyanin and moss masses were strongly correlated ($R^2 = 0.97$ for *P. crista-castrensis* and $R^2 = 0.98$ for *P. schreberi*, Figure 9). This shows that phycocyanin extraction efficiency is not dependent of moss mass in the tested range of 0.25–1.50 g.

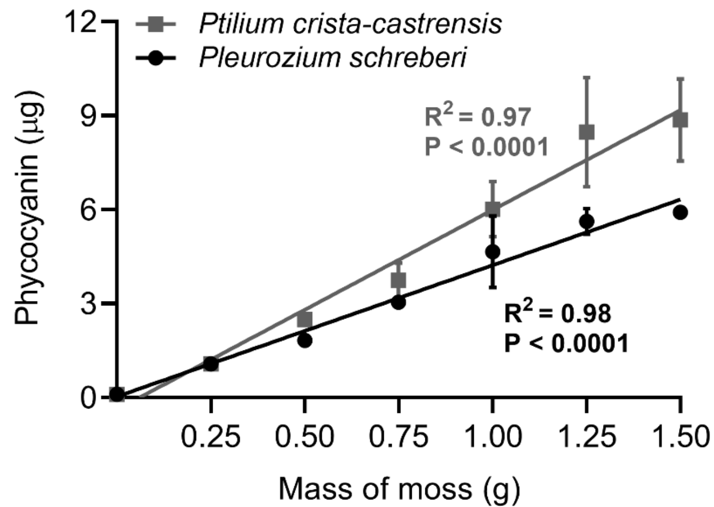

Figure 9. Phycocyanin mass mean ± SD (*n* = 3) linearity in *P. crista-castrensis* (gray squares) and *Pleurozium schreberi* (black circles) feather moss.

Straight lines represent the linear regression between phycocyanin and moss masses, for each moss species. All replicates are from a same moss sample of each species, that were acclimated for 5 days in a growth chamber (30°C, 16 h light, 8 h dark) before being processed.

Using analytical blanks, we found that phycocyanin LOD and LOQ were 3.1 and 4.3 µg.L^{-1} respectively.

We also determined standard phycocyanin and cellular phycocyanin apparent recoveries by spiking a commercial standard and *A. variabilis* cells on moss. The average standard apparent recoveries were 45 ± 7.6% for *P. crista-castrensis* and 69 ± 16.8% for *P. schreberi*. Average cellular apparent recoveries were lower for both moss species, we found 39 ± 3.4% for *P. crista-castrensis* and 50.8 ± 4.2% for *P. schreberi*. No significant matrix effects were observed with an average recovery of spiked C-phycocyanin standard in moss extract of 105.8 ± 10.6% for *P. crista-castrensis* and 100.7 ± 4.5% for *P. schreberi*. Phycocyanin recoveries were similar for the three spike concentrations tested. The repeatability (i.e., inter-day precision) was satisfactory with average relative standard deviations (RSD) of 12.4 and 11.6% for *P. schreberi* and *P. crista-castrensis*, respectively.

Kissoudi et al. (2018) quantified phycocyanin extracted from a cyanobacteria culture by HPLC and found greatly higher LOD and LOQ (670 and 2000 $\mu g.L^{-1}$ respectively), showing that spectrofluorometry is a more suitable quantification technique for phycocyanin. In this study, all samples (cyanobacteria cultures and mosses) had phycocyanin concentration above those limits. Phycocyanin apparent recoveries in moss found in this study are close to the average apparent recovery reported in pure cultures (50–60%, Tavanandi et al., 2018) but optimized methods can reach 90–92% (Kissoudi et al., 2018; Prates et al., 2018; Tavanandi et al., 2018). Phycocyanin concentration measured in cyanobacteria cultures depends greatly on the extraction process (e.g., solvent, extraction time, cell wall disruption technique; Abalde et al., 1998; Reis et al., 1998). A critical step for phycocyanin extraction is the cell membrane disruption step, which, when incomplete, can affect phycocyanin recovery (Stewart and Farmer, 1984). The thermal insulation properties of the moss (Bakatovich and Gaspar, 2019), for example, could prevent an efficient cyanobacteria cell wall disruption. Moreover, we found significantly lower apparent recoveries for cyanobacteria cells addition compared to phycocyanin standard addition (Tableau 2) suggesting that cyanobacteria cellular structures can impact phycocyanin extraction. Cyanobacteria cellular lyse efficiency (cellular apparent recovery/standard apparent recovery, C/B, Figure 7) achieved 86.7% for *P. crista-castrensis* and 73.6% for *P. schreberi*, which confirms that cell wall breaking was not optimal after the phycocyanin extraction procedure.

Tableau 2. Analytical performance parameters tested in this study

	Apparent recovery ± SE (%)	Matrix effects ± SE (%)	Repeatability RSD (%)	Limit of detection (µg.L^{-1})	Limit of quantification (µg.L^{-1})	Range of applicability (g of moss)
Ptilium crista-castrensis	45^{a} ± 7.6 39^{b} ± 3.4	105.8 ± 10.6	11.6	3.1	4.3	0.25-1.50
Pleurozium schreberi	69^{a} ± 16.8 50.8^{b} ± 4.2	100.7 ± 4.5	12.4			

[a]*The apparent recovery after C-phycocyanin standard spikes.*

[b]*The apparent recovery after Anabaena variabilis cells spikes on moss.*

Chittapun et al. (2020) also showed that several species of cyanobacteria had specific cell wall structures which necessitated to using different disruption techniques for each species to obtain an optimal phycocyanin efficiency. Besides the cell wall disruption efficiency, the lower apparent recoveries of cellular phycocyanin could also be explained by the presence of biofilm in cyanobacteria culture, preventing accurate cell counting and pipetting. In addition, apparent recovery might be affected by possible mechanisms of phycocyanin adsorption on moss cell walls after release from cyanobacteria cells. Phycocyanin degradation by temperature (Antelo et al., 2008) and light (Jespersen et al., 2005) over time are potential causes of extraction efficiency loss that we considered minimal in our experiments because moss samples were always kept at cool temperature and in the dark during the extraction. Repeatability reported here are also similar to values reported in literature for pure culture (Kissoudi et al., 2018; Prates et al., 2018). Thus, our results show that the efficiency of cyanobacteria cell wall disruption is the principal factor that could affect the quality of phycocyanin measurements but that, overall, phycocyanin can be accurately and reliably quantified for large cyanobacterial cell density and moss mass ranges.

2.2.4.2. Advantages and limits of the method

Phycocyanin extraction is a quick, simple, and affordable method to assess cyanobacteria quantity living on moss. This method allows estimating cyanobacteria abundance on a great number of samples in limited time and effort (approximately, the extraction procedure takes 6 h30 and the quantification takes 2 h30 for 50 samples). Because of the heterogeneity in cyanobacteria density within and between moss shoots, many leaves and/or shoots need to be processed to achieve a reasonable estimate of cyanobacteria number per shoots using microscopic counting. With phycocyanin extraction, cyanobacteria quantity can be estimated using many moss shoots (or subsamples of large amounts of homogenized shoots), allowing for an efficient integration of heterogeneity and thus for more reliable estimates of the average number of cyanobacteria. Lastly, phycocyanin extraction is also more environmentally friendly than other pigment extraction techniques because it does not require harmful organic solvents like acetone or hexane (Papadaki et al., 2017). Phycocyanin extraction in a water-based solvent also has the advantage to limit the co-extraction of other pigments poorly soluble in water, such as chlorophylls, that could interfere with phycocyanin quantification.

However, phycocyanin extraction also has some limitations. Differences in cellular phycocyanin concentration between cyanobacteria strains have been reported in pure cultures (this study, Rippka et al., 1979; Santiago-Santos et al., 2004; Chittapun et al., 2020). This can be explained by variable phycocyanin production per cell, as well as differences in extraction efficiencies due to cell wall thickness and biofilm production, differing among cyanobacteria strains (Chittapun et al., 2020). The composition of cyanobacteria communities colonizing moss can vary with environmental conditions, moss species and time of sampling during the growth season (Ininbergs et al., 2011; Rousk et al., 2013; Warshan et al., 2016). In addition to the inherent variation of phycocyanin quantity among cyanobacteria species, growth phase (McQuaid et al., 2011; Chang et al., 2012) and growth conditions (e.g., culture medium, light, temperature, and nutrient stress) has been shown to affect phycocyanin production (De Morais et al., 2018). For example, N limited availability or high photoperiod can decrease phycocyanin concentration in cyanobacteria cultures (Sloth et al., 2006; Ürek and Tarhan, 2012; Prates et al., 2018). Thus, comparison of phycocyanin data, used as cyanobacteria cell number estimates, from sites characterized by contrasted environmental conditions and from moss samples with different cyanobacteria communities should be made with caution. These limitations, due to species-specific phycocyanin cell content varying with environmental conditions, were also reported to impact the quantification of cyanobacteria using phycocyanin in aquatic ecosystems (Seppälä et al., 2007). Calibration of the method (e.g., phycocyanin linearity and apparent recovery) using strains isolated from moss species and sampling sites of interest could alleviate this potential bias.

2.2.4.3. Phycocyanin concentration and nitrogen fixation in boreal feather moss

Direct comparison of phycocyanin extraction with other cyanobacteria quantification methods is delicate and would be poorly informative since each method (e.g., microscope counting, qPCR, and echinenone extraction) has its own flaws. Thus, we decided to evaluate how phycocyanin extraction compares to other methods using an independent measurable: the BNF. Several studies suggested that in low N deposition areas, mosses control the colonization of cyanobacteria based on their N demand for growth (DeLuca et al., 2007; Gundale et al., 2011; Rousk et al., 2013). For *Pleurozium schreberi* collected in Scandinavia, BNF activity was showed to be closely related to cyanobacteria quantity measured by microscopic counting (DeLuca et al., 2007; Rousk et al., 2013; Warshan et al., 2016). Another study from Chile reported a similar linear relationship between

BNF and moss-associated cyanobacteria quantity using echinenone, a pigment produced by cyanobacteria (Arróniz-Crespo et al., 2014). Thus, assuming that phycocyanin is a reliable proxy for quantifying cyanobacteria, phycocyanin, and BNF should be correlated in moss samples collected in low N deposition (< 3 $kg.ha^{-1}.yr.^{-1}$) forest sites in Eastern Canada. We measured phycocyanin and BNF of *P. schreberi* and *P. crista-castrensis* from four sites at the beginning (June) and the end (September) of the growth season. Phycocyanin concentrations varied between 0.45–1.26 $\mu g.g^{-1}$ in June and 0.56–0.96 $\mu g.g^{-1}$ in September for *P. crista-castrensis* and 0.42–0.48 $\mu g.g^{-1}$ in June and 0.61–0.88 $\mu g.g^{-1}$ in September for *P. schreberi* (Tableau S1, Annexe 1). We found no significant effect of the sampling month on phycocyanin concentration (value of p = 0.075) but we report a significant effect of the moss species on phycocyanin content (value of p = 3.3×10^{-4}) confirming the need for testing and calibrating the phycocyanin extraction method on the chosen moss species, as discussed earlier. Phycocyanin concentrations of both moss species collected in June and September were positively correlated with BNF activity (R^2 = 0.30, Figure 10). We found a correlation coefficient relatively similar to those reported by Arróniz-Crespo et al. (2014) using echinenone quantification (R^2 = 0.44) and DeLuca et al. (2007) using microscopic counting (R^2 = 0.58).

This result suggests that phycocyanin extraction is a valuable semi-quantitative method, allowing for the rapid assessment of cyanobacteria abundance living on moss. Using phycocyanin for the quantification of cyanobacteria in moss suffers from the same major limitation as the quantification of cyanobacteria in aquatic ecosystems: cellular phycocyanin concentrations vary among species and strains. For moss, as in aquatic systems, evaluating the effects of environmental factors on the number of cyanobacteria requires the rapid analysis of large numbers of samples. Phycocyanin extraction allows such rapid and high throughput analysis in aquatic systems. While further studies on other moss species, sites, and environmental conditions, are required to validate the potential of phycocyanin quantification as a proxy of cyanobacteria quantity, our results strongly suggest that phycocyanin also represents an easy, rapid, and affordable way to evaluate moss-associated cyanobacteria quantity. Phycocyanin quantification can guide the use of more costly and time-consuming complementary methods to estimate cyanobacteria abundance (e.g., qPCR). Further work should be done on better characterizing cyanobacteria communities associated with feather

moss to improve our understanding of its impact on cyanobacteria quantification estimated with phycocyanin measurements.

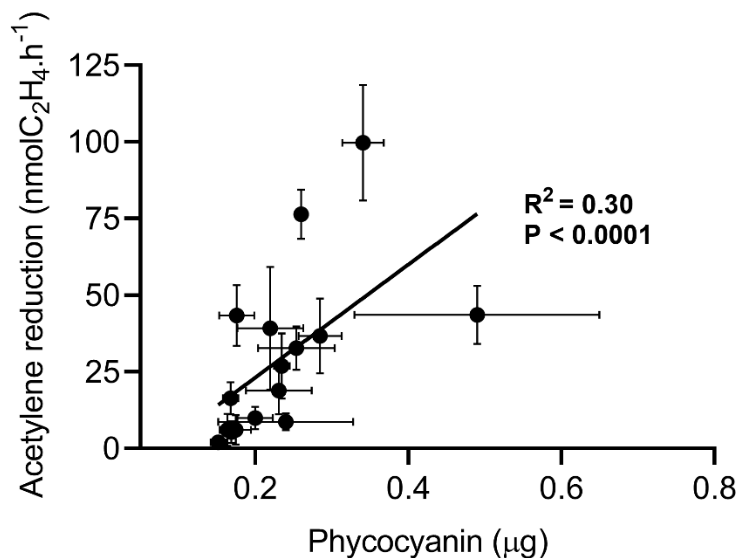

Figure 10. Linear regression between acetylene reduction mean ± SD (*n* = 4) and phycocyanin mass mean ± SD (*n* = 3) of feather moss *P. crista-castrensis* and *P. schreberi* collected in Quebec, Canada in June and September 2019.

The linear regression regroups a total of 64 observations for acetylene reduction and 48 observations for phycocyanin measurements. All samples were acclimated for 5 days in a growth chamber (18°C; 16h light, 8h dark) before being processed for acetylene reduction and phycocyanin, consecutively.

2.2.5. Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

2.2.6. Author contributions

MR, RD, and J-PB designed the study and the experiments. MR performed all the experiments and data analyses and wrote the manuscript. RD and J-PB revised the manuscript. All authors contributed to the article and approved the submitted version.

2.2.7. Funding

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2.2.8. Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.2.9. Acknowledgments

We want to thank Pierre-Olivier Danis, Marick Gagnon, Stevan Roué, and Charlotte Blasi for their help with field sampling and lab experiments. We also thank Dr. Ólafur Sigmar Andrésson for providing cyanobacteria strains used in this study.

2.2.10. Supplementary material

Voir Annexe 1.
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.611792/full#supplementary-material.

2.2.11. References

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CHAPITRE 3 : FACTEURS ENVIRONNEMENTAUX ET CLIMATIQUES CONTRÔLANT LA BIOMASSE CYANOBACTÉRIENNE ET LA FIXATION D'AZOTE ASSOCIÉES AUX MOUSSES EN FORÊT BORÉALE

3.1. Informations préliminaires

L'article présenté dans ce chapitre a été accepté et publié en ligne dans le journal *Journal of Ecology* le 31 mars 2022. Voir la référence : Renaudin, M.; Blasi, C.; Bradley, R. L.; Bellenger, J. P. New Insights into the Drivers of Moss-Associated Nitrogen Fixation and Cyanobacterial Biomass in the Eastern Canadian Boreal Forest. *Journal of Ecology*. **2022**. https://doi.org/10.1111/1365-2745.13881.

Les modifications effectuées dans ce chapitre par rapport à la version publiée sont de natures typographiques. Seule la responsabilité de l'étudiante est engagée dans les modifications effectuées.

3.1.1. Auteurs et affiliations

Marie Renaudin[1*], Charlotte Blasi[1], Robert L. Bradley[2], Jean-Philippe Bellenger[1*]

[1] Centre Sève, Département de Chimie, Université de Sherbrooke, Sherbrooke, QC, Canada

[2] Centre Sève, Département de Biologie, Université de Sherbrooke, Sherbrooke, QC, Canada

[*] Corresponding authors:

Marie Renaudin; marie.renaudin@usherbrooke.ca

Jean-Philippe Bellenger; jean-philippe.bellenger@usherbrooke.ca.

3.1.2. Participation de chaque auteur

M.R., R.L.B. et J-P.B. ont conçu cette étude et les expériences associées. M.R. a réalisé l'ensemble de l'échantillonnage, expériences et analyses de données, et a écrit le manuscrit. C.B. a contribué

à l'échantillonnage et aux mesures de FBN. R.L.B. et J-P.B. ont révisé le manuscrit. Tous les auteurs ont contribué à cet article et ont approuvé la version soumise.

3.2. Article « New insights into the drivers of moss-associated nitrogen fixation and cyanobacterial biomass in the eastern Canadian boreal forest »

3.2.1. Abstract

1. Moss-associated cyanobacteria dinitrogen (N_2-) fixation can contribute to support moss growth and constitutes a major source of new N in boreal forest ecosystems. The biomass of moss-colonizing cyanobacteria and their N_2-fixation are usually considered linearly correlated. Yet, recent evidence has shown that cyanobacterial biomass and N_2-fixation can be decoupled, suggesting that they are not necessary affected by the same environmental and ecological drivers. Climate and nutrients were reported as affecting moss-associated N_2-fixation, with equivocal results, whereas drivers of moss cyanobacterial biomass remain unclear. In addition, these drivers are often determined through manipulative experiments (e.g., fertilization, incubation) and remain to be validated with complementary observational studies to help us better understand future impacts of global change on the moss-cyanobacteria symbiosis.
2. We hypothesized that moss-associated cyanobacterial biomass is controlled *in situ* by factors affecting bacterial growth, whereas N_2-fixation is controlled by factors affecting enzymatic reactions.
3. Using random forests, Spearman correlations and linear mixed-effects models, we determined the main drivers of cyanobacterial biomass and N_2-fixation of two feather moss species, which were collected over three years along a 1000-km latitudinal transect in the eastern Canadian boreal forest.
4. We found that temperature, precipitation, and phosphorus were the main positive drivers of moss cyanobacterial biomass, and that temperature and molybdenum were the main positive drivers of N_2-fixation. Vanadium was a negative driver of N_2-fixation, suggesting the use of alternative nitrogenases by cyanobacteria.
5. Both cyanobacterial biomass and N_2-fixation were strongly influenced by the moss species and were negatively correlated with moss C:N stoichiometry, highlighting the role of N_2-fixation in moss N-enrichment.

6. Synthesis. We identified for the first time some environmental drivers of moss-associated cyanobacterial biomass and showed that they contrast with the drivers of N_2-fixation, which should be considered in further research. This is an important advance in our knowledge of moss-cyanobacteria associations, which would greatly help in better predicting the impacts of global change on this symbiosis and on nitrogen inputs in boreal forest ecosystems.

Keywords: Boreal forest, Biogeochemistry, Cyanobacteria, Ecosystem function and services, Environmental drivers, Feather moss, Nitrogen Fixation.

3.2.2. Introduction

Feather mosses are ubiquitous cryptogamic plants that can cover up to 70-100% of boreal forest floors (Oechel & Van Cleve, 1986). Feather mosses affect the functioning of boreal forest ecosystems (see review by Lindo *et al*., 2013) by controlling soil chemistry (Cornelissen et al., 2007; Stuiver et al., 2014), regulating soil moisture and temperature (Gornall et al., 2007), and storing carbon (C) (Turetsky et al., 2010). Some filamentous cyanobacteria can establish epiphytic symbioses with feather mosses (Ininbergs et al., 2011; Warshan et al., 2017), transfer nitrogen (N)-rich compounds from dinitrogen (N_2-) fixation to the moss and, therefore, contribute to support the moss' N needs (Bay et al., 2013). In addition, N_2-fixation by moss-associated cyanobacteria constitutes a major source of new N inputs in high-latitude ecosystems (DeLuca *et al*., 2002; Stewart et al., 2011). Fixed N is retained over a long period of time in moss tissue but is gradually released into forest soil when the moss decomposes (DeLuca et al., 2022). Thus, moss-cyanobacteria associations are important for the long-term N budget of boreal forests.

N that is transferred to the host moss is considered the primary cyanobacterial commodity (Stuart et al., 2020). Several authors have suggested that mosses can control cyanobacteria colonization based on their N needs by attracting cyanobacteria when N is limiting (Bay et al., 2013) and inhibiting colonization in N-rich environments (DeLuca et al., 2007; Gundale et al., 2011; Leppänen et al., 2013). A positive linear relationship between the number of cyanobacteria living on moss and N_2-fixation activity was reported for various moss species at numerous locations (DeLuca et al., 2007; Rousk et al., 2013a, 2017a; Arróniz-Crespo et al., 2014; Renaudin et al., 2021), suggesting that higher N_2-fixation is correlated with higher cyanobacterial biomass on moss.

While significant, this relationship only partially explained variability (9-50%) in N_2-fixation and is not always observed (Goth et al., 2019). In plant-cyanobacteria symbioses, about 80% of the N_2 that is fixed by cyanobacteria is transferred to the host (Meeks, 2007) and, therefore, does not contribute to cyanobacteria growth. Furthermore, Warshan *et al*. (2016) found evidence that some cyanobacteria genera that were living on moss do not contribute to N_2-fixation. For example, *Nostoc* was the most abundant genus that was associated with the moss *Pleurozium schreberi*, but it had low N_2-fixation-related gene expression compared to *Stigonema*, which was less abundant but contributed most to N_2-fixation. These findings indicate that in the moss-cyanobacteria symbiosis, some cyanobacteria could be “cheaters” by taking advantage of favourable growth conditions and C and sulphur (S) that are provided by the moss (Warshan *et al*., 2017; Stuart *et al*., 2020), without fixing N_2 in return. This implies that cyanobacterial N_2-fixation and biomass might not be necessarily always correlated and could be affected by different sets of environmental factors.

Numerous studies have investigated the effects of climatic and nutrient variables (especially N) on moss-associated N_2-fixation. However, some factors (e.g., micronutrients, tree density) remain mostly unexplored and research regarding variables affecting cyanobacterial biomass on moss is scarce. Better understanding the environmental and ecological factors controlling cyanobacterial biomass and their N_2-fixation is not only important for our comprehension of moss-cyanobacteria symbioses, but it is also essential to predict how global change will affect the moss-cyanobacteria system and, ultimately, moss growth and N input in the boreal forest ecosystem. Global change (e.g., temperature increase, extension of the growth season length; Price et al., 2013; Yeung et al., 2019) is projected to be particularly intense in high-latitude ecosystems (Post et al., 2018). Modification of the atmospheric deposition regime that is linked to the development of human activity is likely to affect nutrient inputs affecting both N_2-fixation and cyanobacterial growth. For instance, atmospheric deposition of iron (Fe), molybdenum (Mo) and vanadium (V), the metal cofactors of the nitrogenase enzyme catalyzing the N_2-fixation reaction (Bishop et al., 1980), have substantially increased since the beginning of the Industrial Revolution and are expected to continue to rise (Schlesinger et al., 2017; Hamilton et al., 2020; Wong et al., 2021).

Many studies have investigated the effects of simulated climate change on moss N_2-fixation (see review by Hupperts *et al*., 2021), but no work has been conducted to explore these effects on moss cyanobacterial biomass. Increasing temperature has been consistently shown to promote moss N_2-fixation (Smith, 1984; Gentili et al., 2005; Gundale et al., 2012a), up to an optimum of 27-30 °C above which activity starts to decline, but only when moisture was not limiting (Gundale et al., 2012a, b). This temperature optimum is cyanobacteria species-specific and is influenced by the moss identity (Gentili et al., 2005; Jean et al., 2012). Moisture levels are also critical for the activity of N_2-fixers given that water addition and moisture limitation can respectively increase or decrease N_2-fixation (Gundale et al., 2009; Jackson et al., 2011). Overall, extreme temperature and drought can lead to cellular stress and to a drastic decrease in N_2-fixation (Gundale et al., 2012b; Whiteley & Gonzalez, 2016). Similarly, warmer temperatures were shown to promote cyanobacterial biomass in lakes (Verschoor et al., 2017; Cremona et al., 2018), while reduced precipitation decreased cyanobacterial biomass in biocrusts (Fernandes et al., 2018). Thus, climatic variables (i.e., temperature, precipitation) appear to exert comparable effects on cyanobacterial biomass and N_2-fixation.

Nutrients also play an important role in regulating moss N_2-fixation in boreal environments. Nutrients are supplied to cyanobacteria mainly through atmospheric deposition, but they could also be provided by the moss. Mosses are known to bioaccumulate large quantities of P and trace metals, especially on their cell walls (Bates, 1992; Renaudin et al., 2018) and can release substantial quantities of organic nutrients through leaching (Carleton & Read, 1991). Given that N_2-fixation has a high energetic cost, the addition of bioavailable N (Zackrisson et al., 2004) or fertilization by atmospheric deposition (Ackermann et al., 2012) can suppress moss-associated diazotroph activity. It is generally affirmed that high-N availability stops N_2-fixation, but N addition experiments have revealed contrasted results. For example, artificial addition of 3 $kgN.ha^{-1}.yr^{-1}$ inhibited moss N_2-fixation (Gundale et al., 2011; Sorensen et al., 2012). In contrast, additions of 10, 12.5 and 50 $kgN.ha^{-1}.yr^{-1}$ did not affect diazotrophic activity (Ackermann, 2013; Gundale et al., 2013), while supplies of 5 $kgN.ha^{-1}.yr^{-1}$ even promoted moss N_2-fixation (Rousk & Michelsen, 2016). Thus, the response of N_2-fixation to N appears complex and is likely influenced by the local environmental conditions that are experienced by moss-associated N_2-fixers. Only a few studies tested the effect of N fertilization on both N_2-fixation and cyanobacterial biomass but N addition to moss carpets

alone (Gundale et al., 2011; Sorensen et al., 2012), or in combination with leaf litter (Alvarenga & Rousk, 2021), appeared to reduce N_2-fixation and cyanobacteria colonization in Swedish forests. This suggests that N deposition affects cyanobacterial N_2-fixation and biomass in a similar manner, possibly due to strong control that is exerted by the host over cyanobacteria populations, modulated by its N needs (Bay et al., 2013; Leppänen et al., 2013). Like N, the effect of phosphorus (P) on moss N_2-fixation is highly variable, with fertilization experiments reporting positive (Zackrisson et al., 2004; Rousk et al., 2017a), negative (Smith, 1984; Scott et al., 2018), or no effects (Zackrisson et al., 2009). The effect of P on moss-colonizing cyanobacterial biomass has been mostly underlooked. Field additions of P at low concentration were correlated with higher numbers of cyanobacteria that were counted on feather moss leaves in Canada (Rousk et al., 2017a). The high P cost of cyanobacteria metabolism could lead to a more robust control of P over cyanobacteria growth than over N_2-fixation. Although less frequently studied, the nitrogenase metal cofactors Mo, V and Fe can also influence N_2-fixation rates. Mo that was supplemented by artificial additions stimulated moss N_2-fixation (Rousk et al., 2017a), but Mo that was derived from road traffic had no effect on moss N_2-fixation (Scott et al., 2018). This suggests that Mo can be a limiting nutrient for moss-associated cyanobacterial N_2-fixation in isolated areas of the boreal forest, as observed in cyanolichens (Darnajoux et al., 2019). To our knowledge, only one study tested the effects of Mo on moss-associated cyanobacterial biomass. Field and laboratory additions of Mo had a positive effect on moss cyanobacterial numbers, but it has been suggested that Mo first promoted N_2-fixation, which ultimately increased cyanobacterial growth (Rousk et al., 2017a). Diazotrophs require Mo for different metabolic pathways, but in overwhelmingly lower quantities than for N_2-fixation (Williams & Fraústo da Silva, 2002). Moreover, a substantial portion of fixed N_2 is transferred to the host and does not contribute to cyanobacterial growth. Thus, the control of Mo over cyanobacterial growth is probably much less acute than it is over N_2-fixation. In Mo-limited boreal forests, V can support cyanobacterial N_2-fixation (Darnajoux et al., 2014, 2019), but no study has yet tested the effect of V on moss-associated N_2-fixation and cyanobacterial biomass. The role of V in cyanobacteria metabolism is less clear than Mo, but V is thought to be mostly used in enzymatic reactions such as N_2-fixation (Rehder, 2015). Thus, like Mo, V is primarily expected to control N_2-fixation rather than cyanobacterial growth. Fe is also an important nutrient for bacteria metabolism (e.g., photosynthesis, respiration). However, moss N_2-fixation displayed contrasting responses to Fe fertilization. Akther & Rousk (2019) demonstrated a positive linear

relationship between moss Fe concentrations and N_2-fixation activity on a roadside gradient. However, Goth et al. (2019) reported no correlation between railroad-derived Fe, N_2-fixation and cyanobacteria numbers on moss leaves. Conclusive evidence of Fe limitation of N_2-fixation in unmanaged ecosystems has only been documented in alkaline soils where Fe solubility and availability are very low (Marschner, 2012). In boreal forest soils, which are often acidic, Fe is unlikely to limit either N_2-fixation or bacterial growth. More generally, nutrient inputs can indirectly be correlated with tree density, through the intermediary of the canopy. Throughfall or tree litter can provide nutrients (e.g., N, P, metals) to cyanobacteria that are living on moss and promote or reduce their N_2-fixation (DeLuca et al., 2008; Rousk & Michelsen, 2017; Jean et al., 2020; Alvarenga & Rousk, 2021), but direct effects of tree density on moss N_2-fixation and cyanobacterial biomass are poorly documented.

So far, the drivers of moss-associated cyanobacterial biomass and N_2-fixation were mostly determined by manipulative experiments (e.g., fertilization, moss carpet relocation, open-top chambers) that are very effective in isolating and examining the effects of one specific variable. Yet, these experiments introduce bias, especially when testing nutrient variables. Fertilization experiments use nutrient species that are more readily available for micro-organisms, and which are less complex chemically than species that are naturally present in the environment. This leads to overestimating the effects of nutrient addition on N_2-fixation, as has been suggested for N (Rousk et al., 2013b). Using natural gradients of climate or nutrients and seasonal variability (i.e., observational study) is a complementary approach for evaluating the potential role of environmental factors on cyanobacterial growth and N_2-fixation activity and validate observations that are made in manipulative experiments. Studies on factors influencing moss-cyanobacteria association also disproportionally originate from northern Europe where forest management practices, climate and human activities contrast with those of North American boreal forests.

Therefore, the objective of this study was to determine and compare the *in situ* environmental and ecological drivers of moss-associated cyanobacterial biomass and N_2-fixation, using a climatic and nutrient gradient in the eastern Canadian boreal forest. We hypothesized that moss-associated cyanobacterial biomass and N_2-fixation have contrasted sets of drivers, the cyanobacterial biomass being controlled by factors impacting bacterial growth and N_2-fixation being controlled by factors

impacting enzymatic reactions. Based upon previously cited studies, we predicted temperature and precipitation to have similar positive effects and N deposition to have a similar negative effect on moss cyanobacterial biomass and N_2-fixation. However, we predicted nutrients to have different effects on cyanobacterial biomass and N_2-fixation. We expected P to be an important positive driver of cyanobacterial biomass, Mo, and V to be positive drivers of N_2-fixation, and Fe to have limited or no effect on either. We measured cyanobacterial biomass, using a pigment-based method that was recently validated for mosses, and N_2-fixation of two dominant feather moss species collected over three years, at the beginning and end of the growth season in the eastern Canadian boreal forest, along a 1000-km latitudinal transect constituting a natural gradient of temperature, precipitation, and nutrient deposition.

3.2.3. Materials and methods

3.2.3.1. Moss sampling

Pleurozium schreberi (Brid.) Mitt. and *Ptilium crista-castrensis* (Hedw.) De Not., two dominant moss species in the eastern Canadian boreal forest, were collected on eight sites (S1-S8) located along a latitudinal transect in Quebec, Canada, and which were sampled in June, September and October of 2017, 2018, and 2019 (Figure 11, Tableau S2, Annexe 2). The sampling sites consisted of 150 × 150 m plots, which were located at least 50 m from roads to avoid direct anthropogenic influence. All eight sites were located between 47° and 55°N and are distributed within the three larger bioclimatic zones across the eastern Canadian boreal forest: balsam fir-white birch forest (S1 and S2); black spruce-moss forest (S3-S6); spruce-lichen forest (S7 and S8). On the transect, black spruce (*Picea mariana*) and balsam fir (*Abies balsamea*) were the most abundant tree species, and the understory was dominated by feather mosses and ericaceous shrubs. On each site, whole shoots of moss were collected randomly in mixed-species or mono-species carpets. One 3.5 L plastic bag per species and site was filled with moss and kept in the dark at ~12°C until being processed, within one week following collection. For all the following analyses, only green parts (i.e., photosynthetically active) of the moss shoots were used.

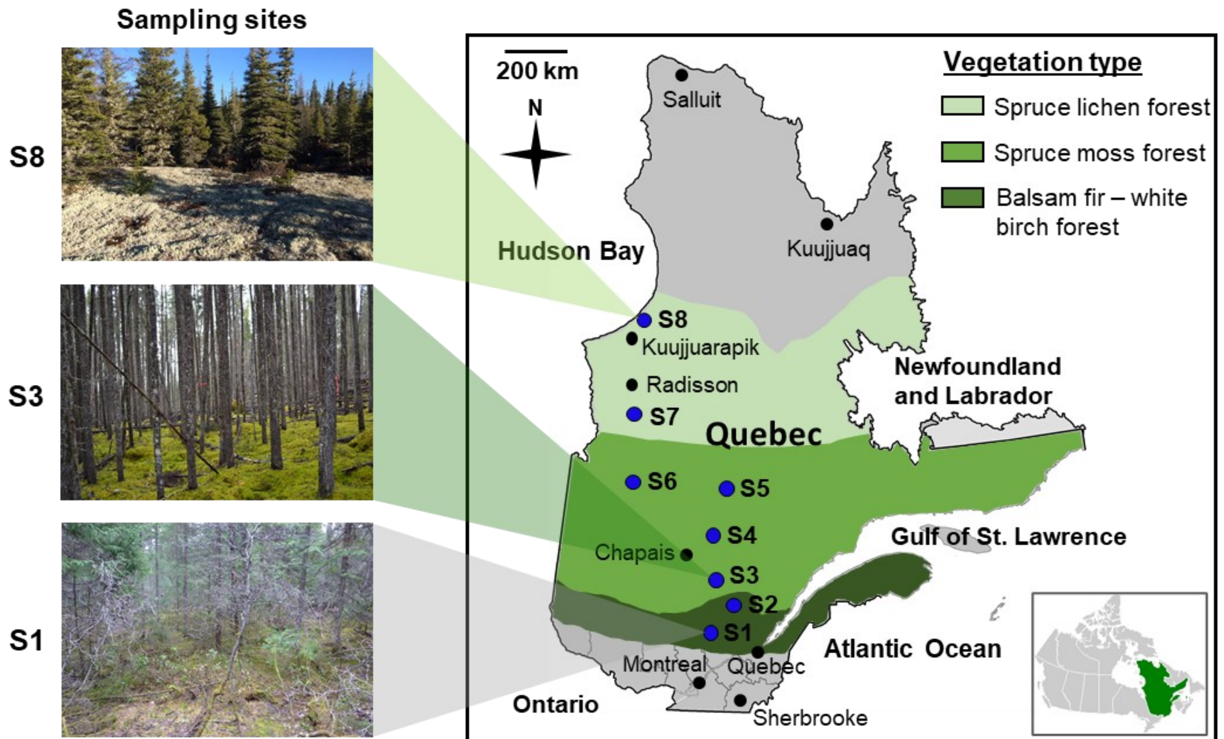

Figure 11. Map of the sampling sites that are located along a 1000-km latitudinal transect in Quebec, Canada. Feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis*, two dominant species in the eastern Canadian boreal forest, were collected in June, September, and October of 2017, 2018 and 2019.

3.2.3.2. Sampling site environmental parameters

Mean monthly temperature and precipitation data were provided by a network of meteorological stations that are managed by the Government of Quebec (MELCC, 2019). Measurements at the closest meteorological station were selected to estimate temperature and precipitation at each sampling site (Tableau S2, Annexe 2). We used mean monthly temperature and precipitation for May, August, and September when the sampling was performed in early June, September and October, respectively. Total N deposition was estimated using mean annual nitrate deposition data (which accounted for most of total N deposition) that were collected by Marty et al. (2019). Tree density was determined by counting the total number of trees (deciduous and coniferous) taller than 1 m on each sampling site.

3.2.3.3. Nitrogen fixation and cyanobacterial biomass assessment

Moss N_2-fixation was estimated using the Acetylene Reduction Assay (ARA; Hardy et al., 1968) on four replicates per species, site, and time of sampling according to Renaudin et al. (2021). Moss shoots were cleaned, and brown parts were discarded. Between 1.8 and 3 g (dry weight) of moss were sprayed with deionized water to homogenize the hydration states of the samples, placed in 250 mL glass jars, and acclimated for 3 days in a growth chamber (18 °C, 16 h light/8 h dark cycle; Biochambers SPC-56, Winnipeg, MB, Canada). Then, samples were incubated with 20% acetylene for 24 h at 18 °C and continuous light. Then, ethylene concentrations were measured with a GC-FID (Shimadzu 8A, Kyoto, Japan) equipped with a Supelco column 01282011 (Supelco Analytical, Bellefonte, PA, USA).

Following the ARA, moss samples were milled and separated in triplicates for phycocyanin extraction and subsequent cyanobacterial biomass estimation. The method for phycocyanin extraction and quantification on moss samples is described in detail by Renaudin et al. (2021). Briefly, between 0.30 and 0.70 g (dry weight) of samples were placed in sodium phosphate buffer and subjected to a series of freeze-thaw cycles, sonicated in an ultrasound bath, and centrifuged. Phycocyanin was measured using a spectrofluorometer (PTI QuantaMaster 400 Phosphorimeter, Horiba Ltd., Kyoto, Japan) at excitation and emission wavelengths of 585 nm and 643 nm, respectively.

3.2.3.4. Moss nutrient concentration analyses

Moss samples were oven-dried at 50 °C for 48 h and ground in liquid N_2. Between 30 and 35 mg of dried sample were weighed and placed in a trace metal-free tube (SCP Sciences, Baie-d'Urfé, QC, Canada) with 2 ml of nitric acid (trace metal-free grade, ThermoFisher Scientific, Waltham, MA, USA) and 200 µl of hydrogen peroxide (trace metal-free grade, MilliporeSigma, St. Louis, MO, USA). Samples were digested at room temperature for 30 min followed by 1 h at 45 °C and 2 h at 65 °C in a heating block digestion system (DigiPREP, SCP Sciences). Digested samples were diluted with Milli-Q water (MilliporeSigma) to reach a 2% acid concentration. Mo, V, Fe and P concentrations were measured by ICP-MS (X-Series II, ThermoFisher Scientific) using rhodium (Rh) as the internal standard. To determine moss C:N ratios, between 2.5 and 5 mg of dried sample

were placed into 8 × 5 mm tin capsules (Elemental Microanalysis Ltd., Okehampton, Devon, UK), which were folded following analysis facility recommendations. Total C and N concentrations were measured with an elemental analyzer (PDZ Europa ANCA GSL, Sercon Ltd., Crewe, UK) coupled with an IRMS (PDZ Europa 20-20, Sercon Ltd.) at the UC Davis Stable Isotope Facility (Davis, CA, USA). All moss nutrient analyses were carried out in triplicates and concentrations were reported as ppm (µg of element per g of moss DW).

3.2.3.5. Statistical analyses

Statistical analyses were performed with R software version 4.1.1 (R Core Team, 2021). Collinearity of climatic and nutrient variables was assessed by examining the matrix of Pearson's correlation coefficients (package *corrplot* version 0.90; Wei & Simko, 2021). Only Mo, V and Fe concentrations in moss were correlated. To further study collinearity between Mo and V in linear models, we calculated their Variance Inflation Factors (VIFs; package *car* version 3.0-11; Fox & Weisberg, 2019) with a tolerance threshold of 3 (Zuur *et al*., 2010). Residual distribution and homoscedasticity were also checked and, to approach a normal distribution of residuals, N_2-fixation and cyanobacterial biomass were log-transformed when needed.

We first performed exponential regression and Spearman correlations (package *stats* version 4.1.1; R Core Team, 2021) between all our data of log-transformed mean N_2-fixation and log-transformed mean cyanobacterial biomass to characterize the relationship they share.

To determine the drivers of moss-associated cyanobacterial biomass and N_2-fixation, we used regression type random forest models (package *randomForest* version 4.6-14; Liaw & Wiener, 2002). Random forests are robust algorithms even when data are not normally distributed or when there is some degree of multicollinearity among variables (Cutler *et al*., 2007). In the models, moss species, month and site of sampling, monthly mean temperature and precipitation, tree density, total nitrogen deposition, moss C:N ratio and Mo, V, Fe and P concentrations were used as predictors of log-transformed N_2-fixation activity and cyanobacterial biomass. We used a leave-one-out cross validation (LOOCV) method to evaluate the performance of the models (package *caret* version 6.0-90; Kuhn, 2021). We ran six iterations of each model with *ntree* = 5000 (i.e., number of trees to build in the model) and *mtry* = 6 (i.e., number of predictors randomly picked at

each split). A variable importance score was then calculated for each predictor and all the predictors were ordered based upon their importance scores.

To confirm and characterize the effects of the predictors on N_2-fixation and cyanobacterial biomass, we conducted linear regressions and Spearman correlations between log-transformed mean N_2-fixation, log-transformed mean cyanobacterial biomass, and the most important quantitative predictors that were determined by the random forest models (i.e., temperature, C:N, Mo, V and Mo:V for N_2-fixation; temperature, precipitation, C:N and P for cyanobacterial biomass). We also performed Kruskal-Wallis tests (Kruskal & Wallis, 1952) to explore any potential moss species effect on nutrient content (Mo, V, P, C, N and C:N), N_2-fixation and cyanobacterial biomass.

Finally, linear mixed-effects models (package *lme4* version 1.1-26; Bates et al., 2015) were used to test more specifically (i) the effects of the nitrogenase cofactors Mo and V on log-transformed N_2-fixation and cyanobacterial biomass, and (ii) the effects of C and N on log-transformed N_2-fixation. Fixed effects were set as log-transformed Mo and V, or C and N concentrations, while random effects were set as the period (combination of month and year) and site of sampling, to have a better control on the effects of the sampling variables on moss nutrient content. Model fit was assessed by performing the Likelihood Ratio Test (LRT; package *lmtest* version 0.9-38; Zeileis & Hothorn, 2002) on the full models (i.e., candidate models) and null models (i.e., intercept and random effects-only models). We considered the fixed effects to be relevant when the LRT output of the full model was significantly different than the LRT output of the null model. Statistical differences between LRT outputs were tested with the Chi-Squared test.

3.2.4. Results

3.2.4.1. Latitudinal transect environmental conditions and moss nutrient concentrations

The latitudinal transect used for sampling constitutes a temperature, precipitation, and atmospheric deposition gradient. Between the two extreme sites of the transect (i.e., S1 and S8), mean monthly temperature and precipitation decreased by 7.7 °C and 67.8 mm, respectively (Figure 12).

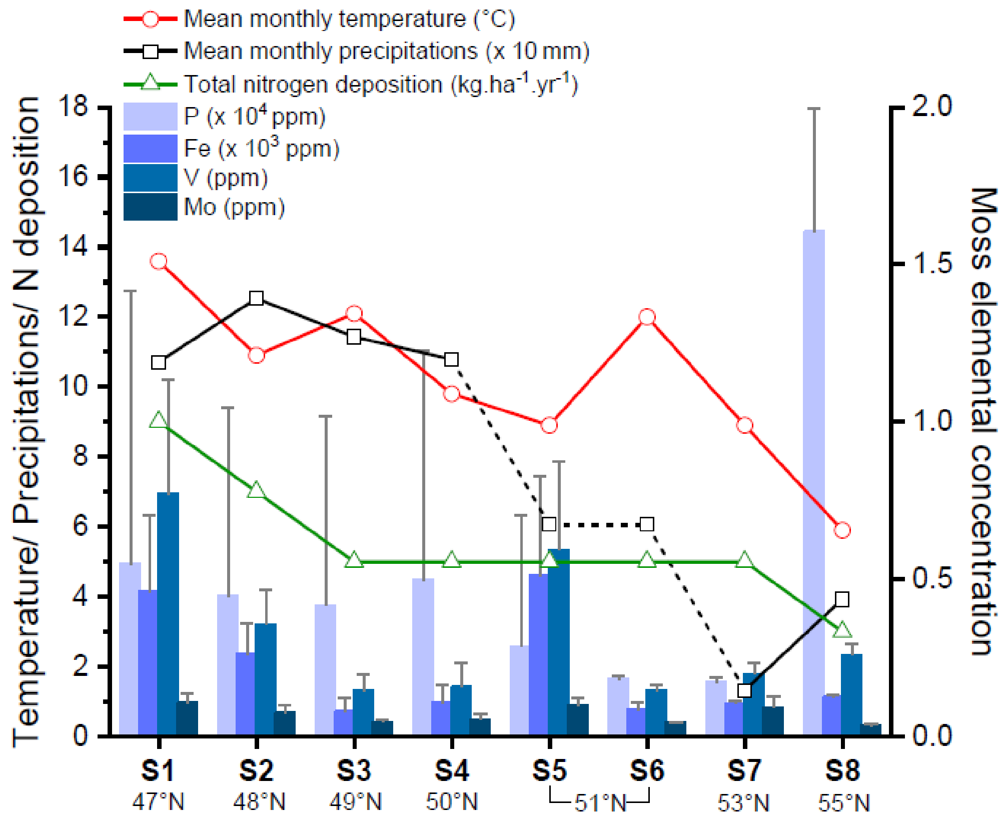

Figure 12. Climatic parameters (© Gouvernement du Québec, ministère de l'Environnement et de la Lutte contre les changements climatiques, 2019), total nitrogen atmospheric deposition and mean nutrient concentrations (*n* = 3) measured in the feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis* along a latitudinal transect in Quebec, Canada.

Each point (climatic variable) or bar plot (nutrient variable) represents the mean of all the observations made at the same site. Error bars represent standard deviation. Units in the figure were adjusted for the sake of clarity.

Temperature and precipitation increased throughout the growth season and were higher in September than in June. Mean monthly temperatures ranged between 5.4 (June 2019) and 17.2 °C (September 2017) and mean monthly precipitation ranged between 13.3 (June 2018) and 182.5 mm (September 2019; Tableau S2, Annexe 2).

Total N deposition also decreased along the transect, from 9 $kg.ha^{-1}.yr^{-1}$ at S1 to 7 $kg.ha^{-1}.yr^{-1}$ at S2 and 5-3 $kg.ha^{-1}.yr^{-1}$ at S3-S8. However, all the other nutrient concentrations in moss (i.e. P, Mo,

V, Fe) did not clearly decline with latitude, despite varying significantly between sites. For example, on average, S3 and S6 were the sites with the lowest metal concentrations, whereas S1 and S5 were the sites with the highest metal concentrations. In addition, moss P concentrations were the lowest at S7 and the highest at S8 (0.17×10^4 and 1.60×10^4 ppm, respectively).

3.2.4.2. Determination of the environmental drivers of moss-associated nitrogen fixation and cyanobacterial biomass

Combining all of the data that were collected on the latitudinal transect, we found a significant positive exponential relationship between moss-associated N_2-fixation and cyanobacterial biomass ($\rho = 0.60$, $P = 0.0005$; Figure 13).

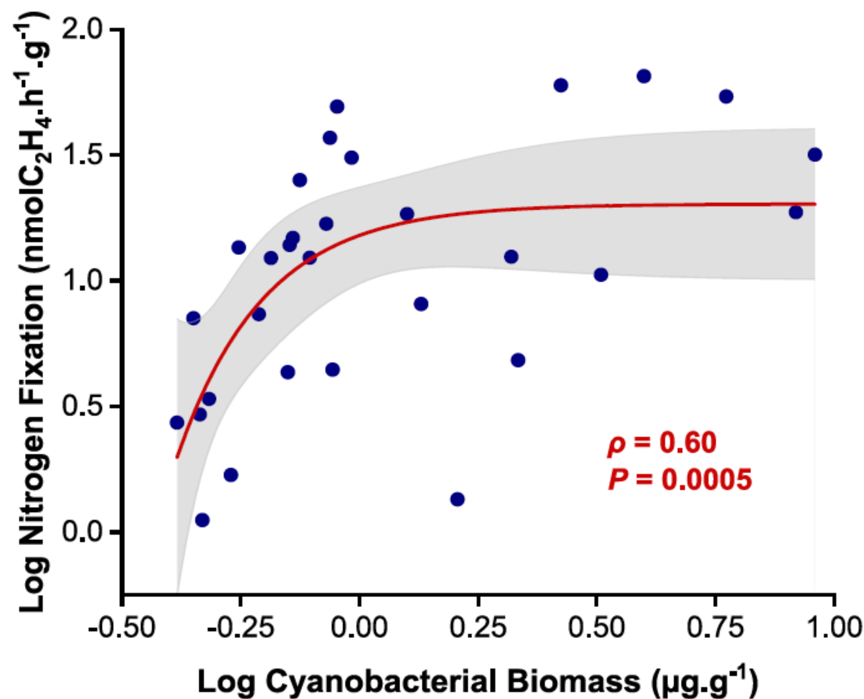

Figure 13. Spearman correlation between log-transformed mean nitrogen fixation (*n* = 4) and log-transformed mean cyanobacterial biomass (*n* = 3) that was measured in feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis* that were collected along a latitudinal transect in Quebec, Canada.

Blue dots indicate observations (30 in total) and the solid red line indicates the best-fitting exponential regression. The grey zone represents the 95 % confidence interval.

LOOCV of the random forest models showed that overall error (root mean square and mean absolute errors) was < 1 for N_2-fixation and < 0.5 for cyanobacterial biomass, while the associated R^2 values were 0.34 and 0.80, respectively. In the first random forest model (Figure 14a), all the predictors collectively accounted for 39% of the N_2-fixation variability. Moss species was the best predictor (highest variable importance score) of N_2-fixation, followed by sampling month. Temperature was the best climatic predictor of N_2-fixation, while the most important nutrient variables were, in order, V, C:N and Mo. P and Fe concentrations, N deposition, tree density and precipitation had importance scores close to or less than 0 (Figure 14a), indicating that these factors did not influence N_2-fixation activity.

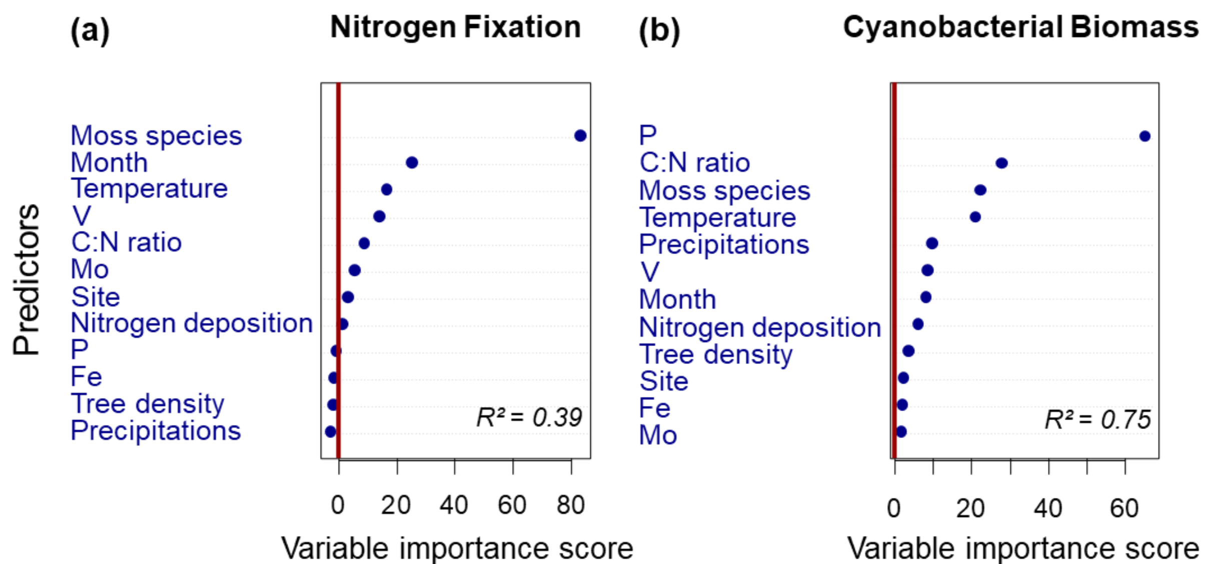

Figure 14. Predictive variable importance plots of log-transformed nitrogen fixation (a) and log-transformed total moss-associated cyanobacterial biomass (b) measured in *Ptilium crista-castrensis* and *Pleurozium schreberi* collected on a latitudinal transect in Quebec, Canada. Variable importance scores were calculated by combining 6 iterations of each regression random forest model. Each model regroups a total of 66 observations for nitrogen fixation and 30 observations for cyanobacterial biomass. The number of variables randomly sampled as candidates at each split was set as 6 and the total number of trees grown was set as 5000. The red vertical line was placed at a variable importance score equal to 0 to help visually separate predictors which have an impact on response variables (variable importance score above 0) and predictors which do not (variable importance score below 0).

However, all variables that were included in the second model apparently affected cyanobacterial biomass colonizing moss (variable importance scores > 0; Figure 14b) and collectively, accounted for 75% of the variability. P concentration was the best predictor of cyanobacterial biomass with a variable importance score exceeding 60 and, as for N_2-fixation, temperature was the best climatic predictor. The most important nutrient predictors, besides P, were C:N followed by V. Cyanobacterial biomass was also influenced by mean monthly precipitation and moss species identity. Tree density, the sampling site and Fe and Mo concentrations had low variable importance scores and did not appear to be important drivers of moss-associated cyanobacterial biomass.

Using Spearman correlations (Figure 15), we found that temperature was positively correlated with N_2-fixation ($\rho = 0.30$, $P = 0.014$) and cyanobacterial biomass ($\rho = 0.59$, $P = 5.31 \times 10^{-4}$), but that moss C:N ratio was negatively correlated with the two response variables ($\rho = -0.36$, $P = 0.003$ for N_2-fixation; $\rho = -0.54$, $P = 0.002$ for biomass). V and Mo concentrations were negatively correlated ($\rho = -0.23$, $P = 0.056$; Figure S2, Annexe 2) and positively correlated ($\rho = 0.18$, $P = 0.141$; Figure S2, Annexe 2) with N_2-fixation, respectively. However, both correlations were not statistically significant and only reflected a tendency. In contrast, there was a significant positive correlation between Mo:V and N_2-fixation ($\rho = 0.46$, $P = 0.0001$; Figure 15b). Precipitation and P were strongly positively correlated with moss-associated cyanobacterial biomass ($\rho = 0.47$, $P = 0.021$ for precipitation; $\rho = 0.77$, $P = 2.38 \times 10^{-6}$ for P). However, the linear regression between cyanobacterial biomass and P was driven by two groups of samples (Figure 15g). The groups corresponded to data from different time points. In September 2018, both P and phycocyanin concentrations were higher, which could be explained by a warmer summer (Tableau S2, Annexe 2).

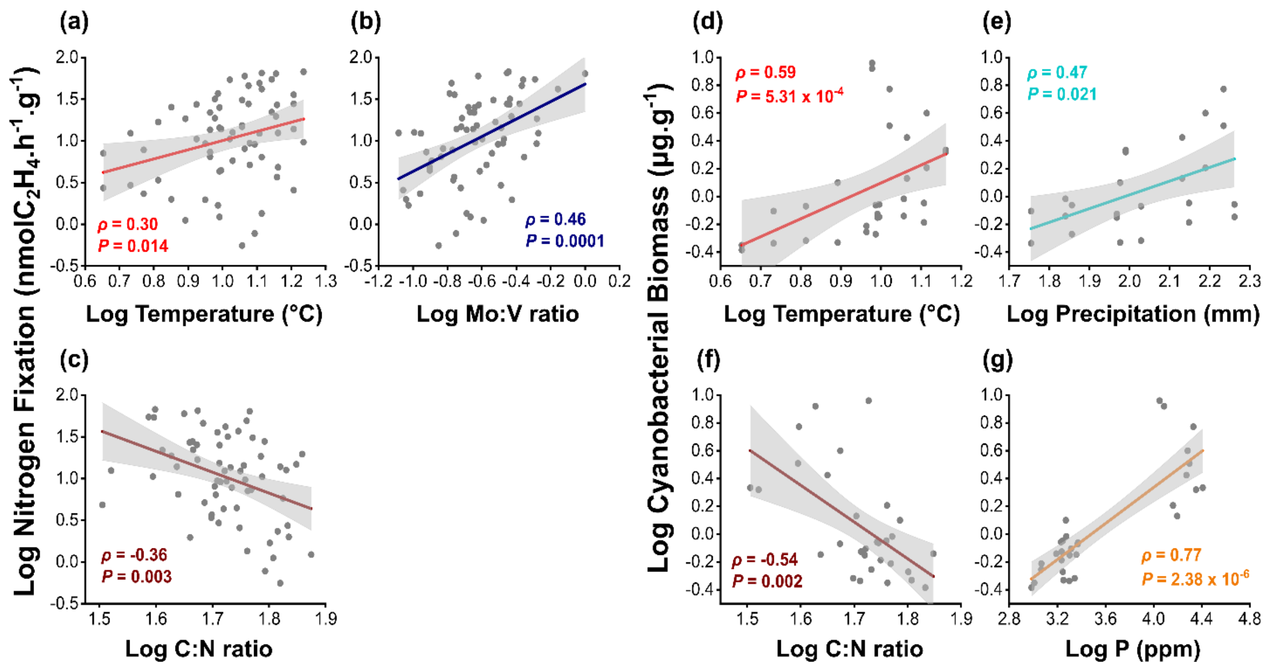

Figure 15. Spearman correlations between log-transformed mean nitrogen fixation (*n* = 4), measured in feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis*, and temperature (a), molybdenum:vanadium (Mo:V) ratio (b), carbon:nitrogen (C:N) ratio (c); and log-transformed mean cyanobacterial biomass (*n* = 3) associated with the same feather mosses and temperature (d), precipitation (e), carbon:nitrogen (C:N) ratio (f) and phosphorus (P) (g).

Grey dots indicate observations (66 for nitrogen fixation and 30 for cyanobacterial biomass) and solid lines indicate best-fitting linear regressions. Grey zones represent 95 % confidence intervals.

Given this two-group linear regression and because moss species was a major driver of cyanobacterial biomass and N_2-fixation (Figure 14), we determined whether there was a potential moss species effect on nutrient concentrations, potentially creating bias in our statistical analyses and interpretation. Kruskal-Wallis tests revealed a clear difference in ARA rates between the two moss species. On the latitudinal transect, *Ptilium crista-castrensis* N_2-fixation was higher than *Pleurozium schreberi*'s on average ($\chi^2 = 23.31$, $P = 1.38 \times 10^{-6}$; Tableau 3). This strong effect of the moss species on N_2-fixation was confirmed by the random forest analysis (Figure 14a). We also showed significant differences in Mo ($\chi^2 = 5.12$, $P = 0.023$), C ($\chi^2 = 11.77$, $P = 0.0006$), N ($\chi^2 = 5.36$, $P = 0.021$) concentrations and C:N ratio ($\chi^2 = 6.42$, $P = 0.011$) between the two mosses.

Tableau 3. Nutrient concentrations (*n* = 33) consisting of molybdenum (Mo), vanadium (V), phosphorus (P), carbon (C), nitrogen (N) and C:N ratio; acetylene reduction (AR; used to estimate nitrogen fixation; *n* = 33) and phycocyanin concentrations (PC; used to estimate moss-associated cyanobacterial biomass; *n* = 15) that were measured in the feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis,* collected along a latitudinal transect in Quebec, Canada in 2017, 2018 and 2019. The moss species effect on the eight variables was assessed using the Kruskal-Wallis test.

	Mo ± SE (ppm)	**V ± SE (ppm)**	**P ± SE (ppm)**	**C ± SE ($mg.g^{-1}$)**	**N ± SE ($mg.g^{-1}$)**	**C:N ± SE**	**AR ± SE ($nmolC_2H_4.h^{-1}.g^{-1}$)**	**PC ± SE (ppm)**
P. schreberi	0.0648 ± 0.0048	0.4189 ± 0.0582	4252.9 ± 1044.5	438.28 ± 10.70	7.98 ± 1.51	56.72 ± 1.76	8.10 ± 1.20	1.50 ± 0.40
P. crista-castrensis	0.0841 ± 0.0146	0.3534 ± 0.0615	4923 ± 856.9	428.82 ± 11.94	8.70 ± 1.32	50.39 ± 8.77	28.46 ± 4.96	2.13 ± 0.55
χ^2	*5.12*	*0.90*	*1.90*	*11.77*	*5.36*	*6.42*	*23.31*	*2.29*
-	-	-	-	-	-	-	-	-
***P*[a]**	***0.023 ****	***0.342 ns***	***0.168 ns***	***0.0006 ******	***0.021 ****	***0.011 ****	***1.38 × 10^-6 ******	***0.130 ns***

*[a] *, P< 0.05; **, P< 0.01; ***, P<0.001; ns, not significant.*

However, there were no significant differences for the other nutrients and for cyanobacterial biomass. Thus, there is a potential interaction between the moss species and Mo, C and N content, which could bias statistical analyses of N_2-fixation if not considered.

As Mo and V relationships with N_2-fixation were unclear (Figure S2, Annexe 2), and both variables were found as relatively important predictors of N_2-fixation and cyanobacterial biomass (Figure 14), we more closely studied the effects of these nitrogenase cofactors on cyanobacterial biomass and N_2-fixation by using linear mixed-effects models, which controlled for the effects of time and location of sampling (Tableau 4). We also performed a similar linear mixed-effects model using C and N as fixed effects (Tableau S3, Annexe 2). VIFs of Mo and V were below three and, thus, we considered this moderate collinearity acceptable for the model. For the three linear mixed-effects models, LRTs showed that full models were significantly different from null models (models in Table 2: $\chi^2 = 26.37$, $P = 1.87 \times 10^{-6}$ for N_2-fixation and $\chi^2 = 8.48$, $P = 0.014$ for cyanobacterial biomass; model in Table S2: $\chi^2 = 19.58$, $P = 5.60 \times 10^{-5}$). This means that full models fit the data better and were, therefore, used for further analysis. The first model explained 46% of the variation in N_2-fixation and 82% of the variation in cyanobacterial biomass, with fixed effects accounting for 32% and 7% of the explained variance, respectively. Mo was positively correlated with N_2-fixation ($t = 5.44$, $P = 1.23 \times 10^{-6}$) and cyanobacterial biomass ($t = 3.48$, $P = 0.002$) but V was negatively correlated with N_2-fixation ($t = -5.50$, $P = 2.57 \times 10^{-5}$) and biomass ($t = -3.68$, $P = 0.001$). This confirmed the positive relationship between N_2-fixation and Mo:V (Figure 15b). The second model explained 28% of the variation in N_2-fixation, with the fixed effects accounting for 16% of the explained variance. We found a negative correlation between C and N_2-fixation ($t = -2.44$, $P = 0.019$), but a positive correlation between N concentration and N_2-fixation ($t = 2.04$, $P = 0.048$). This model confirmed the negative correlation between N_2-fixation and C:N (Figure 15c).

Tableau 4. Linear mixed-effects models using nitrogenase cofactors molybdenum (Mo) and vanadium (V) as predictors of log-transformed (a) nitrogen fixation and (b) moss-associated cyanobacterial biomass.

Each model regroups a total of 66 observations for nitrogen fixation and 30 observations for cyanobacterial biomass. Mo and V concentrations were measured in the feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis* that were collected along a latitudinal transect in Quebec, Canada in 2017, 2018 and 2019. Mo and V concentrations were set as fixed effects whereas the period (combination of month and year) and site of sampling were set as random effects.

	(a) Nitrogen fixation[c]				
		Estimate[a]	SE	*t*	***P***[b]
Predictive variables	**Mo**	2.14	0.39	*5.44*	***1.23 × 10⁻⁶ ******
	V	-1.31	0.24	*-5.50*	***2.57 × 10⁻⁵ ******
	(b) Cyanobacterial biomass[c]				
		Estimate[a]	SE	*t*	***P***[b]
Predictive variables	**Mo**	0.97	0.28	*3.48*	***0.002 *****
	V	-0.60	0.16	*-3.68*	***0.001 *****

[a]*The estimate represents the slope of the relationship between the response and the predictive variables (i.e., fixed effects).*

[b]**, P< 0.05; **, P< 0.01; ***, P<0.001; ns, not significant.*

[c]*The models respectively explain 46% and 82% of the variation in nitrogen fixation (a) and in cyanobacterial biomass (b).*

3.2.5. Discussion

3.2.5.1. Moss identity is a major driver of cyanobacterial biomass and nitrogen fixation

Over the three years of sampling, *Ptilium crista-castrensis* had significantly higher N_2-fixation rates than did *Pleurozium schreberi* (Tableau 3), explaining why moss species was an important driver of N_2-fixation in our study. Similar differences in N_2-fixation between moss species have been previously reported (Leppänen et al., 2013, 2015; Darnajoux et al., 2018) and, in a recent study including 34 moss species from Alaska, Stuart et *al.* (2021) confirmed that the moss family was a major driver of N_2-fixation. This apparent widespread relationship between moss identity and N_2-fixation could be explained by (i) a moss species-specific number of colonizing cyanobacteria, supported by the fact that moss species was a major driver of cyanobacterial biomass in our study, or by (ii) a moss species-specific microbiome, comprising cyanobacteria with different N_2-fixation rates.

In our study, we reported a significant positive exponential relationship between moss-associated N_2-fixation and cyanobacterial biomass (Figure 13). Several studies demonstrated a positive linear relationship between N_2-fixation and the quantity of cyanobacteria on moss in Sweden (Rousk et al., 2013a; DeLuca *et al*., 2007), Chile (Arróniz-Crespo et al., 2014) and Canada (Rousk et al., 2017a; Renaudin et al., 2021), confirming that N_2-fixation and cyanobacterial biomass are linked. It has been hypothesized that this relationship is the result of the moss controlling the number of colonizing cyanobacteria as a function of its N demand (Bay et al., 2013; Leppänen et al., 2013). Moss N demand is likely to be species-specific and depends on the moss growth rate and N supplies. Several studies have reported that moss species had different growth rates (Zechmeister, 1998; Pouliot et al., 2010). In contrast, N supply depends on the ability of moss to scavenge N from different sources, such as atmospheric deposition, soil, and N_2 that is fixed by diazotrophs (Ayres et al., 2006; Liu et al., 2013). Studies comparing the efficiency of different moss species in using various sources of N are limited, but they indicate that N acquisition strategies vary between species (Ayres et al., 2006; Song et al., 2016). Thus, the influence of moss identity on N_2-fixation could result from different N acquisition strategies among moss species. Some moss species, in our case *P. crista-castrensis*, could rely more on N_2-fixation to meet their needs, and would allow a higher level of cyanobacteria colonization. However, we showed that cyanobacterial biomass explained a

significant proportion, but not all variability in N_2-fixation (ρ = 0.60, P = 0.0005, Figure 13), suggesting that factors other than moss N demand can affect moss cyanobacterial biomass.

Warshan et al. (2016) showed that some cyanobacteria genera, despite being abundant on feather mosses, do not contribute substantially to N_2-fixation and vice versa, and that their contributions to N_2-fixation fluctuate over the growth season. This could explain the relatively weak relationship between N_2-fixation and cyanobacterial biomass. Moss species have distinct morphological and physiological features, such as number of leaves per shoot, density of shoots in the carpet, specific surface area and water retention capacity (Elumeeva et al., 2011; Jonsson et al., 2015). These functional traits could lead to host-specific micro-habitat conditions and affect light and water availability to cyanobacteria colonies on moss leaves. Recently, Liu and Rousk (2021) showed that the moss hydration rate, which was related to species morphology, was a key trait controlling N_2-fixation and cyanobacterial colonization on feather moss leaves. The link between moss identity and N_2-fixation could also result from the structure of the moss microbiome. Indeed, the composition of cyanobacteria communities is known to vary among moss species (Ininbergs et al., 2011; Warshan et al., 2016; Holland-Moritz et al., 2018, 2021) and cyanobacterial species have different N_2-fixation rates (Gentili et al., 2005). Further work is needed to explore global and diazotrophic moss microbiomes and their relationships with moss N demand, micro-habitat, and N_2-fixation.

3.2.5.2. Climatic and site-specific predictors of cyanobacterial biomass and nitrogen fixation

Temperature was the best climatic predictor of both moss-colonizing cyanobacterial biomass and N_2-fixation (Figure 14). We found that higher mean monthly temperatures were correlated with higher cyanobacterial biomass and higher N_2-fixation rates. The month of sampling was the second highest predictor of N_2-fixation and appeared to be relatively important for cyanobacterial biomass. This may be caused by the strong correlation between the month of sampling and temperature, especially because we collected mosses at the beginning and the end of the growing season, which was characterized by a 4 °C difference on average. While we are not aware of any study testing the effect of temperature on total moss-associated cyanobacterial biomass, but temperature has been shown to be positively correlated with cyanobacterial biomass in lakes (Cremona et al., 2018) and in cultures (Thomas & Litchman, 2016). Moreover, temperature can affect the composition of the

moss microbiome and the relative abundance of cyanobacteria. Recently, a two-year warming experiment reported a substantial increase in *Nostoc* relative abundance on *Sphagnum* mosses (Carrell *et al*., 2019). In contrast, moderate warming of 1.4 °C for 20 years led to a decrease of *Nostoc* relative abundance that was associated with the moss *Racomitrium* sp. in Iceland (Klarenberg *et al*., 2021). The effect of temperature elevation on moss-associated cyanobacterial relative abundance may thus depend on the severity and duration of warming, as well as the location and moss species being considered. In similar warming experiments, a positive effect of temperature on feather moss N_2-fixation was observed in the Swedish boreal forest (Gundale et al., 2012a) and in arctic tundra (Rousk & Michelsen, 2017). The average temperature optima for cyanobacterial growth and terrestrial N_2-fixation are respectively 25-35 °C (Paerl, 2014) and ~ 25 °C (Houlton et al., 2008). Moreover, moss N_2-fixation was shown to positively respond to temperatures increasing up to 22 °C, followed by being inhibited at ~ 30 °C (Gundale et al., 2012a). In the eastern Canadian boreal forest, where average monthly temperatures are low (< 20 °C, Figure 12), moss cyanobacterial biomass and N_2-fixation, therefore, are unlikely to be negatively affected by long-term high summer temperatures. Even with global warming, monthly average temperature is not expected to rise above 20-25 °C during the boreal growth season in boreal regions, suggesting that warming could stimulate cyanobacterial growth and N_2-fixation activity. However, in the field, temperature and moisture cannot be dissociated from one another (Rousk et al., 2017b): and the stimulation of cyanobacterial growth and N_2-fixation by warmer temperatures could be counteracted by moisture limitation.

Mean monthly precipitation was a highly ranked predictor of cyanobacterial biomass (Figure 14b), validating the importance of water availability for bacterial growth. Precipitation also contributes to nutrient inputs (e.g., Ca, K, Mn, P), especially through canopy leaching during throughfall (Gauslaa et al., 2021), which could favour moss-associated cyanobacterial growth. However, precipitation had no significant effect on N_2-fixation (Figure 14a). Similarly, Dynarski et al. (2019) reported no significant effect of precipitation on N_2-fixation in litter but other studies showed that reduced precipitation had negative effects on N_2-fixation in moss (Gundale et al., 2009, 2012b; Whiteley & Gonzalez, 2016; Zheng et al., 2020). The impact of drought on moss N_2-fixation likely depends on the intensity, the duration, and the frequency of the events (Gundale et al., 2012b). Our study only included moss that was collected during wet periods (i.e., early and late growing season)

and it is not excluded that precipitation would have had a greater effect on N_2-fixation if we had included samples that were collected during the drier period (i.e., July-August). Overall, our findings are consistent with the general observed pattern that higher temperature and moisture conditions promote N_2-fixer activity and biomass (Reed et al., 2011). Precipitation events are predicted to be more intense, but less frequent in eastern Canada (Ouranos, 2015), which might reduce cyanobacterial growth and N_2-fixation. However, some regions of the eastern Canadian boreal forest experience high precipitation rates and could be less affected by drought periods in the future (D'Orangeville et al., 2016).

Despite a significant increase in standing tree numbers from S1 to S5 (Tableau S2, Annexe 2), tree density was a poor predictor of cyanobacterial biomass and N_2-fixation. Tree density is linked to key drivers of moss N_2-fixation, such as light availability (Gentili et al., 2005) and nutrient inputs through litter or throughfall (DeLuca et al., 2008; Jean et *al.*, 2020). Thus, other variables, such as canopy openness, light or throughfall composition, which are more directly related to N_2-fixation, could be better predictors of moss N_2-fixation and cyanobacterial biomass.

3.2.5.3. The nutrients driving moss-associated cyanobacterial biomass and nitrogen fixation

Random forest analyses revealed that N deposition had a very low impact on moss-associated cyanobacterial biomass and N_2-fixation (Figure 14). It is worth noting that N deposition experienced on our study sites (3-9 $kg.ha^{-1}.yr^{-1}$) was high relative to boreal locations at higher latitudes (e.g., 0.8-2 $kg.ha^{-1}.yr^{-1}$ in Finland; Leppänen *et al*., 2013). Therefore, the effect of N deposition on N_2-fixation could be more pronounced at these locations. Field additions on mosses that were as low as 3 $kgN.ha^{-1}.yr^{-1}$ inhibited N_2-fixation and decreased cyanobacterial numbers on leaves (Gundale et al., 2011; Sorensen et al., 2012). Recently, Alvarenga and Rousk (2021) suggested that the deleterious effect of N fertilization on moss-associated cyanobacterial abundance could be due to acidification of the moss by ammonium additions. With a lower pH, the moss surface would become a less favourable habitat for cyanobacterial colonization or growth. Yet, we did not observe a decrease in N_2-fixation or cyanobacterial biomass when N deposition were higher in the southern part of the transect (7-9 $kg.ha^{-1}.yr^{-1}$). The discrepancy between our findings (observational study) and those from field additions (manipulative study) could be due to the form of N and how it is supplied to mosses. Fertilization experiments often apply readily

available inorganic forms of N (e.g., nitrate, ammonium) in pulses, whereas atmospheric depositions have compositions that are more complex, and contain both organic and inorganic forms of N. Moreover, the composition and concentration of N deposition is strongly altered by processes occurring in the tree canopy prior to reaching the moss carpet (Houle et al., 2015). Therefore, there is a significant difference between available N concentrations in simulated and actual N deposition, potentially contributing to an over-estimation of N deposition effects by fertilization experiments (Rousk et al., 2013b). Nevertheless, our data suggest that the inhibition of moss N_2-fixation by N deposition could be less acute than what has been reported in manipulative experiments. In the eastern Canadian boreal forest, where N deposition is low and not expected to increase drastically in subsequent decades (Houle et al., 2015; Gilliam et al., 2019), it is likely that N_2-fixation will not be significantly inhibited by N deposition. On the contrary, moderate increase in atmospheric N deposition could even be beneficial to N_2-fixation, by alleviating N-limitation of nitrogenase enzyme production (Reed et al., 2011). This is supported by our data where moss N_2-fixation was even higher at the site that was exposed to the highest levels of N deposition (S1; Tableau S2, Annexe 2), and by Rousk & Michelsen (2016), who demonstrated that low supplies of N (5 $kg.ha^{-1}.yr^{-1}$) can promote moss N_2-fixation.

Unlike N deposition, we identified that moss C:N ratio was an important predictor of cyanobacterial biomass and N_2-fixation (Figure 14). N_2-fixation was negatively correlated with C and positively correlated with N concentration in moss (Tableau S3, Annexe 2), which led to a negative correlation between N_2-fixation and C:N (Figure 15c). This finding contradicts those of Leppänen et al. (2013) and Salemaa et al. (2019), who showed a positive relationship between N_2-fixation and C:N in feather mosses that were collected along a N deposition gradient in Finland. We suggest that these discrepancies result from differences in N deposition regimes that are coupled with contrasting forest management practices between eastern Canadian and northern European boreal forests. Compared to eastern Canada, northern European forests are generally more densely populated, with greater industrial and agricultural activities and are more intensively managed (which impacts tree density and canopy coverage). All of these dissimilarities potentially lead to higher N deposition levels (Dentener et al., 2006) and lower relative contributions of N_2-fixation to the moss N budget in northern European forests compared to eastern Canadian forests. Under high atmospheric deposition conditions (e.g., northern Europe), moss N concentrations

increase and N_2-fixation decreases, resulting in a positive relationship between N_2-fixation and C:N. Indeed, ARA rates were significantly lower in Finland mosses than in our study and N_2-fixation was inhibited at the sites where N deposition was higher (Leppänen et al., 2013; Salemaa et al., 2019). Under very low N deposition regimes, as is the case in eastern Canada, N_2-fixation constitutes a major contributor to the moss N budget and leads to an enrichment of the moss host and to a lower C:N ratio.

Our data indicated that P was a major driver of cyanobacterial biomass (Figure 14b). We also determined that warmer summer temperatures could lead to higher moss P content and higher cyanobacterial biomass (Figure 15g). Temperature elevation could favour moss activity (e.g., photosynthesis) and growth (Street *et al*., 2018; Thiemer *et al*., 2018), which could lead to an increase in moss P requirements and, therefore, to higher P uptake by the moss tissue. This is also supported by studies suggesting translocation of P from old to young moss segments to support their growth (Lang *et al*., 2014; Thiemer *et al*., 2018). However, in our study, P did not seem to affect N_2-fixation (Figure 14a). This is consistent with two recent meta-analyses which regrouped fertilization experiment results and reported the lack of impact of P on boreal moss N_2-fixation, suggesting that N_2-fixation was not limited by P in boreal ecosystems (Dynarski & Houlton, 2018; Zheng et al., 2019). Cyanobacteria could mine P-rich compounds at the surface of the moss leaves by using extracellular phosphatase enzymes (Houlton et al., 2008) and thus avoiding P shortages. Mosses could also actively transfer P to the cyanobacteria, as has been already demonstrated for C and S (Stuart et al., 2020).

We showed that Mo concentrations in moss and N_2-fixation were significantly positively correlated (Figure 14a; Tableau 4). Several studies have reported that Mo can limit N_2-fixation in various matrices as soil, leaf litter and lichens, across tropical, temperate and boreal biomes (Barron et al., 2009; Wurzburger et al., 2012; Jean et al., 2013; Darnajoux et al., 2019). Fertilization experiments (Rousk et al., 2017a; Rousk & Rousk, 2020) and meta-analyses (Dynarski and Houlton, 2018; Zheng et al., 2019) have also shown Mo limitation of N_2-fixation in boreal and arctic mosses. Reed et al. (2013) found that N_2-fixation was Mo-limited when Mo concentrations in leaf litter were below 200 ppb. More recently, similar Mo thresholds were reported in boreal cyanolichens (Darnajoux et al., 2019) and feather mosses (Darnajoux et al., unpublished data). Along our

transect, average Mo concentrations were 65 ppb for *Pleurozium schreberi* and 84 ppb for *Ptilium crista-castrensis* (Tableau 3). This shows that Mo depositions are generally low in pristine high-latitude boreal forests and that Mo limitation of N_2-fixation is likely widespread in boreal feather mosses. Mo deposition is expected to increase globally with human activities (Wong et al., 2021), which could possibly alleviate the Mo limitation and promote moss N_2-fixation. Ma et al. (2019) found that Mo addition increased cyanobacteria relative abundance in paddy soils, and Rousk et al. (2017a) observed higher cyanobacteria colonization of moss that had been supplemented with Mo. However, our results did not indicate that Mo was an important predictor of cyanobacterial biomass. Because Mo concentration was correlated with higher N_2-fixation rates, this suggests that Mo initially promotes N_2-fixation, which can subsequently favour cyanobacterial growth (Rousk et al., 2017a).

For the first time, we report a significant relationship between V concentration and N_2-fixation in moss (Figure 14a; Tableau 4). To our knowledge, only one other study had shown a correlation between V and N_2-fixation in tropical tree litter and phyllosphere (Moreira et al., 2021). V is a co-factor of the alternative V-nitrogenase (-Nase) enzyme that has been discovered in the genome of many N_2-fixers in tropical, temperate and boreal ecosystems (Betancourt et al., 2008; McRose et al., 2017; Villarreal et al., 2021), including cyanobacteria associated with mosses (Nelson et al., 2019). Given that V is much more abundant than Mo in the environment, including in feather mosses (Tableau 3), the V-Nase enzyme can support N_2-fixation, which represents an advantage for diazotrophs living in Mo-limited ecosystems. For example, the V-Nase was shown to contribute between 15 and 50% of the N_2-fixation when Mo was limiting in boreal cyanolichens (Darnajoux et al., 2019). In our study, the negative relationship between V and N_2-fixation (Tableau 4), as well as the positive relationship between Mo:V and N_2-fixation (Figure 15b), can be explained by the relative efficiencies of the nitrogenase isoforms to reduce ethylene. At 18 °C (i.e., the ARA incubation temperature), the V-Nase is three-fold less efficient than Mo-Nase in reducing acetylene (Eady, 2003). Therefore, a greater contribution of the V-Nase to N_2-fixation would translate into lower ARA rates. This result strongly suggests that moss-associated cyanobacteria use the V-Nase and that V availability could potentially regulate cyanobacterial N_2-fixation.

In our study, Fe was not an important predictor of either N_2-fixation or cyanobacterial biomass. In terrestrial ecosystems, Fe-limitation usually occurs in alkaline soils and was only reported in intensive agricultural systems (Zuo et al., 2004) and in a karst tropical forest (Winbourne et al., 2017). This indicates that Fe-limitation in terrestrial ecosystems mostly emerges in intensive production systems or in soils where Fe availability is naturally low, and is unlikely to appear, at a large scale, in boreal forests. This was confirmed by studies showing no effect, thus assuming no limitation, of railroad- and roadside-derived Fe on feather moss N_2-fixation in the Swedish boreal forest (Scott et al., 2018; Goth et al., 2019).

In conclusion, according to our hypothesis, we found that feather moss-associated cyanobacterial biomass and N_2-fixation had contrasting drivers in the eastern Canadian boreal forest (see Figure 16).

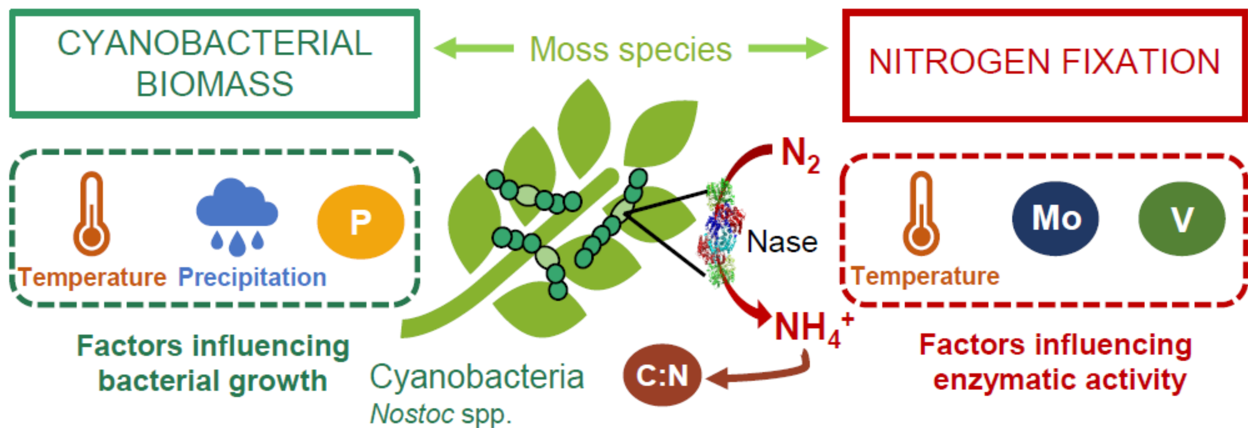

Figure 16. Summary of the main environmental and ecological drivers of moss-associated cyanobacterial biomass and their nitrogen fixation, determined using feather moss samples collected on a latitudinal transect across the eastern Canadian boreal forest.

Filamentous cyanobacteria colonizing moss can fix nitrogen (N) with the nitrogenase enzymes ("Nase") in specialized cells called heterocysts (light green). The ammonium produced by nitrogen fixation can lead to moss N enrichment and to lower C:N ratio (brown). Nitrogen fixation (red) is positively influenced by the temperature and the nitrogenase cofactor molybdenum (Mo) but negatively influenced by vanadium (V). The cyanobacterial biomass associated with moss (green) is positively influenced by temperature, precipitation, and phosphorus (P).

This modifies the common assumption that N_2-fixation and cyanobacterial biomass are directly linked and that low N_2-fixation rates always indicate low cyanobacterial biomass on moss. For example, P could be beneficial for cyanobacterial growth without promoting their ability to fix N_2. Given that cyanobacterial biomass and N_2-fixation are influenced by different environmental factors strongly suggests that they will not respond similarly to global change. The development of human activities at higher latitudes and the modification of fire regimes will lead to increased nutrient deposition in the boreal forest (Price et al., 2013; Yeung et al., 2019), potentially affecting moss-associated cyanobacteria. Further work focusing on the influence of environmental conditions on the moss diazotrophic microbiome, including non-cyanobacterial N_2-fixers, is required to better understand the consequences of global change on N_2-fixation.

3.2.6. Acknowledgements

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3.2.7. Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2.8. Authors' contributions

MR, RLB and J-PB designed the study and the experiments. MR performed all of the sampling, experiments and data analyses, and wrote the manuscript. CB contributed to sample collection and to nitrogen fixation measurements. RLB and J-PB revised the manuscript. All authors contributed to this article and approved the final submitted version.

3.2.9. Data availability statement

The data that support the findings of this study are available in the supplementary material of this article (Annexe 2) and have been deposited on Figshare https://doi.org/10.6084/m9.figshare.19127780.v1 (Renaudin, 2022). The authors are fully responsible for climatic data (mean monthly temperature and precipitation) used and displayed in this article, which were provided by the MELCC (Government of Quebec).

3.2.10. Supporting information

Voir Annexe 2.
En raison de la taille des données, le Tableau S2 a été déposé sur Figshare et sera disponible après publication avec le lien suivant : https://doi.org/10.6084/m9.figshare.19127780.v1.

3.2.11. References

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CHAPITRE 4 : CARACTÉRISATION ET INFLUENCE DE L'ENVIRONNEMENT SUR LE BACTÉRIOME GLOBAL ET DIAZOTROPHIQUE DES MOUSSES EN FORÊT BORÉALE

4.1. Informations préliminaires

L'article présenté dans ce chapitre a été accepté et publié en ligne dans le journal *Science of the Total Environment* le 6 mai 2022. Voir la référence : Renaudin, M.; Laforest-Lapointe, I.; Bellenger, J. P. Unraveling Global and Diazotrophic Bacteriomes of Boreal Forest Floor Feather Mosses and their Environmental Drivers at the Ecosystem and at the Plant Scale in North America. *Science of the Total Environment*. **2022**. https://doi.org/10.1016/j.scitotenv.2022.155761.

Les modifications effectuées dans ce chapitre par rapport à la version publiée sont de natures typographiques. Seule la responsabilité de l'étudiante est engagée dans les modifications effectuées.

4.1.1. Auteurs et affiliations

Marie Renaudin[1*], Isabelle Laforest-Lapointe[2], Jean-Philippe Bellenger[1*]
[1] Centre Sève, Département de Chimie, Université de Sherbrooke, Sherbrooke, QC, Canada
[2] Centre Sève, Département de Biologie, Université de Sherbrooke, Sherbrooke, QC, Canada
[*] Corresponding authors:
Marie Renaudin; marie.renaudin@usherbrooke.ca
Jean-Philippe Bellenger; jean-philippe.bellenger@usherbrooke.ca.

4.1.2. Participation de chaque auteur

M.R. et J-P.B. ont conçu cette étude et les expériences associées. M.R. a réalisé l'ensemble de l'échantillonnage, expériences, analyses de données, et a écrit le manuscrit. I.L-L. a contribué aux analyses bio-informatiques et statistiques des données de séquençage. J-P.B. et I.L-L. ont révisé le manuscrit. Tous les auteurs ont contribué à cet article et ont approuvé la version soumise.

4.2. Article « Unraveling global and diazotrophic bacteriomes of boreal forest floor feather mosses and their environmental drivers at the ecosystem and at the plant scale in North America »

4.2.1. Highlights

- Feather moss bacteriome was studied on a climate and nutrient deposition gradient.
- We performed cDNA amplicon sequencing on 16S rRNA and *nifH* genes.
- Cyanobacteria are major contributors to nitrogen fixation in feather mosses.
- Bacteria occupy ecological niches linked to their functions along the moss shoot.
- Climate and nutrients shape the feather moss bacteriome composition and structure.

4.2.2. Abstract

Feather mosses are abundant cryptogams of the boreal forest floor and shelter a broad diversity of bacteria who have important ecological functions (e.g., decomposition, nutrient cycling). In particular, nitrogen (N_2-) fixation performed by feather moss-associated diazotrophs constitutes an important entry of nitrogen in the boreal forest ecosystem. However, the composition of the feather moss bacteriome and its environmental drivers are still unclear. Using cDNA amplicon sequencing of the 16S rRNA and *nifH* genes and cyanobacterial biomass quantification, we explored the active global and diazotrophic bacterial communities of two dominant feather moss species (i) at the ecosystem scale, along a 500-km climatic and nutrient deposition gradient in the North American boreal forest, and (ii) at the plant scale, along the moss shoot senescence gradient. We found that cyanobacteria were major actors of the feather moss bacteriome, accounting for 33% of global bacterial communities and 65% of diazotrophic communities, and that several cyanobacterial and methanotrophic genera were contributing to N_2-fixation. Moreover, we showed that bacteria were occupying ecological niches along the moss shoot, with phototrophs being dominant in the apical part and methanotrophs being dominant in the basal part. Finally, climate (temperature, precipitation), environmental variables (moss species, month, tree density) and nutrients (nitrogen, phosphorus, molybdenum, vanadium, iron) strongly shaped global and diazotrophic bacteriomes. In summary, this work presents evidence that the feather moss bacteriome plays crucial roles in supporting moss growth, health, and decomposition, as well as in the boreal forest carbon and

nitrogen cycles. This study also highlights the substantial effects of climate and nutrients on the feather moss bacteriome, suggesting the importance of understanding the impacts of global change on moss-associated bacterial growth and activity.

Keywords: Boreal forest floor; Feather moss; Moss-bacteria interactions; Diazotrophic microbiome; Cyanobacteria; Nitrogen fixation.

4.2.3. Introduction

Feather mosses are ubiquitous cryptogams that have important ecological and biogeochemical functions in the boreal forest (e.g., buffering soil temperature and moisture, influencing nutrient availability; Gornall et al., 2007; Turetsky et al., 2010; Slate et al., 2019), including hosting bacteria playing key roles in nutrient cycles, such as methane oxidation (Kostka et al., 2016), organic matter decomposition (Turetsky, 2003), denitrification (Wicaksono et al., 2021), and nitrogen (N_2-) fixation (DeLuca et al., 2002). However, to date, the nature and diversity of feather moss-associated bacteria remain unclear. Thus, studying the moss bacteriome is critical (i) to improve our understanding of microbial processes and nutrient cycling in boreal forest ecosystems and (ii) to assess how they will potentially affect and be affected by global change.

Cyanobacteria can establish symbioses with feather mosses and support their growth by providing them with ammonium through N_2-fixation (Bay et al., 2013). At a larger scale, moss-associated cyanobacterial N_2-fixation constitutes an important input of N in boreal forests (0.1-7 $kgN.ha^{-1}.yr^{-1}$; DeLuca et al., 2007, 2002; Zackrisson et al., 2009) as fixed N is gradually released in soil when the moss is decomposing (DeLuca et al., 2022) or is leached during drying-rewetting cycles (Slate et al., 2019). These diazotrophic associations have been observed in dominant boreal feather moss species (e.g., *Pleurozium schreberi*, *Ptilium crista-castrensis*, *Hylocomium splendens*) in Alaska, Canada, and Northern Europe (DeLuca et al., 2002; Houle et al., 2006; Holland-Moritz et al., 2021). Moreover, a recent study on 34 moss species showed that N_2-fixation was a widespread trait of the moss bacteriome in high-latitude habitats (Stuart et al., 2021). However, despite efforts in characterizing moss-associated cyanobacteria communities, much remains to be understood about the entire feather moss diazotrophic bacteriome. Until now, most studies that explored the feather moss diazotrophic bacteriome relied on fluorescence microscopic observations (DeLuca et al.,

2002; Houle et al., 2006; Jean et al., 2012) or culture-dependant experiments (Gentili et al., 2005; Ininbergs et al., 2011), which are valuable but limited methods as they cannot fully capture the composition and diversity of bacterial communities in environmental samples. Furthermore, the small number of studies on feather moss-associated bacteria that used sequencing or quantifying techniques (i.e., amplicon sequencing, qPCR) targeted 16S rRNA or *nifH* genes in environmental samples DNA (Ininbergs et al., 2011; Leppänen et al., 2013; Arróniz-Crespo et al., 2014; Warshan et al., 2016; Holland-Moritz et al., 2021, 2018). These procedures allow us to draw a general picture of moss-associated bacterial communities but include dormant or dead individuals and give little information about metabolically active bacteria, including N_2-fixers. Alternatively, RNA-based techniques have been efficiently used to give information on putatively active bacteria (i.e., bacteria potentially able to synthesize proteins; Blazewicz et al., 2013) and could thus be used to expand our knowledge of moss microbiome ecological and biogeochemical functions (Ma et al., 2017; Tian and Li, 2017).

Two studies in Northern Europe have attempted to identify the diazotrophs colonizing feather mosses by sequencing *nifH* gene in DNA (Ininbergs et al., 2011; Leppänen et al., 2013) but, to our knowledge, no data is available for the North American boreal forest. North European and North American boreal forests are submitted to contrasted climates, atmospheric deposition regimes, and management policies (Boonstra et al., 2016) . These differences in environmental conditions could lead to contrasted moss bacteriomes between the two regions, supporting the need of bringing new information about the feather moss bacterial communities in the North American boreal forest. Cyanobacteria are often considered the main contributors to moss N_2-fixation. For example, Leppänen et al. (2013) found that 96% of *nifH* sequenced amplicons isolated from Finnish feather mosses were cyanobacterial. Yet, recent studies brought evidence that other bacteria (e.g., *Alphaproteobacteria*, especially *Methylocystaceae*, *Bradyrhizobiaceae*, and *Acetobacteraceae* families) are potential N_2-fixers associated with feather and *Sphagnum* mosses (Bragina et al., 2015; Holland-Moritz et al., 2021).

Despite still being largely unknown, the feather moss bacterial communities appear to be moss species-specific (Holland-Moritz et al., 2018, 2021) and to vary with location and time (Warshan et al., 2016). Climatic conditions and nutrients are known to impact moss-associated cyanobacterial

N_2-fixation and abundance (Rousk et al., 2017; Stuart et al., 2021). Temperature and precipitation, as well as, molybdenum (Mo), vanadium (V), iron (Fe) (i.e., metal cofactors of the nitrogenase enzymes catalyzing N_2-fixation), and P, were reported as drivers of N_2-fixation or cyanobacterial biomass in the eastern Canadian boreal forest (Renaudin et al., 2022). N availability also strongly regulates N_2-fixation and moss colonization by cyanobacteria as they are both promoted in N-poor environments but become inhibited when N availability increases (Zackrisson et al., 2004; Bay et al., 2013; Salemaa et al., 2019; Alvarenga and Rousk, 2021). However, the influence of climate and nutrients on moss bacterial communities is still unclear and has rarely been addressed. Recent studies reported that temperature impacts bacteria living on feather (Jean et al., 2020; Holland-Moritz et al., 2021) and *Sphagnum* mosses (Carrell et al., 2019; Tveit et al., 2020). In addition, trees can influence light and nutrient inputs by the intermediate of leaf litter and canopy coverage, and, therefore, shape the feather moss bacteriome (Jean et al., 2020; Holland-Moritz et al., 2021; Rodríguez-Rodríguez et al., 2022).

At the plant scale, little is known about the distribution of bacteria along the moss shoot. The moss shoot constitutes a senescence gradient with the aerial green apical part being metabolically active and the belowground brown basal part being decomposing dead tissues. Moreover, nutrient concentrations vary along the moss shoot and are more reflective of the atmospheric deposition in the apical part but are closer to the composition of the surrounding soil in the basal part (Leblond et al., 2004). Therefore, this range of micro-conditions on the shoot could create specific niches for bacteria which growth and activity are expected to change importantly along the moss senescence gradient. Accordingly, cyanobacteria were reported to colonize mostly the apical part (Reksten, 2014; Chen et al., 2019) whereas alphaproteobacteria were shown to colonize preferentially the basal part of the shoot (Park et al., 2013). Studying the distribution of bacterial communities along the shoot would allow to better link moss metabolism, bacterial processes, and micro-habitat. The moss shoot also represents a valuable micro-scale system to examine the effects of several variables, such as nutrients, on microbial communities (i.e., discarding the influence of other major *in situ* parameters like precipitation or canopy).

The objectives of this study were (i) to characterize metabolically active global and diazotrophic bacterial communities of dominant feather mosses in the North American boreal forest, (ii) to

identify the main bacteria contributing to N_2-fixation, and (iii) to assess the influence of major climatic (temperature, precipitation), environmental (tree density), and nutrient (N deposition, P, Mo, V, Fe) factors on these bacterial communities. Based on previous studies, we hypothesized that global and diazotrophic feather moss bacterial communities would be highly moss-species specific. We also hypothesized that, because we focused on active bacteria, the feather moss bacteriome would strongly shift during the growth season, across the sampling sites and along the moss shoot, as they would be influenced by the environment. Finally, we hypothesized that the active diazotrophic bacteriome would comprise, along with cyanobacteria, less recognized N_2-fixers from other phyla (e.g., *Proteobacteria*).

To reach these objectives, we collected two feather moss species, *Pleurozium schreberi* and *Ptilium crista-castrensis*, along a latitudinal transect in Quebec, Canada, at the beginning and at the end of the growth season (Figure 17). We explored their metabolically active bacterial communities by isolating total RNA and performing high-throughput cDNA sequencing of 16S rRNA and *nifH* amplicons on whole moss shoots and shoot sections.

4.2.4. Material and methods

4.2.4.1. Moss sampling

The two dominant feather moss species *Pleurozium schreberi* (Brid.) Mitt. and *Ptilium crista-castrensis* (Hedw.) De Not. were collected on five sites (S1-S5), consisting of 150 × 150 m plots, located along a latitudinal transect between 47 and 51° N in the boreal forest of the province of Quebec, Canada (Figure 17). This latitudinal transect constitutes a 500-km gradient of temperature, precipitation, and atmospheric deposition (Tableau S4, Annexe 3). Vegetation was dominated by black spruce (*Picea mariana*) and balsam fir (*Abies balsamea*) tree stands and by feather mosses and ericaceous shrubs in the understory. Green apical parts of moss shoots were collected on the five sites in June and September 2017, as well as in October 2017 at S1, S3 and S4. Sections of 0.5 cm were cut with razor blades on the first 6 cm of *P. crista-castrensis* shoots collected in September 2019 at S3. Samples for RNA extraction were cleaned from foreign plant material, placed in sterile RNase-free cryogenic tubes, flash-frozen in liquid N_2 on site within 20 min after collection, and stored at -80 °C until being processed. Samples for N_2-fixation, cyanobacterial biomass and

nutrient measurements were placed in plastic bags in a cooler and stored in the dark at 4 °C until being processed. In total, 38 moss samples were collected for this study. Each sample consisted of a composite sample of moss shoots collected randomly, in mixed- or mono-species carpets, within the square plots. After collection, all samples were homogenized and divided into three or four replicated subsamples for subsequent analyses, resulting in 114 observations.

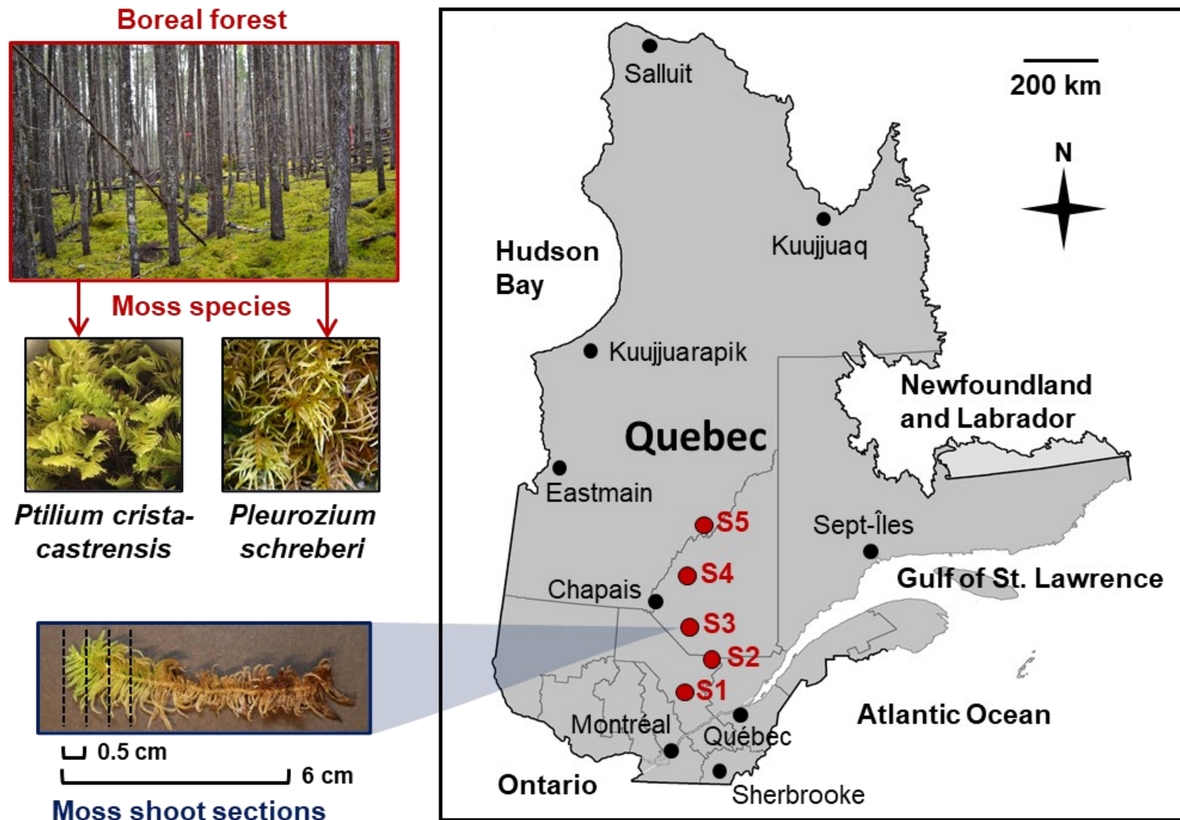


Figure 17. Location of the sampling sites along a latitudinal gradient in Quebec, Canada. Feather mosses *Ptilium crista-castrensis* and *Pleurozium schreberi* were collected in June, September, and October 2017. Sections of 0.5 cm were performed on *P. crista-castrensis* shoots collected on S3 in September 2019.

4.2.4.2. Moss-associated nitrogen fixation, cyanobacterial biomass, and environmental parameters

N_2-fixation and cyanobacterial biomass were both assessed following procedures described in detail in Renaudin et al. (2021). N_2-fixation (n = 4) was estimated by the Acetylene Reduction

Assay (ARA, Hardy et al., 1968). In short, 1.8-3 g (dry mass) of moss apical green parts were placed in 250 ml glass jars and 0.08-0.12 g (dry mass) of 0.5-cm sections of *P. crista-castrensis* shoots were placed in 23 ml glass vials. All samples were incubated with 20% of acetylene for 24 h at 18 °C and continuous light. Then, ethylene concentrations were measured using a GC-FID (Shimadzu 8A, Kyoto, Japan).

Following the ARA, cyanobacterial biomass ($n = 3$) was estimated using a method recently validated for mosses, the phycocyanin pigment extraction (Renaudin et al., 2021). Briefly, whole moss apical parts or shoot sections were ground in a blender and 0.25-0.70 g (dry mass) of samples were placed in 50 ml sterile tubes containing 15 ml of sodium phosphate buffer (MilliporeSigma, St. Louis, MO, 238 USA). Then, samples were submitted to two freeze-thaw cycles and sonicated to break the cyanobacteria cellular membranes. Finally, samples were centrifuged and the extracted phycocyanin was measured using a spectrofluorometer (PTI QuantaMaster 400 230 Phosphorimeter, Horiba Ltd., Kyoto, Japan) at excitation and emission wavelengths of 585 nm and 643 nm, respectively.

Data collection of temperature, precipitation, N deposition, tree density, and nutrient (Mo, V, Fe, P) measurements is also described in detail in Renaudin et al. (2022). Mean monthly temperature and precipitation were supplied by meteorological stations managed by the Government of Quebec (MELCC, 2019; Table S1). For each site, total N deposition was estimated using mean annual nitrate deposition measured by Marty et al. (2019) and tree density was determined by counting the total number of trees taller than 1 m.

For nutrient quantification ($n = 3$), moss samples were oven-dried for 48 h and milled with a mortar and pestle in liquid N_2. Then, 30-35 mg (dry mass) of samples were digested in 3 ml of HNO_3 (trace-metal free grade, ThermoFisher Scientific, Waltham, MA, USA) and 200 µl of H_2O_2 (trace-metal free grade, MilliporeSigma) for 3 h. Mo, V, Fe and P concentrations were determined by ICP-MS (X-Series II, ThermoFisher Scientific) using rhodium as the internal standard.

4.2.4.3. RNA extraction and 16S rRNA and *nifH* amplicon sequencing

Flash-frozen moss samples were ground with a mortar and pestle in liquid N_2, and total RNA was extracted (n = 3) using the NucleoSpin type D steel bead tubes and the NucleoSpin RNA Plant and Fungi kit, following the manufacturer's protocol (Macherey-Nagel, Düren, Germany). Residual DNA was digested during RNA extraction with the rDNase Set (Macherey-Nagel). For all samples, quality control of the extracted RNA (concentration and purity) was checked with a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). RNA was reverse-transcripted into cDNA with the LunaScript SuperMix kit (New England BioLabs Inc., Ipswich, MA, USA) and the cDNA product was cleaned with the QIAquick PCR Purification kit (Qiagen, Hilden, Germany). Then, 16S rRNA (V4-V5 region) and *nifH* genes were amplified in triplicates by PCR with the primer pairs 515F/806R (Caporaso et al., 2012) and Ueda19F/R6 (Ueda et al., 1995; Marusina et al., 2001) leading to amplicons of 253 bps and 394 bps, respectively. We chose the primer pair 515F/806R as it was adopted and extensively used by the Earth Microbiome Project (Gilbert et al., 2010) and the pair Ueda19F/R6 as it was reported to accurately capture the composition and diversity of diazotrophic communities in a large range of environmental samples (Angel et al., 2018). Negative controls were performed for each batch of RNA extraction, reverse transcription, and PCR amplification. PCRs, library preparation, and amplicon sequencing were performed by Genome Quebec in Montreal, Canada. Amplicons were sequenced on the Illumina MiSeq 250 pbs paired-ended platform (Illumina, San Diego, CA, USA).

Raw sequences were processed with the R software version 4.1.1. (R Core Team, 2021) following the DADA2 pipeline (package *dada2* version 1.16.; Callahan et al., 2016). All reads were filtered, trimmed, and truncated at 240 bps. The maximum of expected error allowed per read was set as 2. Pair-ended reads were merged and an ASV (Amplicon Sequence Variant) table discarding singletons was constructed. ASVs indicate sequence variation by tracking single-nucleotide changes and have, therefore, a higher resolution and accuracy than OTUs (Callahan et al., 2017). Chimeric sequences (7.3% of 16S rRNA sequences and 26.5% of *nifH* sequences) were removed and taxonomy was assigned to the ASV table using the data bases Silva version 138 (Quast et al., 2013) for 16S rRNA sequences and nifHdada2 version 1.1.0. (Moynihan, 2020) for *nifH* sequences. Finally, chloroplastic and eukaryotic sequences were removed and only sequences assigned to the Bacteria and Archaea kingdoms were kept for further analysis.

4.2.4.4. Statistical analyses

All statistical analyses were performed with R version 4.1.1. and figures were built using the packages *ggplot2* version 3.3.5. (Wickham, 2016) and *ggbiplot* version 0.55. (Vu, 2011). If needed, data normality was checked with the Shapiro-Wilk test. For all the tests and models ran in this study, each observation represents the mean of a set of three or four replicated subsamples and differences were considered statistically significant for *P* values below 0.05.

To identify changes in bacterial relative abundance, we performed ASV differential abundance analysis between moss species, sites (S1-S5), month of sampling and moss shoot sections (1-6 cm), with the package *DESeq2* version 1.32.0. (Love et al., 2014).

Prior to bacterial alpha-diversity analysis, ASVs were rarefied to the minimum number of reads per sample in both latitudinal transect and moss shoot sections datasets. Alpha-diversity was estimated with the richness index Shannon and Chao1, calculated using the *phyloseq* package version 1.36.0. (McMurdie and Holmes, 2013). To explore sampling site, month, and moss species effects on bacterial alpha-diversity, and to investigate the variation of N_2-fixation, cyanobacterial biomass, and nutrients along the moss shoot, we used Kruskal-Wallis tests followed by the Dunn's post-hoc tests with the package *FSA* version 0.9.1. (Ogle et al., 2021) for non-normally distributed data and ANOVAs followed by Tukey's HSD post-hoc tests for normally distributed data. All *P* values were adjusted for multiple pairwise comparison with the Benjamini-Hochberg method when using the Dunn's test and with the Tukey method when using the Tukey's HSD test.

To determine which bacterial genera were contributing to N_2-fixation across the transect, we ran regression type random forest models (package *randomForest* version 4.6-14; Liaw and Wiener, 2002) in which the log-transformed relative abundances of dominant bacterial genera were used as predictors of log-transformed N_2-fixation. We ran 6 iterations of each model with *ntree* = 2000 (i.e., number of trees to build in the model), and *mtry* = 2 (i.e., number of predictors randomly picked at each split) for global bacterial communities and *mtry* = 3 for diazotrophic communities. Then, bacterial relative abundances were ordered based on their importance scores and we used Spearman's correlations to characterize the relationship between bacterial relative abundance and N_2-fixation (both log-transformed).

We assessed the effects of major environmental variables (i.e., moss species, month, temperature, precipitation, N deposition, Mo, V, Fe, P) along the latitudinal transect and the effects of nutrients (i.e., Mo, V, Fe, P) along the moss shoot, on the composition of moss-associated bacterial communities by normalizing data counts using variance stabilizing transformation (Anders and Huber, 2010) and subsequently calculating Bray-Curtis dissimilarities (sampling site was set as the experimental design formula) using the packages *phyloseq*, *DESeq2* and *vegan* version 2.5-7 (Oksanen et al., 2020). Then, using the *vegan* package, we performed PERMANOVAs on Bray-Curtis dissimilarities (999 permutations). Finally, to further study the relationships between environmental variables, N_2-fixation, and the relative abundance of the dominant bacteria in feather mosses, we carried out Principal Component Analyses (PCAs) and built biplot graphs with the package *ggbiplot.*

4.2.5. Results

4.2.5.1. Feather moss bacterial communities' structure across a latitudinal transect

Combining all the moss samples sequenced in our study, we obtained a total of 1,532,491 sequences and 7806 unique ASVs from 16S rRNA amplicons and 2,493,692 sequences and 1836 unique ASVs from *nifH* amplicons. On average, we recovered 13,443 16S rRNA sequences and 22,068 *nifH* sequences per sample. The ASVs associated with 16S rRNA sequences were assigned to 39 phyla and 290 genera. The more relatively abundant bacterial phyla were: *Cyanobacteria* (33%), *Proteobacteria* (27%), *Actinobacteriota* (10%), *Planctomyceta* (9%), *Acidobacteriota* (7%), and *WPS-2* (5%). The dominant genus was the cyanobacteria *Nostoc* PCC-73102 (i.e., *Nostoc punctiforme*), accounting for a third of the bacterial communities. We identified a total of 12 cyanobacteria genera, the most abundant, after *Nostoc* PCC-73102*,* being *Stigonema* SAG 48.90 (i.e., *Stigonema ocellatum*), *Nostoc* PCC-7107, and *Microcoleus* SAG 1449-1a (i.e., *Microcoleus paludosus*) which represented respectively 5%, 1% and 0.97% of the bacterial communities.

In contrast, ASVs associated with *nifH* sequences were assigned to two phyla only: *Cyanobacteria* (65%) and *Proteobacteria* (35%). *Cyanobacteria*-assigned genera were, in order of relative abundances, *Nostoc* (accounting for almost half of bacterial communities), *Hassallia*, *Scytonema*, *Stigonema*, *Brasilonema*, *Fischerella*, *Calothrix*, *Anabaena*, *Cronbergia*, and *Iningainema*. We

found two genera of *Proteobacteria*, *Methyloferula* and *Methylocapsa*, which represented 28% and 12% of bacterial communities, respectively.

Moss species identity had a significant effect on the bacteriome structure, explaining 2.3% of the global and 2.7% of the diazotrophic bacterial community variation (Tableau 5). *Ptilium crista-castrensis* and *Pleurozium schreberi* harbored the same top 20 (16S rRNA) and top six (*nifH*) dominant genera (Figure 18) but differed in their bacterial community composition for scarce genera. Indeed, *Brasilonema*, *Cronbergia*, *Fischerella*, and *Iningainema* were detected in *nifH* sequences from *P. crista-castrensis* but not from *P. schreberi*. In addition, acidobacteriota and cyanobacteria (*Nostoc* PCC-73102 and *Stigonema* SAG 48.90) ASV relative abundance were significantly higher for *P. crista-castrensis* than for *P. schreberi* (Figure S7, Annexe 3). On the contrary, proteobacteria *Rhizobacter* and *Rhodopila* (assigned with 16S rRNA sequences) and *Methylocapsa* (assigned with *nifH* sequences) ASV relative abundance were higher for *P. schreberi* than for *P. crista-castrensis*.

The bacteriome of the two feather mosses also varied along the latitudinal transect (Figure 18). For the global bacterial community (Figure S8a, Annexe 3), ASV relative abundance of *Microcoleus* SAG 1449-1a and *Nostoc* PCC-7107 were higher at the most northern site (S5) than at the most southern site of the transect (S1). However, ASV relative abundance of *Stigonema* SAG 48.90 was higher at S1 than at S5, and there was no clear pattern of latitude effect on *Nostoc* PCC-73102 ASV relative abundance. For the diazotrophic community (Figure S8b, Annexe 3), ASV differential abundance between S1 and S5 was less clear. *Hassallia*, *Methylocapsa* and *Methyloferula* ASV relative abundance tended to be higher at S1 than at S5 whereas *Nostoc* ASV relative abundance tended to be higher at S5 than at S1.

Tableau 5. PERMANOVA analyses on Bray-Curtis dissimilarities showing the moss-associated global and diazotrophic bacterial community structure variations explained by moss species, month of collection, monthly mean temperature and precipitation, tree density, total N deposition, and phosphorus (P), molybdenum (Mo), vanadium (V) and iron (Fe) concentrations measured in feather mosses collected on a latitudinal transect (a) and along moss shoots (b).

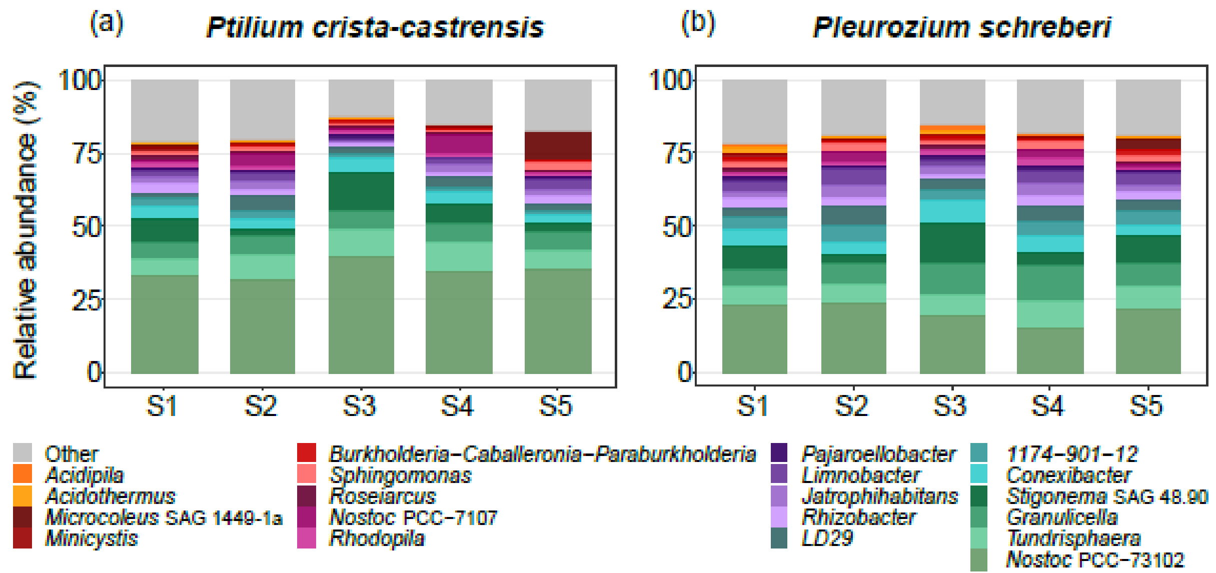
	(a) Latitudinal transect					
	16S rRNA[b]			***nifH***[b]		
Variable	***F***	R^2	P^a	***F***	R^2	P^a
Moss species	1.39	0.023	0.001 *	2.16	0.027	0.013 *
Month	1.15	0.019	0.011 *	2.01	0.026	0.013 *
Temperature	1.24	0.020	0.001 *	5.27	0.067	0.001 *
Precipitation	1.09	0.018	0.071 n.s.	1.37	0.017	0.130 n.s.
Tree density	1.21	0.020	0.001 *	7.70	0.098	0.001 *
N deposition	1.27	0.021	0.001 *	4.02	0.051	0.001 *
P	1.01	0.017	0.450 n.s.	2.12	0.027	0.011 *
Mo	1.07	0.018	0.116 n.s.	1.87	0.024	0.028 *
V	1.06	0.017	0.138 n.s.	1.36	0.017	0.109 n.s.
Fe	1.05	0.017	0.195 n.s.	1.29	0.016	0.136 n.s.
	(b) Moss shoot					
	16S rRNA[c]			***nifH***[c]		
Variable	***F***	R^2	P^a	***F***	R^2	P^a
P	2.07	0.055	0.001 *	11.10	0.225	0.001 *
Mo	1.14	0.030	0.051 n.s.	2.02	0.041	0.028 *
V	1.70	0.046	0.001 *	3.83	0.077	0.001 *
Fe	1.44	0.038	0.001 *	2.44	0.049	0.011 *

[a] **, P< 0.05; n.s., not significant.*

[b] *The models explain 19% and 37% of the variation in global and diazotrophic bacterial community structures, respectively.*

[c] *The models explain 17% and 39% of the variation in global and diazotrophic bacterial community structures, respectively.*

16S rRNA



nifH

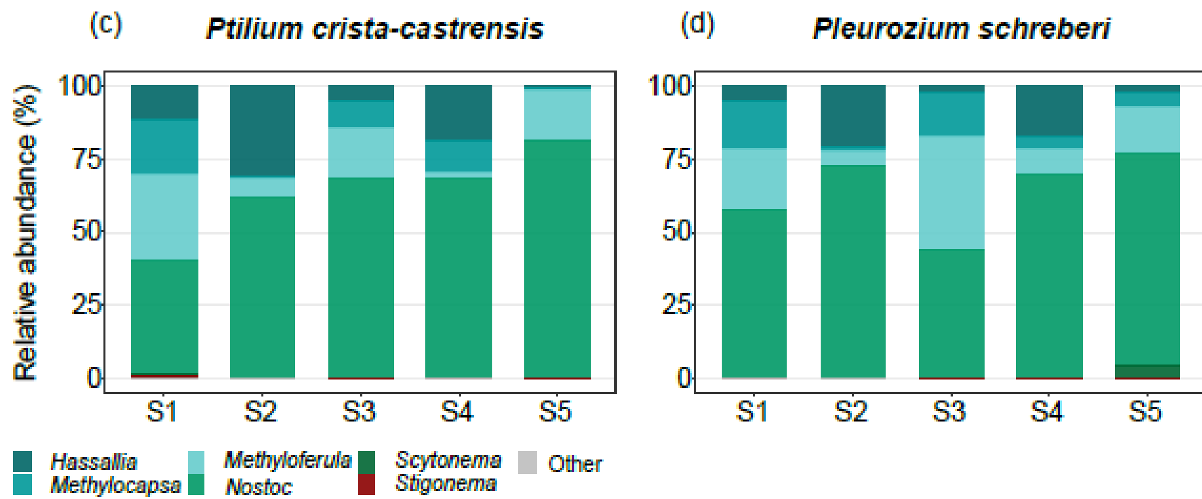


Figure 18. Relative abundance of the dominant bacterial genera detected in 16S rRNA and *nifH* sequences from feather mosses *Ptilium crista-castrensis* (a, c) and *Pleurozium schreberi* (b, d) collected in June and September 2017 (n = 6).

The month of sampling influenced the feather moss bacteriome and explained 1.9% and 2.6% of the variation in global and diazotrophic bacterial communities, respectively (Tableau 5). The bacterial community structure shifted between the beginning (June) and the end (September, October) of the growth season (Figure S3, Annexe 3). Proteobacteria *Rhodopila* and *Sphingomonas*, and cyanobacteria genera *Nostoc* PCC-7107, *Nostoc* PCC-73102 and *Stigonema* SAG 48.90 (assigned with 16S rRNA sequences) ASV relative abundance decreased between June and September/October (Figure S9a and S9b, Annexe 3). Similarly, *Methylocapsa* (assigned with *nifH* sequences) ASV relative abundance was lower in September than in June, but *Methyloferula* and *Nostoc* ASV relative abundance increased at the end of the growth season (Figure S9c and S9d, Annexe 3).

The alpha-diversity of bacteria associated with feather mosses, estimated using Shannon (Figure 19) and Chao1 index (Figure S4, Annexe 3), did not significantly change along the transect (Figure 19b and 19e, Figure S4b and S4e, Annexe 3). However, *P. crista-castrensis*' global bacteriome seemed to display a lower alpha-diversity than *P. schreberi*'s global bacterial communities (χ^2 = 6.88, P = 0.009 for Shannon index but not significant for Chao1; Figure 19a and Figure S4a, Annexe 3). On the contrary, *P. crista-castrensis* was associated with a higher diversity of N_2-fixers than *P. schreberi* (F = 5.28, P = 0.02 for Shannon index; χ^2 = 10.19, P = 0.001 for Chao1; Figure 19d and Figure S4d, Annexe 3). In addition, the alpha-diversity of the global bacterial communities decreased between June and September (χ^2 = 14.28, P = 0.0005 for Shannon; χ^2 = 18.49, $P = 8.79 \times 10^{-5}$ for Chao1; Figure 19c and 19f and Figure S4c and S4f, Annexe 3) but did not change across the growth season for N_2-fixers. We also found that alpha-diversity of the global and diazotrophic bacteriomes were respectively negatively (ρ = - 0.29, P = 0.009) and positively (ρ = 0.29, P = 0.01) correlated with N_2-fixation (Figure S5, Annexe 3).

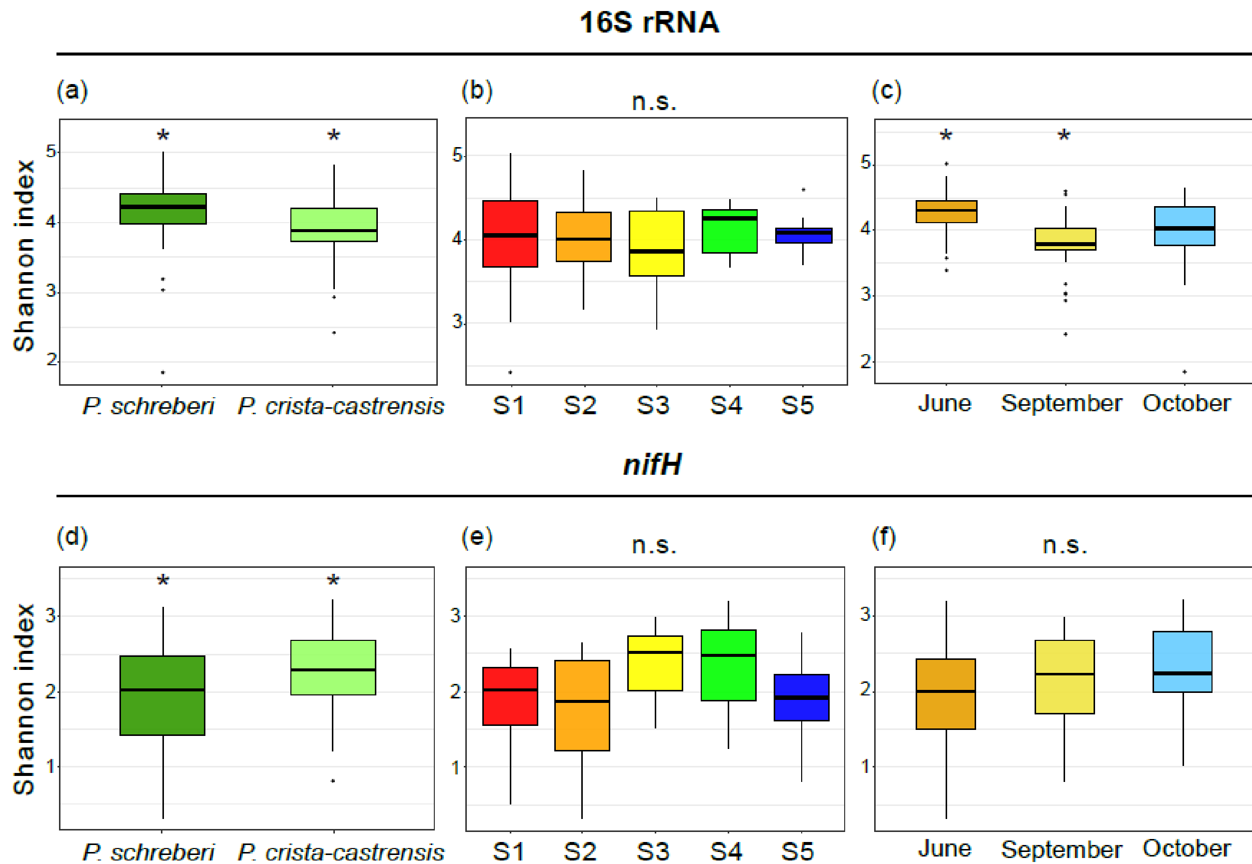


Figure 19. Alpha-diversity (estimated with Shannon index) detected in 16S rRNA and *nifH* sequences from feather mosses *Ptilium crista-castrensis* and *Pleurozium schreberi* collected along a latitudinal transect (S1-S5) in June and September 2017 (a, d) (*n* = 12) and from both species collected in June, September, and October 2017 (b, c, e, f) (*n* = 39).

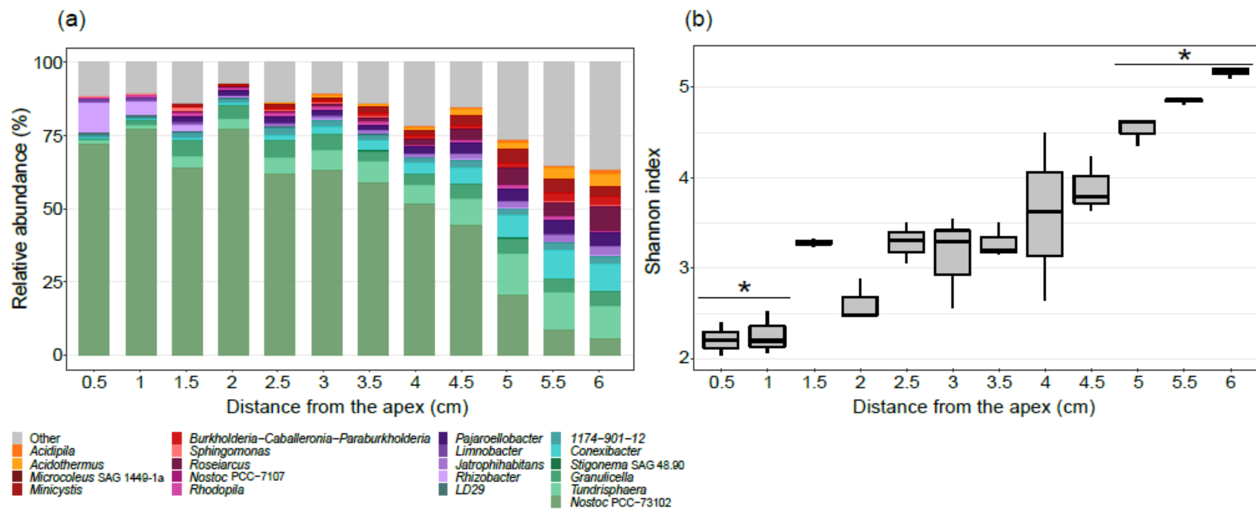
4.2.5.2. Bacterial community distribution along the moss shoot

The structure of the feather moss bacteriome changed along the moss shoot (Figure 20). ASV relative abundance of *Nostoc* PCC-73102 (assigned with 16S rRNA sequences) was higher at 1 than at 6 cm from the moss shoot apex whereas proteobacteria (*Roseiarcus*, *Burkholderia*), acidobacteriota (*Acidipila*, *Bryobacter*) and actinobacteriota (*Acidothermus*, *Conexibacter*) ASV relative abundance were higher at 6 than at 1cm (Figure S10a, Annexe 3). Similarly, cyanobacteria *Hassallia* and *Nostoc* (assigned with *nifH* sequences) were more abundant on the apical part of the shoot and methanotrophic proteobacteria *Methylocapsa* and *Methyloferula* were more abundant on the basal part of the shoot (Figure S10b, Annexe 3).

The alpha-diversity of global bacterial communities increased along the moss shoot and was significantly higher between 5 and 6 cm than in the first two centimeters (χ^2 = 31.47, P = 0.0009 for Shannon index; F = 15, $P = 3.43 \times 10^{-8}$ for Chao1 index; Figure 20b and Figure S6a, Annexe 3). The alpha-diversity of diazotrophic communities estimated by Shannon index did not significantly change with the distance from the apex but it is worth noticing that Chao1 index peaked at 2 cm.

Finally, total cyanobacterial biomass (estimated using phycocyanin) also varied significantly along the moss shoot (F = 16.79, $P = 8.19 \times 10^{-6}$; Figure 21). Cyanobacterial biomass increased with the distance from the apex and reached its maximum at 3 cm, before decreasing.

16S rRNA



nifH

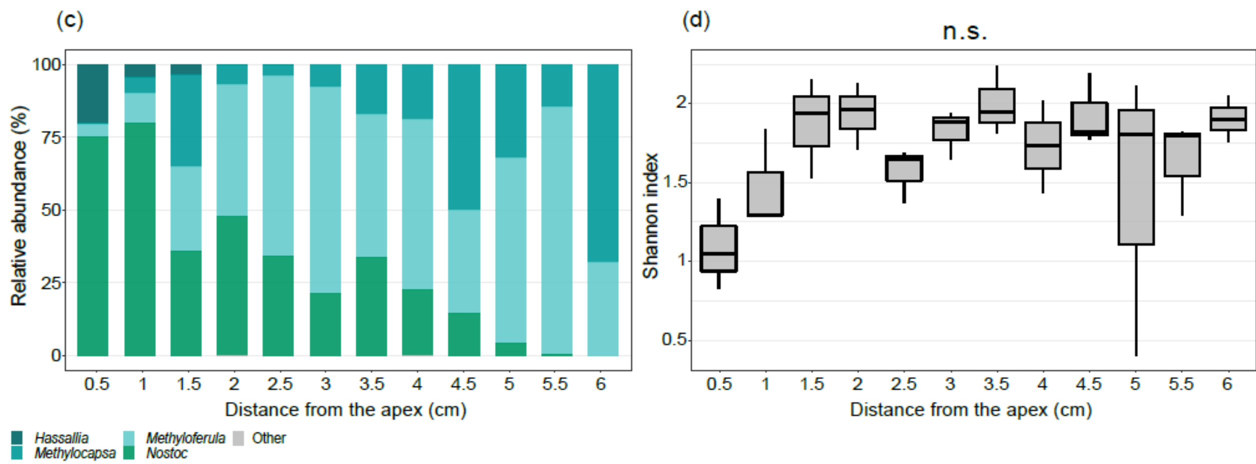


Figure 20. Relative abundance (a, c) of the dominant bacterial genera and alpha-diversity (estimated with Shannon index; b, d) measured in 16S rRNA and *nifH* sequences of 0.5 cm-sections of *Ptilium crista-castrensis* shoots collected on S3 in September 2019 (*n* = 3).

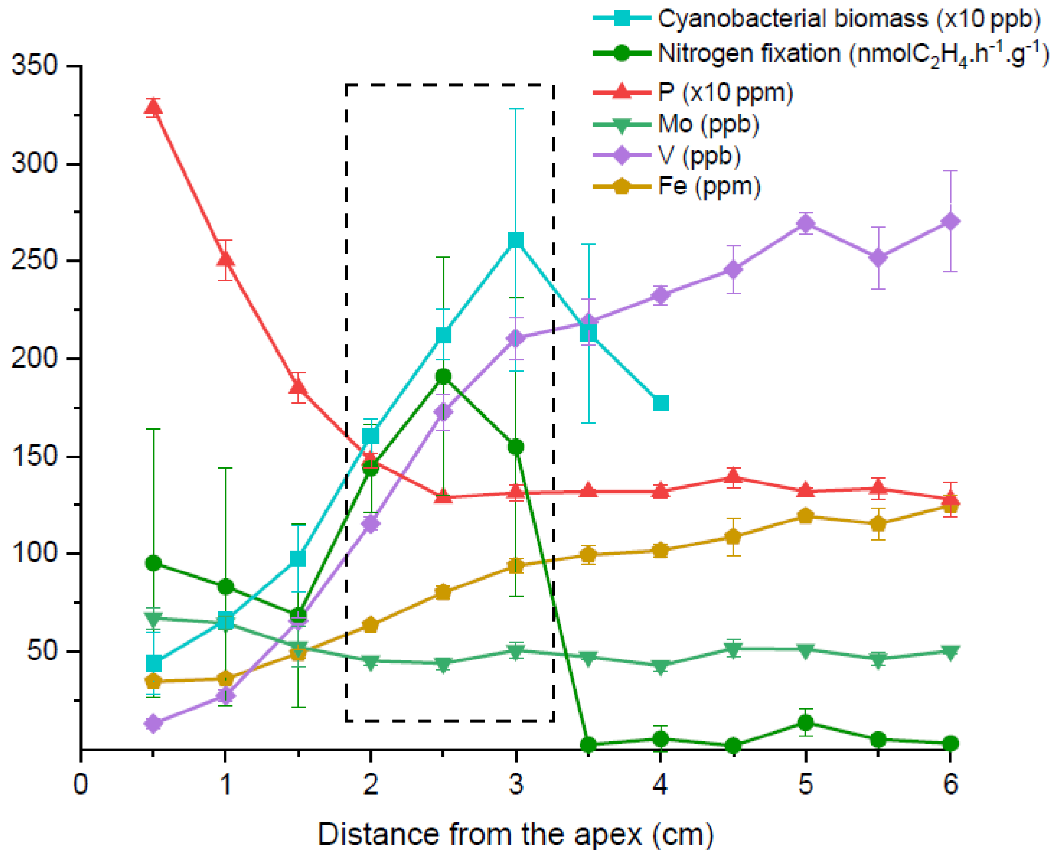


Figure 21. Nitrogen fixation, total cyanobacterial biomass, and phosphorus (P), molybdenum (Mo), vanadium (V) and iron (Fe) concentrations measured along *Ptilium crista-castrensis* shoots collected at S3 in September 2019 (*n* = 3).

The rectangle highlights peaks in nitrogen fixation and cyanobacterial biomass and error bars represent standard deviation.

4.2.5.3. Relationship between bacterial genera and nitrogen fixation

Relative abundances of *Stigonema* SAG 48.90 and *Nostoc* were the top ranked predictors of N_2-fixation for 16S rRNA and *nifH* sequences, respectively (importance scores > 15, Figure 22). *Nostoc*'s relative abundance was negatively correlated with N_2-fixation (ρ = -0.24, P = 0.035) but there was no significant correlation between *Stigonema* SAG 48.90 and N_2-fixation. Relative abundances of *Nostoc* PCC-73102 (for 16S rRNA), *Hassallia* and *Stigonema* (for *nifH*) were, in this order, the positively correlated higher ranked predictors of N_2-fixation (importance scores > 10; Figure 22), which was supported by the PCAs (Figure 23). *Nostoc* PCC-7107 (for 16S rRNA)

and *Methylocapsa* (for *nifH*) were also identified as predictors of N_2-fixation (importance scores of ~4 and ~9 respectively) but were not significantly correlated with it. However, the PCAs suggest that these two genera were positively correlated with N_2-fixation. Finally, of all the genera tested, only *Scytonema* and *Methyloferula* had negative importance scores and did not seem to contribute to N_2-fixation.

Along the moss shoot, N_2-fixation varied significantly ($\chi^2 = 58.62$, $P = 1.67 \times 10^{-8}$; Figure 21). N_2-fixation peaked at 2.5-3 cm from the apex and then drastically decreased at 3.5 cm. This peak corresponds to the location of the highest cyanobacterial biomass. At this distance from the apex, the dominant genera were *Nostoc* PCC-73102, detected in 16S rRNA sequences, and *Methyloferula*, *Nostoc* and *Methylocapsa*, detected in *nifH* sequences.

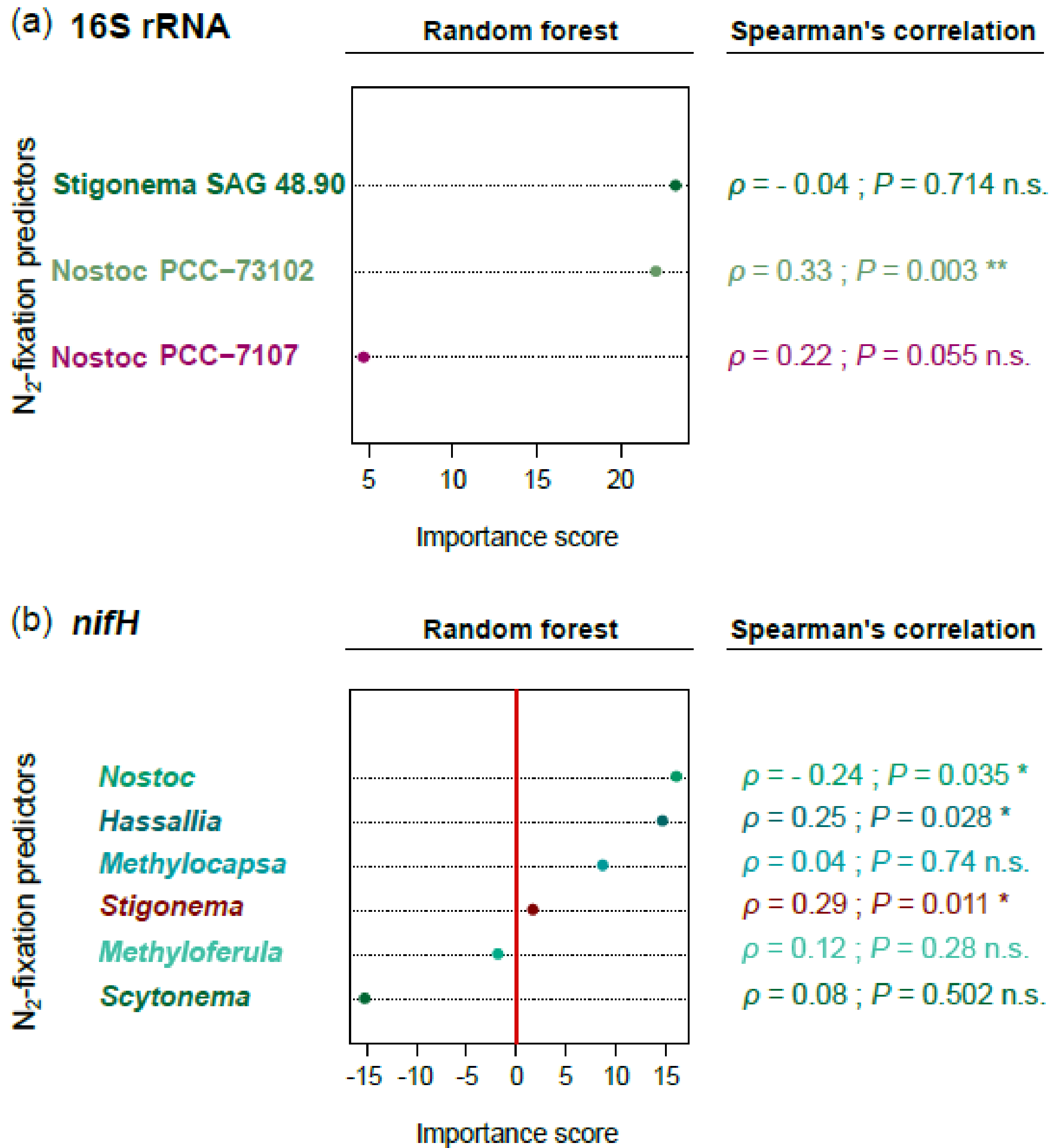


Figure 22. Random forest models and Spearman's correlations between log-transformed nitrogen fixation and log-transformed relative abundances of the dominant cyanobacteria genera identified in 16S rRNA (a) and *nifH* (b) sequences from feather mosses collected in June, September, and October 2017.

Each model and Spearman's correlation regroup 78 observations.

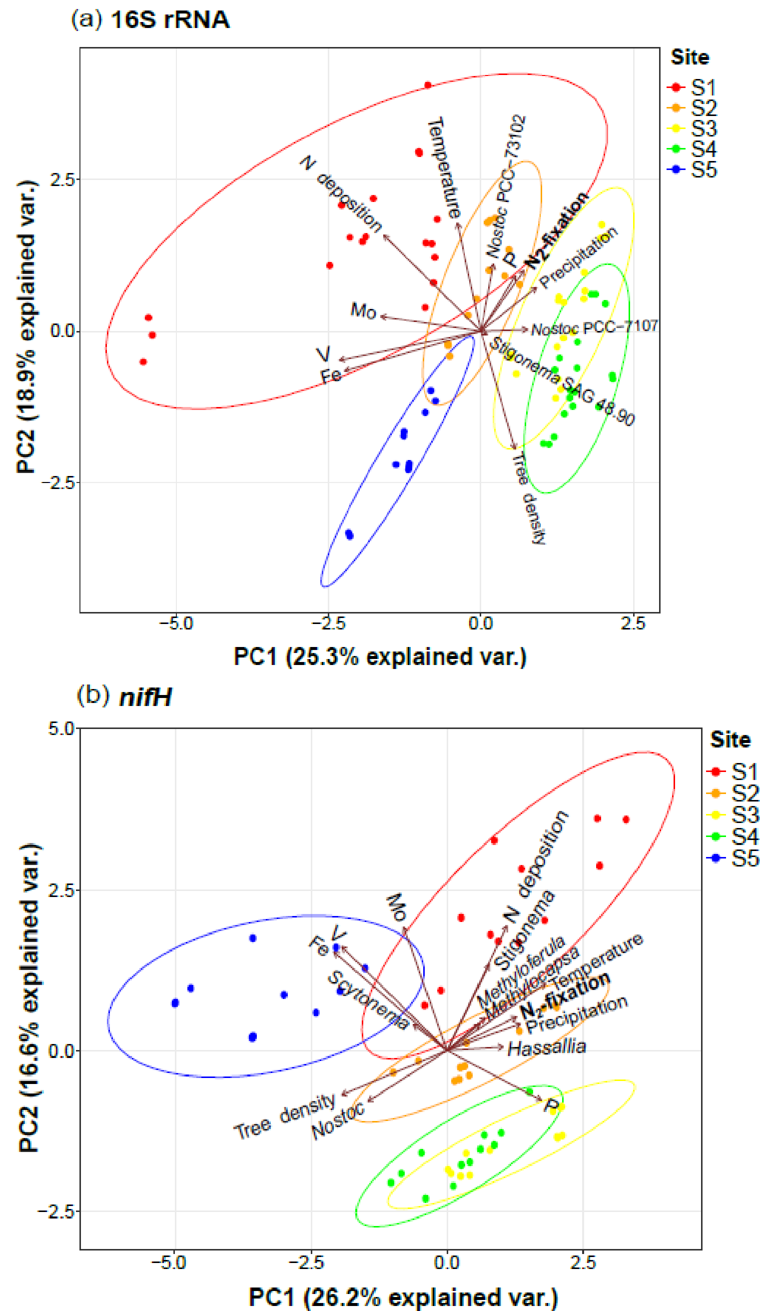


Figure 23. Principal Component Analysis (PCA) biplot showing correlations between environmental variables and relative abundance of the dominant bacteria genera identified in 16S rRNA (a) and *nifH* (b) sequences from feather mosses collected in June and September 2017.

Each PCA regroups 60 observations. Arrows represent variables, points represent each sample and ellipses represent 95% confidence intervals. Colours of points and ellipses were assigned per sampling site.

4.2.5.4. Effects of environmental variables on the feather moss bacteriome

Overall, the ten environmental variables measured along the latitudinal transect explained 19% of the global bacteriome variation and 37% of the diazotrophic bacteriome variation (Tableau 5). Moss species was the parameter explaining the most variation in the global bacteriome (2.3%) and tree density was the variable explaining the most variation in the diazotrophic bacteriome (9.8%). Tree density was positively correlated with relative abundances of *Stigonema* SAG 48.90 (16S rRNA sequences; Figure 23a) and *Nostoc* (*nifH* sequences; Figure 23b). Mean monthly temperature explained 2% of the global bacterial community variation and 6.7% of the diazotrophic bacterial community variation. In addition, temperature was positively correlated with the relative abundance of *Nostoc* PCC-73102 detected in 16S rRNA sequences and *Stigonema*, *Hassallia* and methanotrophs detected in *nifH* sequences, but negatively correlated with *Nostoc* and *Scytonema* in *nifH* sequences (Figure 23). N deposition also had a significant impact on both global and diazotrophic communities (Tableau 5). For 16S rRNA sequences, N deposition were positively correlated with *Nostoc* PCC-73102 (Figure 23a) and for *nifH* sequences, N deposition were positively correlated with relative abundance of methanotrophic genera but negatively correlated with *Nostoc*'s relative abundance (Figure 23b). P and Mo showed contrasted effects as they did not impact global bacterial communities but affected diazotrophic communities (Tableau 5). P seemed particularly strongly correlated with the relative abundance of *Nostoc* PCC-73102 (Figure 23a) and *Hassallia*, whereas *Scytonema*'s relative abundance was strongly positively correlated with Mo, V, and Fe concentrations (Figure 23b). However, precipitation, V and Fe did not affect feather moss-associated bacterial communities.

Along the moss shoot, P concentration was the highest at 0.5 cm and drastically decreased with the distance from the apex until 2.5 cm, where it stabilized (χ^2 = 28.65, P = 0.003) (Figure 21). Similarly, Mo concentration was the highest at 0.5-1 cm (χ^2 = 27.11, P = 0.004) and then slightly decreased and was not significantly different on the rest of the shoot. In contrast, V and Fe concentrations continually increased along the shoot and became significantly different from the top part at 4.5-5 cm (χ^2 = 33.91, P = 0.0004 for V; χ^2 = 33.84, P = 0.0004 for Fe). The nutrients P, Fe, Mo, and V alone explained ~17% of the global bacteriome variation and ~39% of the diazotrophic bacteriome variation (Tableau 5). P was the nutrient explaining the most variation for

both global (5.5%) and diazotrophic communities (22.5%). Mo, V, and Fe also impacted diazotrophic communities, while only Mo did not affect the composition of the global bacteriome.

4.2.6. Discussion

4.2.6.1. New insights into feather moss global bacterial community structure and functions

4.2.6.1.1. Cyanobacteria dominate the feather moss bacteriome

Our results revealed that *Cyanobacteria* was the dominant phylum of global moss bacterial communities, representing more than a third of the ASVs. We found that the dominant cyanobacteria genera comprising the global bacteriome were *Nostoc* PCC-73102 (*N. punctiforme*), *Stigonema* SAG 48.90 (i.e., *S. ocellatum*), *Nostoc* PCC-7107, and *Microcoleus* SAG 1449-1a (i.e., *M. paludosus*) (Figure 18). This confirms previous observations of *Nostoc* and *Stigonema* colonies on *P. schreberi* and *P. crista-castrensis* in the Swedish and Canadian boreal forests (Gentili et al., 2005; Houle et al., 2006; Warshan et al., 2016). However, *Microcoleus*, often present in cyanobacterial biocrusts (Roncero-Ramos et al., 2020), was never detected in the feather moss microbiome. Our findings contrast with recent studies which used the same primer pair for sequencing the 16S rRNA gene (i.e., 515F/806R) showing that cyanobacteria accounted for less than 9% of the bacterial communities and that *Proteobacteria* and *Acidobacteria* were the most abundant bacterial phyla colonizing feather mosses in Alaska (Holland-Moritz et al., 2021, 2018) and *Sphagnum* mosses in the USA (Carrell et al., 2019). These differences could partially be explained by the sequencing of DNA instead of cDNA. The influence of the host moss species identity on cyanobacterial gene expression could further amplify differences in results obtained with DNA vs cDNA. Several studies reported that *N. punctiforme* possess specific genes to establish and maintain symbiosis with a broad variety of plants (Duggan et al., 2013; Campbell et al., 2015) and that the host moss can regulate the expression of these genes (Warshan et al., 2017). For example, the moss seems to control the expression of genes related to *Nostoc*'s motility, nitrogen oxide, and sulfate/phosphate transport. By upregulating certain genes (e.g., production of exoenzymes; Warshan et al., 2017), the host moss could promote directly or indirectly cyanobacterial growth and abundance, which would contribute to cyanobacteria's overrepresentation in the active bacteriome.

4.2.6.1.2. Potential roles of moss-associated bacteria

The active cyanobacteria dominating the feather moss bacteriome could have, in addition to N_2-fixation (see section 4.2.6.2.1.), important functions for moss metabolism and health. Cyanobacteria could induce defence genes in moss, as it was recently demonstrated for *Arabidopsis thaliana* (Belton et al., 2021), and protect their host by producing molecules effective against herbivores or pathogenic bacteria and fungi (e.g., proteins, toxins, siderophores; Neuhof et al., 2005; Årstøl and Hohmann-Marriott, 2019). For example, the antifungal glycosylated lipopeptide Hassallidin A was isolated from *Hassallia* sp. (Neuhof et al., 2005), a genus found in our samples. More generally, bacteria detected amongst the 20 most abundant genera in our samples (Figure 18) were shown to play beneficial roles for their host in other symbioses. For example, *Burkholderia* sp. can participate to *Sphagnum* moss defence against pathogens by producing antifungal molecules (Opelt and Berg, 2004; Shcherbakov et al., 2013) and *Sphingomonas* sp. can improve soybean tolerance to drought stress (Asaf et al., 2017). Both genera can also promote plant growth by producing phytohormones, such as gibberellins or abscisic acid (Shcherbakov et al., 2013; Khan et al., 2014; Luo et al., 2019). Further work is needed to determine if bacteria can play similar beneficial roles in feather moss symbioses.

4.2.6.1.3. Bacteriome shift along the moss senescence gradient

We also found that bacteria were heterogeneously distributed along the moss shoot and that they were occupying different niches, potentially based on their functions (Figure 20 and Figure S10, Annexe 3). There were clear differences in the composition of bacterial communities living on the first two centimeters of the shoot and those living at 5 cm from the apex and below. On the apical part of the shoot, cyanobacteria were dominant but, on the basal decaying part, acidobacteria (e.g., *Acidipila*), actinobacteria (e.g., *Conexibacter*) and proteobacteria (e.g., *Roseiarcus*) became dominant. Accordingly, Chen et al. (2019) found that cyanobacteria were mostly colonizing the green apical part of the moss where they can support the moss N needs (Bay et al., 2013). Moreover, the diversity of global bacterial communities constantly increased along the moss shoot probably because, as moss is decaying, its chemical composition change (Philben et al., 2018) and new decomposer taxa, present in the surrounding soil, colonize dead tissues. For example, actinobacteria and proteobacteria are known to proliferate in forest soils and can degrade

polysaccharides, lignin, and phenolic compounds of dead plant tissues (Větrovský et al., 2014; Lladó et al., 2016). This suggests that the moss shoot is associated with a panel of bacteria helping its growth on the top and participating to its decomposition on the bottom. Chen et al. (2018) drew similar conclusions regarding fungal communities associated with the moss *Dicranum scoparium* and showed that endophytic and epiphytic fungi were colonizing mostly the green apical part and saprotrophic decomposing fungi were colonizing mostly the basal part.

4.2.6.2. Bacteria contributing to nitrogen fixation

4.2.6.2.1. Cyanobacteria are the main contributors to nitrogen fixation

In our study, the active diazotrophic bacteriome of feather mosses only comprised cyanobacteria (65%) and proteobacteria (35%) (Figure 18). Similar cyanobacteria genera were reported on *P. schreberi* in Canada (*Stigonema*, *Nostoc*; Houle et al., 2006) and in Northern Europe (*Nostoc*, *Fischerella*, *Stigonema*, *Calothrix*; Gentili et al., 2005; Ininbergs et al., 2011; Leppänen et al., 2013; Warshan et al., 2016). Carrell et al. (2019) also found that *Nostoc*, *Calothrix*, *Fischerella*, *Stigonema*, and *Scytonema* were assigned with *nifH* sequences from *Sphagnum fallax*. However, we detected several poorly known genera (e.g., *Hassallia* sp., *Cronbergia* sp., *Brasilonema* sp., and *Iningainema* sp.) that were never found associated with mosses. Interestingly, *Iningainema* and *Brasilonema* genera were only found in tropical ecosystems until now (Romanenko et al., 2020; Maltsev et al., 2021). Additionally, *Hassallia* and *Cronbergia* were detected in tropical, temperate, or polar habitats (Komárek et al., 2015; Albrecht et al., 2017; Genuário et al., 2018) but never in boreal ecosystems. Thus, our results indicate that these genera may comprise species that are well adapted to live in the boreal zone. We also found that the cyanobacteria genera *Nostoc*, *Stigonema*, and *Hassallia,* were strongly positively associated with N_2-fixation (Figure 22 and Figure 23). Accordingly, *Nostoc* sp. is generally considered the main contributor to moss N_2-fixation (DeLuca et al., 2002) and *Stigonema* sp. was reported as an active N_2-fixer colonizing feather mosses (Warshan et al., 2016). However, our study brings the first evidence that *Hassallia* sp. can establish associations with feather mosses, actively fix N_2 and, therefore, potentially constitutes a source of N for the moss and the boreal ecosystem.

4.2.6.2.2. Methanotrophs contribute to nitrogen fixation

We also show that methanotrophic bacteria *Methylocapsa* sp. and *Methyloferula* sp. could contribute to N_2-fixation. Indeed, in the basal part of the moss shoot, cyanobacteria's relative abundance become significantly lower than methanotrophs' relative abundance, while N_2-fixation decreases but still occurs (Figure 20, Figure 21 and Figure S10, Annexe 3). *Methylocapsa* and *Methyloferula* are known to be amongst the most active methanotrophic genera associated with *Sphagnum* mosses in peatlands (Esson et al., 2016) and can drive N_2-fixation in these ecosystems (Vile et al., 2014; Carrell et al., 2019). However, Leppänen et al. (2013) did not find methanotrophic *nifH* sequences in the microbiome of *Pleurozium schreberi* in the Finnish boreal forest. This suggests that the North American and the North European boreal forests potentially display different environmental conditions influencing methanotrophic communities (e.g., climate, understory vegetation, forest management practices; Boonstra et al., 2016).

Our findings suggest that methanotrophs are of significant ecological importance for the moss because their N_2-fixation in the basal part of the shoot could contribute to support moss decomposers' activity and moss growth, as N can be relocated in the upper and younger parts of the plant when needed (Eckstein, 2000; Liu et al., 2020). In addition to N, methanotrophs could provide C derived from atmospheric CH_4 to feather mosses, as it was already demonstrated for *Sphagnum* species (Kip et al., 2010), and play a significant role in moss nutrition.

However, because acetylene (used for the ARA to estimate N_2-fixation) inhibits the activity of diazotrophic methanotrophs (Warren et al., 2017), N_2-fixation below 5 cm from the apex could be strongly underestimated and these results should be interpreted with care. Additional work is needed to elucidate the potential roles of methanotrophs in the moss bacteriome using more reliable methods to estimate their N_2-fixation (i.e., ^{15}N incorporation technique).

4.2.6.2.3. Not all cyanobacteria do their part

Several cyanobacteria genera were detected in 16S rRNA sequences but not in *nifH* sequences (e.g., *Microcoleus paludosus, Tolypothrix* sp.; Figure 18), suggesting that they are colonizing feather mosses but do not actively fix N_2, despite being able to. On the other hand, *Stigonema* sp.

was not very abundant in the diazotrophic bacteriome, especially compared to *Nostoc*, but seemed to contribute greatly to moss N_2-fixation (Figure 22 and Figure 23). This indicates that, in the moss-cyanobacteria symbiosis, some cyanobacteria can be specialized in N_2-fixation or can be cheaters by taking advantage of the favorable habitat offered by the moss (e.g., nutrient transfer; Stuart et al., 2020), without providing N in return (Warshan et al., 2016). However, these cheating non-fixing species could contribute to the symbiosis through other functions (e.g., pathogen defense), which remain to be explored.

4.2.6.3. Environmental drivers of the global and diazotrophic feather moss bacteriomes

4.2.6.3.1. Effects of moss species identity

Feather moss species identity had a significant effect on the structure of the global and diazotrophic bacterial communities (Tableau 5). While both moss species were colonized by similar dominant bacterial genera, *P. crista-castrensis* harbored a higher diversity of N_2-fixers than *P. schreberi* (Figure 19 and Figure S4, Annexe 3). This difference was explained by the rarest genera *Brasilonema*, *Cronbergia*, *Fischerella*, and *Iningainema* associated with *P. crista-castrensis* but not with *P. schreberi.* Moreover, on the latitudinal transect, *P. crista-castrensis*' N_2-fixation was, on average, four times higher than *P. schreberi*'s N_2-fixation (Tableau S4, Annexe 3). A similar pattern was found in other studies conducted in the eastern Canadian boreal forest (Darnajoux et al., 2018; Renaudin et al., 2022) and could be linked to the structure of the bacteriome (i.e., higher diversity and higher abundance of N_2-fixing cyanobacteria associated with *P. crista-castrensis*). Indeed, we showed that diazotrophs diversity was positively correlated with N_2-fixation (Figure S5, Annexe 3), which is consistent with Reed et al. (2010) who suggested that N_2-fixers diversity was correlated with hotspots of N_2-fixation in tropical leaf litter. Moreover, Renaudin et al. (2022) showed that the moss species was a major driver of moss-associated cyanobacterial biomass and that *P. crista-castrensis* was colonized by a higher number of cyanobacteria than *P. schreberi*, which could impact N_2-fixation rates. Moss species have morphological and physiological traits (e.g., moisture retention, moss shoot structure, nutrient concentrations, and cationic exchange capacity; Elumeeva et al., 2011; González and Pokrovsky, 2014; Jonsson et al., 2015) that contribute to create specific micro-habitats, potentially affecting bacterial abundance and N_2-fixation. In a recent study, Liu and Rousk (2022) reported that several moss traits, especially moss

hydration rate, were driving cyanobacteria colonization. Therefore, we suggest that *P. crista-castrensis* offers more favorable conditions for N_2-fixers growth and N_2-fixation than *P. schreberi*.

4.2.6.3.2. Effects of temperature and precipitation

Along the transect, mean monthly temperature had a significant effect on feather moss bacterial communities (Tableau 5). Accordingly, Holland-Moritz et al. (2021) showed that temperature was an important predictor of the feather moss bacteriome structure and recent experiments demonstrated major changes in the nature and diversity of moss bacteria following warming (Carrell et al., 2019; Klarenberg et al., 2021). While bacterial diversity did not change much along the transect and across the growth season in our study (Figure 19 and Figure S4, Annexe 3), we detected that several genera were more abundant at higher latitudes and at the end of the growth season (e.g., *Nostoc*, *Microcoleus*) or at lower latitudes and at the beginning of the growth season (e.g., *Stigonema*, *Methylocapsa*) (Figure S8 and Figure S9, Annexe 3), and that the month of sampling had a significant impact on feather moss bacterial communities (Tableau 5). Cyanobacterial relative abundance shifts can be related to the temperature optima of cyanobacterial growth and N_2-fixation, which are species-specific (Gentili et al., 2005; Jean et al., 2012). Thus, some genera could be adapted to specific climate and perform better in northern or southern habitats, or at different times in the season. This is especially the case for *Nostoc* which seemed to prefer colder temperatures (Figure 23b) and was negatively affected by a temperature increase of 1-2 °C on the long term (Klarenberg et al., 2021). This negative relationship between *Nostoc* relative abundance and temperature probably explains why *Nostoc* was negatively correlated with N_2-fixation (Figure 22). Indeed, as the latitude increase, *Nostoc* relative abundance increases but N_2-fixation decreases because of lower temperatures.

In our study, precipitation did not have a significant impact on the feather moss bacteriome (Tableau 5). Mosses were collected at the beginning and at the end of the growth season, characterized by relatively high precipitation, which could have erased a potential effect of water limitation or drought on moss bacterial communities. However, on the same latitudinal transect, precipitation was an important positive driver of moss-associated cyanobacterial biomass (Renaudin et al., 2022). This suggests that, in the North American boreal forest, precipitation may

not alter the composition of the feather moss bacteriome but plays a role in cyanobacterial growth and abundance instead.

4.2.6.3.3. Effects of the tree density

We also found that tree density affected feather moss bacterial communities. Tree density is linked to canopy cover and litter quantity, two variables that are responsible for changes in the structure of feather moss bacteriome because of their influence on light availability (Jean et al., 2020; Holland-Moritz et al., 2021). Thus, higher tree density and canopy cover could lead to decrease light availability and be a disadvantage for phototrophs (e.g., *Nostocaceae*; Jean et al., 2020) but could also be a source of nutrients via litter and throughfall (DeLuca et al., 2008), being potentially beneficial for the bacteria in the understory (e.g., *Stigonema* and *Nostoc* in our study).

4.2.6.3.4. Effects of nutrients

Our study is one of the first to show that macro- (N, P) and micro-nutrients (Mo, V, Fe) had a significant influence on the feather moss bacteriome, especially on the diazotrophic communities, in natural settings (Tableau 5). Several manipulative studies used nutrient addition on moss carpets and demonstrated that N had a negative impact on cyanobacteria quantity and N_2-fixation (Zackrisson et al., 2004; DeLuca et al., 2007; Gundale et al., 2011; Sorensen et al., 2012), suggesting that under N-rich conditions, the moss inhibits cyanobacteria colonization and N_2-fixation stops. However, in our study, N deposition did not affect negatively cyanobacterial biomass and N_2-fixation, even at the transect sites exposed to 9-7 $kgN.ha^{-1}.yr^{-1}$ (Tableau S4, Annexe 3). The inhibition threshold of N_2-fixation is still in debate and the techniques used for N fertilization could cause an overestimation of this phenomenon. Indeed, addition of available inorganic forms of N are poorly representative of the real composition of N deposition, especially in forest ecosystems where atmospheric deposition interacts with the canopy, and artificial N addition can lead to decrease moss pH and be detrimental to bacteria (Alvarenga and Rousk, 2021).

Fertilization experiments also showed that Mo and P had positive impacts on feather moss cyanobacterial abundance (Rousk et al., 2017). Moreover, on the same latitudinal transect and for the same moss species, P was a major driver of cyanobacterial biomass whereas Mo and V were

major drivers of N_2-fixation (Renaudin et al., 2022). However, our results indicated that P and Mo influenced diazotrophic communities but not global bacterial communities. That might be because P is required for nucleic acids, ATP, and protein synthesis and is therefore needed in high quantities for N_2-fixation (Raven, 2012). Mo, V, and Fe are nitrogenase cofactors and can thus influence N_2-fixation. In a similar way, changes in the availability of these nutrients could lead to shift in the feather moss bacteriome composition. Mo is scarce in the environment and very often limits N_2-fixation, especially in remote boreal forest ecosystems (Darnajoux et al., 2019). Some bacterial genera found in our study were shown to possess an alternative V-nitrogenase (Nase) enzyme which can support N_2-fixation when Mo is limited (e.g., *Nostoc* and *Stigonema*; Nelson et al., 2019; Villarreal et al., 2021). Thus, Mo limitation and V availability could select diazotrophs comprising genes coding for the V-Nase and disadvantage bacteria with no alternative Nases. For example, *Scytonema* seemed particularly strongly positively correlated with V (Figure 23b), suggesting that these bacteria possess the V-Nase in their genome.

The micro-environment along the moss shoot senescence gradient also influenced bacterial distribution and biomass (Figure 20 and Figure 21), reinforcing the idea, discussed earlier (section 4.2.6.1.3.), that bacteria live in specific niches on the moss. We showed that nutrient concentrations varied significantly along the moss shoot (Figure 21) and that these fluctuations affected both global and diazotrophic bacteria communities (Tableau 5). P had a significant effect on diazotrophic communities and cyanobacterial relative abundance seemed to follow P variations, both being the highest at 0-1 cm from the apex and then gradually decreasing. However, cyanobacterial biomass and N_2-fixation appeared to be promoted when V and Fe reached higher concentrations, at the 2-3 cm shoot section. Thus, we suggest that cyanobacterial biomass and N_2-fixation peak when P, V, and Fe are all present in satisfying concentrations for bacterial growth and Nases synthesis. Moreover, other major environmental variables potentially change with the distance from the moss shoot apex. Lower parts of the shoot are less exposed to drying factors, such as wind and light, and are located closer or within the soil, which buffers temperature and moisture. Thus, temperature and light availability are expected to decrease along the shoot whereas moisture is expected to increase. Cyanobacteria and phototrophs could be dominant on the top part of the shoot because light is more accessible, whereas non-photosynthetic bacteria (e.g., methanotrophs) could be advantaged on the lower part of the shoot and outcompete phototrophs.

4.2.6.4. Implications for the boreal forest ecosystem

We showed that the active global and diazotrophic bacteriomes were driven by environmental conditions at the ecosystem and at the moss shoot scale. In the North American boreal forest, global change is characterized by warming (+2 °C in 2050; Price et al., 2013), extension of the growth season length (Post et al., 2018), more frequent drought episodes (Ouranos, 2015), higher greenhouse gases atmospheric concentrations (Friedlingstein et al., 2019; Schaefer, 2019) and increasing nutrient deposition (e.g., Mo, V, and Fe; Schlesinger et al., 2017; Hamilton et al., 2020; Wong et al., 2021) due to the development of human activities at northern latitudes. Global change is predicted to be particularly intense in the boreal forest ecosystem (Post et al., 2018) and will presumably modify the structure and the activity of feather moss bacterial communities, leading to profound impacts on boreal forest biogeochemical cycles. Climate change could destabilize the moss microbiome by selecting bacteria that are able to grow at warmer temperatures and that are more resistant to drought. For example, warming was correlated with a decrease of cyanobacterial (especially *Nostoc*) and acidobacterial relative abundances and an increase of proteobacterial relative abundance (Klarenberg et al., 2021). In contrast, warming and increased atmospheric deposition can promote bacterial activity, such as N_2-fixation in the apical part (Lindo and Griffith, 2017; Rousk et al., 2017) and moss decomposition in the basal part (Philben et al., 2018). Moreover, the elevation of CO_2 and CH_4 concentrations can promote bacterial phototrophy and methanotrophy, respectively (Strong et al., 2015; Huang et al., 2018). However, the frequency and the severity of the boreal forest disturbances (e.g., fire, spruce budworm outbreaks), importantly modifying tree density and the canopy, are also predicted to increase (Ouranos, 2015) and could have a negative effect on feather moss global and diazotrophic bacterial communities.

4.2.7. Conclusions

Our work presents new insights into the active feather moss bacteriome and its functions in the North American boreal forest. Our data revealed poorly known diazotrophic taxa that were never found associated with boreal feather mosses (e.g., *Cronbergia*, *Iningainema*) and enabled us to identify the genera *Nostoc*, *Hassallia*, *Stigonema*, and *Methylocapsa* as contributing to N_2-fixation. We also determined that environmental variables (moss species, month, tree density), climate conditions (temperature, precipitation) and nutrients (N, P, Mo, V, and Fe) were driving both global

and diazotrophic bacterial communities. Moreover, we showed that bacteria were inhabiting ecological niches on the moss shoot, cyanobacteria being dominant in the apical part and proteobacteria, acidobacteria and actinobacteria being dominant in the basal part. We advocate that using cDNA-based amplicon sequencing of 16S rRNA and *nifH* genes, combined with quantification of bacterial biomass (phycocyanin) and N_2-fixation, are efficient and complementary methods to study the composition of the active moss bacteriome and to link it to microbial processes. Finally, many bacteria genera we identified are not well known and have uncertain functions. Further work is needed to fully elucidate the moss microbiome, including the study of micro-organisms that have been under-looked (e.g., archaea, virus), to improve our knowledge of their ecological roles in the face of global change and forest ecosystem disturbances.

4.2.8. Data availability

All the raw sequences, datasets and R code used in this study have been deposited on Figshare: https://figshare.com/authors/Marie_Renaudin/11938571. These files will be rendered public upon publication. Raw sequences have also been deposited in the NCBI BioProject database under accession number PRJNA813165 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA813165). The authors are fully responsible for climatic data (mean monthly temperature and precipitation) used and displayed in this article, which were provided by the MELCC (Government of Quebec).

4.2.9. Declaration of competing interests

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

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4.2.11. Supplementary data

Voir Annexe 3.
En raison de la taille des données, le Tableau S4 a été déposé sur Figshare et sera disponible après publication avec le lien suivant : https://doi.org/10.6084/m9.figshare.19127771.v1.

4.2.12. References

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CONCLUSION GÉNÉRALE

Dans cette thèse, nous avons tout d'abord validé une méthode d'extraction et de quantification de la phycocyanine, un pigment photosynthétique cyanobactérien, pour estimer la biomasse de cyanobactéries associées aux mousses (objectif 1). Nous avons démontré que cette méthode était robuste, reproductible et ne comprenait pas d'effets de matrice détectables. Cette méthode est complémentaire aux techniques les plus souvent employées (i.e., comptage au microscope à fluorescence, qPCR) mais permet d'étudier une plus grande quantité d'échantillons, plus rapidement et à moindre coût. L'extraction de la phycocyanine peut notamment être utilisée pour comparer les quantités de cyanobactéries de différentes espèces de mousses ou pour tester les effets de variables environnementales (e.g., lumière, température) sur la biomasse cyanobactérienne des mousses. Cette méthode a donc, par la suite, été utilisée pour réaliser les objectifs 2 et 3.

Nous avons ensuite mis en évidence que la biomasse de cyanobactéries et la FBN, bien que corrélées, n'étaient pas contrôlées par les mêmes facteurs environnementaux (objectif 2). D'une part, la température, les précipitations et P influencent positivement la biomasse et, par extension, la croissance des cyanobactéries colonisant les mousses, et d'autre part, la température et Mo influencent positivement la FBN. Ces résultats apportent donc une nouvelle perspective sur les associations mousses-cyanobactéries puisque certains facteurs peuvent promouvoir la croissance bactérienne sans influencer la FBN, et *vice versa*. Les relations entre Mo, V et la FBN, explorées pour la première fois dans le système mousse-cyanobactéries *in situ* dans notre étude, suggèrent (i) une limitation de la FBN par Mo, qui semble répandue dans la forêt boréale, et (ii) l'utilisation de la V-Nase par les diazotrophes des mousses, permettant de soutenir leur FBN en milieu pauvre en Mo. Ceci souligne l'importance de V, peu souvent pris en compte dans le cycle du N de la forêt boréale.

Dans cette thèse, nous identifions plusieurs genres de cyanobactéries et de protéobactéries méthanotrophes contribuant activement à la FBN des mousses (objectif 3). Ceci souligne le fait que les cyanobactéries, mais aussi les méthanotrophes, sont des acteurs majeurs du cycle du N en forêt boréale, et appelle à prendre en compte l'activité des méthanotrophes dans le futur. Nous

montrons aussi que, comme pour la biomasse cyanobactérienne et la FBN, le bactériome des mousses est fortement contrôlé par son macro- (climat, dépôts atmosphériques, densité d'arbres) et micro-environnement (brin de mousse). Au même titre que les plantes supérieures, les mousses boréales possèdent des communautés bactériennes occupant des niches spécifiques et favorisant potentiellement leur croissance et leur décomposition. Cependant, les fonctions exactes remplies par les bactéries identifiées dans notre étude restent encore, pour la plupart, à être élucidées.

En conclusion, cette thèse démontre l'importance du bactériome des mousses dans le cycle du N de la forêt boréale, permettant sur le long terme de soutenir sa productivité primaire. Ce travail montre également que le climat et les nutriments influencent fortement, mais différemment, la croissance cyanobactérienne, la structure du bactériome et la FBN des espèces de mousses pleurocarpes dominantes en forêt boréale Canadienne. De ce fait, il est encore difficile aujourd'hui d'estimer les conséquences exactes du changement global sur le bactériome des mousses et la FBN. La hausse des températures et des dépôts atmosphériques liés aux activités anthropiques dans la forêt boréale (e.g., exploitation des ressources naturelles) pourrait avoir un effet positif sur la croissance bactérienne (e.g., P) et la FBN en réduisant la limitation en certains nutriments (e.g., Mo). Au contraire, l'augmentation des émissions de N et de la fréquence des perturbations (i.e., épisodes de sécheresse, feux de forêt et maladies) pourraient drastiquement diminuer l'abondance et l'activité des diazotrophes. Il est donc essentiel de poursuivre les efforts vers une caractérisation plus précise des effets du changement climatique, déjà visibles dans les écosystèmes nordiques, sur les communautés de bactéries associées aux mousses et leur FBN, pour pouvoir mieux prédire, à terme, le cycle du N et le futur de la forêt boréale.

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ANNEXE 1. INFORMATIONS SUPPLÉMENTAIRES DU CHAPITRE 2

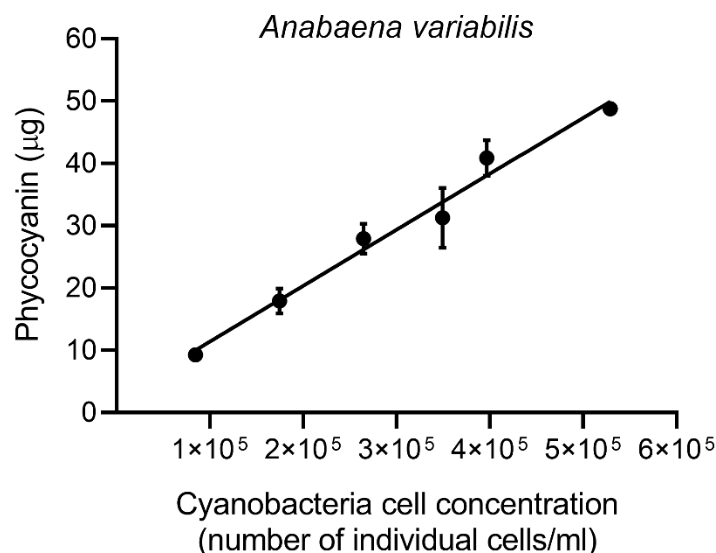


Figure S1. Phycocyanin mass mean ± SD (n=3) linearity in *Anabaena variabilis* culture. The straight line represents the linear regression between phycocyanin mass and the number of individual cyanobacteria cell per ml of culture.

Tableau S1. Phycocyanin concentrations (µg.g^{-1} moss DW ± SD) measured in *Ptilium crista-castrensis* and *Pleurozium schreberi* collected in June and September 2019 in Quebec, Canada (n = 3).

		Sampling site			
		1	**2**	**3**	**4**
June	*Ptilium crista-castrensis*	0.85 ± 0.25	1.26 ± 0.42	0.79 ± 0.02	0.45 ± 0.05
	Pleurozium schreberi	0.48 ± 0.10	0.47 ± 0.08	0.46 ± 0.03	0.41 ± 0.02
September	*Ptilium crista-castrensis*	0.71 ± 0.23	0.96 ± 0.17	0.75 ± 0.08	0.56 ± 0.10
	Pleurozium schreberi	0.88 ± 0.43	0.72 ± 0.04	0.71 ± 0.17	0.61 ± 0.11

ANNEXE 2. INFORMATIONS SUPPLÉMENTAIRES DU CHAPITRE 3

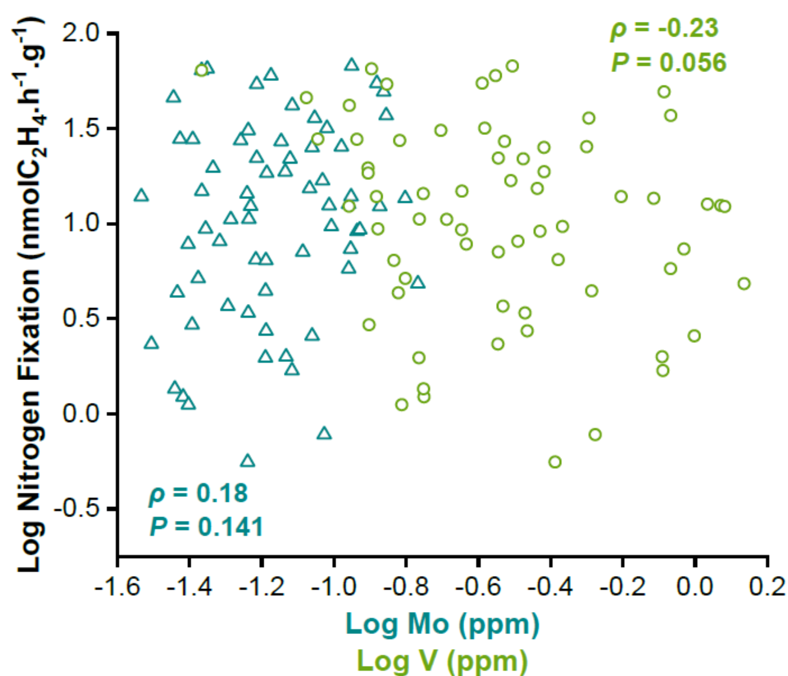


Figure S2. Spearman correlations between log-transformed mean nitrogen fixation (*n* = 4) and log-transformed mean molybdenum (Mo) and vanadium (V) concentrations (*n* =3) measured in feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis* collected on a latitudinal transect in Quebec, Canada.

Blue triangles and green circles indicate observations for Mo and V, respectively. Each regression regroups a total of 66 observations.

Tableau S2. Table summarizing the characteristics of all the moss samples collected (species, date, and location of sampling), the site-specific parameters (mean monthly temperature, precipitation, location of the meteorological stations used for temperature and precipitation estimations, tree density and total nitrogen deposition) and the moss related variables (acetylene reduction, phycocyanin, nutrient concentrations) measured and included in this study.

En raison de la taille des données, ce tableau a été déposé sur Figshare et sera disponible après publication avec le lien suivant : https://doi.org/10.6084/m9.figshare.19127780.v1.

Tableau S3. Linear mixed-effects model using carbon (C) and nitrogen (N) concentrations as predictors of log-transformed nitrogen fixation.

The model regroups a total of 66 observations. C and N concentrations were measured in the feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis,* collected along a latitudinal transect in Quebec, Canada in 2017, 2018 and 2019. C and N concentrations were set as fixed effects whereas the period (combination of month and year) and site of sampling were set as random effects.

	Nitrogen fixation[c]				
		Estimate[a]	SE	*t*	***P***[b]
Predictive variables	**C**	-13.72	5.62	*-2.44*	***0.019 ****
	N	1.98	0.97	*2.04*	***0.048 ****

[a]*The estimate represents the slope of the relationship between the response and the predictive variables (i.e., fixed effects).*

[b]**, P< 0.05; **, P< 0.01; ***, P<0.001; ns, not significant.*

[c]*The model explains 28% of the variation in nitrogen fixation.*

ANNEXE 3. INFORMATIONS SUPPLÉMENTAIRES DU CHAPITRE 4

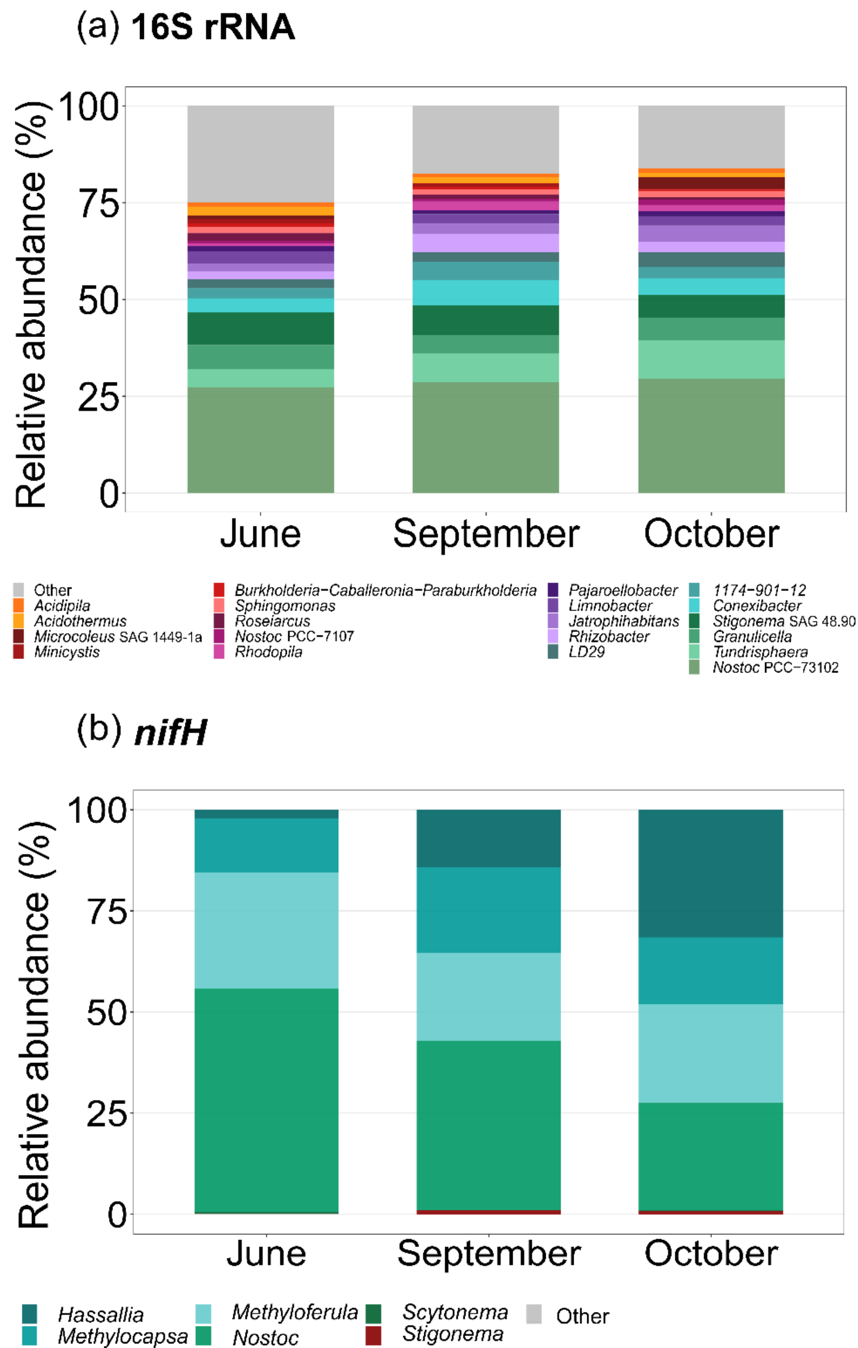


Figure S3. Relative abundance of the dominant bacterial genera detected in 16S rRNA (a) and *nifH* (b) sequences from feather mosses *Ptilium crista-castrensis* and *Pleurozium schreberi* collected on sites S1, S3, S4 in June, September, and October 2017 (*n* = 18).

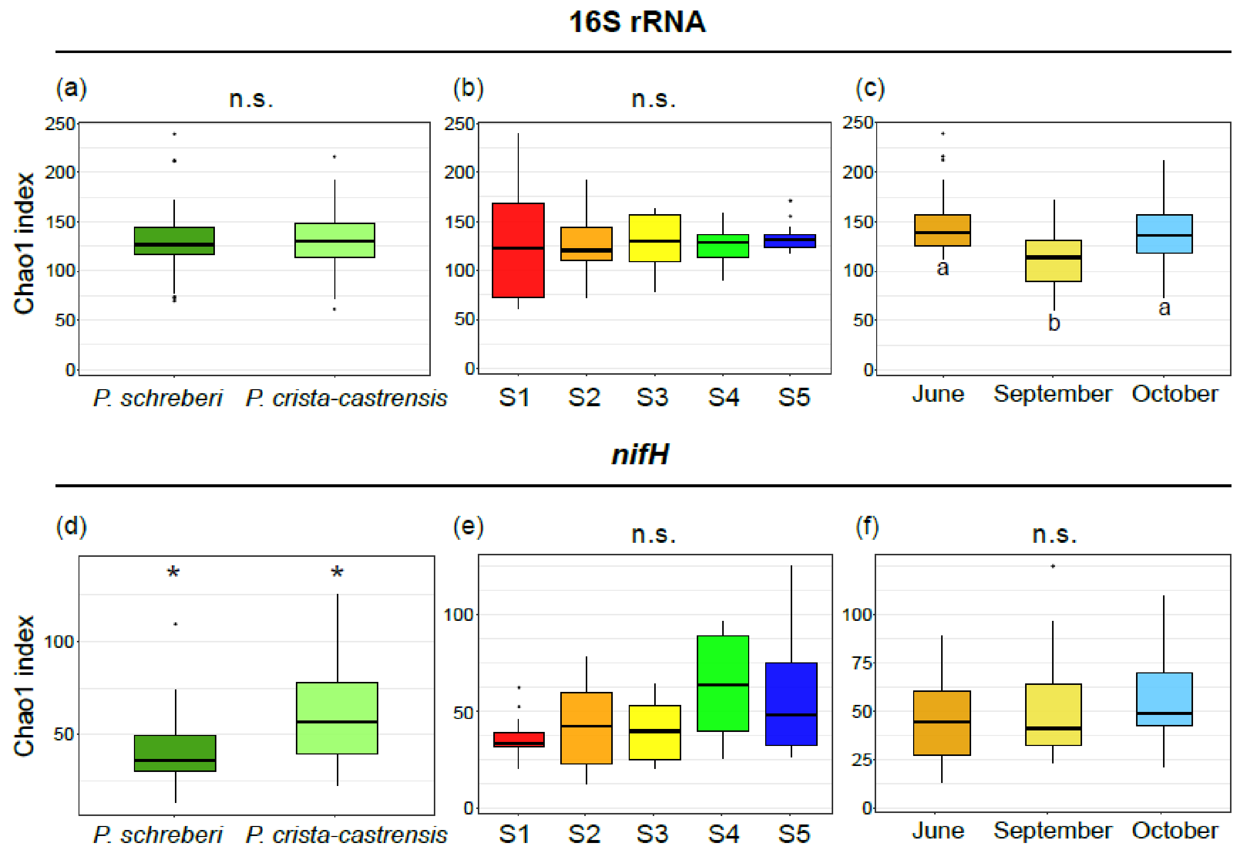
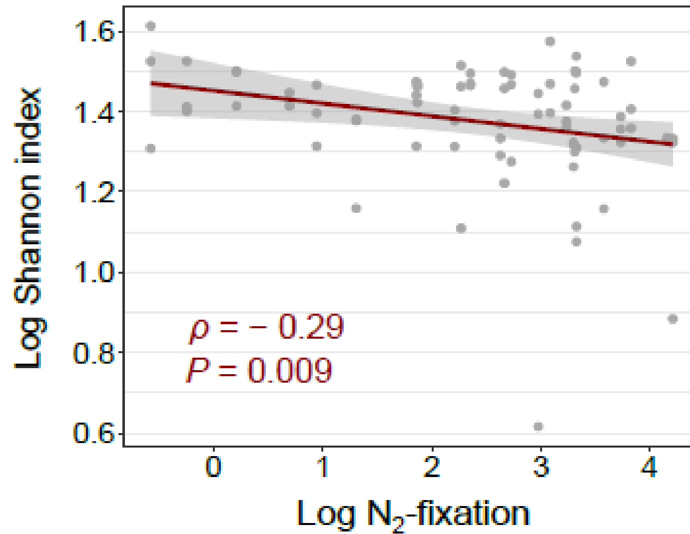


Figure S4. Alpha-diversity (estimated with Chao1 index) detected in 16S rRNA and *nifH* sequences from feather mosses *Ptilium crista-castrensis* and *Pleurozium schreberi* collected along a latitudinal transect (S1-S5) in June and September 2017 (a, d) (*n* = 12) and from both species collected in June, September, and October 2017 (b, c, e, f) (*n* = 39).



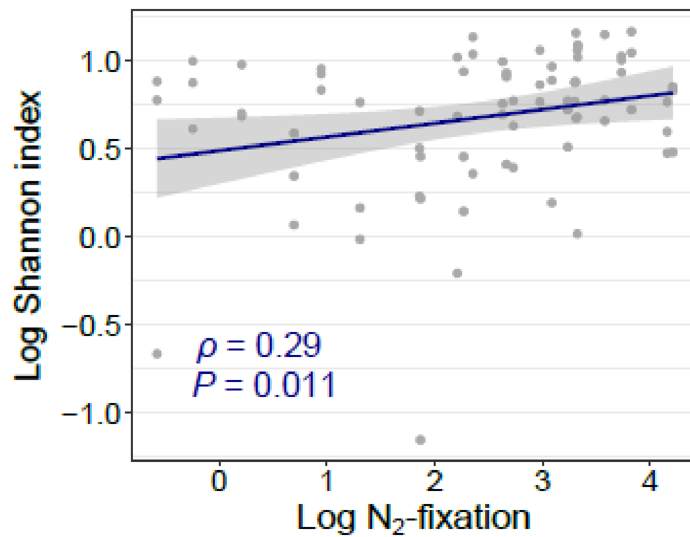


Figure S5. Spearman's correlation between log-transformed nitrogen fixation and log-transformed alpha-diversity (estimated with Shannon index) measured in 16S rRNA (a) and *nifH* (b) sequences from feather mosses *Ptilium crista-castrensis* and *Pleurozium schreberi* collected along a latitudinal transect (S1-S5) in June, September, and October 2017.
Solid lines indicate linear regressions, and each linear regression regroups 78 observations. Grey dots indicate observations and grey zones indicate 95% confidence intervals.

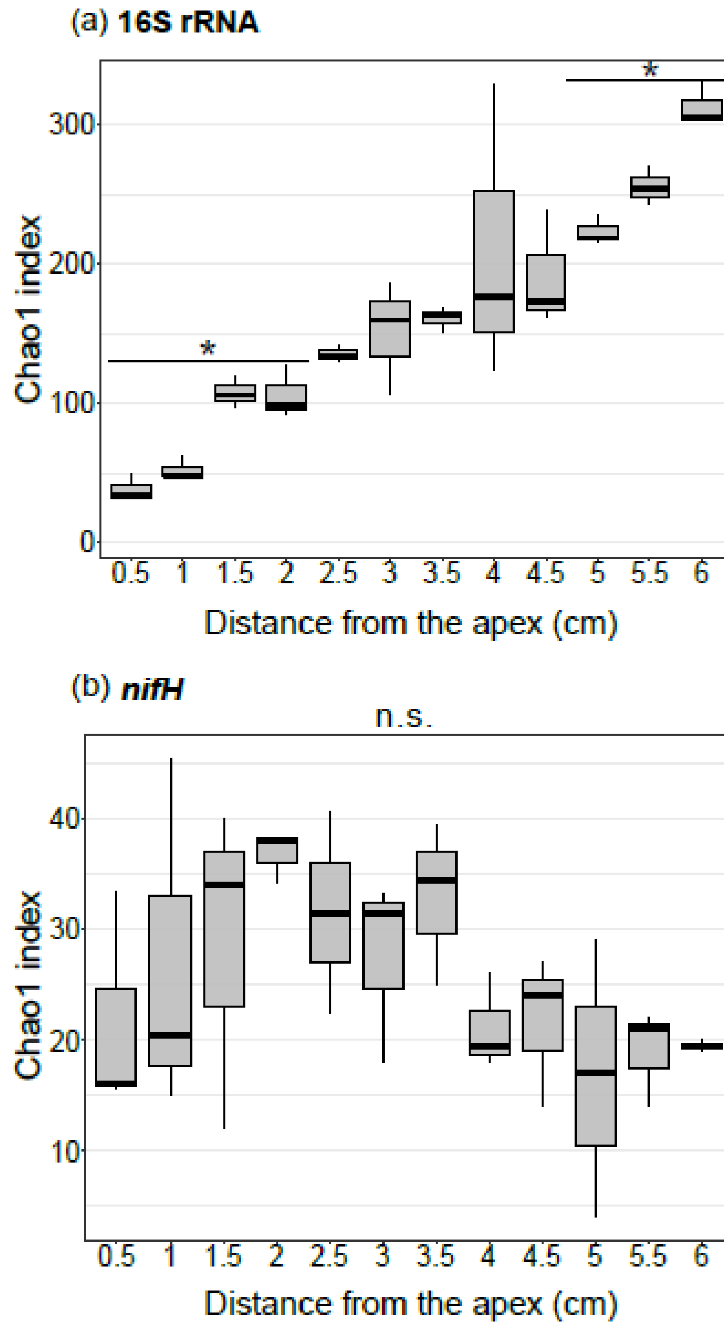
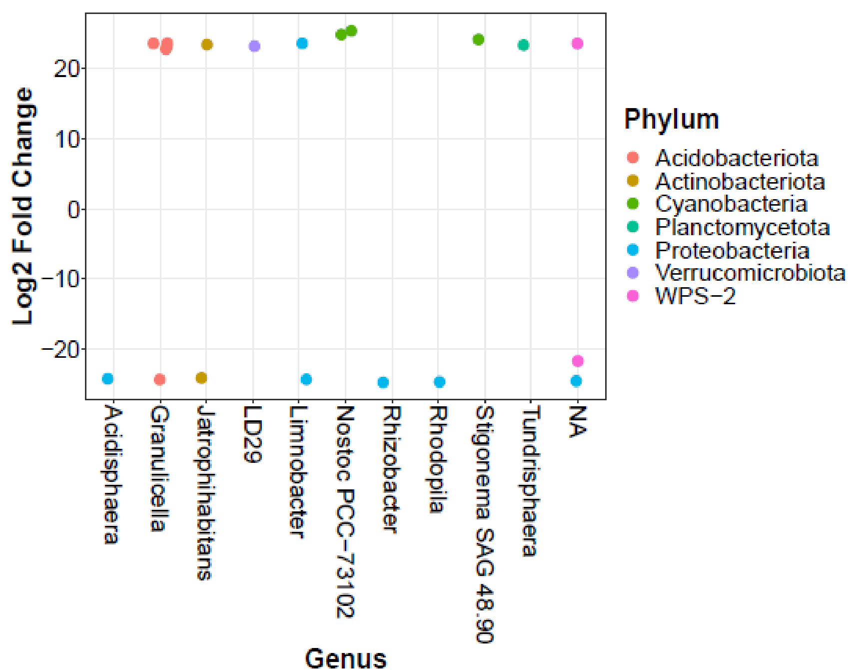


Figure S6. Alpha-diversity (estimated with Chao1 index) measured in 16S rRNA (a) and *nifH* (b) sequences of 0.5 cm-sections of *Ptilium crista-castrensis* shoots collected on S3 in September 2019 (*n* = 3).



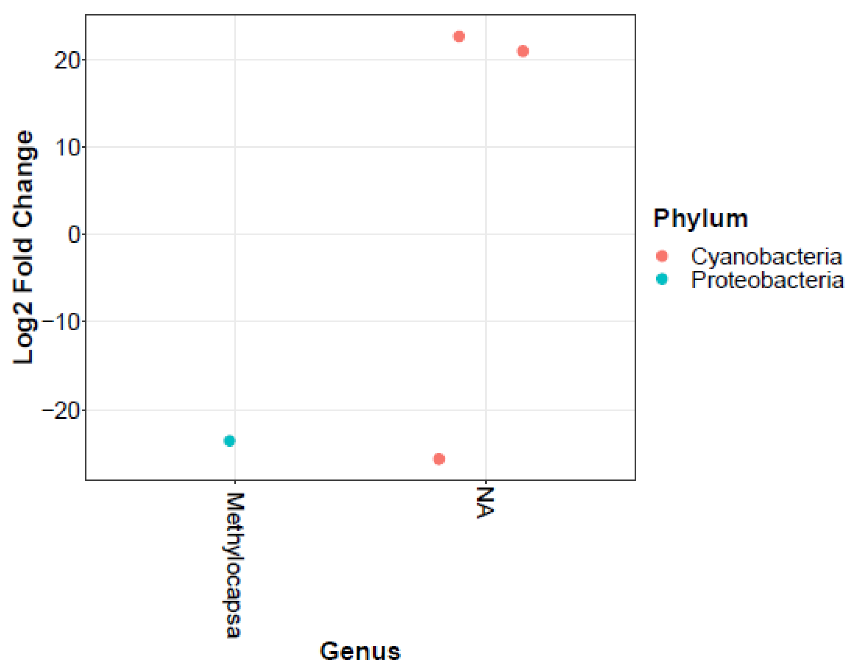


Figure S7. 16S rRNA- (a) and *nifH*-assigned (b) ASV differential abundance analysis between the two moss *Ptilium crista-castrensis* and *Pleurozium schreberi* collected on a latitudinal transect in June, September, and October 2017.

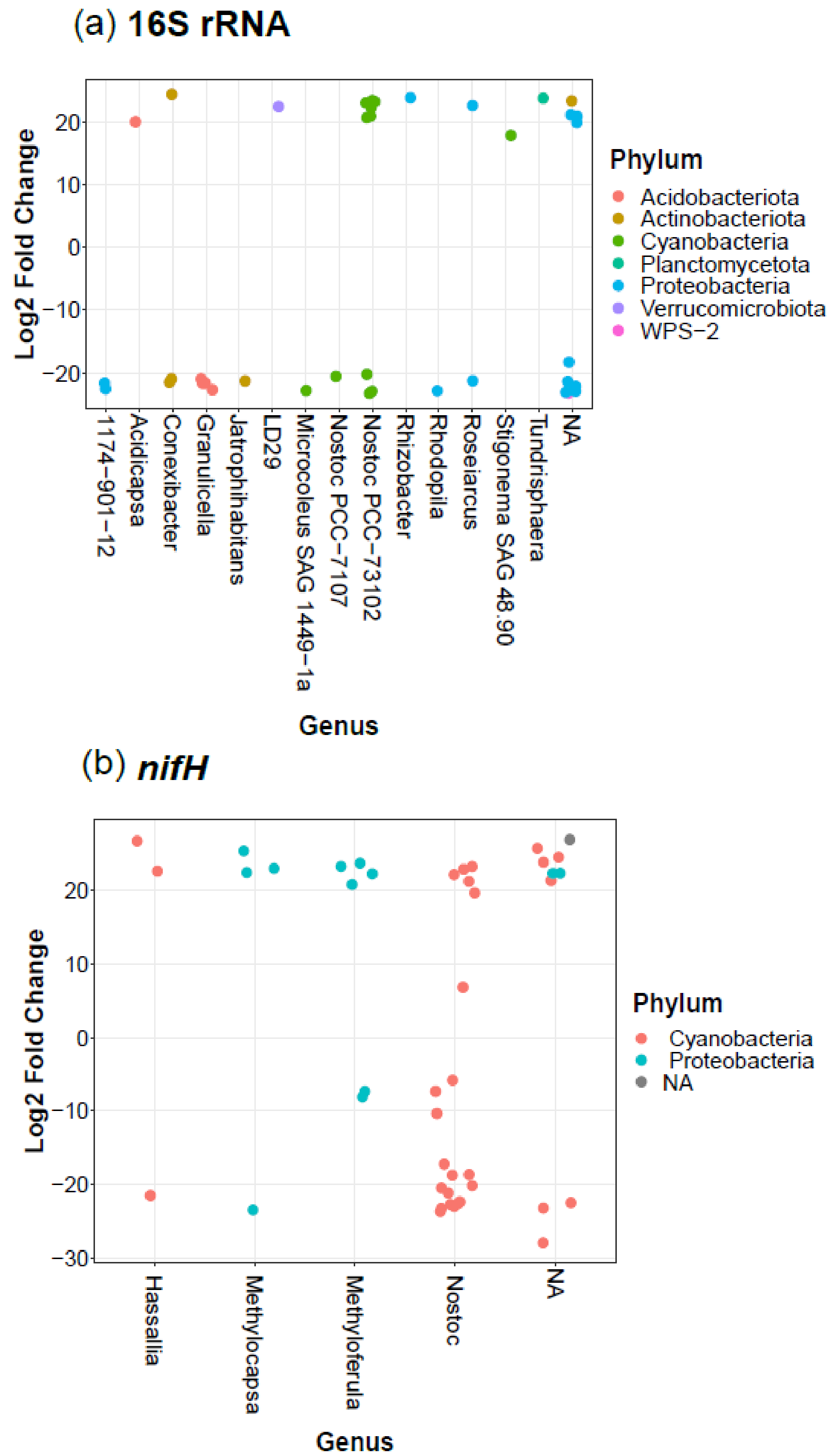
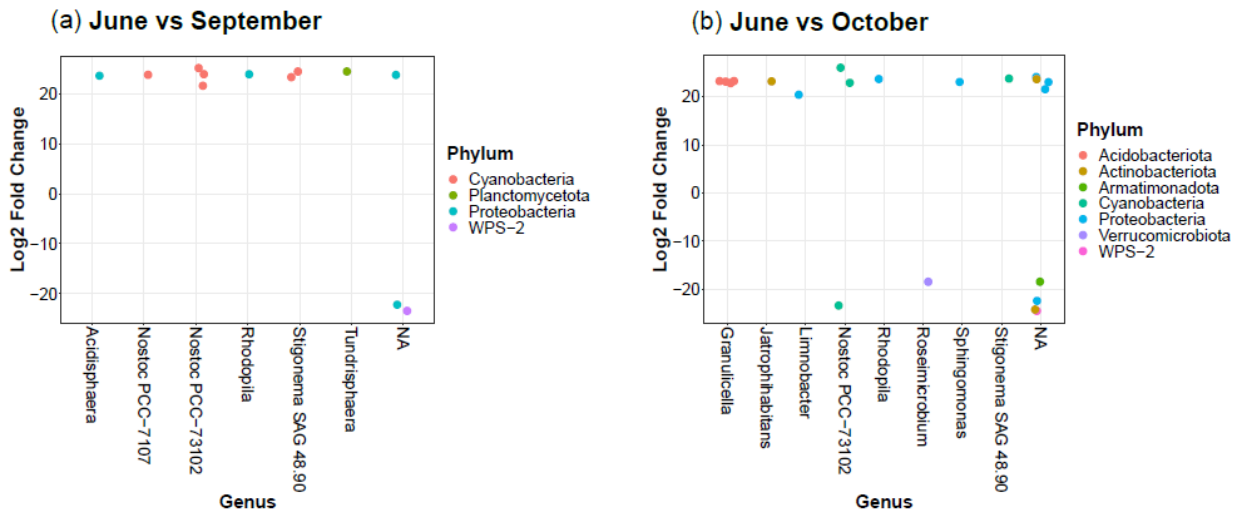


Figure S8. 16S rRNA- (a) and *nifH*-assigned (b) ASV differential abundance analysis between feather mosses collected on the sampling sites S1 and S5, in June, September, and October 2017.



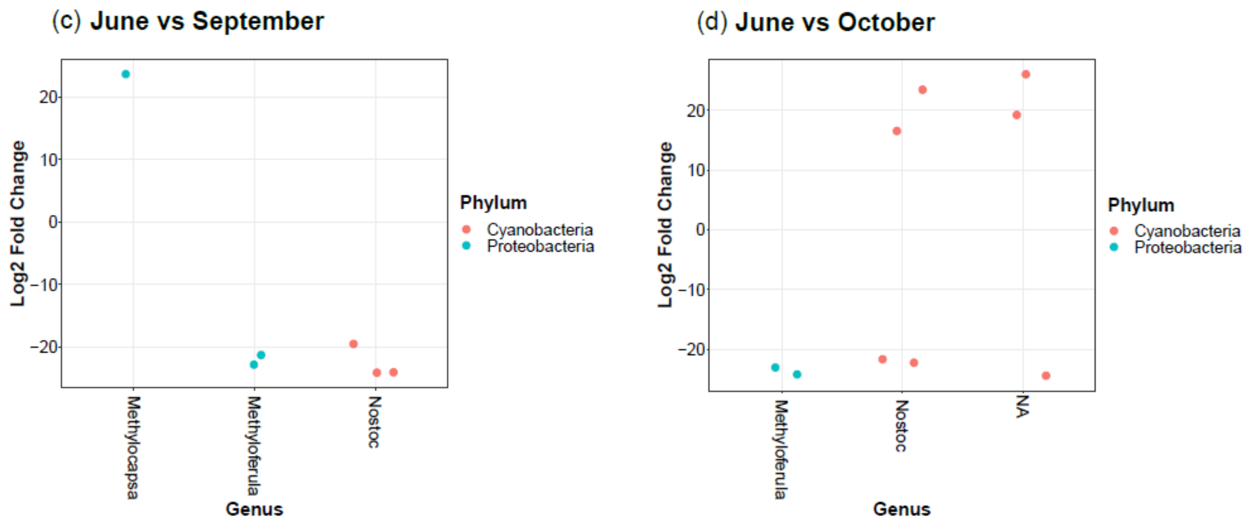


Figure S9. 16S rRNA- and nifH-assigned ASV differential abundance analysis between feather mosses collected on a latitudinal transect in June and September (a, c) or October (b, d) 2017.

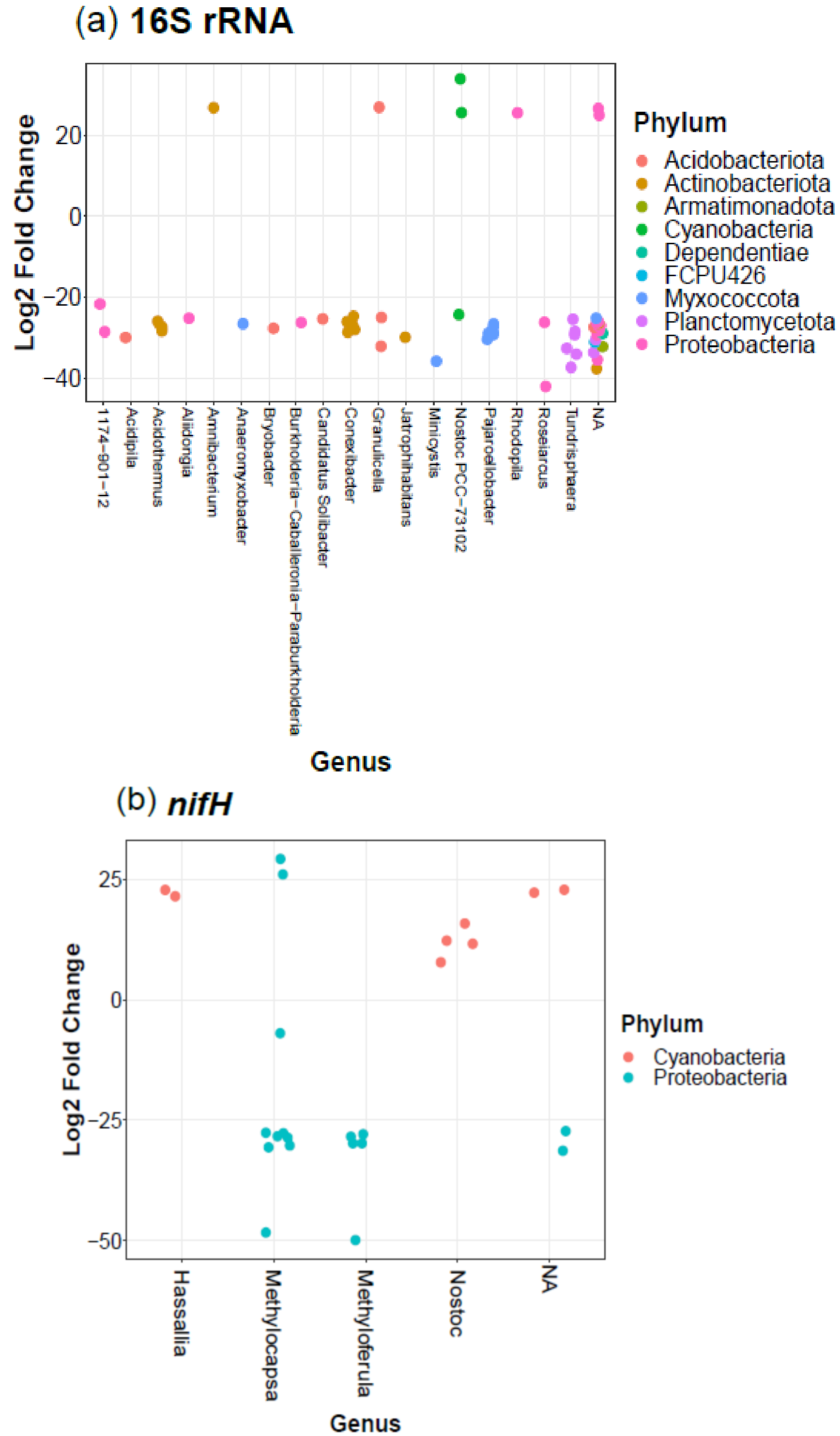


Figure S10. 16S rRNA- (a) and *nifH*-assigned (b) ASV differential abundance analysis between *Ptilium crista-castrensis* shoot sections of 1 cm and 6 cm from the apex. Samples were collected on S3 in September 2019.

Tableau S4. Characteristics of all the moss samples collected (species, date, and location of sampling), site parameters (mean monthly temperature, precipitation, location of the meteorological stations, tree density and total nitrogen deposition) and moss-related variables (nitrogen fixation, cyanobacterial biomass, nutrient concentrations) measured and included in this study.

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