Biological nitrogen fixation in ombrotrophic peatlands

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CONTENTS

List of Figures III

List of Tables VI

Abstract VIII

Résumé X

Acknowledgments XII

Contribution of authors XIV

Chapter 1 - Introduction 1

1.1 Research context 1

1.2 Research objectives 4

1.3 Study sites 6

CHAPTER 2 - Literature review 8

2.1 Nutrient limitation in ombrotrophic bogs 8

2.2 What is biological N_2 fixation? 10

2.3 *Sphagnum* mosses as hosts for microorganisms 11

2.4 The diversity of diazotrophs in *Sphagnum*-dominated peatlands 12

2.5 Measurements of N_2 fixation and potential error sources 15

2.6 Controls on N_2 fixation in peatlands 19

2.7 Conclusions 24

CHAPTER 3 - Seasonal and spatial variability of N_2 fixation at Mer Bleue bog, Ontario, Canada 25

3.1 Context within the thesis 25

3.2 Abstract 25

3.3 Introduction 26

3.4 Materials and Methods 29

3.5 Results 34

3.6 Discussion 35

3.7 Figures and tables 40

CHAPTER 4 - Nitrogen and phosphorus addition affects biological N_2 fixation and *Sphagnum* moss in an ombrotrophic bog 49

4.1 Context within the thesis 49

4.2. Abstract 49

4.3 Introduction 50

4.4. Materials and Methods 54
4.5. Results 58
4.6. Discussion 60
4.7 Figures and tables 67
CHAPTER 5 - Variations in nitrogen, phosphorus and $\delta^{15}N$ in *Sphagnum* mosses along a climatic and atmospheric deposition gradient in eastern Canada 74
5.1 Context within the thesis 74
5.2 Abstract 74
5.3 Introduction 75
5.4 Material and methods 78
5.5 Results 81
5.6 Discussion 84
5.7 Conclusion 89
5.8 Figures and tables 91
CHAPTER 6 - Biogeochemical controls of *Sphagnum*-associated N_2 fixation in bogs along a temperate, boreal and subarctic transect in eastern Canada 101
6.1 Context within thesis 101
6.2 Abstract 102
6.3 Introduction 102
6.4 Material and methods 107
6.5 Results 113
6.6 Discussion 115
6.7 Figures and tables 120
CHAPTER 7 – Conclusions and directions for future research 132
7.1 Chapter synthesis and larger context 132
7.2 Directions for future research 138
REFERENCES 141

List of Figures

Fig. 2.1 Abiotic and biogeochemical factors that may affect nitrogenase activity in peatlands and other high-latitude ecosystems (from Waughman & Bellamy, 1980; Jean *et al*., 2013; Rousk *et al.*, 2013; Lindo *et al*., 2013; Bellenger *et al*., 2014; Larmola *et al*., 2014). 23
Fig. 3.1 Daily means of a) air temperature, b) soil temperature at 5cm depth, c) water table depth and d) daily precipitation at Mer Bleue bog throughout two field seasons. 40
Fig. 3.2 Mean ARA rates over two field seasons across the hydrological transect at Mer Bleue bog. The vertical lines are standard errors. 41
Fig. 3.3 Relationship between a) soil temperature, b) water table depth and c) antecedent precipitation (14 d) and mean ARA per sampling date. Solid line shows the best fit linear regression and gray area is the uncertainty in the regression parameters (± 1 StDev). 41
Fig. 3.4 The relationship between ARA and gravimetric water content (GWC) in *Sphagnum*. Linear regression line: $Log_{10}(ARA) = 0.8 * Log_{10}(GWC) - 1.15$, $R^2 = 0.21$, n = 510, $p < 0.0001$. The gray shaded area indicates the uncertainty of the regression (±1 StDev for slope and intercept). 42
Fig. 3.5 Seasonal variation in slope of the regression between GWC and ARA per sampling date in 2013 (white circles) and 2014 (black circles). The line represents a polynomial fit: $y = -0.00018x^2 + 0.073x - 6.059$. 43
Fig. 3.6 The relationship between the regression slope between GWC and ARA and mean log_{10} ARA per sampling date. Regression line: $y = 2.73*x - 2.29$, $R^2 = 0.69$, n = 16, $p < 0.0001$. The gray area represents ± 95% CI. 44
Fig. S3.1 The relationship between the water table depth and gravimetric water content in *Sphagnum* moss at Mer Bleue in 2013 and 2014. Line represents a best fit model: $GWC = 401.9 + 2501.4 * e^{(-0.07*WTD(cm))}$, R^2 adj = 0.75, $p < 0.0001$. 46
Fig. S3.2 Box and whisker plot showing *Sphagnum* primary productivity over the two years (2013 and 2014). The vertical line depicts 25^{th} and 75^{th} percentile, horizontal line within the box is a median and points above and below vertical lines represent outliers. 47
Fig. S3.3 Primary production of *Sphagnum* mosses of hollow (HO) and hummock (HU) along the transect in 2013 (white circles) and 2014 (black circles) presented as box and whisker plot. The vertical line depicts 25^{th} and 75^{th} percentile, horizontal line within the box is a median. One-way ANOVA showed that there were significant differences between the plots ($F_{3,63} = 8.64$, $p<0.0001$, n = 66) and significant differences between means are depicted with capital letters above box plots (post-hoc Tukey's HSD test). 47
Fig. 4.1 The effects of NH_4 and NO_3 and NH_4NO_3 additions on **A)** N concentration, **B)** P concentration and **C)** N:P ratio in *Sphagnum* mosses. Error bars are standard errors of the mean. The letters above the bars are Tukey's HSD post hoc test: capital letters are differences between the treatments in the NH_4/NO_3 experiment, while italic letters in parenthesis represent differences between the treatments across both experiments. d) The power relationship between N concentration (mean ± SE per plot) in *Sphagnum* after 4 years of fertilization in relation to the annual N input, and calculated homeostatic regulation coefficients (H) for NH_4 and NO_3 treatment. 67

Fig. 4.2 Linear regression of N:P ratio as a function of **A)** N and **B)** P concentration in NH_4/NO_3 experiment..... 68
Fig. 4.3 **A)** *Sphagnum* NPP (mean ±SE, in g m^{-2} y^{-1}) over the field season April – November 2015 across the plots (ANOVA, $F_{6,14}$=4.43, p = 0.01, n = 21), capital letters represent post hoc Tukey's HSD test), **B)** *Sphagnum* NPP as a function of N:P ratio in NH_4 and NO_3 treatments... 69
Fig. 4.4 ARA rates (mean ±SE) plotted on a logarithmic scale as a function of nutrient treatment. Error bars are standard errors and letters above the bars are the result of Tukey's HSD *post hoc* analysis on combined dataset for all three experiments. DW is dry weight. 70
Fig. 4.5 ARA rates expressed as function of **A)** N, **B)** P and **C)** N:P ratio in *Sphagnum* tissue in NH_4 and NO_3 plots (NH_4/NO_3 experiment). 71
Fig. 4.6 Correlations between ARA plotted on the logarithmic scale and **A**) N, **B**) P and **C**) N:P when all three experiments are combined, and **D**) exponential decay model that best describes relationship between ARA and N:P ratio across all three experiments. 72
Fig. 5.1 Location of study sites. Source of information: © 2003. Government of Canada with permission from Natural Resources Canada. Data can be downloaded here: http://geogratis.gc.ca/api/en/nrcan-rncan/ess-sst/b754e4f9-3cdc-439e-9b4a-9aecd057a244.html. 91
Fig. 5.2 Relationships between (a) N concentration (b) P concentration and (c) N:P ratio (mean ± SEM) and latitude in three *Sphagnum* sections (Acutifolia, Cuspidata and Palustria)..... 92
Fig. 5.3 Relationships between *Sphagnum* N:P ratio and (a) N concentration and (b) P concentration in *Sphagnum* sections. 93
Fig. 5.4 Capitulum:stem ratio (mean ± SEM) for *Sphagnum* (a) N concentration, (b) P concentration, and (c) N:P ratio across sites for hummocks and hollows; asterisks (*) indicate significant differences between hummocks and hollows at each site. 94
Fig. 5.5 Relationship between (**a**) $N_{cap:stem}$ ratio and N_{stem} concentration, (**b**) $P_{cap:stem}$ ratio and P_{stem}, and (**c**) $N:P_{cap:stem}$ ratio and $N:P_{stem}$. 95
Fig. 5.6 Relationships between *Sphagnum* $\delta^{15}N$ value and (a) latitude for moss section, (b) N concentration and (c) water table depth. 96
Fig. 6.1 Map of selected bogs along the transect (MB = Mer Bleue, MIR = Mirabel, LLT = Lac à la Tortue, DES = Despinassy, NEL = Nellie, NOR = Norembega, VIC = Victor, SCH = Schefferville). All bogs but VIC and SCH were selected based on Turunen et al. (2004). Source of the base map: https://geoscan.nrcan.gc.ca/starweb/geoscan/servlet.starweb?path=geoscan/fulle.web&search1=R=288786..... 120
Fig. 6.2 The relationship between the mean N_2 fixation rates measured by ARA and $^{15}N_2$ tracer methods plotted on log10 scale. The dashed line represents the best fit linear regression with the bars representing the standard errors for each of the 32 *Sphagnum* samples. 121
Fig. 6.3 N_2 fixation rate (means ± SE) along the latitudinal gradient under optimal environmental conditions in the Phytotron. A two-way ANOVA showed that there was a significant interaction between the sites and microtopography on N_2 fixation rates ($F_{7,109}$ = 10.2, p<0.0001, N = 124). Post-hoc Tukey's HSD test depicted by the capital letters above the bars shows significant differences between the sites and microtopographic features, represented by the Cuspidata and Acutifolia sections, equivalent to hollows and hummocks.. 122

Fig. 6.4 The relationship between ARA rate and a) N concentration (mg g^{-1}), b) P concentration (mg g^{-1}) and c) N:P ratio within two *Sphagnum* sections (Cuspidata and Acutifolia) across the sites. 122
Fig. 6.5 The relationship between $^{15}N_2$ fixation rate and a) GPP and b) GM in sections Acutifolia and Cuspidata. Note that data in a) are shown on log-log scale, and in b) on the log-linear scale (linear x-axis). 123
Fig. 6.6 The distribution of *Sphagnum* N:P ratio in ombrotrophic bogs, derived from the literature and personal communications (N = 205). Literature accessed: Aerts *et al.* (1992), Bragazza *et al.* (2004), Bedford *et al.* (1999), Jiroušek *et al*. (2014), Malmer (1988), Moore (pers. comm.), Pakarinen & Gorham (1984), Pakrinen & Tolonen (1997), Pakarinen (1978a, b), Phuyal *et al.* 2008, van der Heijden *et al.* (2000), Živković *et al.* (2017), Wang (pers. comm.). 124
Fig. 6.7 The range of N and P concentration (mg g^{-1}) from this study (blue circles) superimposed on global distribution on N and P concentration in *Sphagnum* from ombrotrophic bogs (gray circles, for references see Fig.6.6). Blue gradient shows log_{10} scaled ARA N_2 fixation rates. .. 125
Fig. S6.1 The relationship between N concentration (mg g^{-1}) of *Sphagnum* samples obtained by acid digestion and by mass spectrometer. Each value is a mean of 5 subsamples used for ARA and 4 subsamples for $^{15}N_2$, with standard error bars. The dotted line is 95 % confidence interval. 127
Fig. S6.2 The variability of NEE, R and GPP (mean ± SE) of *Sphagnum* sections (Cuspidata and Acutifolia) along the transect. 128
Fig. S6.3 Box and whisker plot showing GM (%) in hollows (HO; Cuspidata) and hummocks (HU; Acutifolia). Student t-test showed that HO > HU ($t(226.1) = -7.02$, $p < 0.0001$, $n = 257$). Horizontal line within the box represents median, while vertical lines represent 25^{th} and 75^{th} percentile, and dots over the vertical lines are outliers. 129
Fig. S6.4 The relationship between the GPP and a) N concentration, b) P concentration and c) N:P ratio in *Sphagnum* sections. 130
Fig. 7.1 Abiotic and biogeochemical factors that affect N_2 fixation in peatlands; **in bold** are the factors studied throughout this thesis. 132

List of Tables

Table 2.1 Studies showing conversion factor (R=ARA/$^{15}N_2$) in different *Sphagnum* spp. SE indicates standard error 19
Table 3.1 Multiple linear regression statistics of N_2 fixation model for Mer Bleue bog. Adj R^2 = 0.52, n = 16, p = 0.0033. 44
Table 3.2 Linear regression statistics of the relationship between GWC (independent variable) and ARA (dependent variable) for each sampling date. 45
Table S3.1 Mean annual ARA rates from both field seasons, 2013 and 2014, calculated for each microform (hummock, hollow and pond) and expressed in µmol m^{-2} d^{-1} units. Small letters (a-c) in the parenthesis represent significant differences between ARA rates among three different microforms (post-hoc Tukey's HSD; one-way ANOVA on log_{10} transformed ARA; $F_{2,502}$ = 48.12, p < 0.0001, n = 505). 48
Table S3.2 Four scenarios to weight surface area and N_2 fixation annual rate estimates at Mer Bleue bog. 48
Table 4.1 The fertilization experimental design and annual nutrient additions (g m^{-2} y^{-1}). 73
Table 4.2 Means (±SE in parentheses) of *Sphagnum* N and P concentration and N:P ratio in PK fertilization plots (Experiment 3; Mann-Whitey's U test significance of P < 0.0001 is depicted in **bold**, n = 15). 73
Table 5.1 Site location (and abbreviation), latitude and longitude, mean annual temperature (MAT, and in parentheses growing degree days above 5° C; GDD), mean annual precipitation (MAP) and in parentheses growing season precipitation (GSP) and snowfall precipitation (in *italics*, *SP*) 1981-2000, pore water pH, estimated total atmospheric N and P deposition, and hummock and hollow water table depth beneath surface at time of sampling (with SD in the parentheses), *Sphagnum* section dominating hummocks and hollows. 97
Table 5.2 The results of multiple regression models predictors for N content a) excluding MAT, b) excluding N_{dep} and for P content in Sphagnum along the gradient c) excluding MAT and d) excluding P_{dep}. 98
Table 5.3 Mean (± SEM) for N and P concentrations and N:P ratio by site and overall. Significant differences among hummocks (Hu) and hollows (Ho) (Mann-Whitney U test, p < 0.05, n = 10) by site and overall are indicated in bold. 99
Table S5.1. Pearson's (rho) correlation between the environmental variables (MAT, MAP) and latitude, and N and P deposition (N_{dep} and P_{dep}). 100
Table S5.2. The results of multiple regressions testing the variance of the content of a) N and b) log10P in *Sphagnum* using environmental (MAT, MAP) variables and atmospheric deposition (N_{dep} or P_{dep}) in moss sections (Acutifolia and Cuspidata). 100
Table 6.1 Linear mixed effect model testing of relationships between N_2 fixation by ARA and a) N concentration, b) P concentration and c) N:P. Models structure is: N_2 fixation ~ X_{1-3} + SECTION + (1 + X_{1-3}|SITE), where X_1=N, X_2=P and X_3=N:P. Significant values are in **bold** (P < 0.05). 126
Table 6.2 Best fit model showing the relationship between $^{15}N_2$ fixation and GPP and GM across the transect ($^{15}N_2$ fixation ~ GPP + GM + (1|SECTION/SITE). Significant values are in **bold** (P < 0.05). 126

Table 6.3 Results showing best fit model $^{15}N_2$ fixation ~ GPP + GM + (1|SITE) for a) Acutifolia and b) Cuspidata. For each model Akaike information criterion (AIC) and Bayesian information criterion (BIC) are presented. Significant values are in **bold** ($P < 0.05$). 126
Table S6.1 Description of variables (mean ± standard deviation (SD), sample size (n)) used for data analyses. 131

Abstract

The main nitrogen (N) sources to *Sphagnum*-dominated, ombrotrophic peatlands (*i.e.* bogs) are biological fixation and atmospheric deposition. N_2 fixing microbes (diazotrophic prokaryotes) associated with *Sphagnum* can supply the N required to support *Sphagnum* primary production and help explain long-term N accumulation rates in peat. Despite the importance of N_2 fixation in ombrotrophic bogs, environmental and biogeochemical controls on N_2 fixation rates are still poorly constrained. Furthermore, it remains unclear how the changes in nutrient status in *Sphagnum* due to increased atmospheric N deposition affect N_2 fixation in ombrotrophic bogs. Therefore, the aims of this thesis were to quantify the importance of biological N_2 fixation associated with *Sphagnum* mosses as N input to ombrotrophic bogs and to identify its main ecological drivers.

I investigated the environmental controls of seasonal and spatial variability of N_2 fixation within the Mer Bleue ombrotrophic bog. Soil temperature and precipitation best explained seasonal variability, whereas gravimetric water content in *Sphagnum* explained spatial variability of N_2 fixation. About 20% of annually accumulated N in *Sphagnum* can be attributed to N_2 fixation at Mer Bleue bog, that together with atmospheric N deposition supports, *Sphagnum* growth. However, in unpolluted bogs, N_2 fixation likely becomes the dominant source of N.

In addition, my thesis focuses on effects of human-induced atmospheric deposition on *Sphagnum* nutrient (N and P) content and N:P ratio, and on the associated diazotrophic activity. N_2 fixation was inhibited by N additions, but the inhibition depended on neither the amount nor N species (NH_4 vs NO_3 vs NH_4NO_3) added in a fertilization at the Mer Bleue bog. However, the addition of P significantly increased N_2 fixation rates. Changes in N:P ratio in *Sphagnum*

represented the best N_2 fixation predictor in this study indicating that changes in atmospheric N and P deposition may be important drivers of N_2 fixation.

These relationships were further tested on a larger geographical scale along a latitudinal ombrotrophic bog transect, where natural variability of N and P concentration and $\delta^{15}N$ in *Sphagnum* was studied. N and P concentrations in *Sphagnum* suggest the existence of a decreasing deposition gradient towards the north, and rising $\delta^{15}N$ values from ~-6‰ in the south to ~-1‰ in the north indicate that N_2 fixation becomes more dominant N source in less polluted bogs. I identified N:P ratio as the strongest biogeochemical control on N_2 fixation in *Sphagnum* with the best fit model explaining about two thirds of the N_2 fixation variance along the transect. N_2 fixation rates decreased with increasing N:P ratio in *Sphagnum*. The range of N:P ratio along the transect covers well the range of N:P ratios found in bogs around the world, suggesting a small potential for N_2 fixation rates when N:P > 16 (indicative of P-limitation in the bog). Additionally, photosynthesis was positively related to N_2 fixation in *Sphagnum* species typical of wet microforms, directly connecting the C and N cycle in bogs. Given that *Sphagnum* N:P ratio can directly be impacted by changes in atmospheric deposition, any changes of N or P inputs into bogs will affect N_2 fixation and potentially alter C cycle and storage dynamics.

The results of this thesis contribute to a better understanding of the main controls on N_2 fixation in ombrotrophic bogs, but also highlight the complexity of *Sphagnum*-diazotrophic interactions. My work demonstrates that the N_2 fixation process intimately couples the C, N and P cycle in peatlands.

Résumé

Les sources principales d'azote (N) dans les tourbières ombrotrophes (bogs) sont la fixation biologique et le dépôt atmosphérique. Les microorganismes fixateurs de N_2 (procaryotes/diazotrophes) associés aux sphaignes peuvent fournir le N qui est requis pour soutenir la productivité primaire des sphaignes et aident à expliquer les taux d'accumulation de N à long terme dans la tourbe. Les contrôles environnementaux et biogéochimiques de la fixation du N_2 sont méconnus, malgré l'importance de ce processus dans les bogs. De plus, les impacts d'une augmentation du dépôt atmosphérique de N sur la fixation du N_2 dans les bogs restent incertains. Les buts de cette thèse sont de quantifier l'importance de la fixation du N_2 associée aux sphaignes comme source de N dans des bogs ombrotrophes et d'identifier les principaux contrôles de cette fixation.

J'ai étudié les contrôles environnementaux de la variabilité saisonnière et spatiale de la fixation du N_2 dans un bog ombrotrophe (Mer Bleue). La température du sol et la précipitation expliquent le mieux la variabilité saisonnière alors que la teneur en eau gravimétrique des sphaignes explique la variabilité spatiale. Dans le bog Mer Bleue, environ 20% du N accumulé dans les sphaignes annuellement provient de fixation du N_2, ce qui soutient la croissance de sphaigne conjointement avec le dépôt atmosphérique. Cependant, dans les bogs non-pollués, la fixation biologique de N_2 devient probablement la source dominante de N.

Ma thèse s'est aussi penchée sur les effets du dépôt atmosphérique d'origine anthropique sur la teneur en nutriments (N et P) et le ratio N:P des sphaignes, et sur l'activité diazotrophe. La fixation du N_2 était diminuée par l'addition de N mais la diminution ne dépendait pas de la quantité ni de l'espèce de N (NH_4 vs NO_3 vs NH_4NO_3) ajoutée dans l'essai de fertilisation au bog Mer Bleue. Au contraire l'addition de P augmentait les taux de fixation du N_2. Les changements

dans le ratio N:P dans les sphaignes représentaient le meilleur indicateur de fixation du N_2, impliquant que les changements dans le dépôt atmosphérique de N et P peuvent être des contrôles importants de la fixation du N_2.

Par la suite, ces liens ont été testés sur une échelle géographique plus étendue, où la variabilité naturelle des concentrations de N et P et de $\delta^{15}N$ dans les sphaignes ont été mesurés le long d'un transect latitudinal de bogs. Les concentrations de N et P dans les sphaignes suggèrent qu'il existe un gradient de déposition décroissant du sud au nord. Les valeurs de $\delta^{15}N$ augmentent de ~-6‰ dans le sud à ~-1‰ dans le nord et indiquent que la fixation du N_2 devient une source dominante de N dans les bogs moins pollués. Le ratio N:P semble être le contrôle biogéochimique majeur de la fixation du N_2, expliquant deux tiers de la variance le long du transect. Les taux de fixation du N_2 diminuaient avec des ratios N:P croissants dans les sphaignes. La gamme des ratios N:P le long du transect est semblable à la gamme trouvée dans les bogs à travers le monde, ce qui suggère qu'il y a un potentiel faible de fixation de N_2 lorsque les ratios N:P > 16 (indiquant une limitation en P). Les taux de photosynthèse étaient en outre liés de façon positive aux taux de fixation du N_2 dans les espèces de sphaigne typiques des microformes humides, ce qui lie directement les cycles du carbone (C) et du N dans les bogs. Le ratio N:P dans les sphaignes étant directement lié aux taux de dépôt atmosphérique, les changements des intrants de N et P dans les bogs ont le potentiel de modifier le cycle et les stocks de C.

Les résultats de cette thèse contribuent à l'avancement des connaissances sur les contrôles de la fixation du N_2 dans les bogs, tout en soulignant la complexité des interactions entres les sphaignes et les diazotrophes. Mon travail montre que le processus de fixation du N_2 participe au couplage des cycles de C, N, et P dans les tourbières.

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Contribution of authors

There are four main chapters (Chapters 3-6) in this thesis that are prepared as manuscripts following the standard requirements for publication in scientific journals. I have developed scientific questions and designed experiments, conducted the field and laboratory analyses, analyzed the data and interpreted results, and wrote manuscripts as a lead author. Dr. Tim R. Moore offered the advice on developing research questions and designing experiments, supported laboratory and field experiments (including designing and maintaining long- and short-term fertilization experiments at Mer Bleue bog), and provided detailed feedback on each manuscript. The roles of other co-authors (for Chapters 3, 4 and 5) are described as follows:

Manuscript 1 (Chapter 3): "**Seasonal and spatial variability of N_2 fixation at Mer Bleue bog, Ontario, Canada**" by Tatjana Živković, Manuel Helbig and Tim R. Moore. Manuel Helbig provided advice on data analyses and delivered feedback on the first draft of the manuscript.

Manuscript 2 (Chapter 4): "**Nitrogen and phosphorus addition affects biological N_2 fixation and *Sphagnum* moss in an ombrotrophic bog**" by Tatjana Živković, Alice Ardichvili and Tim R. Moore. Alice Ardichvili helped with laboratory analyses.

Manuscript 3 (Chapter 5): "**Variations in nitrogen, phosphorus and $\delta^{15}N$ in *Sphagnum* mosses along a climatic and atmospheric deposition gradient in eastern Canada**" by Tatjana Živković, Kristina Disney, and Tim R. Moore. Kristina Disney helped with laboratory analyses on N and P content in *Sphagnum*. Chapter 5 has been published in Botany in 2017, 95(8): 829-839, https://doi.org/10.1139/cjb-2016-0314.

Manuscript 4 (Chapter 6): "**Biogeochemical controls of *Sphagnum*-associated N_2 fixation in bogs along a temperate, boreal and subarctic transect in eastern Canada"** by Tatjana Živković and Tim R. Moore.

Chapter 1

Introduction

1.1 Research context

Peatlands are wetland ecosystems that accumulate at least 30-40 cm deep partially decomposed organic matter (peat). While peatlands cover only 3% of the global land surface they store about 25-30% of world's soil carbon (C) (Yu *et al.*, 2010). Owing to cool temperatures and short growing seasons in northern latitudes (> 45°N), net primary productivity exceeds slow decomposition rates resulting in about 80% of the global peatland C being stored in northern latitudes. Northern peatlands are also large sinks of nitrogen (N) containing between 9 and 18 Pg of N (Limpens *et al.*, 2006; Loisel *et al.*, 2014). However, most N is captured in dead organic matter (peat) that is unavailable to plants and microbes. Given that N represents a primary limiting element for plant growth in peatlands (Aerts *et al.*, 1992), additional sources of N are required to sustain peatland productivity. This is especially true for *Sphagnum*-dominated, strongly acidic, ombrotrophic peatlands (i.e., bogs) that solely depend on nutrients deposited via atmospheric deposition. In Canada, two thirds of peatlands are bogs, which are located mostly in boreal and subarctic regions (Tarnocai, 2006).

Vegetation productivity in bogs that receive low levels of atmospheric N deposition (< 0.1 g N m^{-2} y^{-1}; Vitt *et al.*, 2003) primarily relies on biological nitrogen (N_2) fixation, a microbially-driven process performed by metabolically diverse N_2 fixing (diazotrophic) communities. Diazotrophs live freely or in association within dominant *Sphagnum* mosses though the nature of these microbe-plant interactions remains unknown. However, N_2 fixation sustains primary productivity (Vile *et al.*, 2014) and is responsible for historical N accumulation (Larmola *et al.*, 2014), indicating a strong connection between the C and N cycle in peatlands.

Despite the importance of N_2 fixation in ombrotrophic bogs, environmental controls on N_2 fixation rates are still poorly constrained. Often, parallels to potential drivers and rates of N_2 fixation are drawn from other nutrient-poor, well-studied ecosystems such as feather moss-cyanobacteria interactions in boreal forest and Arctic biocrusts. For example, in the high Arctic, soil moisture and temperature are significant environmental drivers of N_2 fixation (Rousk *et al.*, 2018). Low soil moisture has been identified as the strongest inhibitor of N_2 fixation in both subarctic and arctic regions (Zielke *et al.*, 2005; Stewart *et al.*, 2011c, 2014; Rousk *et al.*, 2018). While warm temperatures (up to 30 °C) can promote N_2 fixation in High Arctic tundra soils (Hobara *et al.*, 2006), Rousk *et al.* (2018) showed that temperatures below 14 °C in the same ecosystem can provide optimal conditions for diazotrophic activity. In *Sphagnum capillifolium* stands in boreal forest, temperature has been found to have a strong positive effect on N_2 fixation while precipitation had no significant effect (Markham, 2009). A temperature optimum of 25°C for N_2 fixation rates in *Sphagnum* peat was found by Kravchenko & Doroshenko (2003), while in subarctic *Sphagnum* this optimum was at 16 °C (Basilier & Granhall, 1978). However, little is known about how these environmental controls affect *Sphagnum*-diazotroph interactions in nutrient-poor bogs and no study thus far has identified the primary controls on N_2 fixation rates within ombrotrophic bogs. Bogs differ from other northern ecosystems in their vegetation and microtopography patterns, water table dynamics, and in the variety of diazotrophic communities involved in N_2 fixation (Bragina *et al.*, 2013), which may promote different environmental drivers of N_2 fixation rates. In Chapter 3, I seek to identify environmental controls on N_2 fixation rates in ombrotrophic bogs by examining *in situ* N_2 fixation rates at Mer Bleue bog peatland with varying microtopography (hummock, hollow and pond) over two field seasons (from May to November).

The post-industrial increase in atmospheric N deposition has impacted nutrient-limited ecosystems world-wide (Galloway *et al.*, 2008), whereby additional N sources often cause a switch from N- to P-limitation (Vitousek *et al.*, 2002). Effects of N deposition on C cycling, vegetation shifts and *Sphagnum* stoichiometry in peatlands have been studied (Wang & Moore, 2014). Larger N concentrations and N:P ratios in *Sphagnum* mosses are directly related to increased N deposition in ombrotrophic bogs along N deposition gradients (Bragazza *et al.*, 2004, 2005) and long-term fertilization experiments (Skinner *et al.*, 2006; Bubier *et al.*, 2007; Juutinen *et al.*, 2010; Sheppard *et al.*, 2013). Given the associative nature between *Sphagnum* and diazotrophs, nutrient changes in moss hosts may impact diazotrophic activity by changing nutrient availability for the N_2 fixation process. Because N_2 fixation is an energetically high-cost enzymatic process, a "nitrostat" theory suggests that when enough N is available, N_2 fixation will be "switched off", and "switched on" if N is lacking (Menge & Hedin, 2009). In addition, diazotrophs require P to store energy in P-rich adenosine-tri-phosphate (ATP) molecules that supports among other metabolic processes also N_2 fixation. P is therefore often a limiting nutrient for diazotrophic functioning across ecosystems. In *Sphagnum*-dominated peatlands, P addition is always associated with larger N_2 fixation rates (Kox *et al.*, 2016; van den Elzen *et al.*, 2017). In boreal forests, a similar effect has been observed (Rousk *et al.*, 2017a). However, only very few studies address the effects of N additions on *Sphagnum*-associated N_2 fixation and results have been inconsistent. While Kox *et al.* (2016) found N additions to decrease N_2 fixation in a laboratory setting, a long-term field N addition showed no effect on N_2 fixation in *Sphagnum* (van den Elzen *et al.*, 2018).

These conflicting results on nutrient limitation of the N_2 fixation process indicate that a new framework for N_2 fixation in peatlands may be required. Rather than investigating the

effects of individual nutrient contents of N and P, the N:P ratio in *Sphagnum* may better explain the variability in N_2 fixation rates. My thesis tests this framework in two separate experiments: 1) investigating the effects of nutrient (NH_4, NO_3 and NH_4NO_3 and PK) addition on N_2 fixation rates at the Mer Bleue bog (Chapter 4), and 2) establishing the relationship between N_2 fixation and N:P ratio in *Sphagnum* from two microtopographic features (hummocks and hollows) along a transect ranging from cool temperate to boreal and subarctic bogs (Chapters 5 and 6).

Lastly, N_2 fixation may be regulated by the host's energy demands to sustain its productivity (net ecosystem exchange [NEE], respiration [R] and gross primary production [GPP]). Assuming a tight association between *Sphagnum* and diazotrophs, there should be a constant host-microbe exchange of energy and nutrients. Such exchange of nutrients and chemical signaling has been recently observed in feather moss-cyanobacteria symbiosis (Bay *et al.*, 2013). Only one study thus far has established that N acquired via N_2 fixation is used toward building new *Sphagnum* biomass (Berg *et al.*, 2012), directly linking C and N cycles in peatlands. It is thus possible that a larger demand for N during the active photosynthesis in *Sphagnum* signals microbes to increase their diazotrophic activity. Alternatively, newly fixed C via photosynthesis could provide additional energy for N_2 fixation activity resulting in both cases in a positive relationship between the GPP and N_2 fixation rates. In my thesis, I test this relationship in *Sphagnum* hummocks and hollows from bogs along the transect in an environmentally-controlled setting (Chapter 6).

1.2 Research objectives

My research aims to elucidate the importance of biological N_2 fixation associated with *Sphagnum* mosses as an N input in ombrotrophic bogs and to identify its main ecological drivers. More specifically, I investigate environmental (water availability, temperature, and precipitation)

and biogeochemical drivers (*Sphagnum*'s N and P content and stoichiometry and photosynthesis) of N_2 fixation in bogs. I also study how human-induced nutrient changes in atmospheric deposition impact *Sphagnum* nutrient (N and P) content and N:P ratio, and the associated diazotrophic activity. Finally, I explore the role of the N_2 fixation process in coupling the C, N and P cycle in peatlands.

I have structured my thesis into four research chapters to address the following research objectives.

(1) To assess the importance of soil moisture, water table, temperature, and precipitation as drivers of N_2 fixation in ombrotrophic bogs, I carried out a systematic two-year, field study along a hydrological gradient at Mer Bleue bog. The hydrological gradient reflects common bog microtopographical features of hummocks, hollows, and pond. (Chapter 3)

(2) Short- and long-term fertilization experiments at the Mer Bleue bog provided a unique opportunity to test how anthropogenic changes of atmospheric deposition (incremental increases in NH_4, NO_3, NH_4NO_3, and P) change nutrient stoichiometry in *Sphagnum* and affect N_2 fixation rates in bogs. Here, the ratio of N and P rather than each nutrient individually emerges as the strongest explanatory variable of N_2 fixation in *Sphagnum* under controlled environmental conditions in the laboratory. (Chapter 4)

(3) Across a geographical transect, I analyzed *Sphagnum* nutrient stoichiometry and $\delta^{15}N$ natural abundance in hummocks and hollows in eight ombrotrophic bogs. In this study, I explore the variability of N and P along the gradient and whether $\delta^{15}N$ in *Sphagnum* carries information on the dominance of N_2 fixation as N input in bogs. (Chapter 5)

(4) Along the transect of temperate, boreal and subarctic bogs in eastern Canada (established in Chapter 5), I assess how *Sphagnum* N and P content and N:P ratio control N_2 fixation

rates. Furthermore, I explore the direct interactions between N and C cycles through diazotrophy by testing the relationship between *Sphagnum* photosynthesis and N_2 fixation activity under controlled conditions. Lastly, I establish a relationship between two widely used measurement techniques for N_2 fixation rates: the $^{15}N_2$ tracer and the acetylene reduction assay (ARA) methods. (Chapter 6)

1.3 Study sites

Part of my research (Chapters 3 and 4) was conducted at the Mer Bleue bog (45.41 °N, 75.52 °W), located 10 km east of Ottawa, Canada. Mer Bleue peatland covers about 28 km^2 and has a mean annual temperature and precipitation of 6°C and 943 mm, respectively (Canadian Climate Normals 1981-2010). The wet inorganic background N deposition was estimated to range from 0.6 – 0.8 g N m^{-2} y^{-1} (Turunen *et al*., 2004; Vet *et al.*, 2014) and P deposition ranges from 6 – 26 mg m^{-2} y^{-1} (Tipping *et al.*, 2014). A large portion of the peatland is a raised ombrotrophic bog dominated by *Sphagnum* moss organized in a typical hummock-hollow microtopography. The bog drains into a beaver pond with floating mosses on its edges. At Mer Bleue bog, dominant vegetation includes shrubs *Chamaedaphne calyculata* Moench, *Rhododendron groenlandicum* (Oeder) K.A. Kron & W.S. Judd, *Vaccinium myrtilloides* Michx. and *Kalmia angustifolia* L. with occasional sedge clusters of *Eriophorum vaginatum* L. Trees are growing sparsely throughout the bog comprising *Larix laricina* (Duroi) K. Koch., *Betula populifolia* Marshall and rarely *Picea mariana* (Miller) BSP (Bubier *et al.*, 2006). Hummocks are predominantly covered by *Sphagnum capillifolium* and *S. magellanicum*, and less by *Polytrichum strictum* (Bubier *et al.*, 2006). Hollows are dominated by *S. angustifolium* and *S. fallax*, while floating mats of *S. majus* and *S. cuspidatum* dominate pond edges. Pond edges are similar to rich fens and freshwater marshes where *Juncus effuses* L. and *Typha latifolia* L. occur

(Bubier *et al.*, 2003). For Chapter 3, I selected a transect that depicts a hydrological gradient from the pond through to the center of the bog dome and includes 3 microtopographic features: hummocks, hollows and floating *Sphagnum* mats in the beaver pond. In Chapter 4, I use plots from established short- and long-term fertilization experiments where increments of NH_4, NO_3 and NH_3NO_3, and PK have been added to study the effects of increased N and P deposition on N_2 fixation rates.

For Chapters 5 and 6, I selected eight ombrotrophic peatlands along a latitudinal transect in eastern Canada (Fig.5.1, Fig.6.1). The peatlands are characterized by an open tree canopy of *P. mariana* (Miller) BSP, and occasional *L. laricina* (Duroi) K. Koch. and *B. populifolia* Marshall. Similar to Mer Bleue bog, most common shrubs were *C. calyculata* Moench, *R. groenlandicum* (Oeder) K.A. Kron & W.S. Judd, *V. myrtilloides* Michx. and *K. angustifolia* L. and sedge, *E. vaginatum* L. (Turunen *et al*., 2004). Hummocks of subarctic and boreal zone bogs are covered by *Sphagnum fuscum* and *S. capillifolium* while most southern bogs have predominantly *S. capillifolium*. Hollows are generally covered by *S. angustifolium*.

CHAPTER 2

Literature review

2.1 Nutrient limitation in ombrotrophic bogs

Ombrotrophic bogs are acidic, *Sphagnum*-dominated peatlands that receive water and nutrients only through wet and dry atmospheric deposition. *Sphagnum* decomposes slowly and productivity exceeds decomposition resulting in the accumulation of organic matter as peat. *Sphagnum* growth in ombrotrophic bogs exposed to low N atmospheric inputs (< 0.1 g m^{-2} y^{-1}) is thought to be N limited (Aerts *et al.*, 1992; Gunnarsson & Rydin, 2000; Vitt *et al.*, 2003). To alleviate N limitation of *Sphagnum* in ombrotrophic bogs, microbes reduce atmospheric, unreactive N_2 gas to bioavailable form of N (ammonium), a process called biological N_2 fixation. N_2 fixing prokaryotes (diazotrophs) have been found in bogs close to the surface associated with *Sphagnum* (Vile *et al.*, 2014) and deeper in the peat profile (Krumholz *et al.*, 1995; Knorr *et al.*, 2015). N_2 fixation thus supports primary production of *Sphagnum* and is responsible for long-term accumulation of N in peat at the rate of 0.5 ± 0.04 g m^{-2} y^{-1} (Larmola *et al.*, 2014; Loisel *et al.*, 2014; Vile *et al.*, 2014).

The second most important macronutrient, P, primarily enters bogs via dust deposition (Vitousek *et al.*, 2010; Tipping *et al.*, 2014; Toberman *et al.*, 2015) and heavily depends on recycling of P within the peat column (Wang *et al.*, 2014). Given that there is no known biological process of P fixation, and no mineral or weathering sources of P, bog vegetation is not only N-limited, but often either NP-co- or P-limited. Consequently, *Sphagnum* growth and NPP has been found to be stimulated by the addition of P in bogs (Limpens *et al.*, 2004). The switch from N to P limitation of *Sphagnum* growth is often triggered in bogs receiving increased atmospheric N deposition since the beginning of the Industrial Revolution in the mid-19th

century. P content has been found to be closely related to peat N content across ombrotrophic bogs in the UK and world-wide, suggesting coupling of N_2 fixation to P availability in bogs (Toberman *et al.*, 2015). P availability can also alleviate the negative effects of N deposition on *Sphagnum* in ombrotrophic bogs (Limpens *et al.*, 2004).

Since the Industrial Revolution, N emissions in the form of dissolved inorganic N (DIN; nitrate NO_3^- and ammonium NH_4^+) and organic N (urea, amines) have increased as a direct consequence of fossil fuel combustion and agricultural practices (Neff *et al.*, 2002; Galloway *et al.*, 2004; Dentener, 2006). DIN is believed to represent one of the most dominant N inputs to ecosystems world-wide (Galloway *et al.*, 2008). The effects of increased N deposition on ecosystem functioning in peat bogs have been extensively studied. *Sphagnum* exposed to increased N deposition is characterized by larger N concentrations and increased N:P ratio of the moss tissue (Bragazza *et al.*, 2004; Wang *et al.*, 2016). At large N deposition rates (> 2 g m^{-2} y^{-1}), *Sphagnum* becomes N saturated (Lamers *et al.*, 2000; Limpens *et al.*, 2006), and leaches N into the rhizosphere where it becomes available to vascular plants (e.g. graminoids and shrubs). Vascular plants can then outgrow and outcompete *Sphagnum* (Bubier *et al.*, 2007; Juutinen *et al.*, 2010). Ultimately, large long-term N deposition depresses C sequestration rates (Berendse *et al.*, 2001; Bubier *et al.*, 2007; Juutinen *et al.*, 2010), lowers *Sphagnum* production (Gunnarsson & Rydin, 2000; Berendse *et al.*, 2001), induces loss of *Sphagnum* (Juutinen *et al.*, 2010), and promotes net C loss from peat bogs (Bragazza *et al.*, 2006).

The effects of increased N deposition on N_2 fixation in ombrotrophic bogs are still not fully understood. This review focuses on identifying: 1) the role of biological N_2 fixation in ombrotrophic bogs and its relationship to *Sphagnum*, 2) current knowledge of diazotrophs involved in N_2 fixation in bogs, 3) main environmental and biogeochemical drivers, including

nutrients (N and P) in *Sphagnum* and their stoichiometry, and 4) possible measurement errors arising from the complexity of the N_2 fixation process.

2.2 What is biological N_2 fixation?

Biological N_2 fixation represents an important biogeochemical reaction across a wide range of ecosystems (Cleveland *et al.*, 1999; Vitousek *et al.*, 2002), in which specialized prokaryotes (diazotrophs) reduce atmospheric N_2 gas into bioavailable ammonium (NH_4^+). In this form, N is used by plants and microbes allowing their survival and increasing their competitiveness in N-poor environments (Houlton *et al.*, 2008).

N_2 fixation is an energetically costly process (dissociation energy of 940 kJ) as six electrons must be transferred to break the triple bond between N atoms in the N_2 molecule to reduce it to 2 NH_3. It requires 16 molecules of adenosine-tri-phosphate (ATP) making the process particularly dependent on P. The overall chemical reaction is:

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

Nitrogenase, an enzyme necessary for this process, has been found in a variety of free-living and symbiotic prokaryotes (Howarth *et al.*, 1988; Fisher & Newton, 2002). The most commonly occurring nitrogenase, molybdenum (Mo-) nitrogenase, is a complex of two distinct metalloproteins, both containing iron (Fe) in their reactive centers, and one also containing molybdenum (Mo). Two alternative nitrogenase enzymes, vanadium (V-) and iron (Fe-) nitrogenase are expressed in some diazotrophs when starved of Mo (Bishop *et al.*, 1982; reviewed in Fisher & Newton, 2002). The cluster of homologous *nif* genes has been identified in encoding Mo-nitrogenase, while *vnf* and *anf* genes are found in diazotrophs with alternative V- and Fe-nitrogenases, respectively. Genes for alternative nitrogenases have been found in

diazotrophs living in soils in terrestrial ecosystems (Bellenger *et al.*, 2014). In peat bogs, most studies focused on identifying *nif* genes (Dedysh *et al.*, 2006; Zadorina *et al.*, 2009; Bragina *et al.*, 2012b) while no study thus far has examined alternative nitrogenases.

2.3 *Sphagnum* mosses as hosts for microorganisms

Sphagnum is often described as an ecosystem engineer due to its unique morphological and physiological properties (van Breemen, 1995; Turetsky, 2003). Morphological features, such as lack of roots and the presence of hyaline cells, give *Sphagna* an advantage over vascular plants in nutrient limited environments. Mature hyaline cells are large (on average 200 μm x 30 μm x 30 μm) and empty dead cells organized around small chlorophyllous cells. Hyaline cells possess pores allowing free passage of water and microorganisms within the *Sphagnum* tissue, and between *Sphagnum* and the surrounding environment. Hyaline cells in *Sphagnum* are responsible for its ability to hold water and nutrients and to maintain moisture during dry periods (Hájek & Vicherová, 2014). Upward capillary water transfer outside the moss tissue is possible due to the large density of *Sphagnum* shoots and branches that radiate around the shoots. *Sphagnum* in peat bogs therefore entraps water and nutrients and provides a suitable environment for microbes. *Sphagnum* is thought to acidify its environment due to the presence of a large number of cation exchange sites in the walls of hyaline cells (Verhoeven & Liefveld 1997; Vicherová *et al.*, 2017). These specialized ion channels take cations from the pore water and replace them with protons (H^+), which are then released into the pore water lowering the pH. As *Sphagnum* decomposes, it releases humic acids that additionally lower the pH to values ranging from 3.5 to 4.5 in bogs (Vitt *et al.*, 2000). Less acidic conditions within hyaline cells compared to the pore water allow microbes to seek refuge inside the *Sphagnum* tissue (Granhall & Hofsten, 1976).

2.4 The diversity of diazotrophs in *Sphagnum*-dominated peatlands

A large diversity of microbial communities associated with *Sphagnum* mosses has been found across peatland types (ombrotrophic bogs, oligotrophic and minerotrophic fens) and microtopographies (Zadorina *et al.*, 2009; Kip *et al.*, 2010). *Sphagnum* mats in peatlands form typical microtopograhical features (microforms) that reflect their distance from the water table (Belyea & Clymo, 2001): 1) hummocks (dry mounds) represent areas with the largest distance between the moss surface and the water table and are usually dominated by *Sphagnum fuscum* and *S. capillifolium* in eastern Canada, 2) hollows and lawns (wet depressions) have the smallest distance to the water table (10-20 cm) and are occupied mostly by *S. angustifolium*, *S. fallax*, and *S. riparium*, 3) intermediate zones (edges of hummocks and lawns) are dominated by *S. magellanicum* and *S. papillosum*, and 4) pools are usually dominated by floating mats of *S. cuspidatum* and *S. majus*. Although *Sphagnum* species occurrence varies between regions and peatland types, morphological similarities and similar water and nutrient availability lead to similar *Sphagnum* species assemblage patterns. Similarly, the diversity of microbiota within *Sphagnum* seems to be host-specific rather than driven by their geographic location (Opelt *et al.*, 2007; Bragina *et al.*, 2012a, 2013). Secondary metabolites excreted by *Sphagnum* species together with acidity and nutrient availability seem to drive microbial community structure within *Sphagnum* (Opelt *et al.*, 2007; Bragina *et al.*, 2012a).

The community structure of diazotrophs in peatlands have only been revealed in the last decade using molecular techniques by targeting nitrogenase-encoding *nif* genes (Dedysh *et al.*, 2004; Zadorina *et al.*, 2009; Kip *et al.*, 2011; Bragina *et al.*, 2012b). Diazotrophy is present in metabolically diverse microbial groups including phototrophic, heterotrophic and methanotrophic bacteria and Archea. Most abundant diazotrophic groups belong to

Cyanobacteria, *Alphaproteobacteria*, *Deltaproteobacteria* and to a lesser extent to *Gammaproteobacteria* and Archea. *Sphagnum*-associated diazotrophs are often found in the photosyntetically active parts of *Sphagnum* in hyaline cells or living endophytically. Free-living diazotrophs are commonly found deeper within the peat profile (Knorr *et al.*, 2015). *Sphagnum* biomass gain (Berg *et al.*, 2012) and primary productivity (Larmola *et al.*, 2014; Vile *et al.*, 2014) have been attributed to diazotrophic activity in peatlands, pointing toward an active exchange of C, N and other nutrients between mosses and diazotrophs. However, the nature of these associations between *Sphagnum* and diazotrophs and their controls remain largely unknown.

Cyanobacteria have been found living endophytically (Granhall & Selander, 1973; Granhall & Hofsten, 1976) and epiphytically (Granhall & Selander, 1973; Blasco & Jordan, 1976; Chapman & Hemond, 1982) on *Sphagnum*, or free in peatland pore water (Granhall & Selander, 1973; Schwintzer, 1983). Intracellular *Nostoc* cyanobacteria-*Sphagnum* moss associations have been detected as early as 1890 by Limpricht (see Basilier, 1980). Granhall & Selander (1973) established that three dominant *Sphagnum* mosses (*S. lingbergii*, *S. riparium*, *S. jensenii*, pH ranged from 4.2 – 6.9) from wet, minerotrophic depressions harbored a diverse community of cyanobacteria living either endo- or epiphytically in Abisko region, Sweden. The average N_2 fixation for *Sphagnum* with epiphytic cyanobacteria was 600-, 60- and 30-times higher than the rates of *Sphagnum* with no cyanobacteria, *Sphagnum* surrounded by free-living cyanobacteria, and *Sphagnum* with mainly intracellular cyanobacteria, respectively (Granhall & Selander, 1973). However, little is known about the mechanisms behind these cyanobacteria-*Sphagnum* associations. Partly, the limited mechanistic understanding is due to the complex

micro-environment of hyaline cells in *Sphagnum* where, besides cyanobacteria, a diverse microbial community resides.

The largest bacterial group found in the top 10-20 cm within the peat column in a bog is Alphaproteobacteria (Zadorina *et al.*, 2009), which includes phototrophic, heterotrophic, and methanotrophic genera. Other groups like *Gammaproteobacteria, Deltaproteobacteria, Clostridia*, *Chlorobea* and *Bacilli* have also been identified, but their abundance is smaller (Zadorina *et al.*, 2009). Methanotrophic bacteria alongside other groups (e.g. Bacilli, *Klebsiella*, *Clostridium* and some sulfate-reducing bacteria) are capable of diazotrophy, and are known to live across a range of O_2 conditions (from aerobic and micro-aerobic to strict anaerobes). Frequent fluctuations in water table depth and varying O_2 conditions within the peat column may influence the relative importance of water table dynamics on N_2 fixation activity in peatlands. Recently, Larmola *et al.* (2014) have shown that methanotrophy induces N_2 fixation along a peatland succession gradient. Methanotrophs play an important role in the live, submerged *Sphagnum* plants in minerotrophic fens but less in drier ombrotrophic bogs (Larmola *et al.*, 2014; Vile *et al*., 2014). Due to their ability to oxidize methane (CH_4) to CO_2, methanotrophs contribute about 30% of the C within floating *Sphagnum* (Raghoebarsing *et al.*, 2005; Larmola *et al.*, 2010). Thus, methanotrophs can play an important role in connecting the C and N cycle in peatlands. However, the mechanisms behind the co-occurrence of large N_2 fixation rates and methane-oxidation levels are yet to be described.

The nature of interactions between different microbes within hyaline cells and *Sphagnum* mosses remains uncertain. Granhall & Hofsten (1976) proposed that nitrogenous compounds and carbohydrates released by *Sphagnum*'s chlorophyllus cells and cyanobacteria stimulate bacterial respiration in hyaline cells. The resulting increased CO_2 levels would decrease O_2 levels and

further stimulate cyanobacteria to fix N_2. The by-products of N_2 fixation (H_2 and NH_3) could be used as substrates by methane-producing bacteria within the hyaline cells (Granhall & Hofsten, 1976; Solheim & Zielke, 2002). Recently, Larmola *et al.* (2014) proposed that CH_4 could directly induce methanotrophic diazotrophs to fix N_2, or that CO_2 produced through the methane-oxidation pathway would further stimulate both photosynthesis in phototrophic microbes and *Sphagnum.* Consequently, decreased O_2 could increase the efficiency of oxygen-sensitive nitrogenase in cyanobacteria or in other present diazotrophs (Granhall & Hofsten, 1976; Larmola *et al.*, 2014). Any changes in microbial community structure within *Sphagnum* could therefore alter N_2 fixation rates in peatlands at the ecosystem level. The microbial diversity makes it not only difficult to measure N_2 fixation rates but also to clearly identify the main drivers involved in the process.

2.5 Measurements of N_2 fixation and potential error sources

N_2 fixation has been measured: 1) indirectly, via acetylene reduction assay (ARA), in which a gross activity of nitrogenase is measured, or 2) directly, using the stable isotope $^{15}N_2$ tracer enriched method ($^{15}N_2$ method) that gives the net rate of N_2 incorporation into biomass (Zehr & Montoya, 2007).

First evaluated by Hardy *et al.* (1968), ARA has been widely used in a range of ecosystems to estimate nitrogenase activity and sometimes to quantify N_2 fixation. It is an inexpensive and quick method. In addition to reducing N_2 to NH_3, nitrogenase enzyme reduces other substrates (acetylene, hydrogen cyanide, azides) in competing reactions (Hardy *et al.* 1968). Due to lower energy requirements for the reaction, nitrogenase preferentially reduces acetylene to ethylene rather than N_2 to NH_3. The linear production of ethylene is recorded over time providing a rate of ethylene production. In order to convert an acetylene reduction rate to a

N_2 fixation rate, a mole ratio (R: how many moles of acetylene are reduced to ethylene versus how many moles of N_2 are reduced to NH_3, R = ARA rate/$^{15}N_2$ rate) must be calculated. The theoretical value of R = 3 was calculated by Hardy et al. (1968) using chemical stoichiometry of these two reactions whereby 2 electrons (e^-) are required to reduce acetylene to ethylene, and 6 e^- to reduce N_2 to NH_3. This value has been later adjusted to 4 because of the production of at least 1 mole of H_2 during in the process of N_2 reduction by nitrogenase (Schwintzer & Tjepkema, 1994). Thus, instead of 6, 8 e^- are needed to reduce 1 molecule of N_2 to ammonia. However, the measured R often diverges from the theoretical value (Bellenger *et al.*, 2014). Thus, to derive the mole ratio, consecutive or parallel reactions are carried out: one using ARA and another using a direct $^{15}N_2$ method. In the latter, $^{15}N_2$ gas is introduced to the closed chamber and after the incubation, the difference in ^{15}N enrichment of the sample versus the control is determined with the use of stable isotope mass spectrometry (Postgate, 1982).

The widely used R of 3 has been determined in earlier studies in feather moss-cyanobacteria associations (DeLuca *et al.*, 2002; Zackrisson *et al.*, 2004). A wide range of R (from around 0.1 – 11) has been reported in different systems such as oceans, symbiotic N_2 fixation in higher plants and soil samples (reviewed in Bellenger *et al.*, 2014). High variability in R has also been found in tundra ecosystems where biological soil crusts dominated by cyanobacteria exhibit R from 0.022 – 0.073 (Liengen, 1999) and 1.3 – 3.5 (Stewart *et al.*, 2011c). For *Sphagnum* mosses, R has been determined in only a few studies (Table 2.1), while other studies used a ratio of 3 (Waughman & Bellamy, 1972, 1980; Alexander & Schell, 1973; Blasco & Jordan, 1976; Basilier, 1979; Schwintzer, 1983; Rousk *et al.*, 2018) except for Granhall & Selander (1973) that used 1.5. Ratios that diverge from the theoretical R = 3 are often justified by different methodological issues as a possible explanation. For example, R larger than the

theoretical (3-4) was attributed to 60-times higher solubility of acetylene than $^{15}N_2$ in water (Hardy *et al.*, 1973) making acetylene more accessible thus favoring ARA over $^{15}N_2$. Alternatively, large amounts of H_2 produced by nitrogenase activity may inhibit reduction of $^{15}N_2$. However, H_2 does not affect the reduction of acetylene to ethylene (reviewed in Liengen, 1999). A $R < 3$ is usually explained by nutritional and/or N limitation, inhibition of microbial growth by acetylene, and O_2 sensitivity of N_2 fixation (reviewed in Liengen, 1999). Hardy *et al.* (1973) suggested that the partial pressure of O_2 in incubation chambers (pO_2) represents the most common reason for differing R and proposed that caution must be used for field measurements where pO_2 values in the chambers should match the ones of the ambient environment. Finally, an R of 0.5 – 2 may be a result of the activation of alternative nitrogenase enzymes (V- and Fe-nitrogenase) in certain microorganisms as suggested by Liengen (1999) and shown by Smith *et al.* (1987) and Bellenger *et al.* (2014).

Lower than theoretical R can also be attributed to ARA limitations to measure nitrogenase activity of certain groups of N_2 fixing bacteria. While several studies reported the presence of heterotrophic bacteria in *Sphagnum* detected under the microscope or grown on N-free medium (Granhall & Hofsten, 1976), earlier studies reached a consensus that cyanobacteria are the main N_2 fixing community (Waughman & Bellamy, 1972, 1980; Blasco & Jordan, 1976; Basilier & Granhall, 1978; Schwintzer, 1983). Only recently, the development of molecular techniques and the growing knowledge of the ecological significance of certain heterotrophic bacteria have revealed the abundance of the *nif*H gene across microbial groups previously linked to other functions in these ecosystems (Dedysh *et al.*, 2004; Zadorina *et al.*, 2009; Bragina *et al.*, 2012b). The ARA method, for example, does not entirely capture nitrogenase activity of methane oxidizing bacteria in fens (Larmola *et al.*, 2014). Given that acetylene inhibits the methane

monooxygenase enzyme, methane oxidizing bacteria only show nitrogenase activity via ARA, if given other substrates like methanol or ethanol (De Bont & Mulder, 1976). ARA should be used cautiously in environmental samples, especially where more than one group of N_2 fixing organisms is present, such as in *Sphagnum* mosses and peat (Zadorina *et al.*, 2009). Since dominant N_2 fixing communities may vary across sites, types of peatlands, microtopography within a peatland, depth and moss-host species, ARA measurements should be calibrated with the $^{15}N_2$ tracer method. It is important to note that due to the natural variability of N_2 fixation rates most conversion factor studies are carried out in laboratory conditions capturing nitrogenase activity under set environmental conditions and thus, may cause over- or underestimation of N_2 fixation in the field in some cases. The variability of R between sites (Table 2.1) calls for further research and better quantification of N_2 fixation rates in peatlands.

More recently a few studies used the $^{15}N_2$ method without ARA to study N_2 fixation activity in peatlands or tundra (Krumholz *et al.*, 1995; Gavazov *et al.*, 2010; Larmola *et al.*, 2014; Knorr *et al.*, 2015; Novak *et al.*, 2018). In this thesis, I have used both ARA and $^{15}N_2$ tracer methods to measure N_2 fixation rates. I derived R across the wide geographical transect that includes the Mer Bleue bog (Chapter 6) and applied R to derive N_2 fixation rates at Mer Bleue in Chapters 3 and 4. Despite methodological issues in measuring and estimating N_2 fixation rates, it is evident that *Sphagnum* mosses in peatlands still harbor diverse microbial communities involved in diazotrophy living both epi- and endophytically despite the highly acidic environment.

Table 2.1 Studies showing conversion factor ($R=ARA/^{15}N_2$) in different *Sphagnum* spp. SE indicates standard error

R (SE)	Biome	Site	Organism	Author
4 and 5.1	Subarctic peatland	Abisko, Sweden	*Sphagnum riparium*/cyanobacteria	Basilier (1980)
2.8 and 3.4	Subarctic peatland	Abisko, Sweden	*Sphagnum angustifolium*/cyanobacteria	Basilier (1980)
3.5	Temperate blanket bog	Thoreau's bog, USA	*Sphagnum* spp.	Chapman and Hemond (1982)
0.85 (0.12)	Low Arctic tundra	Daring Lake, NWT	*Sphagnum* spp.	Stewart et al. (2011)
0.32 (0.05)	Boreal bogs	Alberta, Canada	*Sphagnum spp.*	Vile et al. (2014)

2.6 Controls on N_2 fixation in peatlands

Because N_2 fixation is an enzymatic process, it greatly depends on abiotic physical and chemical factors in the environment. Studies of N_2 fixation in northern biomes focus mainly on biological crusts in tundra or feather moss in boreal forest. These studies have identified several important abiotic and biogeochemical factors regulating N_2 fixation: temperature, pH, N and P availability, moisture, light, and metals (Fig. 2.1; Rousk *et al.*, 2013; Lindo *et al.*, 2013). It is important to note, however, that most of these drivers and their interactions have been rarely explored in *Sphagnum* dominated peatlands. While N_2 fixation in northern, nutrient-poor, bryophyte-dominated environments may be to some extent comparable to N_2 fixation in peatlands, the unique diazotrophic community structure, hydrology and microtopography in peatlands warrants further investigations of important N_2 fixation controls.

a) Temperature

Nitrogenase activity in terrestrial ecosystems is strongly driven by temperature with an optimum at around 25 ºC (Vitousek *et al.*, 2002; Houlton *et al.*, 2008) leading to the assumption that N_2 fixation in northern biomes would be low or negligible (Cleveland *et al.*, 1999; Vitousek *et al.*, 2002; Houlton *et al.*, 2008). A few studies on bryophyte-cyanobacteria associations in high latitudes showed that the temperature optimum for N_2 fixation ranges between 20 and 30 ºC (Chapin *et al.*, 1991; Zielke *et al.*, 2002; Gundale *et al.*, 2012), with a sharp decline above 30 ºC (Jean *et al.*, 2012). However, *Sphagnum* mosses were not analyzed in these studies. The optimal temperature for nitrogenase activity in peat from Ontario in a laboratory study was 20ºC, with very low rates at 5ºC and above 20-25ºC (Blasco & Jordan, 1976). Similarly, Basilier (1979) found that at temperatures between 16 and 20ºC nitrogenase activity was at its peak in a Swedish subarctic fen. Kravchenko & Doroshenko (2003) demonstrated in a laboratory experiment using peat from a Russian bog that N_2 fixation occurs in the range of 5 to 45 ºC with an optimal temperature of 25-30 ºC. Increasing N_2 fixation in *S. capillifolium* followed the temperature rise from 5 to 26 ºC over the growing season in Manitoba (Markham, 2009).

b) Moisture

Moisture and water availability play a critical role in nitrogenase activity in soils (Vitousek *et al.*, 2002). In the Arctic and in boreal forests, N_2 fixation by cyanobacteria associated with bryophytes strongly depends on moisture (Zielke *et al.*, 2002, 2005; Stewart *et al.*, 2011c; Jackson *et al.*, 2011; Rousk *et al.*, 2014a; Rousk & Michelsen, 2016; Rousk *et al.*, 2018). Rewetted cyanobacteria seem to have the ability to restore N_2 fixation after desiccation within 4-14 h (cf. Vitousek *et al.*, 2002), allowing post-drought recovery of these ecosystems. The direct moisture effect on N_2 fixation activity in *Sphagnum*-dominated peatlands has not yet

been studied, though a rising water table seems to increase N_2 fixation rates in fens (Leppänen *et al.*, 2015). In ombrotrophic bogs, the water table depth fluctuates throughout the field season and differs between microforms within a peatland. Furthermore, a complex diazotrophic community in bogs may have different demands for water availability than the more uniform cyanobacteria communities found in feather mosses in boreal forest stands. Both precipitation and water table level could therefore have an effect on N_2 fixation in peatlands.

c) pH

The optimal pH range for N_2 fixation seems to be between 5.9 and 6.2 (Smith, 1984). However, pH can also modify the nature of *Sphagnum*-cyanobacteria associations in peatlands (Solheim & Zielke, 2002). While no cyanobacteria were detected in a bog with a pH of 3.9 (Schwintzer, 1983), in pH of 4.0, 4.2 and 4.9 only intracellular cyanobacteria were found within *Sphagnum* hyaline cells (Granhall & Hofsten, 1976; Chapman & Hemond, 1982). In contrast, with pH > 5, cyanobacteria were found only as epiphytes on *Sphagnum* or free-living in pore water (Granhall & Selander, 1973). It has been proposed that buffering properties of hyaline cells offer refuge to cyanobacteria in very acidic peat bog conditions (Solheim & Zielke, 2002). However, a wide range of acidophilic and other dizatrophs have been found in bogs along the peat profile (Waughman & Bellamy, 1980; Bragina *et al.*, 2012b) suggesting that these may replace cyanobacteria in strongly acidic environments.

d) Biogeochemical controls

Terrestrial N and P cycles are closely related and intrinsically connected through the N_2 fixation process (Vitousek *et al.*, 2002, 2013). Because N_2 fixation is an energetically expensive process where 16 ATP are needed to fix one N_2 molecule from the atmosphere, P is essential for

nitrogenase activity. While terrestrial ecosystems are often thought to be N-limited, recent evidence suggests that peatlands may be either NP co- or P-limited (Wang & Moore, 2014). If the N_2 fixation process is P-limited, its activity increases with P additions to *Sphagnum* (Kox *et al.*, 2016; van den Elzen *et al.*, 2017, 2018). Greater P availability in fens could also explain larger N_2 fixation rates in fens compared to bogs along peatland succession gradients (Waughman & Bellamy, 1972; Larmola *et al.*, 2014). N additions on the other hand result in less consistent effects on N_2 fixation in peatlands. According to the "nitrostat" theory, microbes would cease their diazotrophy (Menge & Hedin, 2009) when other sources of N are available. In this way, the high energetic costs of N_2 fixation can be avoided. In contrast, the lack of additional N will result in increased N_2 fixation activity. Larger N additions slower or inhibit N_2 fixation in cyanobacteria associated with feather mosses (Zackrisson *et al.*, 2004; Gundale *et al.*, 2011; Ackermann *et al.*, 2012; Rousk & Michelsen, 2016) though in a laboratory setting N addition had no effect on the same moss-diazotroph association (Rousk *et al.*, 2014a). Similarly, N_2 fixation in *Sphagnum* was either inhibited (Kox *et al.*, 2016) or there was no effect after N was added in a peatland (van den Elzen *et al.*, 2018). These diverse results indicate that effects of N additions on N_2 fixation in peatlands might be moderated by the availability of P or other nutrients.

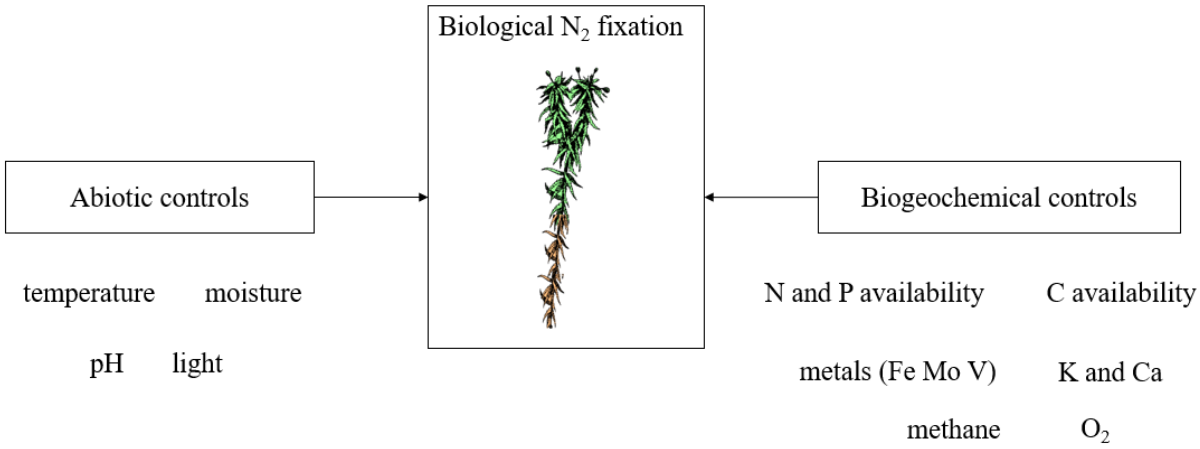

Fig. 2.1 Abiotic and biogeochemical factors that may affect nitrogenase activity in peatlands and other high-latitude ecosystems (from Waughman & Bellamy, 1980; Jean et al., 2013; Rousk *et al*., 2013; Lindo *et al*., 2013; Bellenger *et al*., 2014; Larmola *et al.*, 2014).

Other potential biogeochemical controls on N_2 fixation rates have been rarely tested in peatlands. Waughman & Bellamy (1980) found that an increase in potassium (K) availability and a decrease in calcium (Ca) in 17 peatland systems across a latitudinal gradient in Europe explain 12% and 16% of the increase in nitrogenase enzyme activity, respectively. The availability of Fe, Mo and V, which are building blocks for nitrogenase enzymes, may also impact N_2 fixation activity in peatlands. However, these relationships have rarely been tested. For example, Fe in combination with P was the best explanatory variable for methanotrophic N_2 fixation across a fen-bog gradient (Larmola *et al.*, 2014). The addition of Mo to feather moss-cyanobacteria associations increased N_2 fixation rates (Rousk *et al.*, 2017a), while both Mo and P were limiting N_2 fixation in boreal forest stands throughout the growing season (Jean *et al.*, 2013). Finally, the effects of gases such as CH_4, O_2, and CO_2 on N_2 fixation in peatlands are still unclear. The addition of CH_4 stimulated N_2 fixation in fens in one study (Larmola *et al.*, 2014), but there was no effect in the same fen in another study (Leppänen *et al.*, 2015). The most recent evidence suggests that the addition of CO_2 and CH_4 had no effect on diazotrophy but that O_2 suppressed

N_2 fixation rates up to 90% in a Minnesota bog (Warren *et al.*, 2017). Evidently, the inconsistent results regarding drivers of N_2 fixation call for more in-depth studies in peatlands.

2.7 Conclusions

Diazotrophic activity is strongly driven by abiotic factors in the environment (e.g. temperature, moisture, and the availability of nutrients within *Sphagnum*) but may also be affected by *Sphagnum* nutrient status and photosynthetic activity. This review reveals the knowledge gap in understanding of how these abiotic factors regulate N_2 fixation in peatlands, especially in ombrotrophic bogs. The major focus of my thesis is to improve the current knowledge about the main controls affecting N_2 fixation in bogs, and to shed light on how nutrient availability (specifically N and P content in *Sphagnum*) affects diazotrophy in a manipulated environment but also across a transect covering temperate, boreal and subarctic ecosystems.

CHAPTER 3

Seasonal and spatial variability of N_2 fixation at Mer Bleue bog, Ontario, Canada

3.1 Context within the thesis

As discussed in Chapter 2, *Sphagnum*-associated N_2 fixation in peatlands depends on environmental controls, but little is known about ecosystem level N_2 fixation drivers in ombrotrophic peatlands. In this thesis, I focus on two groups of environmental factors: abiotic (temperature, water moisture content and precipitation) and biogeochemical (*Sphagnum*'s nutrient status and photosynthetic activity). A better understanding of abiotic drivers of N_2 fixation in peatlands can elucidate the potential changes of this process due to ongoing global change and help with better predictions of future N_2 fixation in ombrotrophic peatlands.

In Chapter 3, I aim to identify the most important abiotic controls on N_2 fixation within the ombrotrophic bog. This study measures N_2 fixation rates at Mer Bleue bog for the first time and investigates temporal and spatial drivers of N_2 fixation within a bog with a typical hummock-hollow-pond microtopography. Taking into account microtopography and seasonal changes in N_2 fixation rates throughout two growing seasons, I derive an N_2 fixation estimate for Mer Bleue and discuss the fate of fixed N and its incorporation into the new *Sphagnum* biomass.

3.2 Abstract

Northern peatlands are globally important carbon (C) and nitrogen (N) sinks due to slow decomposition rates and organic matter accumulation. Despite their large N storage, peatlands depend on sources of bio-available N to sustain their biomass production. In addition to human-induced atmospheric N deposition, di-nitrogen (N_2) fixation represents an important biological N source in ombrotrophic bogs, but, its environmental controls are still poorly understood. In this

study, we examined seasonal and spatial variability of *Sphagnum* associated N_2 fixation across a hydrological transect (hummock-hollow-beaver pond) in a temperate ombrotrophic bog. We measured N_2 fixation by acetylene reduction assay bi-weekly from May to November over two growing seasons (2013 and 2014) and found that seasonal variability can be best explained by soil temperature and antecedent precipitation (cumulative precipitation over 2 weeks). Peak N_2 fixation rates occur in mid-August, when N_2 fixation rates are about 10 times larger than during the shoulder seasons (May and October). The spatial variability of N_2 fixation is best explained by gravimetric water content in *Sphagnum*, being more pronounced in the peak growing season when N_2 fixation rates are the largest. Finally, we estimate that Mer Bleue bog receives between 0.24 and 0.31g N m^{-2} annually through *Sphagnum*-associated N_2 fixation, which accounts for only 22% of the N accumulated annually into *Sphagnum* production. These results show that while substantial amount of biologically fixed N is incorporated into the *Sphagnum* biomass, a large fraction of N is met by additional N sources at Mer Bleue bog.

3.3 Introduction

Northern peatlands are major global carbon (C) and nitrogen (N) sinks due to slow decomposition rates and long-term organic matter accumulation. Since the last glaciation, northern peatlands have accumulated between 16 – 18 Pg of terrestrial N (Loisel *et al.*, 2014; Wang *et al.*, 2014). Accumulated N, however, is locked into peat organic biomass until the slow microbial decomposition converts it into dissolved inorganic or organic N forms that can be further utilized by plants and microbes. Due to the lack of groundwater input in *Sphagnum*-dominated bogs, N inputs mainly depend on atmospheric deposition, and N exports (denitrification and runoff) are assumed to be small (Limpens *et al.*, 2006). Estimated long-term N accumulation rates (LORNA) and recent N accumulation rates (RERNA) in peatlands range

from 0.34 - 0.61 g N m^{-2} y^{-1} (Loisel *et al.*, 2014) to 1.4 – 3.2 g N m^{-2} y^{-1} (Turunen *et al.*, 2004), respectively. Neither LORNA (Damman, 1978) nor RERNA (Moore *et al.*, 2005) in ombrotrophic peatlands can be explained by N inputs from precipitation only. Another important source of N in peatlands, which could explain the discrepancy in N inputs, is microbially-driven biological N_2 fixation.

With the exception of a few studies (Rosswall & Granhall, 1980; Hemond, 1983), peatland N budgets have rarely been quantified due to methodological challenges in measuring N_2 fixation rates, fast turnover of bio-available N and or due to the uncertainties related to the measurements of N inputs and outputs (Limpens *et al.*, 2006). Recently, biological N_2 fixation rates in dominant *Sphagnum* moss species were shown to be large enough to sustain primary productivity of *Sphagnum* in peatlands and to explain N accumulation rates in peat (Larmola *et al.*, 2014; Vile *et al.*, 2014). However, despite its important role in peatland functioning, little is known about how N_2 fixation responds to changes in environmental (abiotic) conditions.

Environmental controls on N_2 fixation have been rarely tested in ombrotrophic peatlands, though both moisture and temperature are known drivers of N_2 fixation in similarly nutrient limited ecosystems (Rousk *et al.*, 2017b). Seasonal patterns of N_2 fixation have been observed in feather moss-cyanobacteria associations in boreal forests with N_2 fixation rates peaking late in the growing season in September (DeLuca *et al.*, 2002). Seasonality of *in-situ* N_2 fixation rates was also observed in *Sphagnum* in Manitoba (Markham, 2009) with N_2 fixation rates linearly increasing as temperature rose from 6 to 25 ºC. Though variable, optimal temperatures for N_2 fixation range between 20 and 30 ºC in boreal forests and subarctic tundra (Chapin *et al.*, 1991; Zielke *et al.*, 2002; Gundale *et al.*, 2012). The modeled N_2 fixation optimum across all ecosystems is assumed to be 25 ºC (Houlton *et al.*, 2008), indicating that in peatlands N_2 fixation

peaks would be expected mid-summer. However, optimal mid-summer temperatures in temperate and boreal regions may coincide with drier conditions in bogs, often resulting from a deep water table. Water availability was found to be a critical driver of N_2 fixation in soils across terrestrial ecosystems (Vitousek *et al.*, 2002). Similarly, a strong dependence of N_2 fixation by bryophyte-cyanobacteria association on soil moisture was found in the Arctic and in boreal forest ecosystems (Zielke *et al.*, 2002, 2005; Stewart *et al.*, 2011c; Jackson *et al.*, 2011; Rousk *et al.*, 2014a; Rousk & Michelsen, 2016; Rousk *et al.*, 2018). Water content within *Sphagnum* depends on the water table depth and precipitation (Hayward & Clymo, 1982) thus N_2 fixation may be driven by these variables within a bog. The direct moisture effect on N_2 fixation activity in *Sphagnum*-dominated ombrotrophic peatlands has not yet been studied, though a rising water table seems to increase N_2 fixation rates in fens (Leppänen *et al.*, 2015). Larger N_2 fixation rates were measured in wet, submerged *Sphagnum* mosses in minerotrophic wet depressions or pools in fens compared to much lower N_2 fixation activity in dry, raised ombrotrophic parts of the same subarctic peatland complex in Sweden (Granhall & Selander, 1973). In most ombrotrophic bogs, the water table depth fluctuates throughout the field season and differs between microforms (hummocks, hollows) within a peatland, with drier hummocks and wetter hollows. Ponds at the bog margin often remain wet throughout the growing season with continuously submerged floating *Sphagnum* mats on their edges (Bubier *et al.*, 2006).

Here, we test the role of moisture availability on N_2 fixation across three distinct microforms at a temperate ombrotrophic bog: pond, hollows and hummocks. In addition, we examine the importance of other environmental controls (air and soil temperature, precipitation and light availability) on N_2 fixation across two growing seasons (May – November) using bi-weekly measurements of N_2 fixation rates. The objectives of this paper were: 1) characterize

seasonal and spatial variability in N_2 fixation activity, 2) to test if moisture availability and other environmental drivers control the spatial and temporal variability of N_2 fixation and 3) to derive an estimate of biological N_2 uptake over the growing season at the Mer Bleue ombrotrophic bog.

3.4 Materials and Methods

Field site and experimental design description

Mer Bleue is a raised cool-temperate ombrotrophic bog (45.41 °N latitude, 75.48 °W longitude) near Ottawa, Ontario. The mean annual temperature is 6.6°C with a mean precipitation of 919.5 mm (Canadian Climate Normals 1981-2010). The growing season of 2013 (May - Oct) was drier than average with rainfall amounts of 492 mm compared to 587.6 mm for the same period in 2014 (Fig. 3.1). Mer Bleue bog is characterized by a typical hummock-hollow microtopography and features a beaver pond at the bog margin. Most common microforms at Mer Bleue are hummocks (51.2%), followed by hollows (12.7%), while trees cover 33.6%, and open water with unclassified mosses cover 2.4% of the surface area (Arroyo-Mora *et al.*, 2018).

Mer Bleue ground cover is dominated by *Sphagnum* species. Most common *Sphagnum* mosses in hummocks are *Sphagnum capillifolium* and less *S. magellanicum*, while wetter hollows are dominated by *S. fallax* and *S. angustifolium* (Bubier *et al.*, 2006). *S. majus* and *S. cuspidata* are found on the beaver pond edges submerged or floating (Bubier *et al.*, 2006). Dominant plant communities in the bog include evergreen and deciduous ericaceous shrubs (*Chamaedaphne calyculata, Rhododendron groenlandicum, Kalmia angustifolium and Vaccinium myrtilloides*), sporadically occurring sedges (*Eriophorum vaginatum*) and trees (*Picea mariana* and *Larix laricina*) (Bubier *et al.*, 2006).

We established five 2x2-m permanent plots in May 2013 tracing a hydrological gradient from the wettest (pond) via intermediate hollows (one in transitional and one in the bog area) to the driest hummock plots (one in transitional and one in bog area) to capture the wide range of moisture availability at the ombrotrophic bog (Fig. S3.1). In the plots, we installed perforated tubes to measure water table depth (WTD) and recorded the WTD throughout the growing season in 2013 (June – October) and in 2014 (May – November; Fig. 3.1c). Continuous measurements of temperature (air and peat at depth of 5cm), photosynthetically active radiation (PAR), relative humidity (RH) and precipitation were made at a micrometeorological eddy covariance tower (Lafleur *et al.*, 2001). We used 30-min averages of meteorological variables to derive daily means (Fig 3.1a, b and d).

N_2 fixation measurements and gravimetric water content in *Sphagnum*

Bi-weekly to monthly measurements of nitrogenase activity using acetylene reduction assays (ARA; Hardy *et al.*, 1968) and of gravimetric water content (GWC) in *Sphagnum* were conducted throughout two field seasons 2013 and 2014 resulting in 536 individual measurements on 17 sampling dates. We incubated the top 6-cm live *Sphagnum* moss in acid washed 250 mL Mason jars with lids adapted for gas exchange (tubing with the 3-way stopcock). *Sphagnum* cores were placed into jars with capitula oriented towards the bottom of the jar. Enclosed jars were then buried into the peat to minimize heating within the jar with the bottom of the jar facing upward exposing capitula to the sunlight over the course of the incubation. We made fresh acetylene gas before each field experiment using calcium carbide and DI water, and replaced 10% (v/v) of headspace in jars with acetylene. We confirmed the linear production of ethylene over 24 and 48 h in the field incubations allowing us to use a 24 h incubation period for individual ARA measurements. We sampled after 24 h of incubation using 10 mL syringes with

stopcocks, and transported samples to the laboratory where they were analyzed for ethylene production on a GC-2014 Shimadzu FID (HayeSep N 80/100 mesh, 2m-column, Injector and Detector at 175°C and column at 70°C). We ran controls (moss without acetylene added) to ensure there was no production of ethylene by plants or microbes. We used blanks (acetylene in Mason jar but no moss) to ensure acetylene's purity: no ethylene was produced without acetylene addition and no ethylene was detected in acetylene gas.

After each field experiment, we measured wet moss weight and estimated headspace in each jar in the laboratory replacing the air with DI water. We dried moss at 60°C, and ground moss for further analyses. We calculated GWC in *Sphagnum* moss (%) using the following equation:

$$Gravimetric\ water\ content\ (\%) = \frac{(wet\ moss\ (g) - dry\ moss\ (g))}{dry\ moss\ (g)} \times 100\%$$

Estimating N_2 fixation rates for Mer Bleue bog

To estimate N_2 fixation rates, we used a conversion ratio (R) of 1.16 between ARA and N_2 fixation derived in Chapter 6 using parallel ARA and $^{15}N_2$ tracer measurements from 8 similar ombrotrophic bogs along a latitudinal transect in eastern Canada that includes Mer Bleue bog. To define a possible range of N_2 fixation in this study we used the smallest and largest R from previous studies (see Table 2.1). The mean ARA rates (g N m^{-2} d^{-1}) for hummock, hollow and pond over the two field seasons (Table S3.1) were used to estimate annual N_2 fixation per microsite. The annual N_2 fixation was calculated by multiplying mean daily ARA rates with the number of growing season days, 210 days from May to November. To account for differences in surface coverage of each microform of Mer Bleue bog, we used estimates of microform areal

extent at Mer Bleue derived by Arroyo-Mora *et al.* (2018). We used four different scenarios to weight the estimated annual N_2 fixation rates (Tables S3.1 and 3.2):

1. We used two microtopography classes: dry microsites – hummocks and wet microsites – hollows and pond edge. Given that at the pond *Sphagnum* is only found at the pond edge, we assumed that only 10% of open water classified by Arroyo-Mora *et al.* (2018) is covered by *Sphagnum.* This area is then added to hollow area, hummocks represent the remaining bog surface and trees are not included.
2. Hummocks and hollows only are used; open water and trees are omitted.
3. Hummocks, hollows and 10% of open water are included and trees are omitted.
4. Trees are added to the surface area of the hummocks, while hollows represent the remaining surface; open water is omitted.

Net primary production of mosses and N concentration in *Sphagnum*

We used the cranked wire method (Clymo, 1970) to measure moss growth in hummocks and hollows during the 2013 and 2014 growing seasons. 10 cranked wires per plot were placed in May 2013 and measured every 2 months throughout 2013 and 2014 growing seasons until May 2015. Bulk density (BD) was measured using three replicates of known moss volume (10 x 10 x 3 cm; length x width x depth). Each moss core was cut from the permanent plots and moss capitula were removed leaving behind 2 cm length of stems. Wet and dry weight of moss was measured and BD (g cm^{-3}) was estimated per plot and used to calculate primary production of moss in each plot.

To determine N concentration in *Sphagnum*, 3 replicates of *Sphagnum* stems with capitula per plot along the transect were collected in May, July and September in 2014, dried at

50 ºC and finely ground through a 40 mesh sieve (Wiley Mini Mill 3383-L10, Thomas Scientific, USA). Acid digestion was performed following procedures from Parkinson & Allen (1975) using concentrated H_2SO_4 and H_2O_2 with Se and Li_2SO_4 as catalysts. Samples were then analyzed for total N colorimetrically (Murphy and Riley 1962) on a LachatQuick-Chem AE flow injection autoanalyzer (Lachat Instruments, Milwaukee, WI).

We used NPP and N concentration in *Sphagnum* to estimate incorporation of N into new moss growth and to estimate N growth requirements.

Data analyses

To derive the best model explaining seasonal variability of ARA over the two growing seasons, we used stepwise multiple linear regression analyses, in which we tested air and soil temperature, water table depth, antecedent precipitation (3, 5, 7 and 14 d before the measurements), PAR and RH as explanatory variables. Soil temperature and 14-day antecedent precipitation were the only significant effects ($p > 0.05$) that remained in the final model.

Linear regression analysis was used to test the relationship between gravimetric water and ARA across microsites for each sampling date. We then investigated the relationship between the slopes of these regressions to daily mean ARA to investigate the sensitivity and strength of ARA dependence to gravimetric water content.

We tested for the normality of datasets and the residuals in all our regression analyses. Log_{10} transformations were performed if needed to achieve normality. All error bars throughout the manuscript are standards errors, unless indicated otherwise. All statistical tests were performed using R 3.1.2 (R Core Team 2014), JMP 11.2.0 and Matlab 2015b version.

3.5 Results

Seasonal variation of N_2 fixation

There was a large range of ARA rates throughout both field seasons, from 1 to 934 nmol g $(DW)^{-1}$ d^{-1} (Fig. 3.2). The largest mean daily ARA rate was recorded in August 2014 with 94.6 ± 28.1 nmol g $(DW)^{-1}$ d^{-1} (mean ± SE), and was about 14 times larger than the rates in early May and late October (Fig. 3.2).

About 52% of the variance of daily mean ARA rates across the bog transect was explained by antecedent precipitation (cumulative precipitation of 14 d prior to the measurements) and mean soil temperature at 5 cm depth measured at the eddy covariance tower (Table 3.1). Each of these two variables contributed equally to the seasonal variability of ARA rates (Fig. 3.1a and d), while water table depth had no effect (Fig 3.1b). PAR and RH also had no effect on ARA rates and were taken out from further analyses.

Controls on spatial variability of N_2 fixation

Gravimetric water content (GWC) of *Sphagnum* was the strongest explanatory variable of spatial variability of ARA rates at Mer Bleue bog with 21% of the variance explained (Fig. 3.4). The sensitivity of ARA rates to GWC (the steepness of the regression slope) varies throughout the season (Fig. 3.5) and significantly increases with the magnitude of mean daily ARA rates (Fig. 3.6). The strength of the relationship between the daily means of ARA rates and gravimetric water content in *Sphagnum* also varies during the season, being generally strongest in the mid growing season with $R^2 > 0.5$ with weak and statistically not significant relationships in the shoulders of the season (Table 3.2). We note that GWC of the top *Sphagnum* layer has a

non-linear relationship to WTD (Fig S3.1), suggesting that in microsites with the WTD of 25 cm or below, surface *Sphagnum* GWC is no longer hydrologically connected to WTD.

Annual N_2 fixation estimates for Mer Bleue

The estimate of Mer Bleue bog biological N_2 fixation in the top 6cm of *Sphagnum* mosses lies between 0.24 and 0.31 g N m^{-2} annually, based on the mean seasonal ARA values for each microtopographic form, a R of 1.16 and variations in the estimated spatial coverage (Table S3.2). Mean N_2 fixation throughout the two field seasons was about 3 and 4.4 times larger in the pond than in hollows and hummocks, respectively (Table S3.1). but the small surface area of pond mosses substantially reduces their impact on the annual ecosystem-scale N_2 fixation estimate (Table S3.2).

Net primary production of *Sphagnum* estimation of annual N accumulation in *Sphagnum*

The average *Sphagnum* NPP was 190 ± 12.6 g m^{-2} y^{-1} (mean ± SE, Fig. S3.2) over the two years and not significantly different between hollows and hummocks along the transect, though there was a large variability among plots (Fig. S3.3). N% in hummocks *Sphagnum* was 0.75 ± 0.02 % (mean ± SE; n = 18) and in hollows 0.67 ± 0.02 % (mean ± SE; n = 18). To estimate the amount of C and N that is incorporated into new *Sphagnum* biomass each year, we assumed C at 48% (Moore, personal communication) and 0.7% of N (the average of hummocks and hollows along the transect) in *Sphagnum*. We find that about 91 g C m^{-2} y^{-1} and 1.3 g N m^{-2} y^{-1} goes into annual *Sphagnum* growth.

3.6 Discussion

We find that soil temperature and antecedent precipitation best explain seasonal variability while gravimetric water content in *Sphagnum* explains spatial variability of N_2

fixation at Mer Bleue bog. Our findings agree with other studies that identified temperature and soil moisture as important drivers of bryophyte- or biocrust-N_2 fixing microbes associations (Zielke *et al.*, 2002, 2005; Stewart *et al.*, 2011; Jackson *et al.*, 2011; Rousk *et al.*, 2014a; Rousk & Michelsen, 2016; Rousk *et al.*, 2018). However, our study highlights the importance of understanding interactions of abiotic controls on N_2 fixation rates in ombrotrophic peatlands especially across different microforms occurring in bogs. Mer Bleue bog is a typical dome-shaped ombrotrophic bog with hydrologically distinct *Sphagnum*-dominated hummock-hollow patterns and a beaver pond at its margin. *Sphagnum* mosses host N_2 fixing bacteria (diazotrophs) and their moisture status is essential for diazotrophic activity. Unlike in fens, where water table depth was identified as the main driver of N_2 fixation (Leppänen *et al.*, 2015), we found no effect of the bog water table on temporal N_2 fixation dynamics (Fig. 3.3b). Instead, the gravimetric water content of the *Sphagnum* best explained spatial variablity of N_2 fixation at Mer Bleue bog (Figs. 3.4-3.6). Although there is a strong relationship between GWC and water table depth (Fig. S3.1), we find that the top 6-cm *Sphagnum* layer uncouples from the water table when the depth reaches below 25 cm (Hayward & Clymo, 1982). Given that at Mer Bleue bog, the WTD often drops to 40 and 25 cm below the surface in hummocks and hollows, respectively, it is not surprising that precipitation events play an important role in maintaining the moisture necessary for N_2 fixation in the uppermost part of *Sphagnum*. In fens, where the water table is usually closer to the surface, surface moisture is more tightly coupled to water table dynamics. The results of our study indicate that N_2 fixation in bogs may be particularly vulnerable to lowering of the water table and drier conditions.

This study quantifies for the first time N_2 fixation rates at Mer Bleue bog. Our estimates of biological N_2 fixation rates of 0.24-0.31 g N m^{-2} y^{-1} at Mer Bleue bog are about 10 times

smaller than N_2 fixation rates in five Alberta bogs (Vile *et al.*, 2014) and a minerotrophic fen in Finland (Larmola *et al.*, 2014) but similar to a Finnish ombrotrophic bog (0.28 g N m^{-2} y^{-1}; Larmola *et al.*, 2014). The 3 to 4 times larger N_2 fixation rates in the pond *Sphagnum* than hollows and hummocks indicate that bogs with ponds may have much larger N_2 fixation rates than drier bogs. Bogs with large fractions of pond surfaces with floating *Sphagnum* on their margins are not uncommon in eastern Canada and subarctic regions like the Hudson Bay lowlands (Glaser & Janssens, 1986; Macrae *et al.*, 2004; Pelletier *et al.*, 2014; Arsenault *et al.*, 2018). Furthermore, wetter bogs with larger areas covered by hollows may also have larger N_2 fixation rates. Our results highlight the importance of wet pond margins (lagg zone) as hotspots for N_2 fixation in bogs.

At Mer Bleue, the amount of annually accumulated N by hummock and hollows *Sphagnum* mosses tissue (1.1 to 1.3 g N m^{-2} y^{-1}) can be explained by both biological N_2 fixation (0.24 to 0.31 g N m^{-2} y^{-1}) and estimated wet and dry N deposition of 0.8 - 1 g N m^{-2} y^{-1} for the region (Vet *et al.*, 2014). Surface N_2 fixation therefore accounts for about 22 % of the annual N accumulated in live *Sphagnum* tissue.

Furthermore, our study elucidates some of the discrepancy between observed N inputs and the recent accumulated N within the peat column in this ombrotrophic system. Recent N accumulation rates (RERNA) over the last 50 years at Mer Bleue are between 2.3 g N m^{-2} y^{-1} and 2.6 g N m^{-2} y^{-1} in hummocks and hollows, respectively (Turunen *et al.*, 2004). With an annual N deposition input of 0.8 - 1 g N m^{-2} y^{-1} (Vet *et al.*, 2014), and low denitrification and other N exports at Mer Bleue (about 0.3 g N m^{-2} y^{-1}; Moore, personal communication), the "missing source of N" is about 1 g N m^{-2} y^{-1}. N_2 fixation in top 6 cm *Sphagnum* layer only accounts for 30% of this missing N source. There are several possible explanations for these results: 1) there

could be a significant N_2 fixation by free-living diazotrophs occurring lower within the peat profile (Knorr *et al.*, 2015), 2) *Sphagnum* at Mer Bleue strongly depends on N remineralization that is recycled from the deeper peat, or 3) large N deposition together with smaller P availability (Chapter 5 and 6) in *Sphagnum* may slow N_2 fixation rates especially in the surface *Sphagnum* layers.

Recent studies shed light on the sources of the missing N on ombrotrophic peatlands. For example, Knorr *et al.* (2015) showed a high potential for significant N_2 fixation by non-symbiotic diazotrophs in Patagonian bogs. Additionally, the methane oxidizing diazotrophs often found in fens and the wettest areas of oligotrophic peatlands (Larmola *et al.*, 2014) may also play an important role in fixing N in bogs. However, such microbes need a specific niche (e.g. low oxygen and larger methane concentrations) to support their metabolic activity. In bogs, such conditions could be found just above the water table where methane-oxidation has largest rates (Blodau *et al.*, 2007; Juutinen *et al.*, 2018). At Mer Bleue, such conditions would be found between 20-50 cm below the surface in hummocks and in hollows likely closer to the surface (5-15cm). Thus, investigating this portion of N_2 fixation at Mer Bleue may help derive an improved annual estimate of N_2 fixation. The depleted values of natural $\delta^{15}N$ abundance (-8 to -4 ‰, Moore & Bubier, *in press*) in the vascular vegetation at Mer Bleue suggest that the process of N mineralization from peat and vascular plant litter may be an important N source. For example, Mer Bleue *S. capillifolium and S. magellanicum* have capitulum $\delta^{15}N$ values of about -5 ‰; assuming a N_2 fixation of 0‰, the depletion to $\delta^{15}N$ in the *Sphagnum* could come from either uptake of mineralized N with a $\delta^{15}N$ signature of -4 to -8 ‰ or from atmospherically deposited N, which can vary widely in $\delta^{15}N$ signature, depending on source and chemical composition. Lastly, the long-term rate of N accumulation at Mer Bleue bog is estimated to be around 0.9 g N

m^{-2} y^{-1} (Wang *et al.*, 2014) suggesting that in pre-industrial times, when N deposition was low (probably 0.1 – 0.2 g m^{-2} yr^{-1}), N_2 fixation must have been the main source accounting for LORNA. Increased atmospheric N deposition due to human activities has been shown to negatively affect N_2 fixation in boreal forests and subarctic tundra, and change the nutrient content in *Sphagnum* in ombrotrophic peatlands by increasing N and switching *Sphagnum* from N to P limitation. Although contrasting effects of N fertilization on N_2 fixation in *Sphagnum* have been observed (strong negative to no effect; Kox *et al.*, 2016, van den Elzen *et al*. 2017), especially changes in nutrient status and the increase of P limitation could be a reason for relatively small rates at Mer Bleue bog compared to bogs in western Canada. While these biogeochemical controls (*Sphagnum* nutrient status, long-term increased N deposition) may additionally affect N_2 fixation at Mer Bleue bog, our study highlights the importance of abiotic drivers especially the moisture, and identifies a portion of the "missing" N sources at Mer Bleue bog.

3.7 Figures and tables

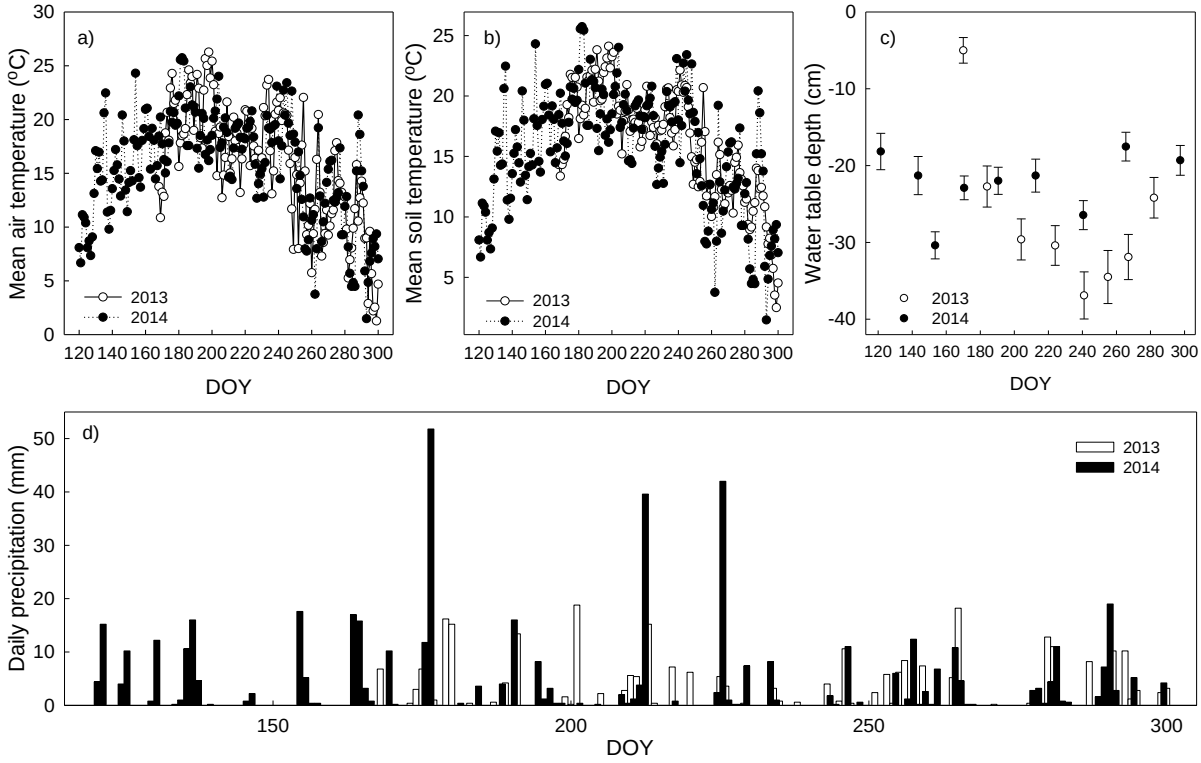

Fig. 3.1 Daily means of a) air temperature, b) soil temperature at 5cm depth, c) water table depth and d) daily precipitation at Mer Bleue bog throughout two field seasons.

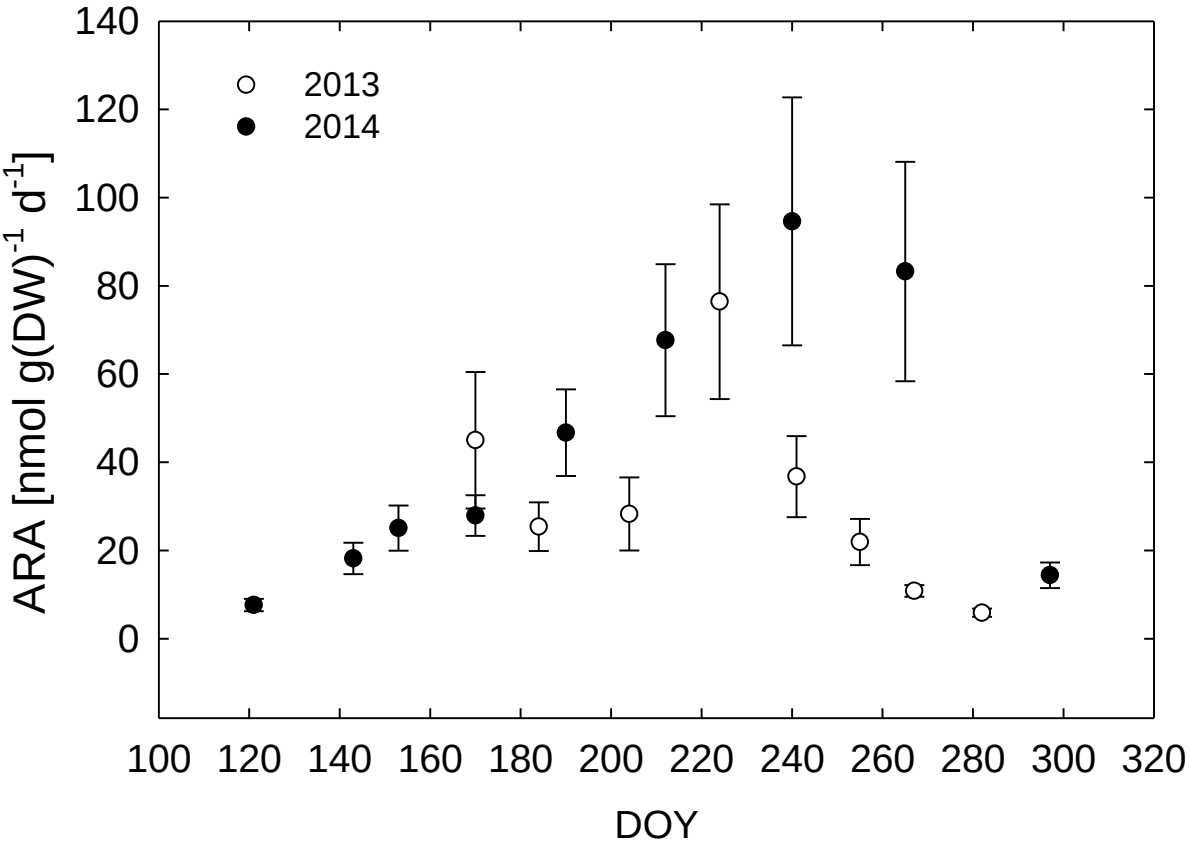

Fig. 3.2 Mean ARA rates over two field seasons across the hydrological transect at Mer Bleue bog. The vertical lines are standard errors.

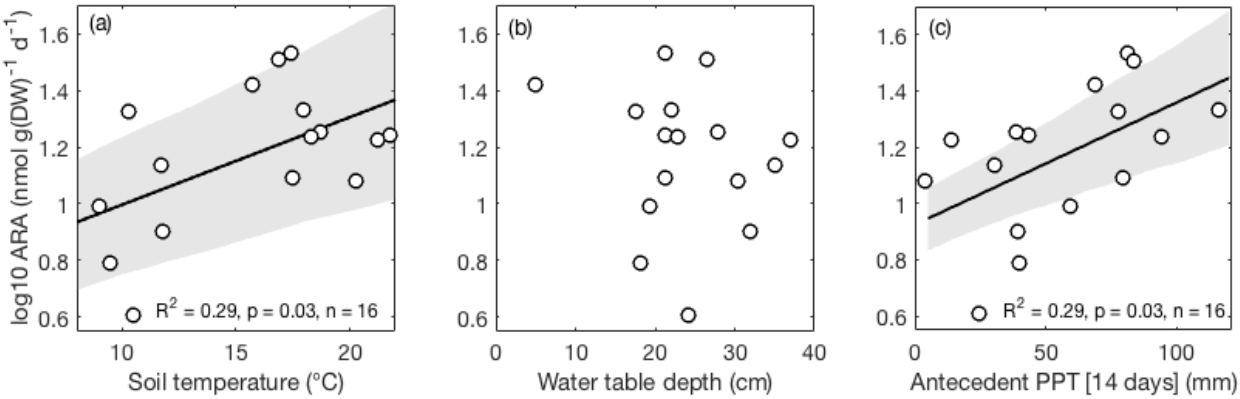

Fig. 3.3 Relationship between a) soil temperature, b) water table depth and c) antecedent precipitation (14 d) and mean ARA per sampling date. Solid line shows the best fit linear regression and gray area is the uncertainty in the regression parameters (± 1 StDev).

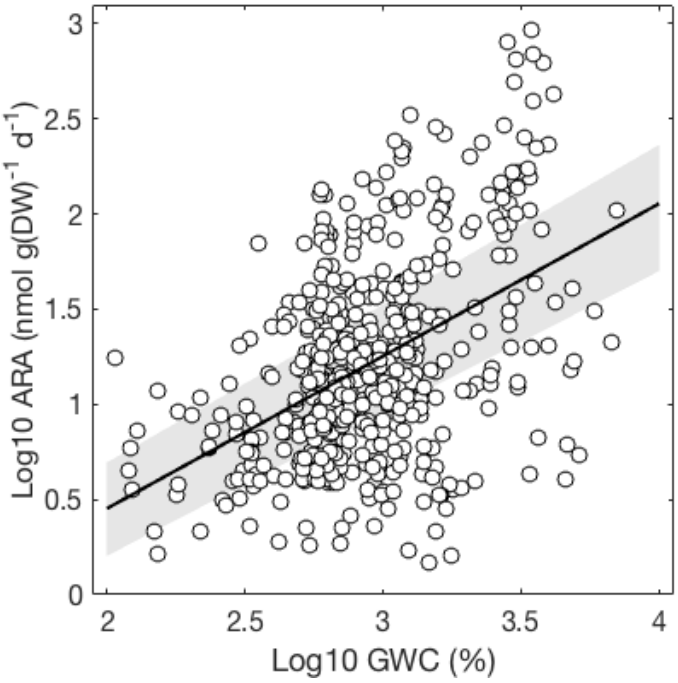

Fig. 3.4 The relationship between ARA and gravimetric water content (GWC) in *Sphagnum*. Linear regression line: $Log_{10}(ARA) = 0.8 * Log_{10}(GWC) - 1.15$, $R^2 = 0.21$, $n = 510$, $p < 0.0001$. The gray shaded area indicates the uncertainty of the regression (±1 StDev for slope and intercept).

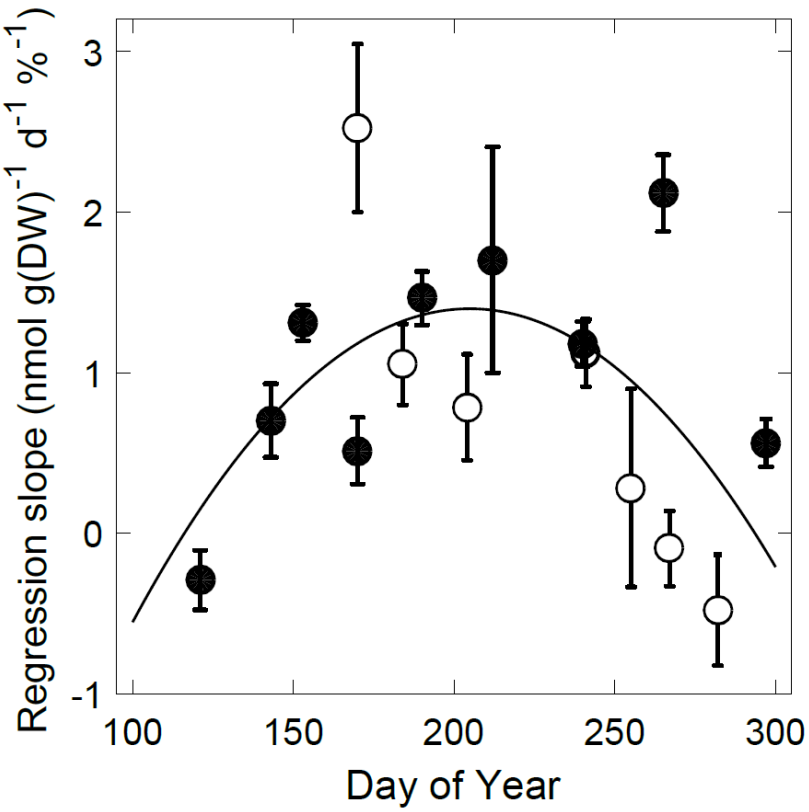

Fig. 3.5 Seasonal variation of the sensitivity of N_2 fixation to moisture, expressed as the slope of the regression between GWC and ARA per sampling date in 2013 (white circles) and 2014 (black circles). The line represents a polynomial fit: $y = -0.00018x^2 + 0.073x - 6.059$ combining both seasons.

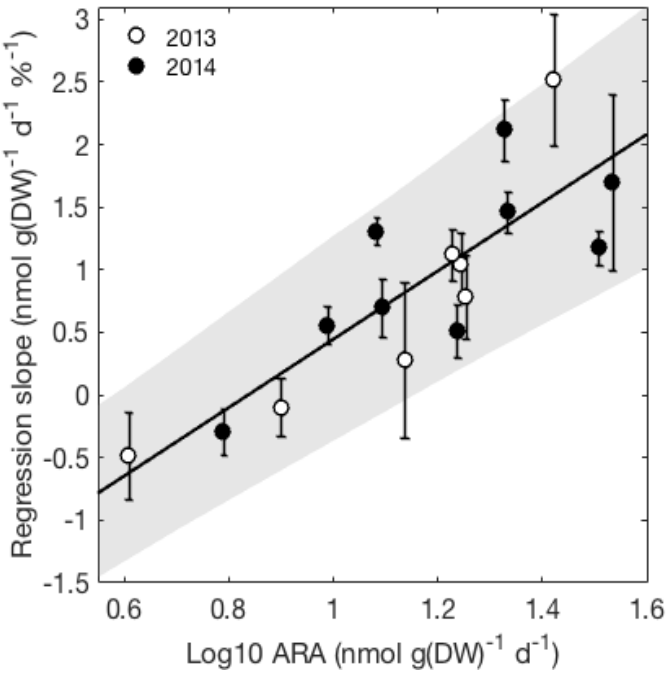

Fig. 3.6 The relationship between the regression slope between GWC and ARA and mean $\log_{10}$ ARA per sampling date. Regression line: y = 2.73*x - 2.29, $R^2 = 0.69$, n = 16, $p < 0.0001$. The gray area represents ± 95% CI.

Table 3.1 Multiple linear regression statistics of N_2 fixation model for Mer Bleue bog. Adj R^2 = 0.52, n = 16, p = 0.0033.

Variable	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**	**Std Beta**	**VIF**
Intercept	0.446	0.183	2.44	0.030*	0	.
Bi-weekly precipitation (mm)	0.004	0.001	3.10	0.009*	0.553	1
Mean soil temperature (°C)	0.029	0.010	2.89	0.013*	0.516	1

Table 3.2 Linear regression statistics of the relationship between GWC (independent variable) and ARA (dependent variable) for each sampling date.

Year	DOY	N	SD (log(GWC))	SD (log(ARA))	Mean (log(ARA))	Mean (log(GWC))	R^2	p-value	Slope (±SD)	Intercept (±SD)
2013	170	9	0.50	1.45	3.27	1165.56	0.77	<0.001	2.52 (0.52)	-15.48 (3.90)
2013	184	25	0.61	0.98	2.86	1123.89	0.43	<0.001	1.05 (0.25)	-4.31 (1.73)
2013	204	24	0.51	0.88	2.89	1155.30	0.20	0.03	0.78 (0.33)	-2.55 (2.29)
2013	224	18	0.21	0.74	3.92	789.55	NaN	NaN	NaN	NaN
2013	241	25	0.82	1.24	2.83	807.54	0.55	<0.001	1.12 (0.21)	-4.39 (1.36)
2013	255	25	0.27	0.79	2.62	922.00	0.01	0.65	0.28 (0.62)	0.70 (4.19)
2013	267	23	0.53	0.57	2.07	787.60	0.01	0.69	-0.10 (0.23)	2.70 (1.53)
2013	282	20	0.46	0.71	1.40	945.01	0.10	0.18	-0.48 (0.35)	4.65 (2.34)
2014	121	25	0.63	0.59	1.82	1771.02	0.10	0.13	-0.29 (0.19)	3.94 (1.35)
2014	143	25	0.71	0.92	2.52	1019.04	0.29	0.01	0.70 (0.23)	-2.16 (1.54)
2014	153	47	0.76	1.15	2.49	838.48	0.75	<0.001	1.31 (0.11)	-5.95 (0.72)
2014	170	47	0.65	0.97	2.85	1275.40	0.12	0.02	0.51 (0.21)	-0.70 (1.44)
2014	190	47	0.62	1.14	3.07	1292.35	0.63	<0.001	1.46 (0.17)	-7.11 (1.16)
2014	212	25	0.31	1.16	3.53	939.51	0.20	0.02	1.70 (0.70)	-8.05 (4.79)
2014	240	46	0.94	1.41	3.47	975.97	0.62	<0.001	1.18 (0.14)	-4.12 (0.91)
2014	265	44	0.58	1.53	3.05	1216.77	0.65	<0.001	2.12 (0.24)	-11.62 (1.66)
2014	297	35	0.85	0.87	2.28	1640.57	0.30	<0.001	0.56 (0.15)	-1.65 (1.05)

Supplementary material

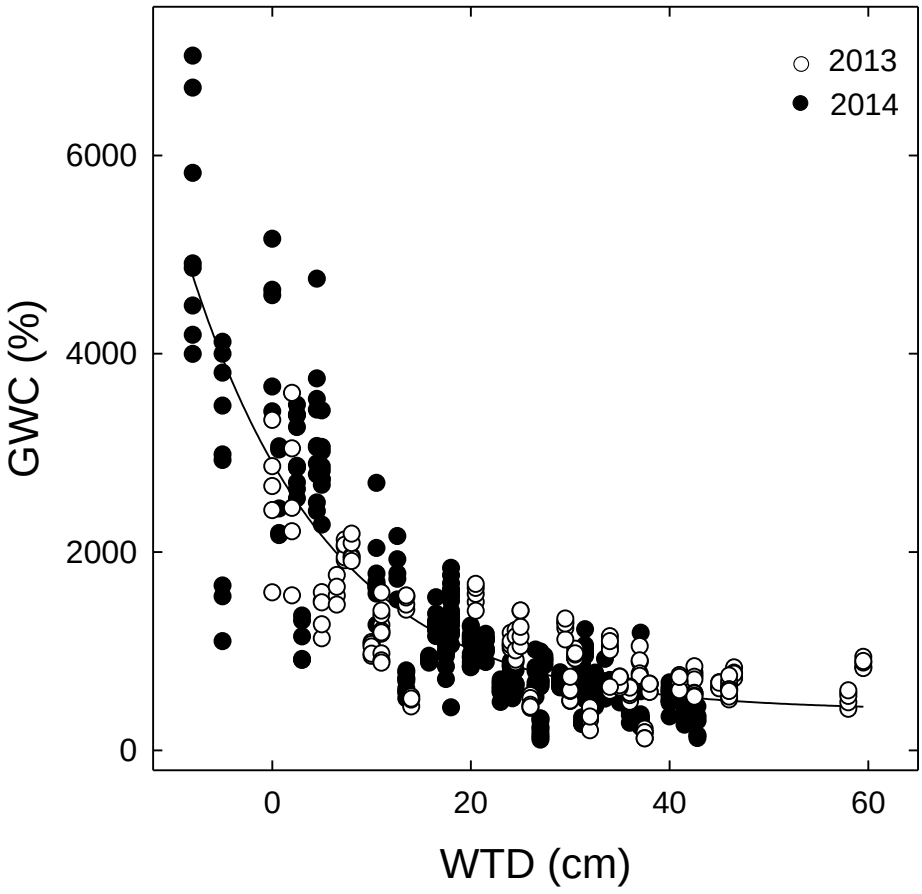

Fig. S3.1 The relationship between the water table depth and gravimetric water content in *Sphagnum* moss at Mer Bleue in 2013 and 2014. Line represents a best fit model: GWC = 401.9 + 2501.4 * $e^{(-0.07*WTD(cm))}$, R^2 adj = 0.75, $p < 0.0001$.

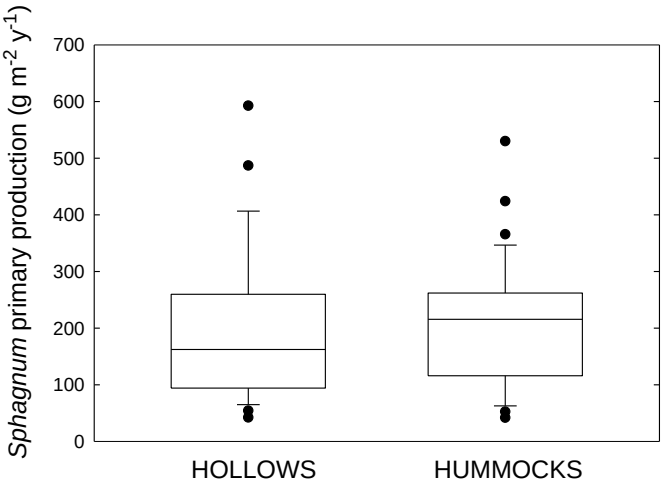

Fig. S3.2 Box and whisker plot showing *Sphagnum* primary productivity over the two years (2013 and 2014). The vertical line depicts 25th and 75th percentile, horizontal line within the box is a median and points above and below vertical lines represent outliers.

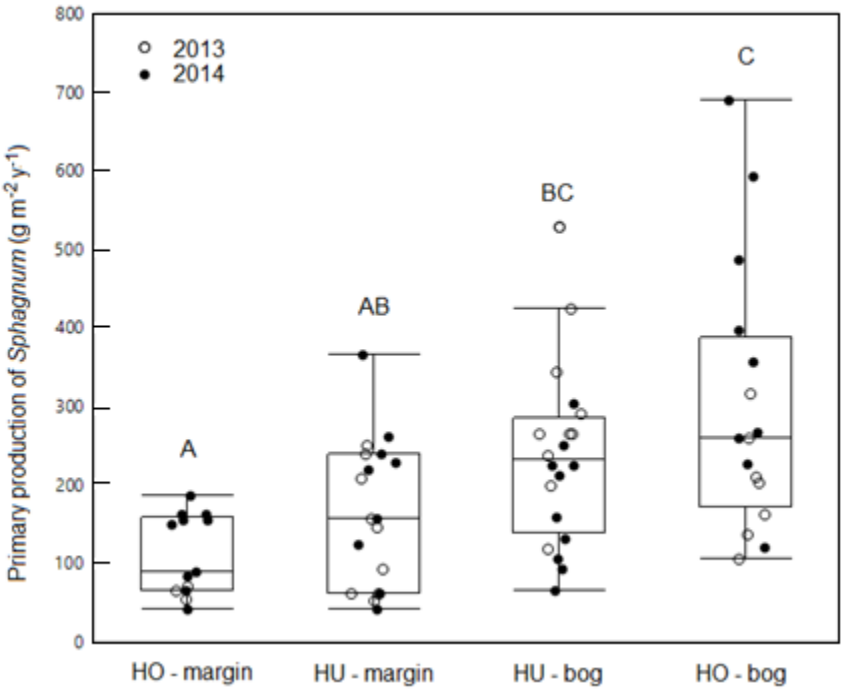

Fig. S3.3 Primary production of *Sphagnum* mosses of hollow (HO) and hummock (HU) along the transect in 2013 (white circles) and 2014 (black circles) presented as box and whisker plot. The vertical line depicts 25th and 75th percentile, horizontal line within the box is a median. One-way ANOVA showed that there were significant differences between the plots ($F_{3,63} = 8.64$, $p<0.0001$, $n = 66$) and significant differences between means are depicted with capital letters above box plots (post-hoc Tukey's HSD test).

Table S3.1 Mean annual ARA rates from both field seasons, 2013 and 2014, calculated for each microform (hummock, hollow and pond) and expressed in µmol m^{-2} d^{-1} units. Small letters (a-c) in the parenthesis represent significant differences between ARA rates among three different microforms (post-hoc Tukey's HSD; one-way ANOVA on log_{10} transformed ARA; $F_{2,502}$ = 48.12, $p < 0.0001$, n = 505).

Microform	N	ARA (µmol m^{-2} d^{-1})	Surface area cover* (%)
Hummock	225	44.7 (a)	51.2
Hollow	221	63.0 (b)	12.7
Pond	105	195.9 (c)	2.4
Trees**	NA	NA	33.6

* Values from Arrojo-Mora et al. (2018), ** Trees can also be above hummocks

Table S3.2 Four scenarios to weight surface area and N_2 fixation annual rate estimates at Mer Bleue bog.

Scenario	Scenario description	N_2 fixation (g N m^{-2} y^{-1})
1	Mean (Hollow + 10% open water) + Hummock	0.31
2	Hollow + Hummock	0.28
3	Hollow + Hummock + 10% open water	0.25
4	Hummock (Tree + Hummock) + hollow	0.24

CHAPTER 4

Nitrogen and phosphorus addition affects biological N_2 fixation and *Sphagnum* moss in an ombrotrophic bog

4.1 Context within the thesis

The industrial revolution and anthropogenic impacts in the last 200 years have resulted in larger N inputs to the biosphere. Elevated atmospheric N deposition, in forms of ammonium (NH_4) and nitrate (NO_3) have affected northern peatlands. Studies on long-term effects of increased N deposition to ombrotrophic bogs showed an increase in N content in *Sphagnum* and vascular plants, and a switch from N- to P-limited systems reflected by larger N:P ratios. The effects on microbial processes, and especially N_2 fixation, remain largely unknown.

In Chapter 4, I investigate the effects of long-term N inputs to an ombrotrophic bog on *Sphagnum* nutrient content and *Sphagnum*-associated N_2 fixation. By using *Sphagnum* from plots treated with gradual increments of different N species (NH_4, NO_3 and NH_4NO_3 combined), and P at Mer Blue bog, I sought to identify changes in *Sphagnum*'s N, P and N:P content and relate them to N_2 fixation activity. I tried to identify the threshold in N input that will inhibit N_2 fixation in ombrotrophic bogs. In particular, I examined whether P-limitation in *Sphagnum*, reflected by the increase in N:P ratio, translates to P-limitation of *Sphagnum* associated N_2 fixation.

4.2. Abstract

Increasing atmospheric deposition of N and P to ombrotrophic bogs may affect *Sphagnum* growth and N_2 fixation and we used a long-term fertilization experiment to examine the effects of N and P deposition on N_2 fixation of *Sphagnum*. We measured nitrogenase activity by acetylene reduction assay in *Sphagnum* from plots treated with long-term addition of different

N amounts and sources (0, 1.6, 3.2 and 6.4 g N m^{-2} y^{-1} as NH_4Cl and $NaNO_3$ and 6.4 g N m^{-2} y^{-1} $NH_4Cl/NaNO_3$), and P (5 g P m^{-2} y^{-1} as KH_2PO_4). *Sphagnum* N and P content and N:P ratio were measured to test nutrient limitation on N_2 fixation.

N_2 fixation significantly decreased with the smallest addition of both N species. NH_4 treatment increased *Sphagnum* N content more than NO_3, while both N species caused N:P ratio to rise above 16, indicating P-limitation. The addition of P increased N_2 fixation < 100 times and < 9 times compared to N treatments and controls, respectively. Ranging from 3 to 40, the N:P ratio best predicted the response of N_2 fixation to N and P additions. Although elevated N deposition substantially decreased N_2 fixation, the N:P ratio in *Sphagnum* may be a good predictor, likely owing to a strong P-limitation.

4.3 Introduction

In nutrient-poor ombrotrophic bogs, the dominant genus *Sphagnum* has developed strategies to compete with vascular plants (van Breemen, 1995). In N-limited bogs, for example, two strategies appear to be physiological adaptations, such as N retention and translocation within *Sphagnum* (Aldous, 2002a,b), while a third may be evolutionary (Kneip *et al.*, 2007) in hosting N_2-fixing microbes (diazotrophs). Biological N_2 fixation is an energetically costly enzymatic process performed by microbes that live free or epi- or endophytically in association with *Sphagnum* mosses. Where atmospheric N deposition is small (< 0.1 g m^{-2} y^{-1}; Vitt *et al.*, 2003), bog ecosystems rely on N_2 fixation as the primary source of N that sustains primary production (Vile *et al.*, 2014). Most of the 18 Pg of N accumulated in northern peatlands (Wang *et al.*, 2014) can be attributed to N_2 fixation by diazotrophs either associated with the live *Sphagnum* (Larmola *et al.*, 2014; Vile *et al.*, 2014) or non-symbiotically in the deeper peat (Knorr *et al.*, 2015).

The industrial revolution and anthropogenic impacts in the last 200 years have resulted in larger atmospheric N deposition as ammonium (NH_4) and nitrate (NO_3) (Galloway & Cowling, 2002), which may alter the N cycle in bogs, though their effects on ecosystem processes are not yet completely understood. One side effect of increased atmospheric N deposition is an increase in phosphorus (P) limitation in terrestrial ecosystems (Vitousek *et al.*, 2010). P enters ecosystems via mineral weathering, dust deposition or local sources such as agriculture (Tipping *et al.*, 2014) but in most ombrotrophic bogs these inputs are small. The large C:P and N:P ratios in northern peatlands suggest strong recycling and P limitation to vegetation and microbes (Bragazza *et al.*, 2004; Wang & Moore, 2014), and the N:P ratio has been used as an indicator of nutrient limitation in plants (Koerselman & Meuleman, 1996).

Biological N_2 fixing organisms may act in terrestrial ecosystems as a 'nitrostat' whereby when the available N is limited, N_2 fixation is turned on and turned off when there is no N limitation (Gutschick, 1981; Menge & Hedin, 2009). Given that elevated N deposition increases N content in *Sphagnum* tissue (Bragazza *et al.*, 2005), the nitrostatic nature of *Sphagnum*-associated N_2 fixation, if it existed, would result in the decline of N_2 fixation with the increase in *Sphagnum* N content. Thus, increased atmospheric N deposition in ombrotrophic bogs would replace N_2 fixation to maintain *Sphagnum* productivity, but evidence suggests a more complicated story.

First, the negative effect of N addition on *Sphagnum*-associated N_2 fixation in ombrotrophic bogs has only recently been established (Kox *et al.*, 2016). Contrasting results on the effects of N deposition on bryophyte associated N_2 fixation have been reported in boreal forests (Rousk & Michelsen, 2016). Increased N deposition decreased N_2 fixation in feather moss-cyanobacteria associations (Gundale *et al.*, 2009; Ackermann *et al.*, 2012) in field

experiments, but no effect was found in the laboratory (Rousk *et al.*, 2014b). Critical loads of N affecting N_2 fixation in these associations range from 0.3 to 1.25 g N m^{-2} y^{-1} and seem to affect two bryophyte species, *Pleurozium schreberi* and *Hylocomium splendens* differently (Gundale *et al.*, 2009; Ackermann *et al.*, 2012; Rousk & Michelsen, 2016). Once N addition ceases, N_2 fixation can be recovered in these associations but it may depend on the amounts of N added (Rousk *et al.*, 2014b; Rousk & Michelsen, 2016). In contrast, N deprivation of *Sphagnum* mosses once exposed to elevated N deposition did not stimulate N_2 fixation (Kox *et al.*, 2016). These inconsistencies in responses suggest the possible importance of drivers other than N in controlling N_2 fixation activity in bryophytes. P is a known prerequisite for nitrogenase activity (Vitousek *et al.*, 2010), and P enrichment maintains larger N_2 fixation after N additions in *Sphagnum* (Kox *et al.*, 2016). In bogs, the availability of P can alleviate the effects of N addition on *Sphagnum* productivity (Limpens *et al.*, 2004) and a tissue N:P ratio >16 indicates P-limitation in *Sphagnum* (Koerselman & Meuleman, 1996; Güsewell, 2004). The 'nitrostat' effect of *Sphagnum*-associated N_2 fixation may therefore depend on the availability of P to moderate the effects of N addition in bogs and *Sphagnum* tissue N:P ratio may be a better predictor of N_2 fixation in ombrotrophic peatlands than N and P concentration alone.

Second, elevated deposition of NH_4 and NO_3 may have different effects on *Sphagnum* mosses. Both can be toxic for *Sphagnum* at the physiological level, but NH_4 seems to have a stronger effect than NO_3 (Press *et al.*, 1986) on reducing *Sphagnum* production (Gunnarsson & Rydin, 2000), with preferential and faster uptake of NH_4 over NO_3 and organic sources of N (Wiedermann *et al.*, 2009; Fritz *et al.*, 2014). *Sphagnum*, however, appears able to adapt to long-term N inputs (Granath *et al.*, 2009), and may have developed NH_4 detoxification mechanisms to buffer the effects of increased NH_4 loads (Fritz *et al.*, 2014). Despite this, *Sphagnum* growth

decreases at critical loads of 1 – 1.5 g m^{-2} y^{-1} (Lamers *et al.*, 2000) and long-term N addition studies in bogs show that *Sphagnum* abundance declines, outcompeted by vascular plants (Bubier *et al.*, 2007; Wiedermann *et al.*, 2009; Juutinen *et al.*, 2010). *Sphagnum* has almost disappeared in European peatlands with large atmospheric N addition (Bobbink *et al.*, 1998). Long-term, elevated (> 1.5 g N m^{-2} y^{-1}) N exposure and overshading by vascular plants, could thus inhibit *Sphagnum* growth. Eventually, the loss of *Sphagnum* could result in the loss of symbiotic diazotrophs.

The aim of this study is to address how long-term, field addition of NH_4 and NO_3 and P affect biological N_2 fixation determined under controlled laboratory conditions, tissue N and P concentrations and N:P ratio, and productivity of *Sphagnum*. At Mer Bleue bog, Ontario, we used a four-year fertilization study in which NH_4Cl and $NaNO_3$ were added separately at 0, 1.6, 3.2, 6.4 g N m^{-2} y^{-1}. We hypothesize that N_2 fixation will be the largest in control plots and will decrease with increased N deposition accompanied by an increase in N concentration and N:P ratio in *Sphagnum* moss. We also hypothesize that the effect of NH_4 on N_2 fixation will be stronger than NO_3 through the differential uptake of NH_4 over NO_3 increasing the N:P ratio. We expect that the growth and net primary productivity (NPP) of *Sphagnum* will decrease with increasing N addition. We used a second experiment in which 6.4 g N m^{-2} y^{-1} of NH_4NO_3 has been added for 10 years to elucidate whether N sources in combination also negatively affect N_2 fixation and we hypothesize that N_2 fixation will not be significantly different at this treatment compared to the addition of NH_4 and NO_3 separately.

To test the effect of P addition on N_2 fixation, we used plots that had received 5 g P m^{-2} y^{-1} as KH_2PO_4 for 15 years compared to controls receiving only distilled water. We hypothesize a larger P concentration, smaller N:P ratio and faster N_2 fixation in *Sphagnum* mosses which had

been treated with P, compared to the controls. Finally, we hypothesize that the N:P ratio from *Sphagnum* across all experiments will be a better predictor of N_2 fixation rates than *Sphagnum* N and P content individually.

4.4. Materials and Methods

Field site

Mer Bleue is a cool temperate, ombrotrophic bog located in Ontario, Canada (45° 40'N, 75° 50'W) with a mean annual temperature and precipitation of 6°C and 943 mm, respectively (Canadian Climate Normals 1981-2010). The hummock vegetation includes *Sphagnum capillifolium, S. magellanicum* and *Polytrichum strictum*, and shrubs *Chamaedaphne calyculata* Moench, *Rhododendron groenlandicum* (Oeder) K.A. Kron & W.S. Judd, *Vaccinium myrtilloides* Michx. and *Kalmia angustifolia* L (Bubier *et al.*, 2006).

Several fertilization experiments have been established at Mer Bleue (Table 4.1), involving the annual addition of nutrients dissolved in distilled water (equivalent to 2 mm addition) seven times from May to August. To eliminate possible interactions with *Sphagnum* growth by shading, vascular plants were clipped each field season in the NH_4/NO_3 experiment but not in plots in the NH_4NO_3 and PK experiments. Plot size was 1 x 1 m in the NH_4/NO_3 experiment and 3 x 3 m in the NH_4NO_3 and PK experiments. We extracted blocks of dominant *S. capillifolium* moss (20 x 20 x 15 cm; width x length x depth) in mid July 2015 (two weeks after a fertilizer application) and transferred them to the laboratory, where they were kept for one week in plastic bags at 4ºC to maintain the field moisture prior to the experiment. Blocks were then sub-sampled (each treatment had 3 plots and for each plot 5 replicates were taken).

N_2 fixation measurements in the controlled environment

We measured N_2 fixation with acetylene reduction assay (ARA, Hardy *et al.*, 1968) in 48-h incubations in a controlled environment comprising 12 h of simulated day cycle, photosynthetically active radiation (PAR) of 400 µmol m^{-2} s^{-1}, temperature 18°C and relative humidity 80%; followed by 12 h of zero PAR.

While ARA method can underestimate the N_2 fixation in *Sphagnum*-dominated peatlands due to potential inactivation of methanotrophic bacteria by acetylene (De Bont & Mulder, 1976), methane (CH_4)-induced N_2 fixation has not been found to be significant in live parts of *Sphagnum* in drier fen-bog ecosystems (Larmola *et al.*, 2014; Leppänen *et al.*, 2015). Mer Bleue hummocks, in which the fertilization experiments were conducted, have a water table varying from 20 to 55 cm below the surface during the growing season (Juutinen *et al.*, 2010). Methane production occurs anaerobically below the water table level, accompanied by methanotrophic activity in aerobic layers at and just above the water table (Juutinen *et al.*, 2018). Methane concentration close to the surface in hummocks is small (Moore *et al.*, 2011) so that if there is CH_4-induced N_2 fixation, it would be lower in the peat, at the interface with water table (Knorr *et al.*, 2015) rather than the top, 5-cm live *Sphagnum*, which is the focus of this study. While ARA does not capture all potential microbial communities involved in N_2 fixation within the peat profile in the bog, this method captures most of the *Sphagnum* associative diazotrophs in the controlled laboratory experiment in this study.

Five sub-replicate *Sphagnum* cores (5 cm deep and 4.5 cm diameter) from each triplicate plot were transferred into 50 mL acid-washed Erlenmeyer flasks which were immediately capped with tight rubber stoppers (Suba-Seal®), reinforced with silicone. 5% (v/v) of the atmosphere was replaced with acetylene (C_2H_2) made fresh from calcium carbide and DI water; no traces of ethylene (C_2H_4) were present in the C_2H_2. We sampled for both CH_4 and C_2H_4 production at 0, 2,

12, 24, 36 and 48 h of incubation. Over the 2 day incubations designed to capture the diurnal cycle, no CH_4 was produced in the flasks, and the linear production of C_2H_4 over time was confirmed. We analyzed gas samples immediately after sampling with a gas chromatograph (GC-2014, Shimadzu) with a flame ionization detector (FID) and HayeSep N (80/100 mesh; 2 m) column and injection/detection and column temperatures of 175 and 75℃, respectively. We used 50, 100 and 300 ppm ethylene standards to build the standard curve.

After incubation, samples were weighed, oven-dried at 60°C and re-weighed and we estimated the headspace volume by replacing the air with DI water. ARA rates are expressed as nmol g^{-1} dry weight (DW) d^{-1}.

Elemental analyses of the Sphagnum tissue

To directly link N_2 fixation and moss tissue N and P concentration, the oven-dried mosses after the ARA were finely ground through a 40 mesh sieve (Wily Mini Mill 3383-L10, Thomas Scientific, USA) and acid digested using concentrated H_2SO_4 and H_2O_2, and Se and Li_2SO_4 catalysts (Parkinson and Allen 1975). Total N and P were colorimetrically analyzed on a LachatQuick-Chem AE flow injection autoanalyzer (Lachat Instruments, Milwaukee, WI).

To estimate the effect of NO_3 versus NH_4 fertilization on N concentration in *Sphagnum* tissue, we calculated homeostatic regulation coefficient (H, used previously in Juutinen *et al.*, 2015; Wang *et al.*, 2016), derived from the following equation:

$$y=c*x^{1/H}$$

where y is N concentration (mg g^{-1}), c is a constant, x is N addition, with an estimated 0.5 g m^{-2} y^{-1} as a background deposition comprising equal contributions of NH_4 and NO_3 resulting in N additions of 0.25, 1.85, 3.45 and 6.65 g m^{-2} y^{-1} as either NH_4 or NO_3.

Sphagnum net primary productivity (NPP) and growth in response to NH_4 and NO_3 fertilization

We installed cranked wires at the end of April 2015 to measure *Sphagnum* growth and to estimate NPP for the growing season in plots that were treated with different increments of NH_4 and NO_3 for four years (NH_4/NO_3 experiment). We measured capitulum height in mid-June and in November at the end of the growing season. One core (10 x 10 x 2 cm; length x width x depth) was extracted from each triplicate plot for each treatment, the moss capitula were cut off and the cores were dried to estimate the bulk density (expressed as g DW cm^{-3}). The *Sphagnum* NPP was calculated by multiplying moss incremental height over the growing season with the bulk density and expressed as g m^{-2} y^{-1}.

Statistical analyses

To test for significant differences among elemental concentrations (N, P and N:P ratio), ARA rates across different treatments in NH_4/NO_3 and NH_4NO_3 experiments, we used nested ANOVA with treatment as a fixed factor, ran as either parametric or non-parametric (nested ANOVA with permutations) analysis as implemented in the R code (nest.anova.perm.R by Borcard and Legendre at adn.biol.umontreal.ca/~numericalecology/Rcode/) followed by *post hoc* Tukey's Honest Significant Difference (HSD) test. The decision between parametric and non-parametric tests was made after Shapiro-Wilk test was performed on raw or transformed datasets and passed or failed the normality and Levene's homogeneity of variance test, respectively. We used Mann-Whitney's *U* test to compare differences in N and P concentration and N:P ratio between control plots and P-treated plots in the PK experiment.

Linear regressions were used to test relationships between variables (N:P *versus* N and P, ARA versus N, *Sphagnum* NPP *versus* N:P ratio) ensuring that the normality of datasets and residuals were met. Owing to non-normal distribution of the variables (P and N:P), Spearman's rank correlations were used to test the relationships between ARA and N, P and N:P across all

treatments. All figures throughout the manuscript are plotted values without transformations, however all equations indicate transformations if they were necessary for the analysis.

Best fit models were used to describe relationships between following variables: power function to derive the homeostasis regulation coefficient (thoroughly defined in Wang *et al.*, 2014), exponential decay for ARA *versus* N for NH_4 treatments, and ARA *versus* N:P ratio in *Sphagnum* tissue. All error bars throughout the manuscript are standards errors.

All statistical tests were performed using R 3.1.2 (R Core Team 2014), JMP 11.2.0 and Matlab 2015b version.

4.5. Results

The effect of N and P addition on N and P concentration, N:P ratio, NPP and growth of Sphagnum

N concentration in *Sphagnum* tissue significantly increased in response to both NH_4 and NO_3 additions (NH_4/NO_3 experiment, nested ANOVA, $F_{6,83} = 28.5$, $P = 0.001$, Fig. 4.1a). There was a non-linear increase of N concentration from ~7 mg g^{-1} in control plots to up to 16 mg g^{-1} in 6.4 NH_4 and 13 mg g^{-1} in 6.4 NO_3 treated plots (Fig. 4.1a and d). Interestingly, plots treated with 6.4 g NH_4NO_3 m^{-2} y^{-1} were not significantly different from the same level of fertilization of NH_4 only, but were significantly larger than the plots receiving 6.4 g NO_3 m^{-2} y^{-1} (nested ANOVA, $F_{8,106} = 23.5$, $P = 0.001$, Fig. 4.1a). The homeostasis regulation coefficient (H) for N concentration in *Sphagnum* tissue was larger in NO_3 than in NH_4 treatments (H = 5.69 and 3.37, respectively; Fig. 4.1d).

P concentration in *Sphagnum* tissue varied among the N treated plots from 0.4 to 0.7 mg g^{-1} with no apparent trends among the treatments nested ANOVA $F_{8,106}$=3.67, *P=0.014*, Fig. 4.1b).

The N:P ratio in *Sphagnum* tissue increased from 13 in controls to above 16 in all N treatments (nested ANOVA, $F_{8,106} = 22.9$, $P = 0.001$, Fig. 4.1c). The largest N:P ratio was 30 in 3.2 g NH_4 treated plots (NH_4/NO_3 experiment; ANOVA, $F_{6,83} = 25.2$, $P = 0.001$, Fig. 4.1c). Notably, all N fertilized plots had N:P >16 while control plots remained < 16 (Fig. 4.1c and 4.2a,b). *Sphagnum* tissue N:P ratio was driven mainly by N concentration rather than P concentration in Experiment 1 (Fig. 4.2a,b).

After 15-years of PK addition (PK experiment, Table 4.1) there were no significant changes in N concentration in *Sphagnum* tissue compared to the control (Table 4.2). However, there was a significant, 3.7 fold increase in *Sphagnum* P concentration in PK treated plots compared to the control, with a decrease in N:P ratio from 14 to 4 (Table 4.2).

Sphagnum NPP significantly decreased in response to 1.6 NO_3, 3.2 NH_4, and 6.4 NO_3 and 6.4 NH_4 treatments compared to the control (Fig. 4.3a), and was best described with the increase in N:P ratio (Fig. 4.3b).

Regulation of N_2 fixation by N and P

There was a significant decrease in ARA rates in plots treated with N compared to controls (nested ARA, $F_{9,119} = 133.1$, $P = 0.001$; Fig. 4.4). The control plots (average 27 ± 4 nmol g^{-1} DW d^{-1}, mean ±SE) from both experiments were not significantly different from each other. The annual addition of 1.6 g N m^{-2} as both NH_4 and NO_3 lowered N_2 fixation up to 30 times compared to controls. The strongest effects on ARA rates were seen in the 3.2 NH_4, 6.4 NH_4 and 6.4 NO_3 treatments, with the smallest rates in the 6.4 NH_4 treatment (0.8 ± 0.1 nmol g^{-1} DW d^{-1}, mean ±SE). ARA rates in the 6.4 NH_4NO_3 treatment were significantly larger than in the 6.4 NH_4 and 6.4 NO_3 treatments. The long-term addition of PK to *Sphagnum* resulted in the largest

ARA rate (287.9 nmol g^{-1} DW d^{-1}), up to 5 times and 110 times larger than controls and N treated plots, respectively.

There was a negative non-linear relationship between ARA and N concentration in *Sphagnum* tissue in NH_4 treatments (exponential decay model, $r^2 = 0.48$, $P < 0.0001$, $n = 88$; Fig. 4.5a), and a significant negative linear relationship between the ARA and N concentration in NO_3 treatments ($r^2 = 0.53$, $P < 0.0001$, $n = 80$; Fig. 4.5a). We also found a significant correlation between ARA and *Sphagnum* tissue N when data from all experiments were pooled (Fig. 4.6a). No correlation was found between ARA and P concentration in the Experiment 1 (Fig. 4.5b) and a weak but significant positive correlation was found across all the experiments (Fig. 4.6b).

An exponential decay model described relationship between ARA and N:P ratio when either N species was added to *Sphagnum* (Fig. 4.5c). A strong negative correlation across N and P treated plots between ARA and N:P (Fig. 4.6c) indicated that N:P ratio could be the best predictor across a wide range of N:P ratios.

Finally, the exponential decay model best explained the relationship between ARA and N:P ratio across all the treatments (model $r^2 = 0.95$, $P < 0.0001$; Fig. 4.6d).

4.6. Discussion

The effect of N and P addition on Sphagnum

This study shows that both NH_4 and NO_3 applied over 4 years at up to 6.4 g N m^{-2} y^{-1} significantly increased N concentration and N:P ratio in photosynthetically active *Sphagnum* tissue in an ombrotrophic bog. Most fertilization studies have focused on the effects of both sources of N on *Sphagnum* stoichiometry, usually applied as NH_4NO_3, a 1:1 ratio (Aerts *et al.*, 1992; Gunnarsson & Rydin, 2000; Limpens *et al.*, 2004; Bubier *et al.*, 2007; Sheppard *et al.*, 2013). We show a larger homeostatic regulation factor for *Sphagnum* in plots receiving NO_3 (H

= 5.7) compared to NH_4 (H = 3.4), the latter being in agreement with (Juutinen *et al.*, 2015) where H = 3.6 when both N species (NH_4NO_3) were added. Our results indicate that NH_4 may have a stronger impact than NO_3 on N allocation mechanisms in *Sphagnum* subjected to increased N deposition. The smaller H and larger increase in N concentration in the NH_4 treatment in our study suggest that NH_4 may be preferentially taken up by *Sphagnum* over NO_3, as noted by Wiedermann *et al.* (2009) and Fritz *et al.* (2014). With elevated concentrations, both NH_4 and NO_3 can be toxic to *Sphagnum* (Press *et al.*, 1986). However, a possible adaptation of *Sphagnum* to elevated NH_4 observed as an increase in N-rich amino acids in live *Sphagnum* tissue (Fritz *et al.*, 2014) may be detected in our study as larger total N in plots receiving elevated NH_4 in comparison to NO_3. On the other hand, the presence and the activity of nitrate reductase shows that NO_3 can be utilized by *Sphagnum* (Woodin *et al.*, 1985; Press *et al.*, 1986), but usually when NO_3 is the primary source. An excess of added NO_3 may leach past the *Sphagnum* and into the rhizosphere, where it may be utilized by vascular plants (Tomassen *et al.*, 2004), resulting in larger H and smaller increase of N concentration in *Sphagnum* in NO_3 treatments.

The addition of 5 g P m^{-2} y^{-1} increased P concentration in *Sphagnum* about 4 times, compared to controls and the decrease in N:P ratio from 14 in controls to 4 in under P treatment suggests a switch from N-P co-limitation at Mer Bleue (Wang & Moore, 2014) to a strong N-limitation (N:P < 14; Koerselman & Meuleman, 1996; Güsewell & Koerselman, 2002). The four-fold increase in P concentration in *Sphagnum* in response to P fertilization is at least twice that of N concentration under 6.4 g N m^{-2} y^{-1} and may indicate that *Sphagnum* stores P in its photosynthetically active parts, and contrary to N, P is not leached downward. Added P in *Sphagnum* may be either quickly used by *Sphagnum* and microbes, or bound to organic matter,

becoming recalcitrant and as such buried into the peat over time. Under the P-limitation, only with the activity of phosphatases (released by plants and microbes) in peat, P becomes unbound from organic material (Pinsonneault *et al.*, 2016) and recycled upwards into *Sphagnum* and plants (Wang *et al.*, 2014). Large P loads may be toxic for plant cells (Bubier *et al.*, 2007) and may hinder the N retention capacity (Xing *et al.*, 2010) which could explain no increase in N in *Sphagnum* from PK plots in comparison to controls. Although not tested in our NH_4/NO_3 experiment, Bubier *et al.* (2007) and Juutinen *et al.* (2010) showed that *Sphagnum* abundance declines at the expense of vascular plants leading to increased N leaching into the rhizosphere (Heijmans *et al.*, 2001; Xing *et al.*, 2010).

Long-term N additions can either increase or decrease *Sphagnum* NPP, depending on which element is limiting its growth: N in the former and P in the latter (Aerts *et al.*, 1992; Heijmans *et al.*, 2001; Güsewell, 2004; Limpens *et al.*, 2004). In this study, four years of N addition resulted in a variable pattern of NPP rates in *Sphagnum* across the treatments: the smallest and largest additions of NH_4, in contrast to NO_3, had no statistically significant effect compared to the control, whereas significant decreases were observed in other treatments. Note, however, that although there were no significant differences due to large variability between the treatment plots, the means of treated plots were half that of the controls. A control other than N treatment may drive this variability (i.e. water table depth), and 55% of NPP variance being explained by the increase in N:P ratio indicates that P can buffer some of the N addition effects. In this study, the largest NPP occurred when the N:P ratio was ~ 14, similar to that for *S. magellanicum* (Fritz *et al.*, 2012).

Regulation of N_2 fixation in Sphagnum by N and P in the controlled environment

N_2 fixation significantly decreased with the addition of both NH_4 (linearly) and NO_3 (non-linearly) and NH_4NO_3. More importantly, ARA rates decreased to < 1 nmol g^{-1} DW d^{-1} in response to the smallest N addition in our study, which becomes an ecologically irrelevant amount of N_2 fixed. Assuming a theoretical ARA:N_2 fixation rate conversion factor of 0.85 (Stewart *et al.*, 2011b) and 0.32 (Vile *et al.*, 2014) in *Sphagnum*, and the length of the field season of 210 days at Mer Bleue, from our laboratory study we estimate a range of maximal rates of N_2 fixation in controls of 0.3 - 0.8 g N m^{-2} y^{-1} and 0.01 - 0.03 g N m^{-2} y^{-1} in the N-amended plots. The mechanisms behind the effects of NH_4 and NO_3 on N_2 fixation may be both physiological and ecological. *Sphagnum* hyaline cells are most likely to host diazotrophs, either via endosymbiotic or passive association, thus the excess of NH_4 would affect both *Sphagnum* and diazotrophs. Generally, NH_4 is incorporated into amino acids glutamine and glutamate in *Sphagnum* (Kahl *et al.*, 1997), and both NH_4 and glutamate are known inhibitors of the expression of nitrogenase enzyme responsible for N_2 fixation. Conversely, NO_3 is at first transformed into NH_4 through the activity of nitrate-reductase in *Sphagnum* then into glutamate (Press *et al.*, 1986). The inhibition of N_2 fixation can therefore be an instantaneous physiological response after the application of NO_3 and NH_4. However, a continuous and long-term addition of both NH_4 and NO_3 may have a toxic effect on cells of *Sphagnum* and diazotrophs, accompanied by the accumulation of amino acids, giving a rise in total N in *Sphagnum* ultimately increasing the N:P ratio and creating strongly P-limited conditions.

Theory suggests that N_2 fixation in some bryophyte-bacteria associations depends on N availability in the environment, acting like a 'nitrostat' (DeLuca *et al.*, 2008; Menge & Hedin, 2009). In boreal forests, for example, the feather moss-cyanobacteria association seems to follow a non-linear response to canopy throughfall N deposition ($N\ fix = 1.71 * e^{(-4.4 * N\ dep)}$; DeLuca *et*

al., 2008, with rates > 1.1 g N m^{-2} y^{-1} leading to no N_2 fixation, confirmed by (Rousk *et al.*, 2014b). Feather moss N_2 fixation may be used as a sensitive indicator for local N deposition (Ackermann *et al.*, 2012). N_2 fixation subjected to N addition or deprivation is regulated by the count and the activity of cyanobacteria suggesting that the effects of N deposition on N_2 fixation may be reversible in boreal forest stands (Rousk *et al.*, 2014b). Recently, N_2 fixation in *S. magellanicum* was shown to decrease as a result of both short- and long-term additions of NH_4NO_3 (Kox *et al.*, 2016) but the levels of added N (4 to 20 g N m^{-2} y^{-1}) are far above current N atmospheric deposition. Our range of N deposition (0.5 to 6.9 g N m^{-2} y^{-1}) is not uncommon (Galloway *et al.*, 2008), though rates of atmospheric N deposition are on the rise in China but declining in most of North America and Europe. Our results suggest that N_2 fixation in the top, live *Sphagnum* will be insignificant above ~2 g N m^{-2} y^{-1} of wet deposition, but it remains unknown if this effect is irreversible in *Sphagnum* mosses exposed to long-term elevated N loading, or if there is an active N_2 fixation lower in the peat profile.

In ombrotrophic bogs, tissue N concentration in *Sphagnum* has been shown to increase with the increased N deposition (this study and e.g. Lamers *et al.*, 2000; Bragazza *et al.*, 2005; Fritz *et al.*, 2014; Wang & Moore, 2014; Juutinen *et al.*, 2015). Given that *Sphagnum* hosts diazotrophic communities, changes in N content in *Sphagnum* tissue may also reflect the effect of N deposition on N_2 fixation activity. In this study, small but significant increases of N concentration from 7.1 mg g^{-1} (control) to 9.4 mg g^{-1} (1.6 NH_4) and 9.8 mg g^{-1} (1.6 NO_3) was accompanied by an order of magnitude decrease in ARA rates, and an increase in N:P ratio, rising from 14 (control) to 23 (1.6 NH_4) and 21 (1.6 NO_3). N_2 fixation in terrestrial ecosystems is strongly P-limited (Vitousek *et al.*, 2010) owing to its large P requirements. Local P deposition (Tipping *et al.*, 2014) may also change *Sphagnum* tissue content, by increasing total P and

significantly decreasing N:P ratio (in this study to an average of 3.7). Thus, P that is taken up by *Sphagnum* may become more available to diazotrophs for their metabolic activity. Interestingly, N:P ratio in control plots never exceeded 15, generally pointing to N and P co-limitation in *Sphagnum*. However, *Sphagnum* tissue N:P > 16 may not only indicate P-limitation on *Sphagnum* productivity (Koerselman & Meuleman, 1996), but it can also point to P-limitation on N_2 fixation also suggested by (Kox *et al.*, 2016). N_2 fixation is driven by N:P ratios in prairie soils (Eisele *et al.*, 1989) and in the aquatic ecosystems (Howarth *et al.*, 1988) especially where changing N:P is caused by eutrophication (Peñuelas *et al.*, 2013). We show that ARA rates exponentially decreased with the increase of N:P ratios and in changing environments, where regional and local N and P inputs vary, N:P ratio in *Sphagnum* may be a good predictor of N_2 fixation activity.

While the smallest addition of N (1.6 g m^{-2} y^{-1}) in the form of NH_4 significantly decreased ARA rates, the NPP of *Sphagnum* was not affected, suggesting that, functionally, increased N deposition may replace N_2 fixation to maintain *Sphagnum* requirements for N. At loads of > 1.6 g N m^{-2} y^{-1} in both NH_4 and NO_3 we recorded a decrease in *Sphagnum* growth and NPP followed by linear increase of N:P ratio. The addition of PK causes a decrease in the abundance of *Sphagnum* at the expense of vascular plants and can be toxic to *Sphagnum* at high levels (Bubier *et al.*, 2007). Moreover, N:P ratio of 3.7 indicates strong N limitation on *Sphagnum* growth and increased need for N to maintain its productivity (Koerselman & Meuleman, 1996). Contrary to expectations however, past research showed that *Sphagnum* from PK plots did not retain any added labelled ^{15}N (Xing *et al.*, 2010), possibly due to large N_2 fixation rates that were not measured at the time. Using the same ARA to N_2 fixation conversion as above, our maximal seasonal N_2 fixation rates range from 1.4 to 3.8 g N m^{-2} y^{-1} for *Sphagnum* in PK treated plots,

relatively large rates for ombrotrophic bogs and comparable to Vile *et al.* (2014). Current evidence of the fate of the fixed N_2 in ombrotrophic bogs indicates that *Sphagnum* incorporates acquired N into its biomass (Berg *et al.*, 2012) and N remains in the mosses without being shared with vascular plants (Rousk *et al.*, 2016). At Mer Bleue, only the plots treated with PK may leach some of the acquired N into the rhizosphere (Xing *et al.*, 2010), where it could be taken up by vascular plants but there is no evidence for such process in current literature. Nevertheless, we show that diazotrophy within *Sphagnum* may be strongly controlled by the relative availability of P in relation to N, which in N-limited environment gives *Sphagnum* a competitive advantage over vascular plants maintaining the main function of bogs as peat-accumulating ecosystems. Long-term anthropogenic N deposition in bogs (as observed by changes in N:P ratios in *Sphagnum*) can induce P-limitation to N_2 fixation process eventually leading to lower productivity and changes in *Sphagnum* functioning in bogs.

4.7 Figures and tables

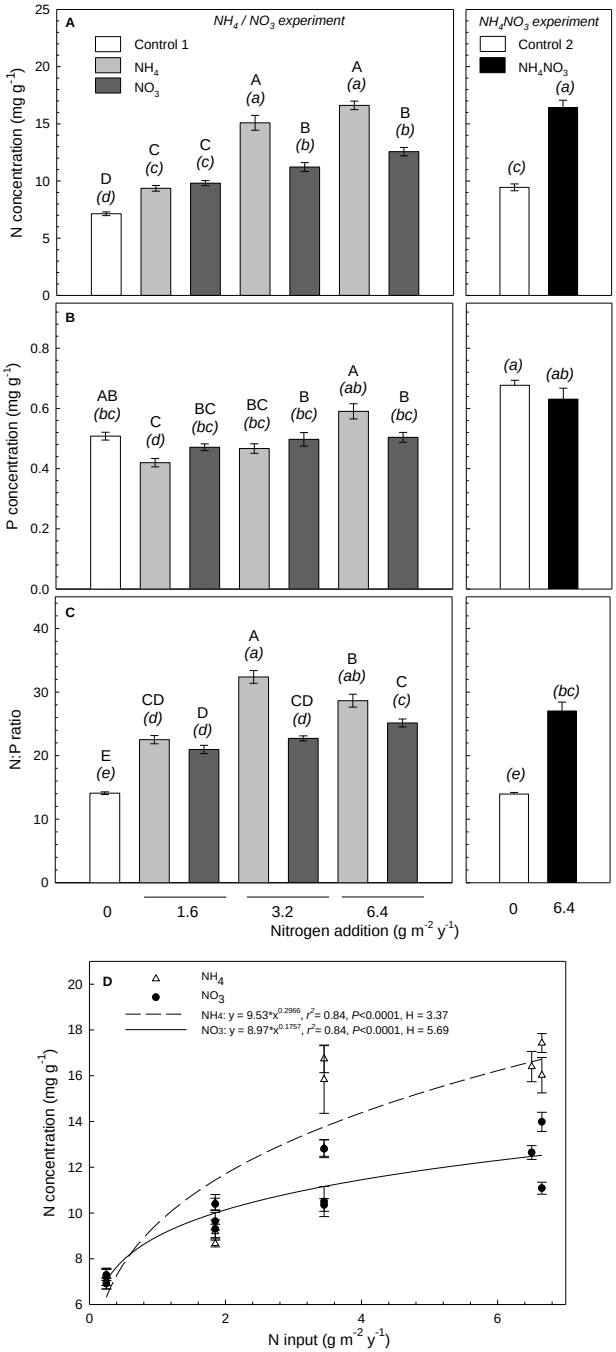

Fig. 4.1 The effects of NH_4 and NO_3 and NH_4NO_3 additions on **A)** N concentration, **B)** P concentration and **C)** N:P ratio in *Sphagnum* mosses. Error bars are standard errors of the mean. The letters above the bars are Tukey's HSD post hoc test: capital letters are differences between the treatments in the NH_4/NO_3 experiment, while italic letters in parenthesis represent differences between the treatments across both experiments. d) The power relationship between N concentration (mean ± SE per plot) in *Sphagnum* after 4 years of fertilization in relation to the annual N input, and calculated homeostatic regulation coefficients (H) for NH_4 and NO_3 treatment.

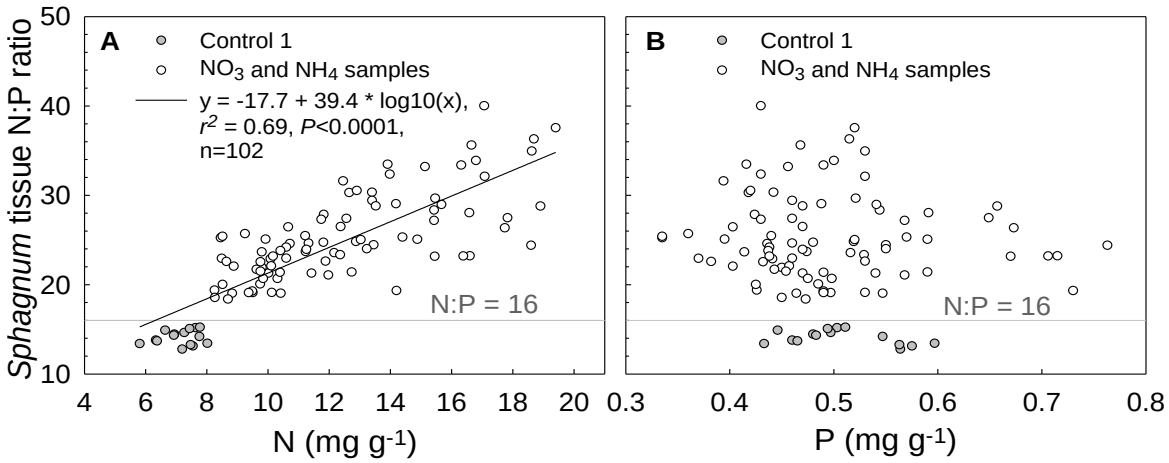

Fig. 4.2 Linear regression of N:P ratio as a function of **A)** N and **B)** P concentration in NH_4/NO_3 experiment.

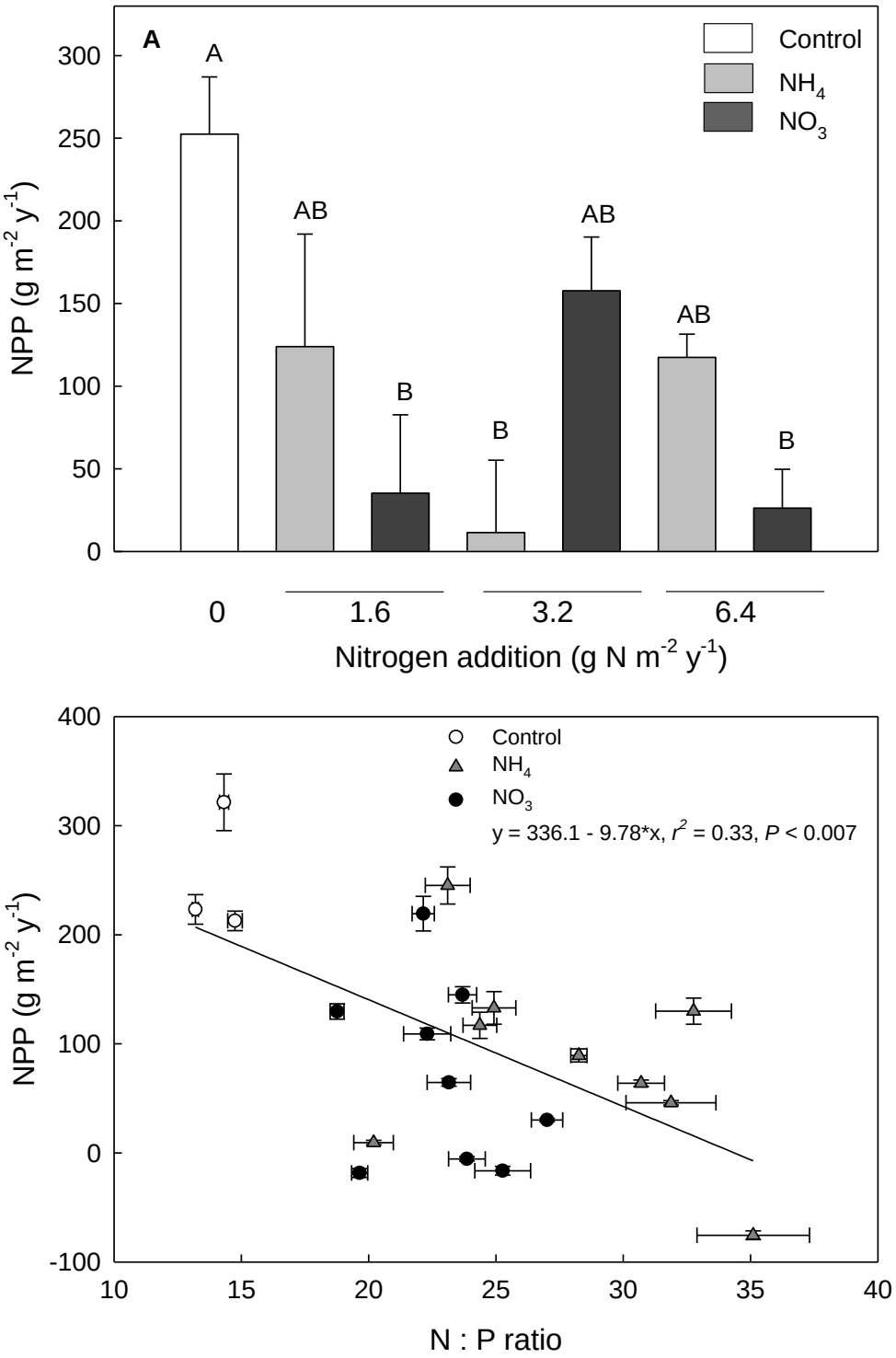

Fig. 4.3 **A)** *Sphagnum* NPP (mean ±SE, in g m^{-2} y^{-1}) over the field season April – November 2015 across the plots (ANOVA, $F_{6,14}$=4.43, p = 0.01, n = 21), capital letters represent post hoc Tukey's HSD test), **B)** *Sphagnum* NPP as a function of N:P ratio in NH_4 and NO_3 treatments.

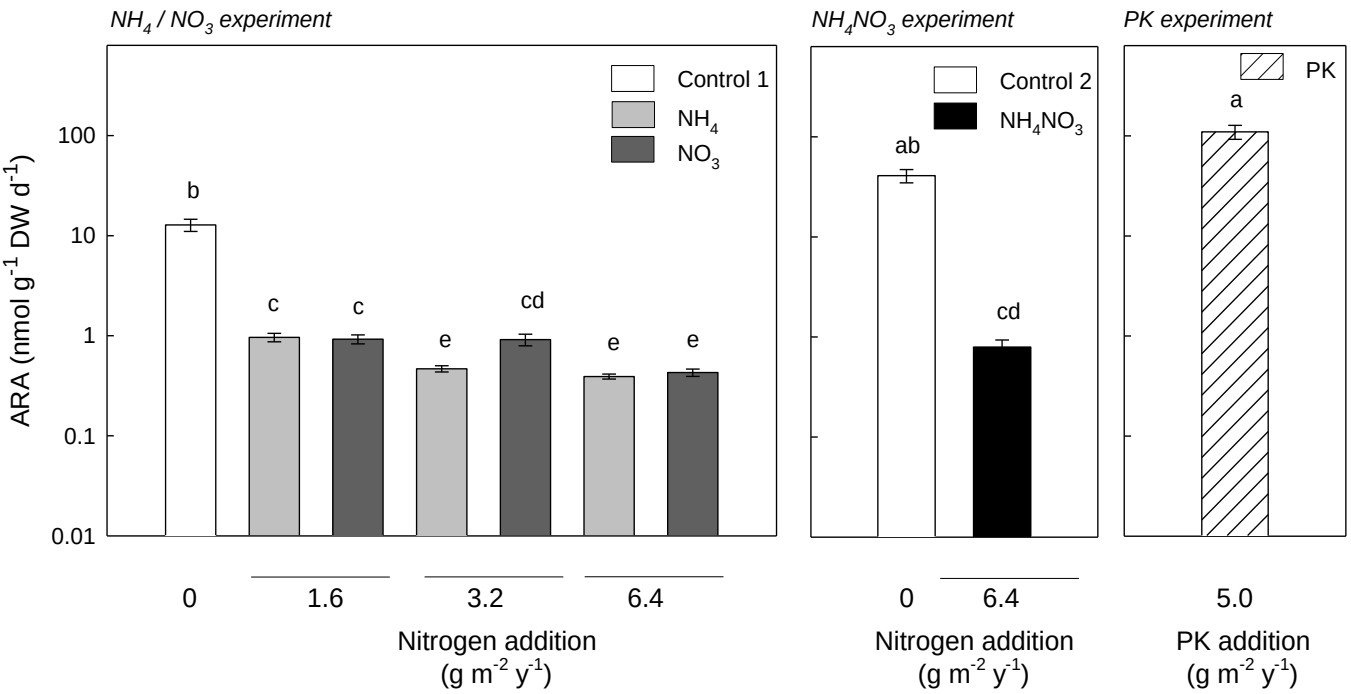

Fig. 4.4 ARA rates (mean ±SE) plotted on a logarithmic scale as a function of nutrient treatment. Error bars are standard errors and letters above the bars are the result of Tukey's HSD *post hoc* analysis on combined dataset for all three experiments. DW is dry weight.

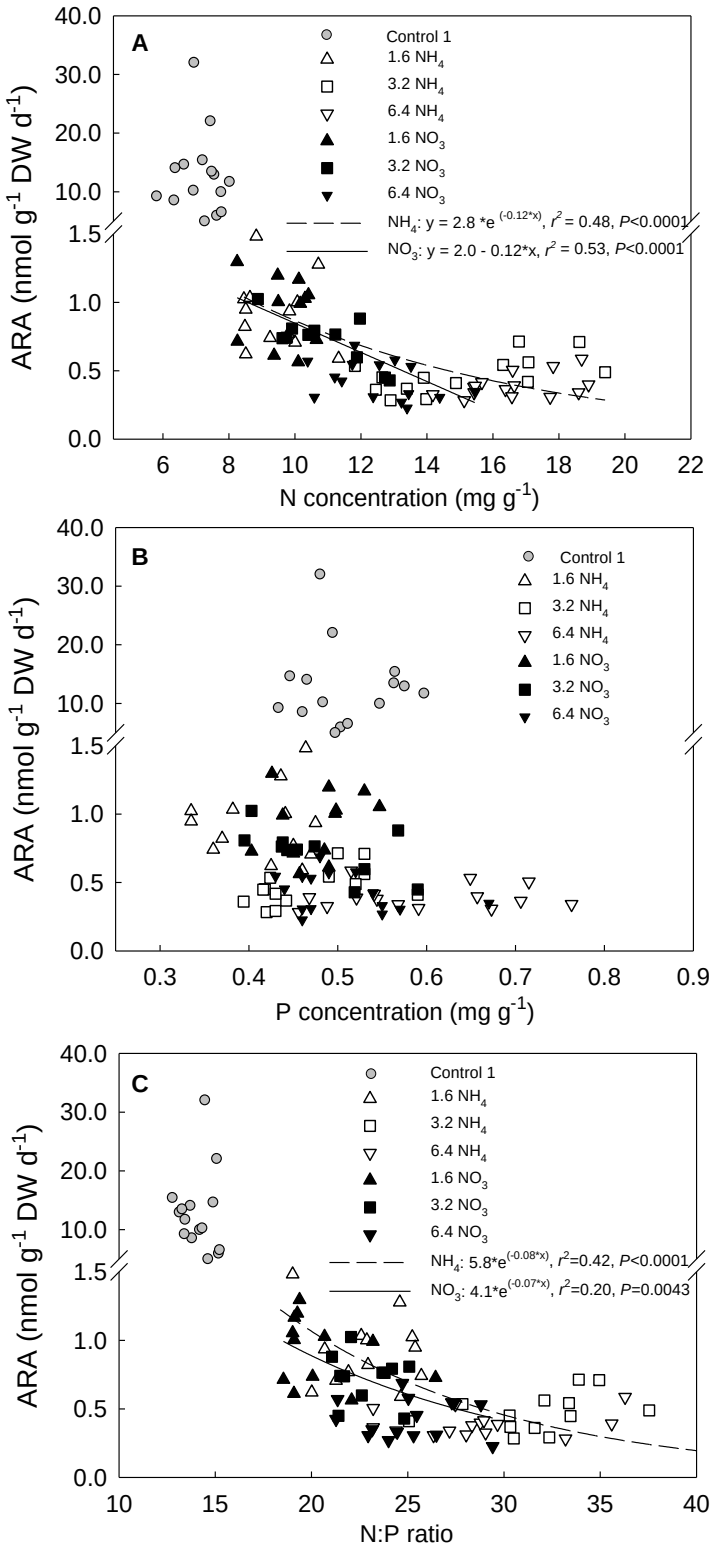

Fig. 4.5 ARA rates expressed as function of **A)** N, **B)** P and **C)** N:P ratio in *Sphagnum* tissue in NH_4 and NO_3 plots (NH_4/NO_3 experiment).

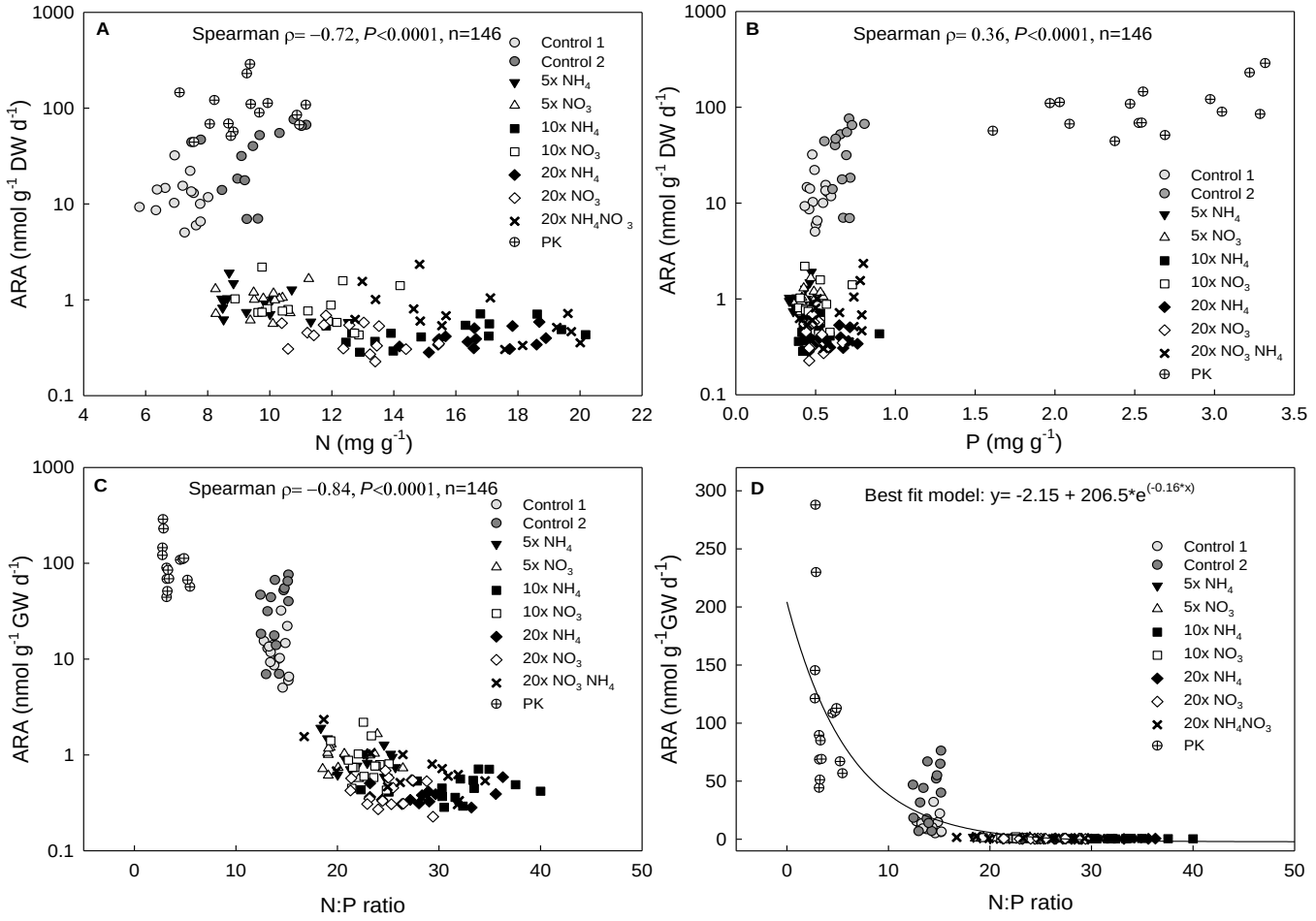

Fig. 4.6 Correlations between ARA plotted on the logarithmic scale and **A**) N, **B**) P and **C**) N:P when all three experiments are combined, and **D**) exponential decay model that best describes relationship between ARA and N:P ratio across all three experiments.

Table 4.1 The fertilization experimental design and annual nutrient additions (g m^{-2} y^{-1}).

Treatment	Start year	N	P	K
NH_4/NO_3 experiment	*2012*			
Control 1		0	0	0
5 NO_3		1.6	0	0
5 NH_4		1.6	0	0
10 NO_3		3.2	0	0
10 NH_4		3.2	0	0
20 NO_3		6.4	0	0
20 NH_4		6.4	0	0
NH_4NO_3 experiment	*2005*			
Control 2		0	0	0
20 NH_4NO_3		6.4	0	0
PK experiment	*2000*			
PK		0	5.0	6.3

Table 4.2 Means (± SE in parentheses) of *Sphagnum* N and P concentration and N:P ratio in PK fertilization plots (Experiment 3; Mann-Whitey's U test significance of $p < 0.0001$ is depicted in **bold**, n = 15).

Property	Control	PK plots
N (mg g^{-1})	9.45 (0.29)	9.18 (0.31)
P (mg g^{-1})	**0.68 (0.02)**	**2.58 (0.13)**
N:P ratio	**13.95 (0.26)**	**3.71 (0.25)**

CHAPTER 5

Variations in nitrogen, phosphorus and $\delta^{15}N$ in *Sphagnum* mosses along a climatic and atmospheric deposition gradient in eastern Canada

5.1 Context within the thesis

Chapters 3 and 4 focused on N_2 fixation rates and controls at Mer Bleue bog in its pristine and experimental setting investigating both abiotic and biogeochemical controls. In Chapter 4, we showed that that the addition of both N and P has an effect on N_2 fixation rates and that best descriptor for N_2 fixation rates could be the ratio between these two elements (N:P) in *Sphagnum* tissue.

The next two chapters (Chapter 5 and 6) are designed to test whether the relationship between N_2 fixation and N:P ratio in *Sphagnum* exists also on the larger geographical scale encompassing temperate, boreal and subarctic bogs. Chapter 5 thoroughly describes this transect, specifically focusing on *Sphagnum* N and P content, and the variability of natural $\delta^{15}N$ abundance which could indicate the main source of N within plants (Robinson, 2001). I investigate these trends in both microtopograhies (hummocks and hollows). I identify that the increase of N and P content in *Sphagnum* could be partially explained by the larger N and P deposition in the South and smaller in the North and suggest that *Sphagnum* in the North may depend more on biological N_2 fixation due to smaller atmospheric N inputs.

5.2 Abstract

We examined concentrations of nitrogen (N) and phosphorus (P) and $\delta^{15}N$ value in *Sphagnum* sections Acutifolia and Cuspidata inhabiting hummocks and hollows from 8 bogs along a transect from ~45 to ~55 °N in Ontario/Quebec. The N concentration in *Sphagnum* declined from South to North, correlating with a decrease in atmospheric N deposition. Although the overall N

concentration was larger in hollows than hummocks, the pattern was inconsistent across the sites. There was a proportionally larger decline in P concentration from South to North and an overall larger P concentration in hollows than hummocks, but there were inconsistent differences across the sites. The N:P ratio ranged from 12:1 to 29:1, driven primarily by the variation in P concentration. Ratios of N and P concentration in *Sphagnum* capitulum:stem averaged 1.2:1, suggesting nutrient resorption from stem to capitulum during growth; the ratio rose with increasing N and P concentration in the capitulum. The $\delta^{15}N$ value of *Sphagnum* rose from ~-6‰ in the South to ~-1‰ in the North, correlated with the decrease in *Sphagnum* N concentration and with a rise in water table. We interpret this to indicate a greater dependence on N_2 fixation for N acquisition in the northern and wetter sites.

5.3 Introduction

Sphagnum mosses control several important processes in the functioning of northern peatlands. Two of these processes are plant production and decomposition, which contribute to *Sphagnum* being a primary driver of peat accumulation (Rydin & Jeglum, 2006). *Sphagnum* is also the location of microbially-driven biological di-nitrogen (N_2) fixation in peatlands (Larmola *et al.*, 2014; Vile *et al.*, 2014). Plant production and decomposition and N_2 fixation are dependent on the nutrient content of the moss tissues, in particular nitrogen (N) and phosphorus (P) (*e.g.* Vitousek & Field, 1999; Bragazza *et al.*, 2009). Human activities have increased the rates of atmospheric deposition of N and P (*e.g.* Holland *et al.*, 1997; Tipping *et al.*, 2014), so that peatlands, particularly ombrotrophic bogs, are subject to 'eutrophication', ultimately resulting in changes in rates of moss production, decomposition, and carbon (C) accumulation (Juutinen *et al.*, 2010).

Atmospheric N deposition increases N content in *Sphagnum* mosses (Aerts *et al.*, 1992;

Bragazza *et al.*, 2005; Granath *et al.*, 2009; Jiroušek *et al.*, 2011). Less is known about rates of atmospheric P deposition, which have a high degree of spatial variation (Tipping *et al.*, 2014) and are thus more difficult to assess in terms of their impact on *Sphagnum* P concentration. Peatland ecosystems can be limited either by N, P, or by N-P co-limitation in combination with a third nutrient, potassium (Walbridge & Navaratnam, 2006). Koerselman & Meuleman (1996) found that plants in peatland ecosystems are most likely limited by N if N:P < 14:1, by P if N:P > 16:1, and N and P co-limited if 14 < N:P < 16, though Güsewell (2004) notes the variable nature of this ratio in influencing plant production. *Sphagnum* mosses are able to translocate nutrients from the stem to the capitulum for photosynthetic activities (Aldous, 2002b) and their exposure to chronically elevated N supply results in a decrease in the difference in N concentration between stem and capitulum (Bragazza *et al.*, 2004; Limpens *et al.*, 2006; Limpens & Heijmans, 2008). Many peatlands have a distinct microtopography resulting in 'hummocks' and 'hollows' with varying distances from the water table, and inhabited by different *Sphagnum* species as well as vascular plants. This microtopographic variability can also affect nutrient acquisition by mosses: *Sphagnum* in hollows may have a larger access to nutrients than hummocks due to their proximity to the water table (Jiroušek *et al.*, 2011).

Analysis of the ^{15}N isotope in *Sphagnum* mosses may provide information on the source of N. N_2 fixation by cyanobacteria and other microbes involves a small change in $\delta^{15}N$ value, close to 0‰ (Kendall & Doctor, 2003; Deane-Coe & Sparks, 2015). Atmospheric deposition of NH_4^+, created mainly by agricultural activities, has a negative $\delta^{15}N$ value, while N emitted from industrial activities (NO_x) has a more positive $\delta^{15}N$ value, though there is considerable variability (e.g., Pearson *et al.*, 2000; Kendall & Doctor, 2003; Bragazza *et al.*, 2005; Skinner *et al.*, 2006; Zechmeister *et al.*, 2008; Novak *et al.*, 2014). Peat in the surface layers has a negative $\delta^{15}N$

value, being derived from the decomposition of plants with a negative $\delta^{15}N$ value (Novak *et al.*, 2014; T.R. Moore, unpublished data). Thus, N (NH_4^+, NO_3^- or dissolved organic nitrogen) absorbed from mineralization of the peat will likely also decrease the $\delta^{15}N$ value in the *Sphagnum* tissue.

We sampled *Sphagnum* mosses from hummocks and hollows at eight sites along a latitudinal transect in Ontario and Quebec from ~45 to ~55 °N, with inferred variations in atmospheric deposition of N and P and climate (Table 5.1). We analyzed the samples to test the following hypotheses:

1. N and P concentration in *Sphagnum* will decrease with increasing latitude, associated with a decline in atmospheric deposition of these two elements along the climatic gradient. N and P concentrations will be larger in hollows than hummocks, as the former are closer to the water table and have a greater access to nutrients mineralized by peat decomposition. The N:P ratio in *Sphagnum* will vary with latitude and microtopography, depending on the relative availability of the two elements.
2. As nutrient availability and concentration in bogs is limited, N and P concentrations in *Sphagnum* capitula will be larger than in stems, suggesting nutrient resorption during growth. Furthermore, the ratio between the capitulum and stem for both N and P will be negatively correlated with N or P concentration in the stem, suggesting the importance of translocation as a mechanism to supply nutrients needed for growth.
3. The $\delta^{15}N$ value of *Sphagnum* will reflect the origin of N and will be less negative with increasing latitude, suggesting a greater dependence of northern sites on N_2 fixation.

5.4 Material and methods

Site selection and sampling

Eight sites in Quebec and Ontario were sampled (Fig. 5.1, Table 5.1). At each site, two hollows and two hummocks were selected, based on the dominant *Sphagnum* species in each microtopographic feature, and moss samples were collected from a block 40 x 30 cm and 20 cm deep. The samples were transported to the laboratory and kept at 4°C until analyzed. As *Sphagnum* species varied across the sites along the gradient, we classified moss into sections. The hummocks comprised mosses from section Acutifolia (*Sphagnum fuscum* and *Sphagnum capillifolium*) and Palustria (*Sphagnum magellanicum*), while hollows were mainly dominated by Cuspidata (*Sphagnum angustifolium*, *Sphagnum fallax*) or Palustria (*S. magellanicum*). Palustria section was represented by *S. magellanicum* at only two sites, inhabiting two hummocks (at LLT) and a hollow (at MB), and thus was not used for statistical analyses owing to a small sample size. We chose to plot Palustria in figures to keep the information available to the reader.

Upon the arrival at the site, we installed perforated PVC wells into each hummock and hollow and used the blow tube method to measure water table depth prior to extracting moss blocks. We collected pore water from the water wells and measured pH in the field with a calibrated pH meter (PC300 waterproof portable meter, Eutech/Oakton Instruments).

We summarized site-specific environmental, climatic and atmospheric N and P deposition data at each site along the gradient (Table 5.1). We used the model ensemble-mean with a resolution of 1 degree for total annual N deposition derived by the Task Force on Hemispheric Transport of Atmospheric Pollutants (HTAP 2010, www.htap.org) summarized by Vet *et al.* (2014). We estimated total annual N deposition based on the model estimates for the

grid-cells covering each site location. P deposition data are estimated values based on models by Mahowald *et al.* (2008) and Tipping *et al.* (2014).

Chemical analyses

Five subsamples (diameter 7.2 cm) were extracted from hummocks and hollows and cut in 6 cm stem lengths, oven dried at 50°C, and then separated into capitulum (the top 1 to 2 cm) and stem (the lower 4 to 5 cm). The *Sphagnum* was finely ground through a 40 mesh sieve (Wiley Mini Mill 3383-L10, Thomas Scientific, USA), dried at 50ºC for 24 h before acid digestion was performed following procedures from Parkinson & Allen (1975) using concentrated H_2SO_4 and H_2O_2 with Se and Li_2SO_4 as catalysts. Samples were then analyzed for total N and P colorimetrically (Murphy & Riley, 1962) on a LachatQuick-Chem AE flow injection autoanalyzer (Lachat Instruments, Milwaukee, WI).

Four additional moss subsamples (6 cm in length and 7.2 cm in diameter; comprised of both capitulum and stem) from two hummocks and two hollows at each site were oven dried at 50^0C, finely ground and analyzed for total N content and δ^{15}N value on a continuous flow Isotope Ratio Mass Spectrometer (IRMS) (UC Davis Stable Isotope facility, USA). The analytical long-term standard deviation for δ^{15}N and N is 0.3‰ and 0.5%, respectively.

Statistical analysis

We averaged the capitula and stem N and P concentrations and N:P ratio for each *Sphagnum* subsample. We used multiple regression method to best predict N and P concentration in *Sphagnum* mosses by considering representative climatic variables (MAT and MAP) and atmospheric deposition (N_{dep} or P_{dep} for N and P concentration, respectively) in two *Sphagnum* sections (Acutifolia from hummocks and Cuspidata from hollows). We $\log_{10}$ transformed

Sphagnum P content data to achieve the normality and all other assumptions for all multiple regression analyses were met.

Linear regressions were performed to evaluate the change in N and P concentrations and N:P ratio in *Sphagnum* mosses with the latitude. We used the Shapiro-Wilk's test for the normal distribution of N, P concentrations and N:P ratio in moss samples. N concentration data were normally distributed ($W = 0.99$, $p = 0.763$) and for P concentration and N:P ratio datasets, normality was achieved after $\log_{10}$ transformation ($W = 0.99$, $p = 0.661$ and $W = 0.99$, $p = 0.650$, respectively). We used linear regression on transformed data, where appropriate. The assumption of homogeneity of variance for each of the variables used in the linear regressions was tested and met using Levene's test. We used the Mann-Whitney *U* test to assess whether the N and P concentrations and N:P ratios were different between hummocks and hollows within each site.

We calculated the ratios between capitulum and stem of N concentration (N_{cap}:N_{stem}), P concentration (P_{cap}:P_{stem}) and N:P ratio [$(N:P)_{cap}$:$(N:P)_{stem}$]. The Kruskal-Wallis test was used on pooled data from hummocks and hollows to compare cap-stem ratios of N, P and N:P ratios among the sites. Because there were no significant differences between the sites, we used the Mann-Whitney *U* test to compare these ratios between hummocks and hollows within each site. Linear regressions were used to test whether stem nutrient concentrations were the main drivers of cap-stem ratios along the gradient. As above, $\log_{10}$ transformations were performed to achieve normal distributions of datasets where appropriate, and all assumptions needed for linear regression analyses were met.

We used linear regression to test the relationship between $\delta^{15}N$ in *Sphagnum* mosses and latitude, and to determine the effect of N concentration in *Sphagnum* tissue and water table depth

on *Sphagnum* $\delta^{15}N$ in hummocks and hollows. All data were normally distributed and the assumption of homogeneity of variance of the variables for each of the linear regression was met.

All statistical analyses were performed using JMP (JMP v.11.2.0, SAS Institute Inc., Cary, NC, USA).

5.5 Results

Study sites were chosen along a climatic gradient spanning from the temperate (MB, MIR, LLT), to the boreal (DES, NEL, NOR) and the subarctic zones (VIC, SCH; Table 5.1). This climatic gradient is indicated by the decrease in mean annual temperature (MAT) and growing degree days (GDD) from the South to the North (Table 5.1). Mean annual precipitation (MAP) generally decreased northwards while snowfall precipitation (SP) increased slightly. Both estimates of N and P deposition followed the decreasing trend towards the North (Table 5.1) most likely associated with the smaller human population and lesser anthropogenic disturbances compared to the southern sites. There is a strong correlation among these atmospheric variables along the transect (supplementary Table S5.1).

Water table depth was variable within and across sites, with hummocks and hollows being 31 to 49 cm and 7 to 25 cm above the water table, respectively; the average difference in elevation between hummocks and hollows was 24 cm (Table 5.1). Water table pH ranged from 3.7 to 4.1 in all but one site, Schefferville (pH 5.1). This was a poor fen, but samples were collected from the peatland margin where vegetation was more similar to bogs.

Sphagnum N and P concentration and N:P ratio

Along the latitudinal gradient where climatic variables (MAT and MAP) and atmospheric chemistry (N_{dep} or P_{dep}) were analysed to explain the variability of N or P nutrient in *Sphagnum* sections, the multiple regressions indicated strong multicollinearity between the variables. This

was shown by Pearson's correlations (Supplementary Table S5.1) and variance inflation factor (VIF) > 10 (Zuur *et al.*, 2010; Supplementary Table S5.2). To test the importance of each of the strongly collinear variables, especially MAT and N_{dep}/P_{dep}, we performed two separate multiple regression analyses. The first excluded MAT, but included N_{dep}/P_{dep} and MAP, and the second excluded N_{dep}/P_{dep} but included MAT and MAP. Each of the analyses was performed on Acutifolia (hummocks) and Cuspidata (hollows) sections separately. We found that N_{dep} explained about 23% and MAT about 26% of the variance in N content in Acutifolia (Table 5.2a, b). Similarly, about 31 and 34% of N content in Cuspidata was explained by N_{dep} and MAT, respectively (Table 5.2a, b). MAP had no explanatory power in any of the regressions testing N content in *Sphagnum* (Table 5.2a, b). The best explanatory variables for P content in *Sphagnum* were P_{dep} and MAT, although only about 10-15% of variance was explained by either (Table 5.2c, d). MAP only had a small influence on P content in Acutifolia and none in Cuspidata (Table 5.2c, d).

The two most represented *Sphagnum* sections across sites (Acutifolia and Cuspidata, hummocks and hollow species, respectively) showed a significant decrease in N concentration with increasing latitude, from ~10 to 6.5 mg g^{-1} between 45 and 55°N, though R^2 values were small (0.28 and 0.25, respectively; Fig. 5.2a). Overall, hummock samples had a significantly smaller N concentration than hollow samples and within the sites, four (VIC, NEL, MIR and MB) had a significantly smaller N concentration in the hummocks than hollows (Table 5.3).

The P concentration in *Sphagnum* decreased with increasing latitude in both Acutifolia and Cuspidata (~0.6 to 0.2 mg g^{-1} from 45 to 55°N, Fig. 5.2b), proportionally larger than the decrease in N concentration. Overall, the hummock samples had a significantly ($p = 0.013$) smaller P concentration than the hollows (0.48 *versus* 0.53 mg g^{-1}; Table 5.3). Three sites had a

significantly smaller P concentration in hummocks than hollows and two other sites, the southernmost MIR and MB, showed a reverse pattern.

The N:P ratio was not significantly related to latitude in both *Sphagnum* sections (Fig. 5.2c). There was no significant difference in N:P ratio between hummock and hollow samples pooled across all the sites. Within sites, significant ($p < 0.05$) differences between hummock and hollow samples occurred at six of the eight sites: at three sites (SCH, NOR and NEL), hummocks had a higher N:P ratio than hollows and the pattern was reversed at VIC, MIR and MB (Table 5. 3). Assuming that the N:P ratio reflects limitations of these two nutrients, in general the hollow *Sphagnum* species are more P limited (N:P ratio > 16), whereas in the hummocks there may be co-limitation of N and P, though the pattern varies across the sites. Concentration of P is more variable than that of N, so that the N:P ratio is driven more by P than N (Fig. 5.3a and b).

Sphagnum N and P concentration and N:P ratio in capitulum and stem

Separation of the *Sphagnum* samples into capitulum (top 1 to 2 cm) and stem (4-5cm in length) revealed cap:stem ratios ≥ 1 for N and P. $N_{cap:stem}$ ratios ranged from 1.0 to 1.5 with hummocks having significantly larger ratios than hollows in the northern sites (Mann-Whitney *U* test, $p < 0.05$ for DES, NEL, NOR and VIC; Fig 5.4a). $P_{cap:stem}$ ratios ranged from 1.1 to 1.9 and showed no significant differences among sites or between hummock and hollow (Fig. 5.4b). $N:P_{cap:stem}$ ratios ranged from 0.8 to 1.32 and were significantly larger in hummocks than hummocks at MB, NOR and VIC (Mann-Whitney *U* test, $p < 0.05$; Fig.5.4c). The $N_{cap:stem}$, $P_{cap:stem}$ and $N:P_{cap:stem}$ ratios were negatively correlated with the N and P concentrations and N:P ratio in the stem, respectively (Fig. 5.5a, b and c).

Sphagnum $\delta^{15}N$ value

Subsampling of the *Sphagnum* (combined capitulum and stem) showed a significant increase in the $\delta^{15}N$ value with latitude in both hummocks and hollows (Fig. 5.6a). Overall, the $\delta^{15}N$ value at 45°N was -5.9 and -4.9 ‰ for hummocks and hollows, respectively, and this increased to -2.5 and -0.9 ‰ at 55°N. There were no significant differences in $\delta^{15}N$ between hummock and hollow at the sites. There was a weak but significant relationship between the $\delta^{15}N$ value and N concentration, with $\delta^{15}N$ becoming more negative (from -2 to -5 ‰) with an increase in N concentration from 5 to 10 mg g^{-1}, but the overall relationship was mostly driven by hummock species (Fig. 5.6b). The $\delta^{15}N$ value increased with a rise in water table depth, increasing from about -5 ‰ at 50 cm to about -3 ‰ at 10 cm beneath the surface (Fig. 5.6c). This relationship was mainly driven by a significant increase of the $\delta^{15}N$ value in hollow species as a response to a lower water table (Fig. 5.6c).

5.6 Discussion

The results of multiple regressions indicate that both MAT and atmospheric deposition chemistry could equally explain the *Sphagnum* nutrient content of N and P along our transect. Mean annual precipitation did not play a role in any of the cases, except in explaining P content in Cuspidata section (Table 5.2). Due to strong collinear relationships between the latitude, climatic variables and atmospheric chemistry (see Supplementary Table S5.1), it is difficult to determine which of the factors plays the strongest role.

We show a significant trend of decreasing N concentration along the latitudinal gradient from South to North in both *Sphagnum* sections, with no differences between the Acutifolia and Cuspidata (Fig. 5.2a). Mosses from Cuspidata section dominate wetter hollows, and Acutifolia as well as Palustria are often found on drier hummocks or in the transitional zones between hummocks and hollows. Studies focusing on similar gradients (Lamers *et al.*, 2000; Bragazza *et*

al., 2005; Zechmeister *et al.*, 2008; Jiroušek *et al.*, 2011) indicate that *Sphagnum* species can be used as good proxies for atmospheric deposition and their nutrient content is less driven by the climate.

Collation of data on N concentration in hummock *Sphagnum* from a wide range of temperate and boreal sites under natural and fertilized conditions (Bragazza *et al.*, 2005; Jiroušek *et al.*, 2011; T.R. Moore, unpublished data) shows a strong correlation between moss N content and atmospheric wet N deposition, represented by equation:

$$\text{N concentration (\%)} = 0.327\ln N_{dep} + 1.103,\ R^2 = 0.75,\ n = 134 \quad (1)$$

The wet atmospheric deposition rates ranged from 0.2 to 0.6 g N m^{-2} yr^{-1} along the gradient of our sites (Turunen *et al*., 2004; Vet *et al.*, 2014), and applying the equation above, the predicted *Sphagnum* N concentration is between 0.6 and 0.9%, similar to the range we observed (0.55 to 1.00%, Table 5.3). The southernmost bogs (MB, MIR and LLT, Fig. 5.1) are located near major cities (Ottawa and Montreal), highways and airport (Mirabel) and have been exposed to larger, anthropogenically-derived N loads from the atmosphere, compared to more pristine bogs in northern latitudes (HBL and SCH) (Turunen *et al*., 2004).

Given that our *Sphagnum* N concentrations fit well within the models by Bragazza *et al.* (2005) and our own, as shown above, we believe that *Sphagnum* nutrient content follows a declining trend from the South to the North along this transect as a result of decreasing nutrient loads from the atmosphere.

Phosphorus concentration follows a similar trend, decreasing along the latitudinal gradient in both Acutifolia and Cuspidata *Sphagnum* sections (Fig. 5.2b). Most of the P in peatlands likely entered these ecosystems during their early formation as a result of bedrock weathering processes (Walbridge & Navaratnam, 2006). The small atmospheric P deposition and

lack of additional P inputs in ombrotrophic bogs results in P recycling and high C:P ratios in the peat profile (Wang *et al.*, 2014) as well as P becoming a limiting nutrient (Toberman *et al.*, 2015). Atmospheric deposition of P is rarely measured, although human activities have been known to increase local P inputs (Tipping *et al.*, 2014) and modeling suggests a decline northwards in Canada (Mahowald *et al.*, 2008). Our results suggest the existence of a latitudinal P gradient, likely due to increased anthropogenic activities in the South and hence larger amounts of P in the moss tissue. The 67% decrease in P concentration (from 0.6 to 0.2 mg g^{-1}) was twice as large as the 35% decrease in N concentration. Walbridge & Navaratnam (2006) report similar ranges for both P (0.25-1.5 mg g^{-1}) and N (5-10 mg g^{-1}) in *Sphagnum spp.* from bogs across North America and Europe.

We found large variations in P concentration between hummocks and hollows (Table 5.3), but the patterns are inconsistent: in the South, hummocks > hollows (MB and MIR), hollows > hummocks (DES, NEL and SCH), and there was no difference in VIC, LLT and NOR. Our analyses excluded species differences across sites as an explanatory variable for these trends, but our interpretation is that recycled P from the decomposition may be more available in the hollows than hummocks due to the proximity of the water table and the form of P that is available to microbes and *Sphagnum*, though the direct mechanisms remain unclear. The activity of phosphatases (enzymes that release P bound to organic matter and make it available to microbes and plants) measured across different *Sphagna* species has been shown to play an important role in P-limited conditions (Press & Lee, 1983) and phosphatase activities could explain some of the observed trends.

Phosphorus is lost at a faster rate than either C and N during peat decomposition resulting in larger C:P and N:P ratios with depth in profiles (Wang *et al.*, 2014). The larger concentration

of P in moss capitulum than in the stem by 1.1 to 2.6 times (Fig. 5.4b) may suggest P transport from the stem into the capitulum in all *Sphagnum* sections along the gradient and the importance of P recycling in these peatlands. We observed a larger N:P ratio in Acutifolia than Cuspidata sections, a trend also described by Jiroušek *et al.* (2011). Assuming that the N:P ratio reflects limitations of these two nutrients, in general the hollow *Sphagnum* species were more P limited (N:P ratio > 16), whereas in the hummocks there may be co-limitation of N and P, though the pattern varied across the sites.

While strong retention and re-translocation of N from the moss stem to the capitulum has been found to play an important role in bogs (Aldous, 2002a,b), under large N loading N is leached downward to the moss stem (Lamers *et al.*, 2000; Novak *et al.*, 2014). Our study shows a similar relationship between capitulum and stem N concentration to that found at the low end of the atmospheric N deposition gradient in Europe (Bragazza *et al.*, 2005). The ratio of N concentration in capitulum to stem in *Sphagnum* was negatively correlated with stem N concentration which suggests that N inputs from the atmosphere are inversely linked with N relocation from the stem to capitulum to maintain *Sphagnum* growth and functioning (Limpens & Heijmans, 2008). The significant differences in the ratio of N concentration in capitulum to stem between hummocks and hollows in more northern sites could be due to hollows in pristine environments having larger N_2 fixation rates than hummocks (Stewart *et al.*, 2011a; Leppänen *et al.*, 2015). The proximity of the water table could physically facilitate the transfer of N from either N_2 fixation or decomposition, decreasing the difference between N concentration in capitulum and stem in hollows. Hummocks, on the other hand, may depend more on the translocation and retention of N in capitula to support the growth and photosynthetic activity

(Aldous, 2002a,b) increasing the $N_{cap:stem}$ ratio. The atmospheric N deposition in our bogs never reached the high values found in parts of Europe (Bragazza *et al.*, 2005).

The N:P ratio has been used as an indicator of N-, P- or NP co-limitation of plant productivity in peatlands (Koerselman & Meuleman, 1996), though the critical value may vary (Bragazza *et al.*, 2004; Walbridge & Navaratnam, 2006). *Sphagnum* from five of the eight bogs in our study had a N:P ratio > 16, ranged between 14 and 16 in two bogs and one bog (NOR) had N:P < 14, when hummocks and hollows were pooled (Table 5.3). This suggests a general P limitation and P is known to control biomass growth and functioning of microbial activities in peatlands, such as N_2 fixation (Fritz *et al.*, 2012; Larmola *et al.*, 2014; Toberman *et al.*, 2015).

The *Sphagnum* $\delta^{15}N$ value can be driven by environmental controls such as water table depth (Asada *et al.*, 2005) and increased N deposition along an altitudinal gradient in Europe (Zechmeister *et al.*, 2008). In our data, latitude explained more variation in *Sphagnum* $\delta^{15}N$ than the water table (Fig.5. 6a and 5.6c). A trend of decreasing $\delta^{15}N$ value with N concentration in *Sphagnum* tissue in hummocks (Fig. 5.6b) supports a similar trend found in European *Sphagnum* sampled along the gradient of increasing atmospheric N deposition (Bragazza *et al.*, 2005). The $\delta^{15}N$ value of the top 5 cm of photosynthetically active *Sphagnum* significantly increased from ~ -6‰ in the South to ~ -1‰ in the North (Fig. 5.6a).

Sphagnum receives N from either wet or dry deposition (as NH_4-N, NO_3-N or dissolved organic nitrogen), biological N_2 fixation, or re-translocation of N from decomposing litter. The $\delta^{15}N$ value may be used as an indicator of the primary source of N to vegetation although reports show a large variability in N fractionation during biologically-mediated processes (*e.g.* denitrification, nitrification and litter decomposition; Nadelhoffer *et al.*, 1996). When the primary source of N is NH_4-N, it generally leads toward more negative $\delta^{15}N$ values. More

positive $\delta^{15}N$ values are attributed to NO_3 as a source of N, while biological N_2 fixation keeps $\delta^{15}N$ values near 0‰. Assuming that there is no fractionation of N to organic N, NH_4 or NO_3, N from litter and peat decomposition in bogs would have a $\delta^{15}N$ value between -4 and -8‰ (T.R. Moore, unpublished data). The low *Sphagnum* $\delta^{15}N$ values in the southernmost bogs (MIR and MB, -6 and -5.4 ‰, respectively) may indicate the combination of two sources, atmospheric NO_3-N deposition associated with locations near cities and translocation of N from decomposition. Bogs in the mid-latitudes (DES, NEL, NOR) with $\delta^{15}N$ values of -4.3, -4.2 and -3.9‰, respectively) may reflect more equal contributions of NH_4-N and NO_3-N, N_2 fixation and recycling of mineralized N, in regions where wet N deposition does not exceed 0.5 g N m^{-2} y^{-1} and at the northernmost site (SCH) the $\delta^{15}N$ value of ~ -1‰ may suggest domination by N_2 fixation. The addition of N downregulates N_2 fixation in boreal forests stands in similar moss-N_2 fixing bacteria associations (DeLuca *et al.*, 2008; Ackermann *et al.*, 2012). Although we suggest that these $\delta^{15}N$ patterns may indicate differences in the source of N acquired by *Sphagnum* mosses, further studies in which different sources of N are systematically added to *Sphagnum* mosses may better explain changes in $\delta^{15}N$ value.

5.7 Conclusion

This study strengthens previously established evidence that N concentration in *Sphagnum* tissues can be used as an indicator of increased background N inputs, especially areas where N deposition data are scarce. Our findings suggest that declining P content in *Sphagnum* mosses may also be a good indicator of decreasing P inputs along the gradient. Most bogs along the South-North gradient seem to be P-limited and *Sphagnum* actively recycles P by re-translocation from the stem to the capitulum. N:P ratios are mostly driven by P content and they are highly variable across sites and microtopography. The $\delta^{15}N$ value increased from South to North,

suggesting different sources of N: peat decomposition and atmospheric NO_3 deposition in the South and N_2 fixation in the North.

5.8 Figures and tables

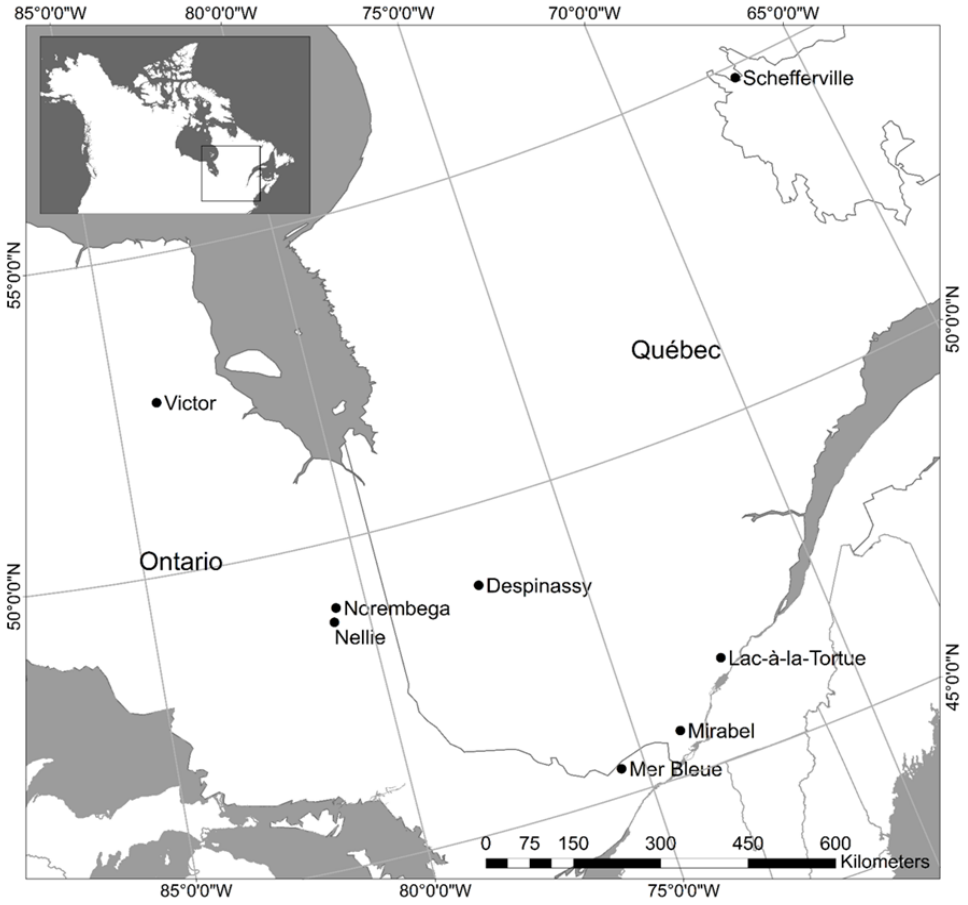

Fig. 5.1 Location of study sites. Source of information: © 2003. Government of Canada with permission from Natural Resources Canada. Data can be downloaded here: http://geogratis.gc.ca/api/en/nrcan-rncan/ess-sst/b754e4f9-3cdc-439e-9b4a-9aecd057a244.html.

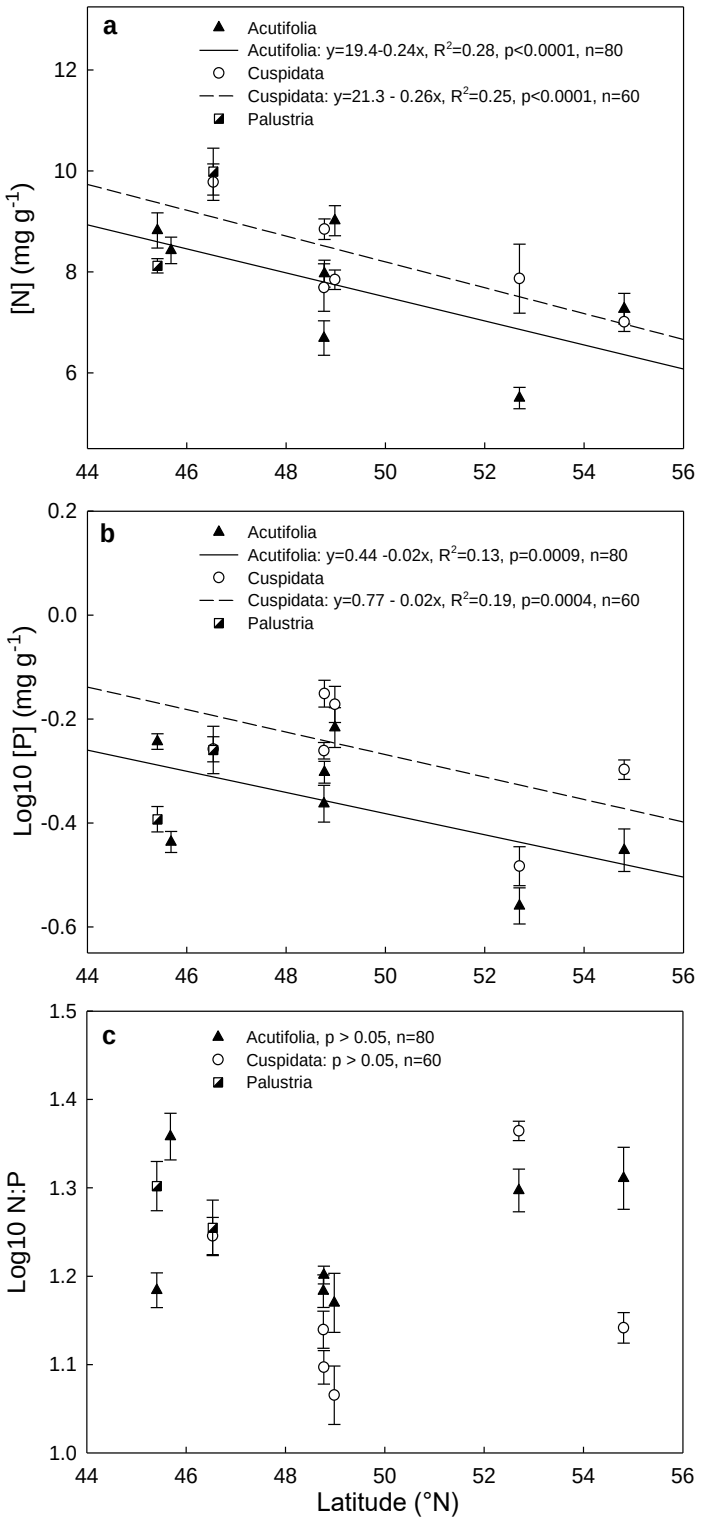

Fig. 5.2 Relationships between (a) N concentration (b) P concentration and (c) N:P ratio (mean ± SEM) and latitude in three *Sphagnum* sections (Acutifolia, Cuspidata and Palustria).

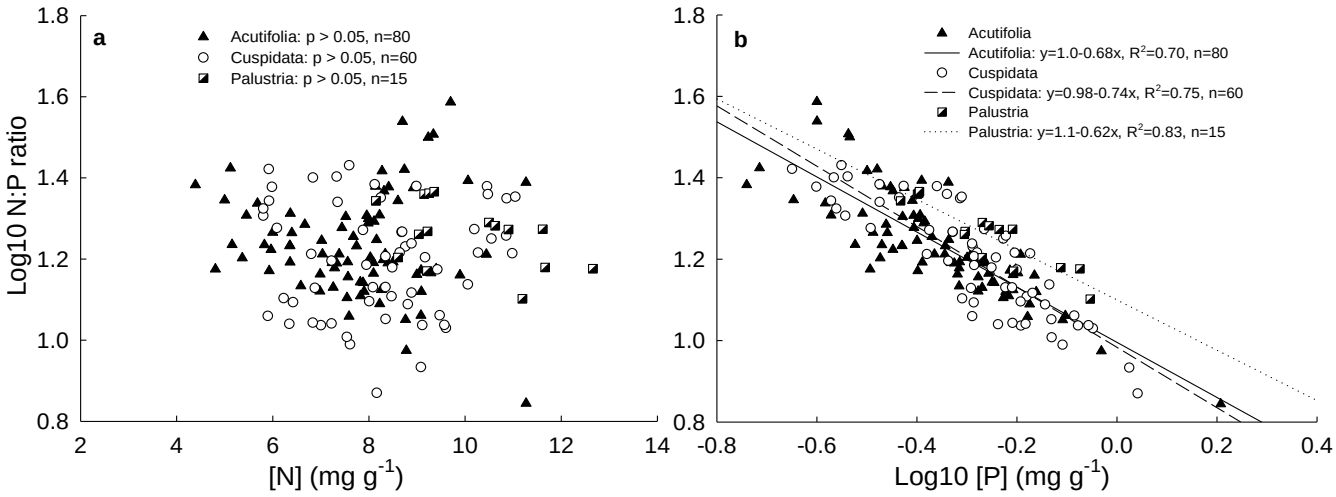

Fig. 5.3 Relationships between *Sphagnum* N:P ratio and (a) N concentration and (b) P concentration in *Sphagnum* sections.

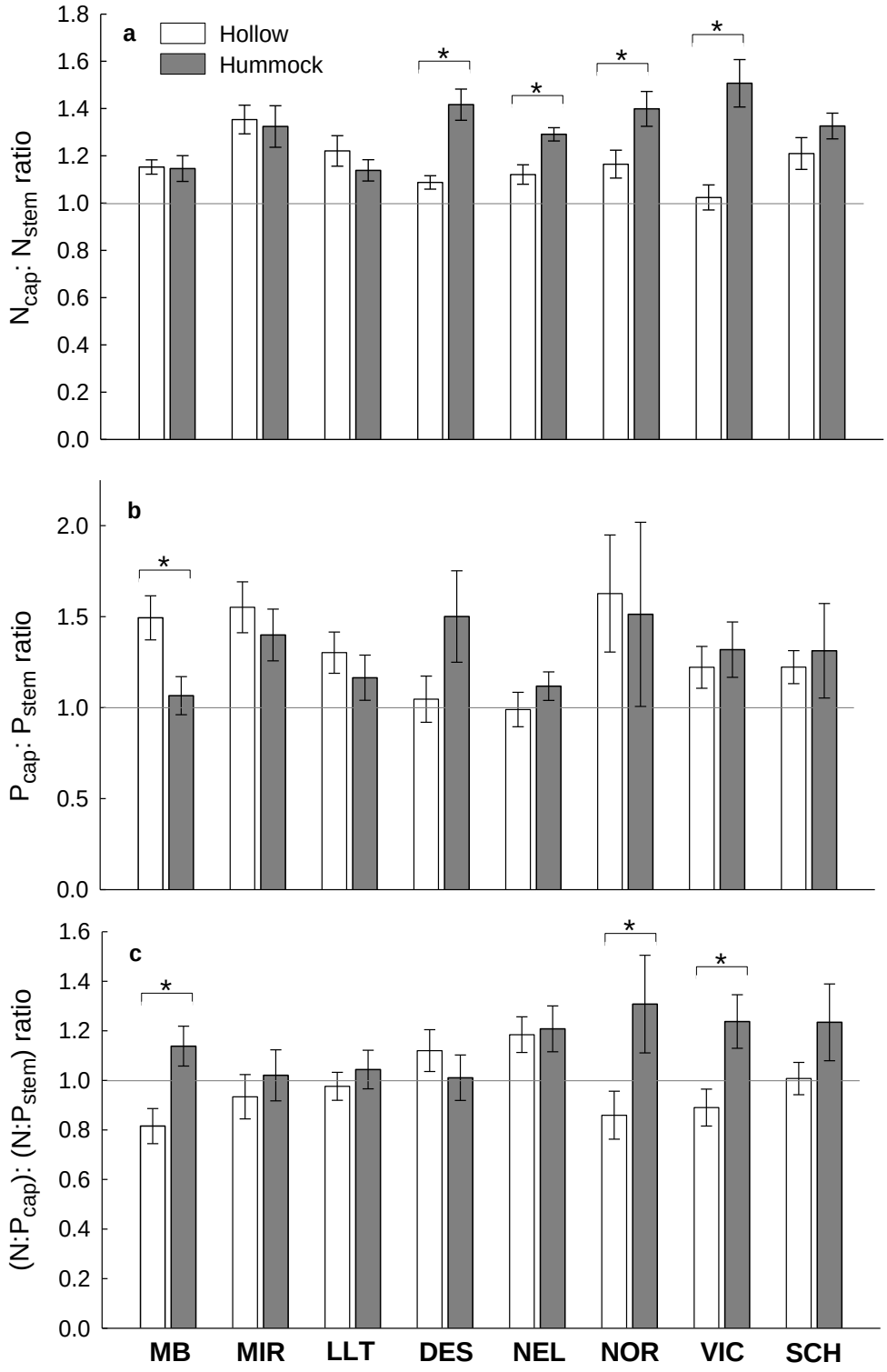

Fig. 5.4 Capitulum:stem ratio (mean ± SEM) for *Sphagnum* (a) N concentration, (b) P concentration, and (c) N:P ratio across sites for hummocks and hollows; asterisks (*) indicate significant differences between hummocks and hollows at each site.

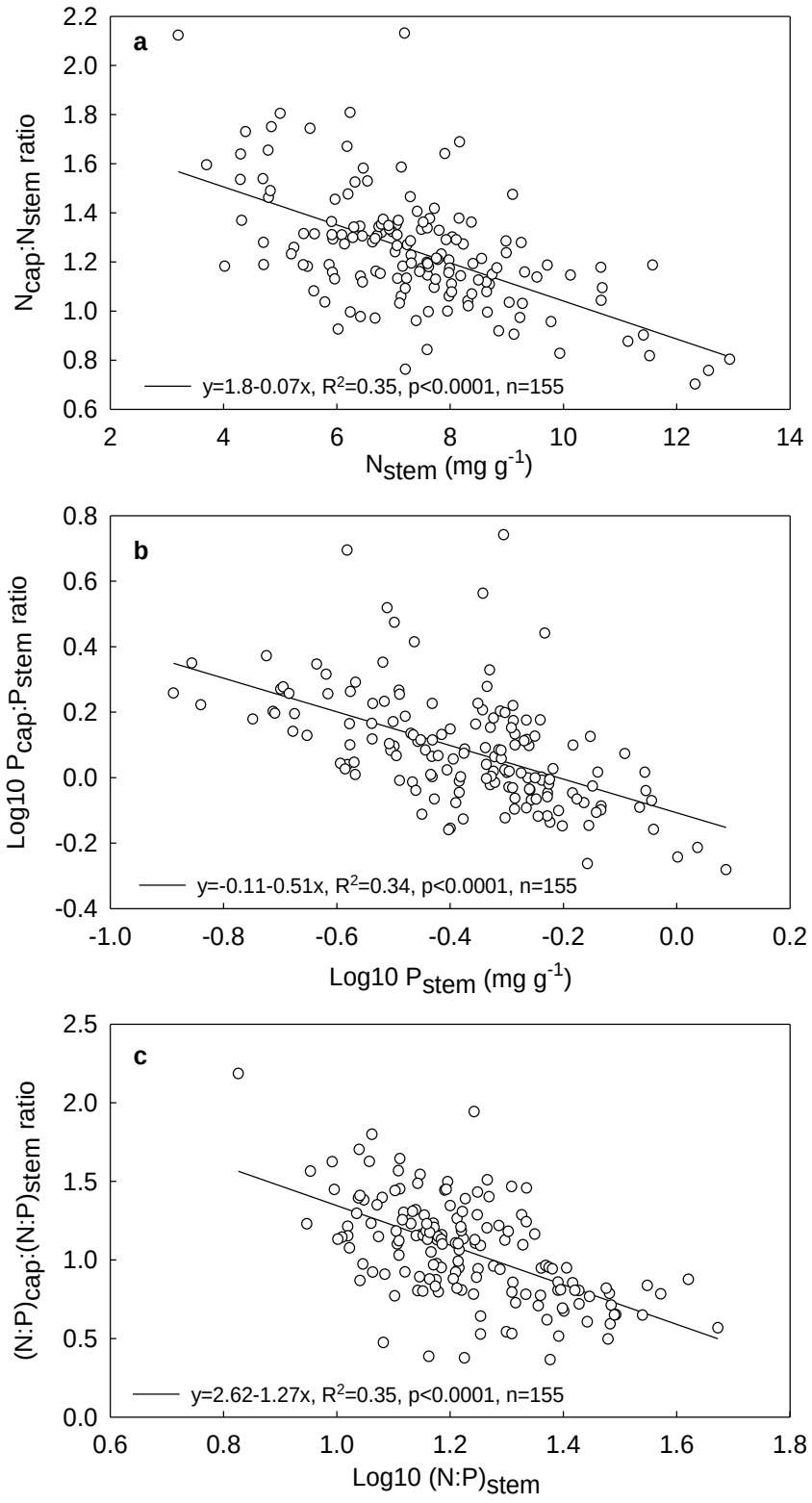

Fig. 5.5 Relationship between (**a**) $N_{cap:stem}$ ratio and N_{stem} concentration, (**b**) $P_{cap:stem}$ ratio and P_{stem}, and (**c**) $N:P_{cap:stem}$ ratio and $N:P_{stem}$.

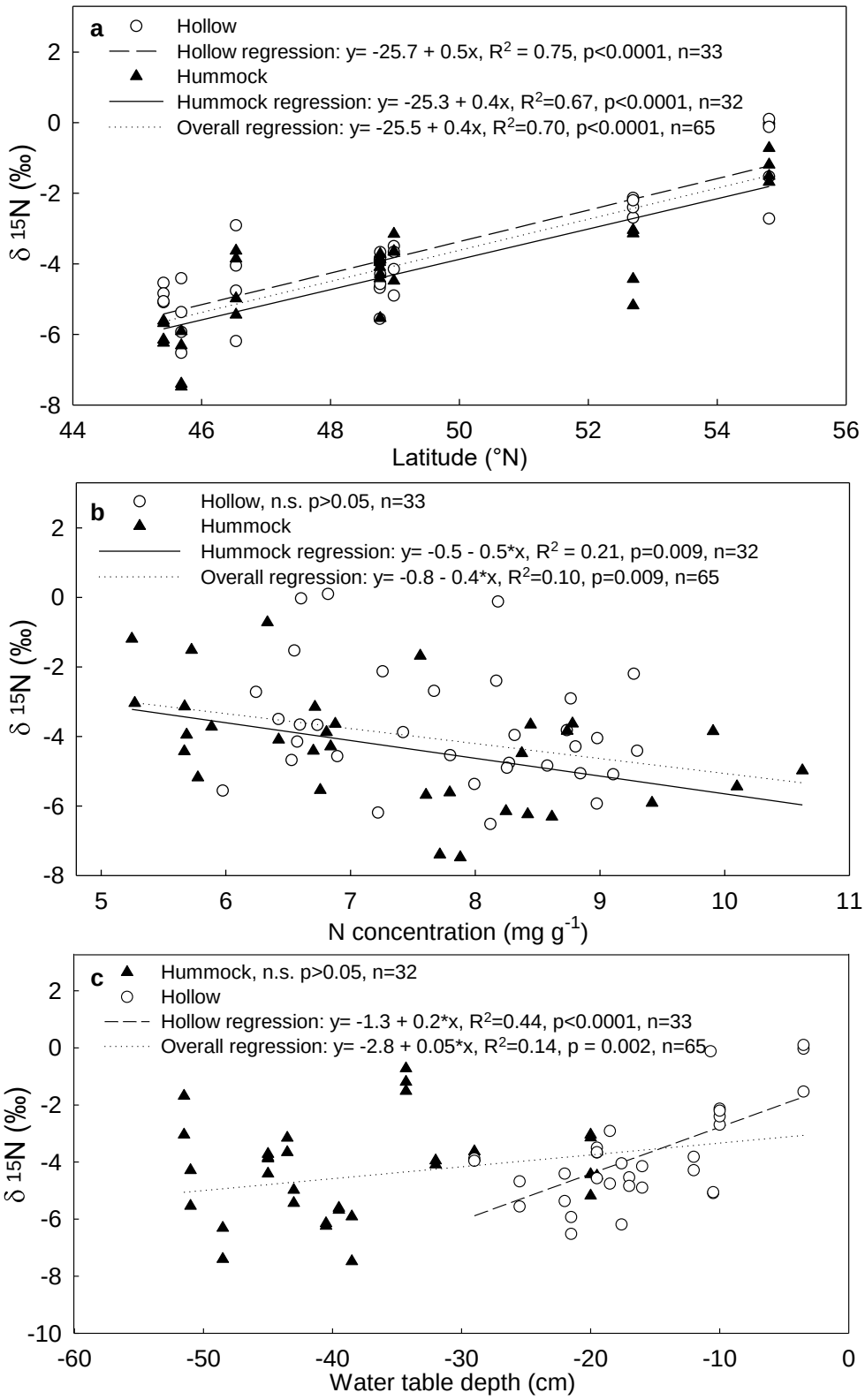

Fig. 5.6 Relationships between *Sphagnum* $\delta^{15}N$ value and (a) latitude for moss section, (b) N concentration and (c) water table depth.

Table 5.1 Site location (and abbreviation), latitude and longitude, mean annual temperature (MAT, and in parentheses growing degree days above 5° C; GDD), mean annual precipitation (MAP) and in parentheses growing season precipitation (GSP) and snowfall precipitation (in *italics*, *SP*) 1981-2000, pore water pH, estimated total atmospheric N and P deposition, and hummock and hollow water table depth beneath surface at time of sampling (with SD in the parentheses), *Sphagnum* section dominating hummocks and hollows.
[a] estimated from Vet et al. 2014, 2017 [b] estimated from modeled P values from Mahowald et al. (2008) and Tipping et al. (2014).

Site	Latitude (N)	Longitude (W)	MAT (GDD) (^{o}C)	MAP (GSP, *SP*) (mm)	pH	N dep[a] ($g\ m^{-2}\ y^{-1}$)	P dep[b] ($g\ m^{-2}\ y^{-1}$)	Water table depth (cm)		*Sphagnum* sections	
								Hummock	Hollow	Hummock	Hollow
Schefferville (SCH)	54°48'23"	66°48'07"	-3.1 (817)	840 (397, *337*)	5.1	0.13	0.01	43 (9)	7 (4)	Acutifolia	Cuspidata
Victor (VIC)	52°41'47"	83°56'55"	-0.5 (1192)	704 (396, *201*)	4.1	0.19	0.01	33 (3)	17 (2)	Acutifolia	Cuspidata
Norembega (NOR)	48°58'55"	80°42'32"	1.8 (1456)	835 (408, *276*)	3.9	0.37	0.02	31 (7)	16 (2)	Acutifolia	Cuspidata
Nellie (NEL)	48°46'13"	80°49'23"	1.8 (1456)	835 (408, *276*)	3.9	0.46	0.02	44 (5)	15 (2)	Acutifolia	Cuspidata
Despinassy (DES)	48°45'50"	77°21'33"	1.5 (1423)	929 (499, *253*)	3.7	0.47	0.02	49 (11)	25 (3)	Acutifolia	Cuspidata
Lac à la Tortue (LLT)	46°31'59"	72°40'00"	5.2 (1927)	1123 (620, *259*)	3.8	0.92	0.04	36 (4)	18 (0)	Palustria	Cuspidata
Mirabel (MIR)	45°41'05"	74°02'50"	5.3 (1931)	1068 (654, *232*)	3.7	1.06	0.04	46 (4)	22 (0)	Acutifolia	Acutifolia
Mer Bleue (MB)	45°24'32"	75°31'03"	6.6 (2182)	920 (597, *164*)	3.7	1.06	0.04	40 (1)	14 (3)	Acutifolia	Palustria

Table 5.2 The results of multiple regression models predictors for N content a) excluding MAT, b) excluding N_{dep} and for P content in *Sphagnum* along the gradient c) excluding MAT and d) excluding P_{dep}.

a)

Variable	Coefficient	St Error	t ratio	Prob < |t|
	Acutifolia (R^2_{adj} = 0.23, p < 0.0001, n = 80)			
Intercept	5.93	1.64	3.62	0.0005
N_{dep}	1.61	0.61	2.63	0.0104
MAP	0.00	0.00	0.4	0.6913
	Cuspidata (R^2_{adj} = 0.31, p < 0.0001, n = 60)			
Intercept	10.53	1.95	5.39	<.0001
N_{dep}	5.30	1.34	3.96	0.0002
MAP	-0.01	0.00	-1.88	0.0654

b)

Variable	Coefficient	St Error	t ratio	Prob < |t|
	Acutifolia (R^2_{adj} = 0.26, p < 0.0001, n = 80)			
Intercept	5.54	1.42	3.91	0.0002
MAT	0.19	0.06	3.11	0.0026
MAP	0.00	0.00	1.13	0.2607
	Cuspidata (R^2_{adj} = 0.34, p < 0.0001, n = 60)			
Intercept	7.95	1.57	5.05	<.0001
MAT	0.32	0.09	3.48	0.001
MAP	0.00	0.00	-0.06	0.9551

c)

Variable	Coefficient	St Error	t ratio	Prob < |t|
	Acutifolia (R^2_{adj} = 0.10, p < 0.0001, n = 80)			
Intercept	-0.06	0.17	-0.34	0.737
P_{dep}	7.16	2.20	3.26	0.0017
MAP	0.00	0.00	-2.3	0.0242
	Cuspidata (R^2_{adj} = 0.09, p < 0.0001, n = 60)			
Intercept	-0.35	0.04	-9.25	<.0001
P_{dep}	4.38	1.72	2.55	0.0133

d)

Variable	Coefficient	St Error	t ratio	Prob < |t|
	Acutifolia (R^2_{adj} = 0.14, p < 0.0001, n = 80)			
Intercept	-0.09	0.15	-0.64	0.52
MAT	-0.02	0.07	3.78	0.0003
MAP	-0.00	0.00	-2.06	0.0424
	Cuspidata (R^2_{adj} = 0.08, p < 0.0001, n = 60)			
Intercept	-0.28	0.02	-15.26	<.0001
MAT	0.02	0.01	2.59	0.0122

Table 5.3 Mean (± SEM) for N and P concentrations and N:P ratio by site and overall. Significant differences among hummocks (Hu) and hollows (Ho) (Mann-Whitney *U* test, $p < 0.05$, $n = 10$) by site and overall are indicated in bold.

Site	N concentration (mg g^{-1})		*p* value	P concentration (mg g^{-1})		*p* value	N:P ratio		*p* value
	Hu	Ho		Hu	Ho		Hu	Ho	
SCH	7.23 (0.33)	7.20 (0.24)	0.447	**0.36 (0.039)**	**0.53 (0.024)**	**0.015**	**21.6 (1.31)**	**13.4 (0.49)**	**0.004**
VIC	**5.50 (0.31)**	**7.86 (0.50)**	**0.007**	0.38 (0.018)	0.34 (0.023)	0.212	**20.4 (1.13)**	**23.7 (0.89)**	**0.049**
NOR	**8.89 (0.43)**	**7.97 (0.25)**	**0.048**	0.63 (0.060)	0.73 (0.075)	0.361	**14.9 (0.98)**	**12.1 (0.69)**	**0.030**
NEL	**7.96 (0.30)**	**8.87 (0.21)**	**0.031**	**0.50 (0.022)**	**0.73 (0.037)**	**< 0.001**	**16.1 (0.58)**	**12.5 (0.51)**	**< 0.001**
DES	6.69 (0.37)	7.69 (0.34)	0.140	**0.45 (0.042)**	**0.55 (0.026)**	**0.049**	15.7 (0.58)	14.2 (0.64)	0.187
LLT	9.99 (0.38)	9.78 (0.37)	0.734	0.58 (0.046)	0.56 (0.032)	0.791	18.7 (1.11)	18.0 (0.65)	0.850
MIR	**7.60 (0.31)**	**9.25 (0.40)**	**0.001**	**0.41 (0.025)**	**0.34 (0.023)**	**0.049**	**19.4 (1.04)**	**29.1 (1.71)**	**< 0.001**
MB	**8.10 (0.25)**	**9.19 (0.36)**	**0.036**	**0.59 (0.030)**	**0.48 (0.031)**	**0.013**	**14.0 (0.49)**	**20.2 (0.92)**	**< 0.001**
Overall	**7.75 (0.15)**	**8.47 (0.14)**	**<0.001**	**0.48 (0.016)**	**0.53 (0.017)**	**0.013**	18.0 (0.38)	17.7 (0.55)	0.521

Supplementary material

Table S5.1. Pearson's (rho) correlation between the environmental variables (MAT, MAP) and latitude, and N and P deposition (N_{dep} and P_{dep}).

	Latitude	MAT	MAP	N_{dep}	P_{dep}
Latitude	1	-0.98	-0.71	-0.93	-0.92
MAT	-0.98	1	0.69	0.96	0.96
MAP	-0.71	0.69	1	0.79	0.84
N_{dep}	-0.93	0.96	0.79	1	0.99
P_{dep}	-0.92	0.96	0.84	0.99	1

Table S5.2. The results of multiple regressions testing the variance of the content of a) N and b) log10P in *Sphagnum* using environmental (MAT, MAP) variables and atmospheric deposition (N_{dep} or P_{dep}) in moss sections (Acutifolia and Cuspidata).

a)

Variable	Coefficient	St Error	t ratio	Prob < |t|	Std Beta	VIF
Acutifolia (R^2_{adj} = 0.26, p < 0.0001, n = 80)						
Intercept	4.40	1.80	2.44	0.017	0.0	.
MAT	0.39	0.21	1.89	0.063	0.8	21.5
N_{dep}	-2.07	2.05	-1.01	0.314	-0.6	34.2
MAP	0.00	0.00	1.50	0.139	0.3	4.9
Cuspidata (R^2_{adj} = 0.31, p < 0.0001, n = 60)						
Intercept	12.3	2.7	4.54	<.0001	0.00	.
N_{dep}	10.1	5.1	1.96	0.0548	1.78	70.57
MAT	-0.3	0.3	-0.96	0.3407	-0.57	30.33
MAP	0.0	0.0	-1.85	0.0701	-0.78	15.43

b)

Variable	Coefficient	St Error	t ratio	Prob < |t|	Std Beta	VIF
Acutifolia (R^2_{adj} = 0.14, p < 0.0001, n = 80)						
Intercept	-0.23	0.19	-1.23	0.2229	0.00	
MAT	0.05	0.03	2.18	0.0327	1.19	27.42
P_{dep}	-10.06	8.20	-1.23	0.2238	-0.87	46.04
MAP	0.00	0.00	-0.08	0.9375	-0.02	6.51
Cuspidata (R^2_{adj} = 0.07, p < 0.0001, n = 60)						
Intercept	-0.55	0.29	-1.91	0.0608	0.00	
P_{dep}	-8.31	13.00	-0.64	0.525	-0.60	56.31
MAT	0.03	0.03	1.02	0.311	0.57	19.44
MAP	0.00	0.00	0.84	0.4031	0.44	16.91

CHAPTER 6

Biogeochemical controls of *Sphagnum*-associated N_2 fixation in bogs along a temperate, boreal and subarctic transect in eastern Canada

6.1 Context within thesis

Northern peatlands store a third of terrestrial soil carbon (C) and are particularly vulnerable to climate change and human-induced increase in atmospheric nitrogen (N) deposition (IPCC, 2014). Biological N_2 fixation is a primary source of N that supports primary productivity in ombrotrophic, *Sphagnum*-dominated peatlands (bogs). N_2 fixing microbial communities (diazotrophs) in tight association with *Sphagnum* mosses directly connect C and N cycles in bogs through the interchange of energy and nutrients, yet biogeochemical controls of N_2 fixation are poorly understood. The relationship between *Sphagnum* CO_2 exchange and N_2 fixation has not been studied yet. Moreover, post-industrial changes in atmospheric N deposition have increased N content and N:P ratio in *Sphagnum*, suggesting a shift from N- to P-limitation in affected bogs. While P is a known driver of N_2 fixation, the effects of the changing N:P ratio in *Sphagnum* on diazotrophy in bogs remains unknown.

Chapter 6 is designed to test whether the N:P ratio that appeared to be an important driver of N_2 fixation in manipulative experiment at Mer Bleue (Chapter 4) also drives N_2 fixation on a larger geographical scale encompassing bogs from temperate-boreal-subarctic transect (Chapter 5). In a controlled laboratory setting I also test the relationship between *Sphagnum* photosynthesis and diazotrophic activity in ombrotrophic bogs for the first time focusing on the two most common microforms (hummocks and hollows) and identify the role of moisture as an important environmental control of N_2 fixation.

6.2 Abstract

Biological nitrogen (N_2) fixation is an important source of N that supports primary productivity in ombrotrophic, *Sphagnum*-dominated peatlands. To address the controls on N_2 fixation in *Sphagnum*, we sampled hummocks and hollows at 8 sites ranging from temperate to subarctic bogs and determined N_2 fixation by $^{15}N_2$ and acetylene reduction assay (ARA) methods under controlled conditions of light, temperature and moisture. We found a strong overall relationship between N_2 fixation rates using the two methods ($R^2 = 0.68$, ARA = 1.16 * $^{15}N_2$), with values ranging from below detection to 33.8 nmol $g(DW)^{-1}$ h^{-1}. N_2 fixation rates were larger in hollow than hummock samples and generally increased northwards along the latitudinal transect.

We found *Sphagnum* N:P ratio to be the strongest biogeochemical control of N_2 fixation, where our best fit model explains about 70% of the N_2 fixation variance along the gradient. N:P ratio therefore can be used as a proxy for a N_2 fixation potential in bogs. The range of N:P ratio in our study is similar to the range of N:P ratios found in *Sphagnum* from bogs around the world, with generally smaller N_2 fixation potential in P-limited bogs. We established a significant positive relationship between *Sphagnum* photosynthesis and associated N_2 fixation in hollows, but no such relationship existed in hummocks where moisture seemed to limit N_2 fixation. Given that *Sphagnum* N:P ratio can directly be impacted by changes in atmospheric deposition, changes of N or P inputs into bogs, will affect N_2 fixation potentially affecting C cycle and storage.

6.3 Introduction

Peatlands contain about a third of the global terrestrial soil carbon (C) (Yu *et al.*, 2010) and about 10-15% of the global soil nitrogen (N; Loisel *et al.*, 2014). The long-term net C and N uptake mainly results from the small imbalance between rates of plant productivity and

decomposition. Productivity is regulated through photosynthesis which, in turn, is influenced by N availability. Prior to the industrial revolution, biological dinitrogen (N_2) fixation was the main input of N in all terrestrial ecosystems (Vitousek *et al.*, 2002). Owing to the lack of surface- or groundwater nutrient influxes in ombrotrophic bogs, the only sources of N are atmospheric deposition and biological N_2 fixation. In undisturbed *Sphagnum* moss-dominated bogs where atmospheric N inputs are small (< 0.1 g N m^{-2} y^{-1}; Vitt *et al.*, 2003), the biological N_2 fixation may exert a major control on *Sphagnum* productivity (Vile *et al.*, 2014) thus coupling C and N cycles.

Biological nitrogen (N_2) fixation is carried out by a metabolically diverse group of microbial organisms (diazotrophs) in peatlands (Dedysh *et al.*, 2006; Bragina *et al.*, 2012a). Common to all diazotrophs is the enzyme nitrogenase that reduces atmospheric N_2 to ammonia, a form of N available for microbes and plants. Diazotrophs depend on phosphorus (P) availability due to large amounts of energy, at least 16 adenosine tri phosphates (ATP) needed to fix one N_2 molecule. P, in contrast to N, cannot be acquired via biological processes (Vitousek *et al.*, 2010) thus its supply in ombrotrophic bogs depends primarily on wet and dry P atmospheric deposition (Tipping *et al.*, 2014). Along with N, P limits plant productivity and functioning, and can control microbial processes like N_2 fixation in peatlands (Larmola *et al.*, 2014). The N:P ratio has been used to infer nutrient limitations in wetland plants (Koerselman & Meuleman, 1996) and changes in N:P ratios have been directly linked to increased atmospheric N and P deposition (Bragazza *et al.*, 2004; Jiroušek *et al.*, 2011; Wang & Moore, 2014). Generally, wetland plants exhibit N-limitation when N:P < 14, P-limitation occurs at N:P > 16, while 14 < N:P < 16 usually implies N-P co-limitation (Koerselman & Meuleman, 1996). Strong internal peat-P recycling (Wang *et al.*, 2014, 2015) indicates that *Sphagnum* and microbially-driven processes within bogs may

often be P- rather than N-limited. The increased atmospheric N deposition over the past century has raised N content and N:P ratio in *Sphagnum* (Bragazza *et al.*, 2004, 2005), pushing *Sphagnum* towards P-limitation. The effects of increased N deposition on N_2 fixation in *Sphagnum* are still not fully understood. While N additions decreased N_2 fixation rates in *Sphagnum magellanicum* in the laboratory setting (Kox *et al.*, 2016), long-term N fertilization showed no effects on N_2 fixation rates in a bog regardless of the type of added N species (van den Elzen *et al.*, 2018). These inconsistent results might be partially explained by the relative abundance of P best described by N:P ratio. With atmospheric N and P deposition both varying over time and across space, *Sphagnum* N:P ratio may be a good indicator of dynamic N_2 fixation potential in bogs (Chapter 5).

Diazotrophs found in *Sphagnum* in peatlands tightly connect C and N cycles through N_2 fixation. While the nature of the interaction between diazotrophic microbes and *Sphagnum* mosses remains unknown, most evidence infers an associative relationship (i.e. Berg *et al.*, 2012). For example, bacterial colonies within hyaline cells in *Sphagnum* were found attached to the cell walls that were shared by photosynthetically active cells allowing for faster and more efficient chemical and nutrient exchange (Bragina *et al.*, 2012a). Whether they are free-living or associated with *Sphagnum* as epi- or endophytes, the activity of diazotrophs seems to be linked to C incorporation in mosses. For example, in a laboratory study, intracellular cyanobacteria in *Sphagnum riparium* fixed N_2, which was then invested into a 35% increase in moss production (Berg *et al.*, 2012).

Recent studies showed that methanotrophs, another type of bacteria capable of diazotrophy, influence C fixation in peatlands (Larmola *et al.*, 2014; Vile *et al.*, 2014). Symbiotic methanotrophic bacteria found in hyaline cells were responsible for 10-30 % increase of

Sphagnum photosynthesis by supplying additional CO_2 through CH_4 oxidation (Raghoebarsing *et al.*, 2005; Kip *et al.*, 2010). However, the specifics of *Sphagnum*-methanotrophic microbe interaction and diazotrophy and its biogeochemical controls are yet to be understood (Ho & Bodelier, 2015). In one study, methane stimulated diazotrophy in peatlands (Larmola *et al.*, 2014), indicating co-occurrence of N_2 fixation and CH_4 oxidation. However, in other studies, methane did not stimulate N_2 fixation (Leppänen *et al.*, 2015; Warren *et al.*, 2017; Kox *et al.*, 2018) suggesting that these processes may only happen under specific environmental conditions such as low O_2 concentration (Kox *et al.*, 2018) or in wetter, minerotrophic peatlands (Larmola *et al.*, 2014).

In drier, well oxygenated surface layers of *Sphagnum* in ombrotrophic bogs, diazotrophs related to methanotrophy, although present, are not likely to be active (Larmola *et al.*, 2014). There, the activity of phototrophic and heterotrophic bacteria may dominate N_2 fixation. Non-photosynthetic diazotrophs (heterotrophs and methanotrophs) would be able to provide fixed N to photosynthetically active *Sphagnum* for its C fixation, while photosynthetic N_2 fixers (e.g. cyanobacteria) would invest newly gained energy from their own photosynthesis into N_2 fixation and supply *Sphagnum* with additional N required for C fixation. In both cases, a positive relationship between *Sphagnum* photosynthetic rate and N_2 fixation could be expected. Limited C supply can also limit N_2 fixation: endophytic diazotrophs most likely depend on photosynthates produced by mosses. Under unfavorable environmental conditions, for example low moisture availability, both *Sphagnum* photosynthesis (Silvola, 1990; Schipperges & Rydin, 1998; Chong *et al.*, 2012) and diazotrophic activity (Chapter 3) may be inhibited. Diazotrophy can be thus particularly vulnerable to changing precipitation and extended droughts impacting C

fixation in peatlands. Although potentially important, the relationship between *Sphagnum* photosynthesis and diazotrophic activity has not yet been studied in peatlands.

Due to its enzymatic nature, N_2 fixation is affected by water table depth, moss species and microtopography (Leppänen *et al.*, 2015), and other environmental factors such as temperature and precipitation (Chapter 3), that could conceal important biogeochemical controls, such as P availability and N:P ratio in *in situ* studies.

In this study, we measured N_2 fixation in a controlled environment by incubating two *Sphagnum* sections (Acutifolia and Cuspidata, occupying hummocks and hollows, respectively) collected from ombrotrophic bogs along a temperate, boreal and subarctic transect in eastern Canada. We hypothesized that:

1) H1: N_2 fixation will be best explained by *Sphagnum* tissue N:P ratio rather than by N and P content individually;
2) H2: The relationship between N_2 fixation and N:P ratio will differ between two *Sphagnum* sections, and will be stronger in Cuspidata compared to Acutifolia;
3) H3: there will be a positive relationship between *Sphagnum* gross primary production (GPP) as a measure of photosynthesis and N_2 fixation indicating a connection between C and N cycles in bogs;
4) H4: we expect stronger relationship between *Sphagnum* GPP and N_2 fixation in hollows than hummocks owing to the greater moisture availability in the former.

6.4 Material and methods

Site selection and experimental design

Four cores of two hummocks and two hollows (40 x 30 x 20cm; length x width x depth) were harvested in July and August 2014 from eight ombrotrophic bogs along a latitudinal transect in eastern Canada (Fig. 6.1; site descriptions in Živković *et al.*, 2017**)**. *Sphagnum* species varied across the transect, but hummocks were populated by section Acutifolia and hollows by section Cuspidata, while Palustria occurred only in two bogs inhabiting both a hummock and a hollow (at MB and LLT, respectively). We excluded Palustria from the dataset due to its small sample size. Therefore, only sections Acutifolia and Cuspidata represent hummocks and hollows, respectively. Cores were transferred to McGill University in iced coolers and kept at 4°C prior to the start of the experiment.

Moss cores were fitted into acid washed plastic containers (40 x 30 x 30 cm; length x width x depth) and placed in controlled environment chambers (Phytrotron, McGill University) to acclimatize for five days prior to the analyses. Acclimatization conditions were temperature of 16°C, 85% relative humidity, and a diurnal light schedule of 12 h photosynthetic photon flux density (PPFD) of 400 μmol m^{-2} s^{-1} and 12 h of darkness. We used DI water to keep mosses moist and photosynthetically active. However, due to the different water holding properties of the moss samples, the gravimetric moisture content (GM) varied between samples. Thus, we use GM as one of the effects in statistical analyses.

CO_2 exchange, ARA and $^{15}N_2$ measurements were performed using incubation vessels with a temperature of 20°C, continuous light at PPFD = 600 μmol m^{-2} s^{-1}, relative humidity of 80%, and ambient CO_2 level (410 - 460 μmol mol^{-1}). Continuous light was used to enable photosynthetically active N_2 fixing diazotrophs and PPFD of 600 μmol m^{-2} s^{-1} was selected after

a series of light curve tests on random samples of Acutifolia and Cuspidata at 0, 400, 600 and 800 μmol m^{-2} s^{-1} confirmed light saturation at that level, as shown by Chong *et al.* (2012)

CO_2 exchange measurements

We measured net CO_2 exchange (NEE) on 10 subsamples from each plastic container following the protocol developed by Juutinen *et al.* (2015). We transferred each subsample (top 6 cm of moss, diameter 7.2 cm) into the plastic vessel (7.2 x 3 cm; diameter x height) that was placed onto a small platform with the groove filled with DI water to ensure gas tight seal. A small chamber (10 x 10 cm; diameter x height) was used to cover the vessel containing mosses. We recorded change in headspace CO_2 concentrations using CO_2/H_2O analyzer LI-6262 (LI-COR) every 10 s within the first minute and every 15 s within next two minutes at 600 μmol m^{-2} s^{-1}. Measurements of NEE under dark conditions represent respiration (R). We calculated CO_2 exchange rates (NEE and R) from linear changes in CO_2 concentration over the three-minute incubation period, excluding regressions with the R^2 lower than 0.95. CO_2 values were corrected for the temperature and chamber volume. We calculated gross primary production (GPP) by subtracting NEE and R and henceforth refer to it as photosynthesis. We expressed all CO_2 fluxes (NEE, R and GPP) per unit ground area (mg C m^{-2} h^{-1}) and per unit moss dry weight (DW) mass (mg C $g(DW)^{-1}$ h^{-1}). 6 subsamples were thereafter used for ARA and the remaining 4 for $^{15}N_2$ analyses.

Measurements of N_2 fixation, gravimetric moisture and nutrients (N, P and N:P)

N_2 fixation was measured in parallel incubations using both acetylene reduction assay (ARA) and $^{15}N_2$ tracer methods. Each of the moss cores that was used for measurements of CO_2 exchange was immediately placed into either a 250-mL Mason jar for ARA or a 50-mL Erlenmeyer flasks for $^{15}N_2$ tracer assay. Mason jars and Erlenmeyer flasks were capped

immediately to capture the moisture content in mosses. For ARA, each lid was equipped with the tubing and a stopcock which was tested for air tightness prior to analyses. For the $^{15}N_2$ tracer method, we used rubber septa reinforced with silicone. In five of the subsamples we replaced 10% (v/v) headspace air with pure acetylene (freshly made mixing CaC_2 and DI water in the laboratory) for ARA and $^{15}N_2$ enriched gas (98.9%, Cambridge Isotopes). One subsample was used as a control to ensure that there was no ethylene production over the course of incubation in ARA, and two subsamples of mosses with no $^{15}N_2$ gas additions were used as controls for $^{15}N_2$ enrichment method and their average was taken when $^{15}N_2$ rate calculation was performed. We sampled for ethylene production at 0, 2, 12, 24, 36 and 48 h of incubation to ensure the linearity of the ethylene production in ARA incubations. We also measured CH_4 concentrations in ARA gas samples collected over the 48 h and detected a range of 1.6 - 5.6 ppm. We analyzed gas samples immediately after sampling with gas chromatograph (GC-2014, Shimadzu) with flame ionization detector (FID) and HayeSep N (80/100 mesh; 2-m column; temperature of the injector and detector at 175℃, column temperature at 75℃). We used 50, 100 and 300 ppm ethylene standards to build the standard curve. $^{15}N_2$ fixation rates were calculated following the equation (Liengen, 1999; Stewart *et al.*, 2011c):

$$Y = \frac{atom\%\ 15N\ excess}{100} \times \frac{total\ Nsample\ \times 10^9}{t \times 28} \times \frac{100}{\%15Nair}$$

where Y (nmol N g dw $^{-1}$ h^{-1}) is amount of N_2 fixed, $^{15}N_{excess}$ is calculated as a difference between atom%^{15}N sample and atom% ^{15}N control, total N is the amount of total N in the sample, *t* represents the incubation time, 28 (g mol^{-1}) is the molecular weight of N_2 and the $\%^{15}N_{air}$ is the amount of ^{15}N in the Erlenmeyer flasks (%).

We measured the GM content of *Sphagnum* from the wet and dry weight of mosses used in both ARA and $^{15}N_2$ tracer assays:

$$\text{GM (\%)} = \frac{wet\ weight\ (g) - dry\ weight\ (g)}{dry\ weight\ (g)}$$

Concentrations of N and P and the N:P ratio were obtained by grinding, digesting and analysing dry mosses that were used in ARA assays with methods described in Živković *et al.* (2017). Dried and ground mosses from the $^{15}N_2$ tracer method assay were used to obtain N concentration and $\%^{15}N$ at the Stable Isotope Facility at UC Davis, California and to calculate $^{15}N_2$ fixation rates. Owing to the small sample amount (5 mg) used in the $^{15}N_2$ tracer method, we were unable to determine P directly thus all data analyses involving nutrients were performed on ARA values. There was a strong significant linear relationship ($R^2 = 0.68$) between *Sphagnum* N concentration values obtained from the ARA and $^{15}N_2$ treatment samples (Fig. S6.1).

Calculations of ARA and $^{15}N_2$ fixation rates and conversion ratio (CR)

Means and standard errors for each of the two *Sphagnum* sections (Acutifolia and Cuspidata) sampled from the 8 bogs were calculated for rates of ARA (n = 5 samples/section) and $^{15}N_2$ fixation (n = 4 samples/section). To evaluate the relationship between ARA and $^{15}N_2$ fixation, we used linear regression that accounts for standard errors in both variables following the method described in York *et al.* (2004) as implemented in the "york_fit" Matlab code (Matlab R2015b). The 95% confidence interval (CI; 0.95, 1.68) of the linear regression was derived by regressing 1000 bootstrap realizations of the dataset. The CR represents the slope of the linear regression: ARA = CR * $^{15}N_2$ fixation (CR = 1.16, [CI]: 0.95, 1.68, Fig. 6.2).

Statistical methods

To show differences in $^{15}N_2$ fixation rates among sites and between Sphagnum sections, we used two-way ANOVA ('aov' function in the R 'base' package: (R Core Team 2013) and post-hoc Tukey's HSD test.

We used a linear mixed-effect model (LMM) approach through maximum likelihood to test relationships between N_2 fixation and GPP, GM and N, P and N:P across the transect but also to tease apart relationships within two *Sphagnum* sections (Acutifolia in hummocks and Cuspidata in hollows). A series of models were built to describe best fits for these relationships (Tables 6.1-6.3). LMM was implemented using the "lmer" function within the R (v3.1.2) package "lme4" (Bates *et al.*, 2015). When necessary, we $\log_{10}$ transformed data to achieve normality. We used z-correction ($z = \frac{x - mean\,(x)}{std\ \ dev(x)}$) to avoid scaling issues between variables that can impact LMM analyses. To find the best fit model we built models with all possible permutations of the fixed and random effects. We used the Akaike Information Criterion (AIC) as implemented in the R function 'aictab', 'AICcmodavg' package (Mazerolle, 2015) to select the best-fit model. AIC is a measure of the predictive ability of a model that can be used to compare models whereby the model with the strongest predictive value has the smallest AIC (Zuur *et al.*, 2009). All models throughout the study were checked for assumptions of heterogeneity, independence and normality of residuals (Zuur *et al.*, 2009).

H1: The relationship between N_2 fixation and Sphagnum nutrient content a) N, b) P and c) N:P

We used LMM to test H1 and to evaluate the relationship between ARA (as a proxy for N_2 fixation) and N, P and N:P ratio (fixed effects) within two *Sphagnum* sections (Acutifolia and Cuspidata; fixed effects) across eight sites (random effect). The best fit model had the following structure: N_2 fixation ~ $X_{1\text{-}3}$ + SECTION + (1 + $X_{1\text{-}3}$|SITE), where X_1=N, X_2=P and X_3=N:P. To

determine which of the three variables (N, P or N:P) was the best predictor for N_2 fixation, we compared the three best fit models for N, P, and N:P, respectively (A0, B0 and C0, Table 6.1) using the AIC method. Because the relationship between N_2 fixation and N:P was the strongest, next we tested the strength of this relationship relative to the two *Sphagnum* sections.

H2: The importance of Sphagnum sections on the relationship between N_2 fixation and N:P

To test the effect of *Sphagnum* sections on the relationship between N_2 fixation and N:P across the transect (H2) we built two LMM. In the first model, *Sphagnum* section was a fixed and sites a random effect (C0 model: N_2 fixation ~ N:P + SECTION + (1 + N:P|SITE). In the second model, we excluded sections (C0.null model: N_2 fixation ~ N:P + (1 + N:P|SITE) and kept all other model parameters the same. To test the differences between the models, we used ANOVA ('aov' function in the R 'base' package: (R Core Team 2013). We calculated the marginal and conditional R^2 for the overall best fit model (C0 model) through restricted maximum likelihood (REML). Marginal R^2 describes the proportion of variance explained by the model's fixed factors while conditional R^2 considers both fixed and random effects (Nakagawa & Schielzeth, 2013).

H3: Testing overall relationship between the $^{15}N_2$ fixation and GPP and GM across the transect

LMM were built to test the relationship between $^{15}N_2$ fixation and GPP and GM (fixed effects), across 8 sites (random effect) in 2 *Sphagnum* sections (Acutifolia and Cuspidata; random effects; Table 6.2). The best fit model was selected with the following structure: N_2 fixation ~ GPP + GM + (1|SECTION/SITE).

H4: The relationship between N_2 fixation and GPP and GM in a) *Acutifolia* and b) *Cuspidata*

To further investigate the relative importance of GPP and GM (fixed effects) on N_2 fixation of each *Sphagnum* section, we used a model N_2 fixation ~ GPP + GM + (1|SITE) for each of the *Sphagnum* sections (i.e., using sites as a random effect, Table 6.3). We then calculated the marginal and conditional R^2 for each model through restricted maximum likelihood (REML).

6.5 Results

Variability of N_2 fixation, CO_2 exchange, GM and Sphagnum nutrient concentrations across the transect

N_2 fixation rates measured by the $^{15}N_2$ tracer method showed large variability ranging from below the detection limit up to 33.8 nmol $g(DW)^{-1}$ h^{-1} (Table S6.1). Neither latitude nor *Sphagnum* section alone explained N_2 fixation variability across the transect. We found that the interaction term between sites and *Sphagnum* sections had a significant effect on N_2 fixation rates (Fig. 6.3). Overall, N_2 fixation rates were significantly larger in hollows dominated by *Sphagnum* section Cuspidata (7.05 ± 0.95 nmol $g(DW)^{-1}$ h^{-1}, mean ± SE) than in hummocks dominated by Acutifolia (1.73 ± 0.26 nmol $g(DW)^{-1}$ h^{-1}, Student t-test, $p < 0.0001$, Table S6.1) indicating that *Sphagnum* mosses from hollows may provide a more suitable environment for diazotrophic communities. N_2 fixation in hollows showed a variable but overall increasing trend from the south to the north (Fig. 6.3), with the smallest rates at MIR (0.25 ± 0.1 nmol $g(DW)^{-1}$ h^{-1}) and the largest at SCH (12 ± 3.7 nmol $g(DW)^{-1}$ h^{-1}). There was no such trend in hummocks (Fig. 6.3).

The rates of CO_2 exchange also varied among the sites and within the two *Sphagnum* sections (Table S6.1, Fig. S6.2). Two-way ANOVA showed a significant interaction effect between sites and *Sphagnum* sections on GPP, R and NEE (Fig. S6.2). In five out of eight sites,

there was a significant NEE at a PPFD of 600 μmol m^{-2} s^{-1} in Cuspidata section (hollows) but there was negligible NEE in Acutifolia (hummocks) at any of the sites (Fig. S6.2). Hummocks showed smaller GPP than hollows (0.35 ± 0.02 vs 0.46 ± 0.02 mg $g(DW)^{-1}$ h^{-1}, respectively; Fig. S6.2) and hollows were significantly wetter (1260.31 ± 21.16 %, mean ± SE) than hummocks (1049.44 ± 22.08 %; Fig S3).

N concentration along the transect varied from 4.4 to 11.6 mg g^{-1}, while P concentration ranged from 0.2 to 1.6 mg g^{-1}. N:P ratios of individual samples ranged from strongly N-limited [6.9 (NOR)] to strongly P-limited [38.6 (MIR)]. Variability across the transect (range 31.6) was larger than variability within individual sites, which was most pronounced at MIR (range 24.1; Table S6.1).

Relationship between N_2 fixation and nutrient content in Sphagnum from two different microtopographies

The best predictive model of N_2 fixation in *Sphagnum* across the transect includes N:P ratio rather than N and P individually (H1, smallest AIC, Table 6.1, Fig. 6.4). N_2 fixation is decreasing with increasing N:P ratio (i.e., decreasing P availability, Fig. 6.4c). However, the relationship between N_2 fixation and N:P ratio differed between *Sphagnum* sections, Acutofolia and Cuspidata (H2, ANOVA, $\chi^2(1) = 50.01$, $p < 0.0001$, Fig. 6.4c). The relationship was stronger in Cuspidata (hollows) compared to Acutofolia (hummocks; Tukey's test, $t_{(129.68)} = 7.415$, $p < 0.0001$, Fig. 6.4, Table 6.1). The model (C0, see Table 6.1c) explained 45% of the variance (marginal $R^2 = 0.45$) taking into account only fixed effects (N:P and microtopography). However, when random effects (i.e., sites) are added, the model explained 68% of the variance (conditional $R^2 = 0.68$), indicating that these relationships vary among the sites.

The relationship between N_2 fixation and GPP and GM in a) *Acutifolia* and b) *Cuspidata*

We found that N_2 fixation was driven by both GPP and GM, while no significant interaction between GPP and GM was detected (H3, Table 6.2a; Fig. 6.5). The best explanatory variable differed between the two *Sphagnum* sections (H4): N_2 fixation was only positively related to GM in hummocks (section Acutifolia) (Table 6.2a and Fig.6.5a, R^2 of the overall model) explaining 18% of the variance, whereas in hollows (Cuspidata) GM had no effect. In hollows, GPP was positively related to N_2 fixation explaining 20% of the variance, while the relationship was not significant in hummocks (Table 6.2b and Fig.6.5b).

6.6 Discussion

N_2 fixation rates in peatlands

We used both $^{15}N_2$ tracer and ARA N_2 fixation methods and found that under controlled laboratory conditions these two methods were highly correlated with an ARA:$^{15}N_2$ ratio, or conversion factor (R), of 1.16. This is smaller than the theoretical R of 3-4.5 (Hardy *et al.*, 1968), but is consistent with the few reported R values for *Sphagnum*-associated N_2 fixation rates. R values ranged from 0.32 in Alberta bogs (Vile *et al.*, 2014) to 0.85 in Arctic tundra (Stewart *et al.*, 2011a) and 3.9 in a Minnesota bog (Warren *et al.*, 2017). The small R values (<3) have been attributed to the presence of non-cyanobacterial N_2 fixing communities, most likely methanotrophs (Larmola *et al.*, 2014; Vile *et al.*, 2014). Methanotrophs are known to be inhibited by acetylene and thus, their activity is not fully captured by the ARA method (De Bont & Mulder, 1976). However, methane-induced N_2 fixation is not found in all peatlands (Leppänen *et al.*, 2015; Warren *et al.*, 2017) and the small R values could be due to other processes such as oxygen levels (Warren *et al.*, 2017) or the presence of alternative N_2 fixing enzymes in diazotrophs (Bellenger *et al.*, 2014). Although we cannot identify the reason for the small R

values in this study, the strong relationship between the two methods indicates that primary diazotrophs seem to have similar activity and responses under the controlled environmental conditions.

The $^{15}N_2$ fixation rates in our study were about 4 to 7 times larger than rates measured at a Minnesota bog (Warren *et al.*, 2017) and in both studies, rates in hollows were up to 4 times larger than in hummocks. Our rates also fall within the range of other reported *Sphagnum*-related N_2 fixation rates under similar environmental conditions (Gavazov *et al.*, 2010; Larmola *et al.*, 2014; van den Elzen *et al.*, 2018) suggesting a large potential for N_2 fixation in ombrotrophic bogs in Eastern Canada though we cannot scale laboratory to ecosystem N_2 fixation rates. However, our study provided a unique opportunity to test biogeochemical controls (*Sphagnum* N and P concentration and N:P ratio and GPP) that can often be concealed by abiotic controls.

N:P ratio as an important driver of N_2 fixation

Our study using *Sphagnum* samples from the transect shows that the strongest biogeochemical driver of N_2 fixation is the concentration of P relative to N in *Sphagnum* tissue, indicated by the N:P ratio (Table 6.1 and Fig 6.4c). *Sphagnum* N:P ratio in the literature ranges widely from 2.3 to 40.7 across ombrotrophic bogs world-wide (Fig. 6.6) representing both N- (N:P < 14) and P-limitation (N:P > 16). The majority of *Sphagnum* from Cuspidata section has a N:P ratio smaller than 14, while mosses from Acutifolia seem to be more P-limited. Both N and P concentrations from the literature exceed the concentrations measured in our study (Fig. 6.7.) owing to several long-term disturbed bogs across Europe (e.g. Bragazza *et al.*, 2004, 2005). However, the N:P ratio in this study covers well the range of the global distribution in ombrotrophic bogs (Fig. 6.6 and Fig. 6.7). The largest N_2 fixation values (dark blue, Fig. 6.7.) in overlaid datasets fall within N:P < 15 indicative of *Sphagnum* N-limitation, and oppositely, the

smallest N_2 fixation values fall within the P-limited *Sphagnum* (light blue, N:P > 20). We show that *Sphagnum* N:P ratio thus can be used as a proxy for diazotrophy potential in ombrotrophic bogs, which is particularly important for northern bogs that are vulnerable to local, regional and global changes in N and P atmospheric deposition.

The link between Sphagnum productivity and N_2 fixation and the role of microtopography and moisture

Our study highlights the link between C and N cycle in ombrotrophic bogs through a *Sphagnum* – diazotroph association. Once the effects of environmental factors were minimized (though as mentioned in Methods moisture was not controlled in this experiment), we were able to show a small, but significant positive relationship between *Sphagnum* photosynthesis and N_2 fixation though moisture had a positive effect too (Table 6.2).This link suggests a bi-directional flow of nutrients (C and N) between *Sphagnum* and diazotrophs during photosynthesis (GPP), though the actual mechanisms remain unclear. One possible explanation is that *Sphagnum*'s photosynthetic activity requires additional N, which is obtained through associative diazotrophy. N in plants is a major component of chlorophyll and rubisco enzyme (e.g., Weston *et al.*, 2015). Oppositely, it is possible that newly fixed C from active photosynthesis in *Sphagnum* stimulates diazotrophs to invest newly gained energy towards N_2 fixation. No study thus far explored the relationship between the photosynthesis and N_2 fixing activity in *Sphagnum* mostly due to large variability in N_2 fixation rates in non-controlled in-situ settings, large diversity of diazotrophic communities involved in N_2 fixation (Zadorina *et al.*, 2009; Kip *et al.*, 2011; Bragina *et al.*, 2012b), and the differences in spatial and temporal scales at which these two processes are studied (Weston *et al.*, 2015). Our study warrants further investigation of this relationship and the drivers that could be involved in regulating the C-N link in ombrotrophic bogs.

The relationship between N_2 fixation, and GPP and moisture differed between hollows (*Sphagnum* section Cuspidata) and hummocks (section Acutifolia; Table 6.3). N_2 fixation rates in hollows were not limited by moisture but had a positive relationship with GPP suggesting that diazotrophy may be an important source of N for *Sphagnum* in hollows. In contrast, moisture was the only significant driver of N_2 fixation rates in hummock mosses and no relationship with the GPP was detected. Interestingly, there was no significant difference in GPP between hummocks and hollows (Table S6.1), and N content in *Sphagnum* explained about 40% of the variance in GPP in hummocks (Fig. S6.4a), suggesting that hummocks may rely on different sources of N than hollows to sustain their GPP. It is possible that hummocks use N deposited via the atmosphere and N from remineralization (Živković *et al.*, 2017; Moore and Bubier, in press) for photosynthesis without the need for additional energetically costly N from biological N_2 fixation.

The difference in N_2 fixation rates between hummocks and hollows also depends on moisture availability. Moisture is a limiting factor of N_2 fixation in northern latitude ecosystems (Belnap, 2001; Stewart *et al.*, 2011b; Rousk & Michelsen, 2016; Rousk *et al.*, 2018) and in *Sphagnum* in peatlands (Leppänen *et al.*, 2015). Hummocks are often exposed to dry conditions during the summer months in ombrotrophic peatlands and while *Sphagnum* can tolerate desiccation (Hájek & Vicherová, 2014), moisture availability may have a stronger effect on the diazotrophic microbiome. Our study was carried out in the laboratory setting where the lowest detected gravimetric moisture was 690 % (g (DW) g^{-1}; equivalent to 88% of water content in the *Sphagnum* sample). However, Acutifolia may have had smaller N_2 fixation rates due to lower in-field potentials caused by longer drying conditions prior to the sampling date (i.e. water table depth at the time of sampling was 31 - 49 cm in hummocks and 7 - 25 cm in hollows; see

Živković *et al.*, 2017). As a result of differences in moisture conditions across microtopographic features, it is possible that the diazotrophic community associated with *Sphagnum* in hummocks differs in size and structure from the one in hollows. Additionally, *Sphagnum* has adapted to such differences by obtaining and utilizing N from different sources.

Overall, our results suggest that *Sphagnum* in ombrotrophic bogs obtains most of its N through N_2 fixation in hollows and atmospheric deposition and mineralization of organic matter in hummocks (also in Živković et al. 201; Chapter 4). However, biogeochemical controls, such as N:P ratio within *Sphagnum*, represent an important limiting factor for associated N_2 fixation in ombrotrophic peatlands. P-limited *Sphagnum* (N:P > 20; here and Chapter 4) have small N_2 fixation rates despite favourable environmental conditions in the laboratory, indicating P limitation of the N_2 fixation process.

6.7 Figures and tables

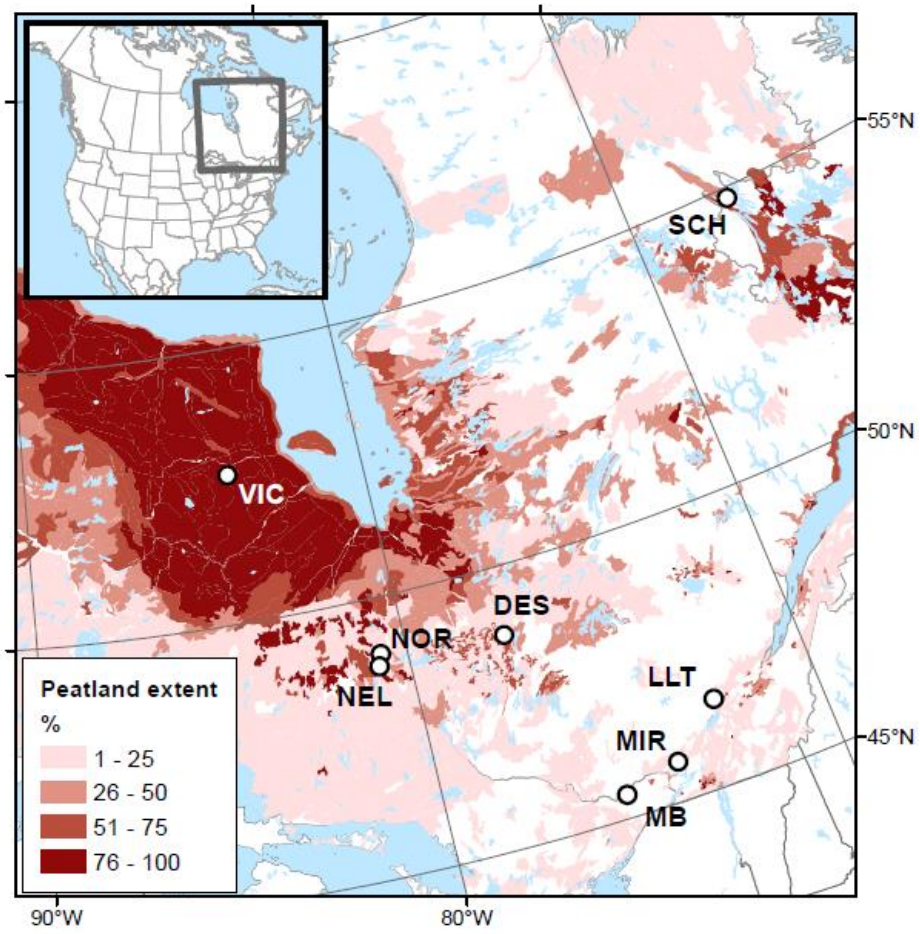

Fig. 6.1 Map of selected bogs along the transect (MB = Mer Bleue, MIR = Mirabel, LLT = Lac à la Tortue, DES = Despinassy, NEL = Nellie, NOR = Norembega, VIC = Victor, SCH = Schefferville). All bogs but VIC and SCH were selected based on Turunen *et al.* (2004). Source of the base map: https://geoscan.nrcan.gc.ca/starweb/geoscan/servlet.starweb?path=geoscan/fulle.web&search1=R=288786

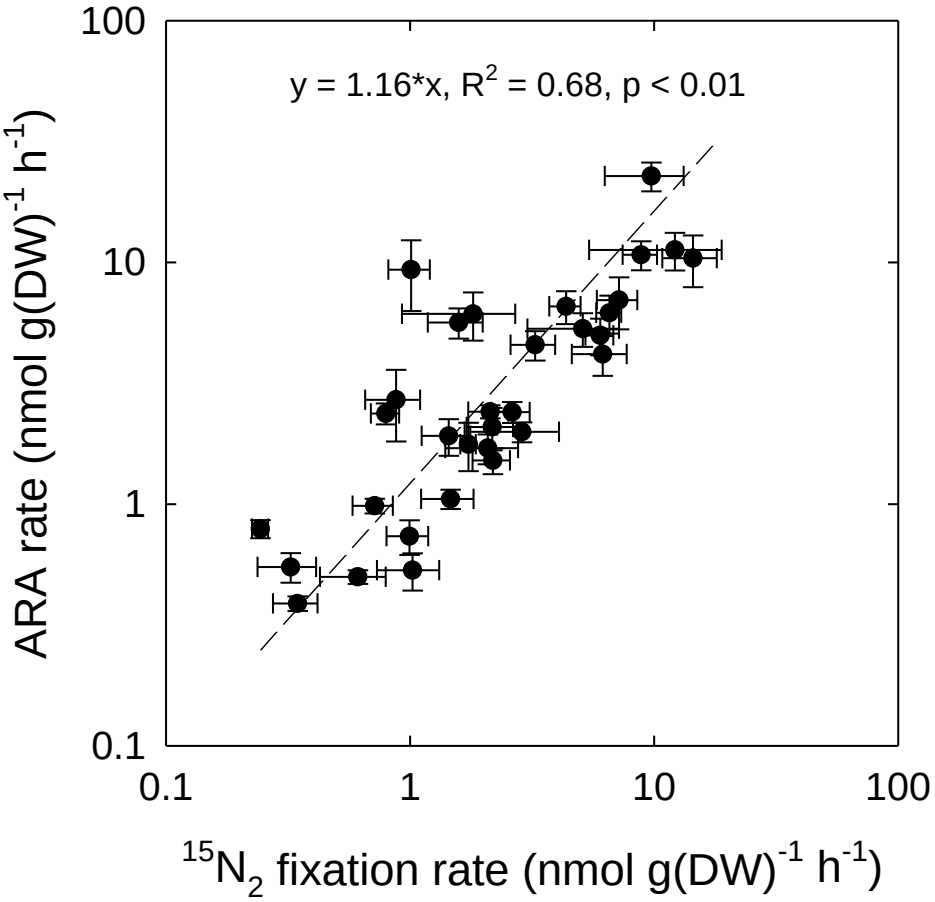

Fig. 6.2 The relationship between the mean N_2 fixation rates measured by ARA and $^{15}N_2$ tracer methods plotted on $\log_{10}$ scale. The dashed line represents the best fit linear regression with the bars representing the standard errors for each of the 32 *Sphagnum* samples.

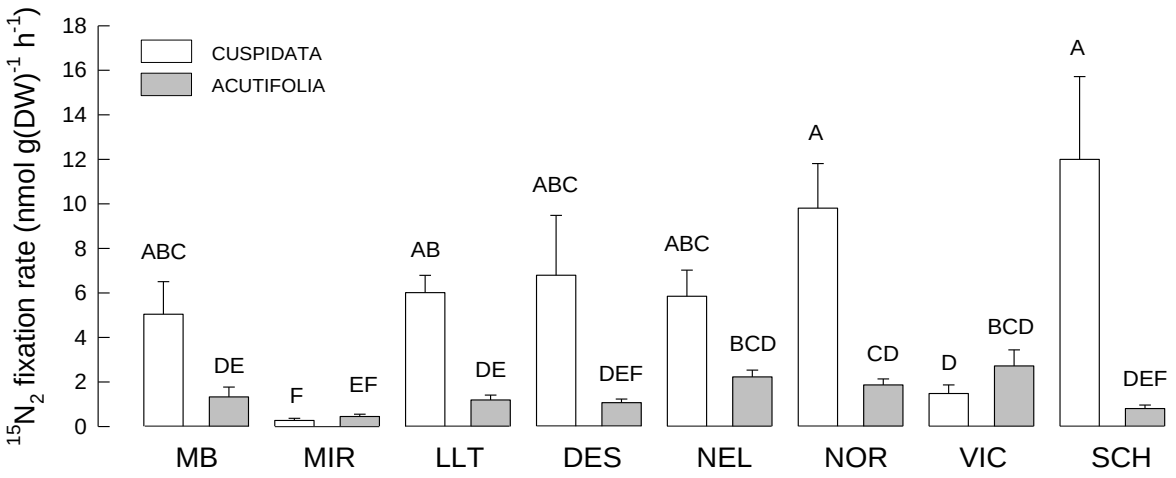

Fig. 6.3 N_2 fixation rate (means ± SE) along the latitudinal gradient under optimal environmental conditions in the Phytotron. A two-way ANOVA showed that the interaction term between sites and microtopography had a significant effect on N_2 fixation rates ($F_{7,109} = 10.2$, $p<0.0001$, N = 124). Post-hoc Tukey's HSD test depicted by the capital letters above the bars shows significant differences between the sites and microtopographic features, represented by the Cuspidata and Acutifolia sections, equivalent to hollows and hummocks.

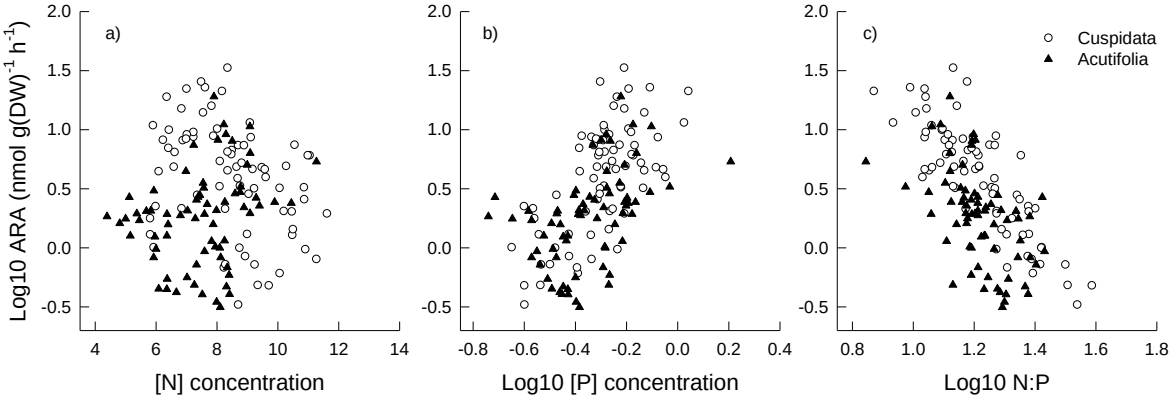

Fig. 6.4 The relationship between ARA rate and a) N concentration (mg g^{-1}), b) P concentration (mg g^{-1}) and c) N:P ratio within two *Sphagnum* sections (Cuspidata and Acutifolia) across the sites.

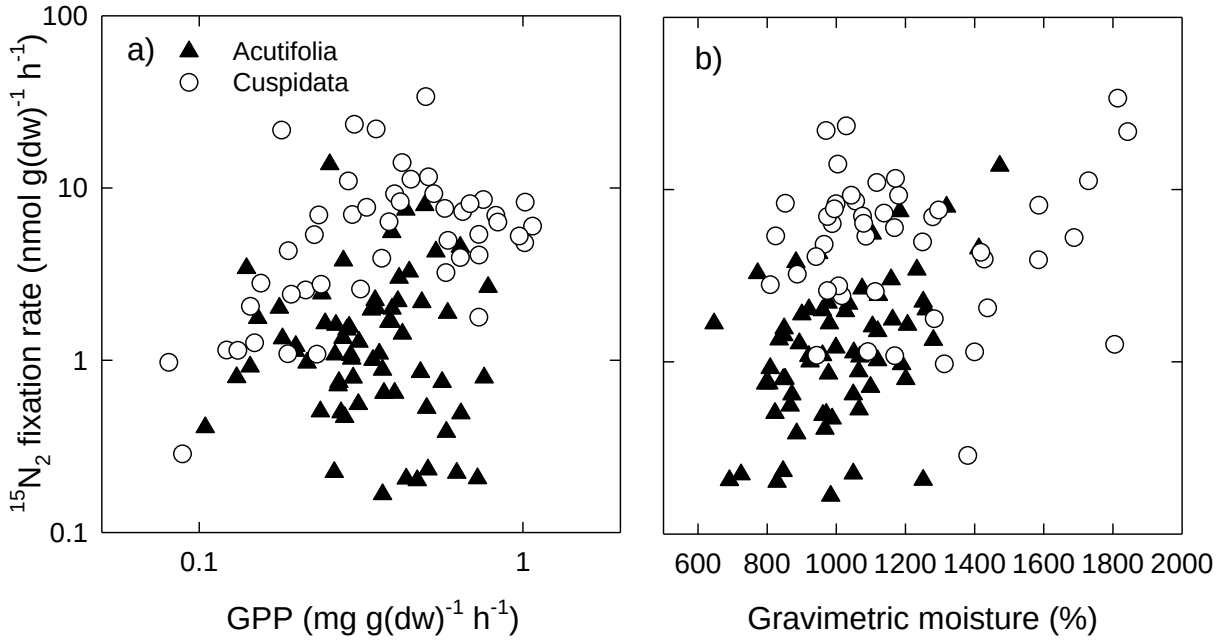

Fig. 6.5 The relationship between $^{15}N_2$ fixation rate and a) GPP and b) GM in sections Acutifolia and Cuspidata. Note that data in a) are shown on log-log scale, and in b) on the log-linear scale (linear x-axis).

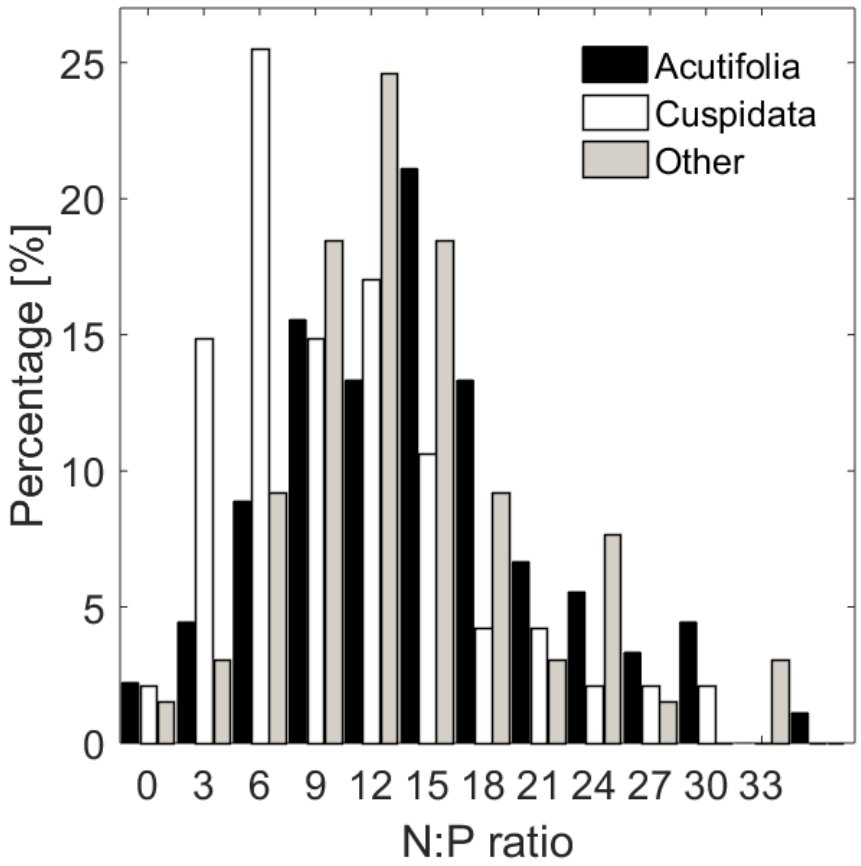

Fig. 6.6 The distribution of *Sphagnum* N:P ratio in ombrotrophic bogs, derived from the literature and personal communications (N = 205). Literature accessed: Aerts *et al.* (1992), Bragazza *et al.* (2004), Bedford *et al.* (1999), Jiroušek *et al*. (2014), Malmer (1988), Moore (pers. comm.), Pakarinen & Gorham (1984), Pakrinen & Tolonen (1997), Pakarinen (1978a, b), Phuyal *et al.* 2008, van der Heijden *et al.* (2000), Živković *et al.* (2017), Wang (pers. comm.)

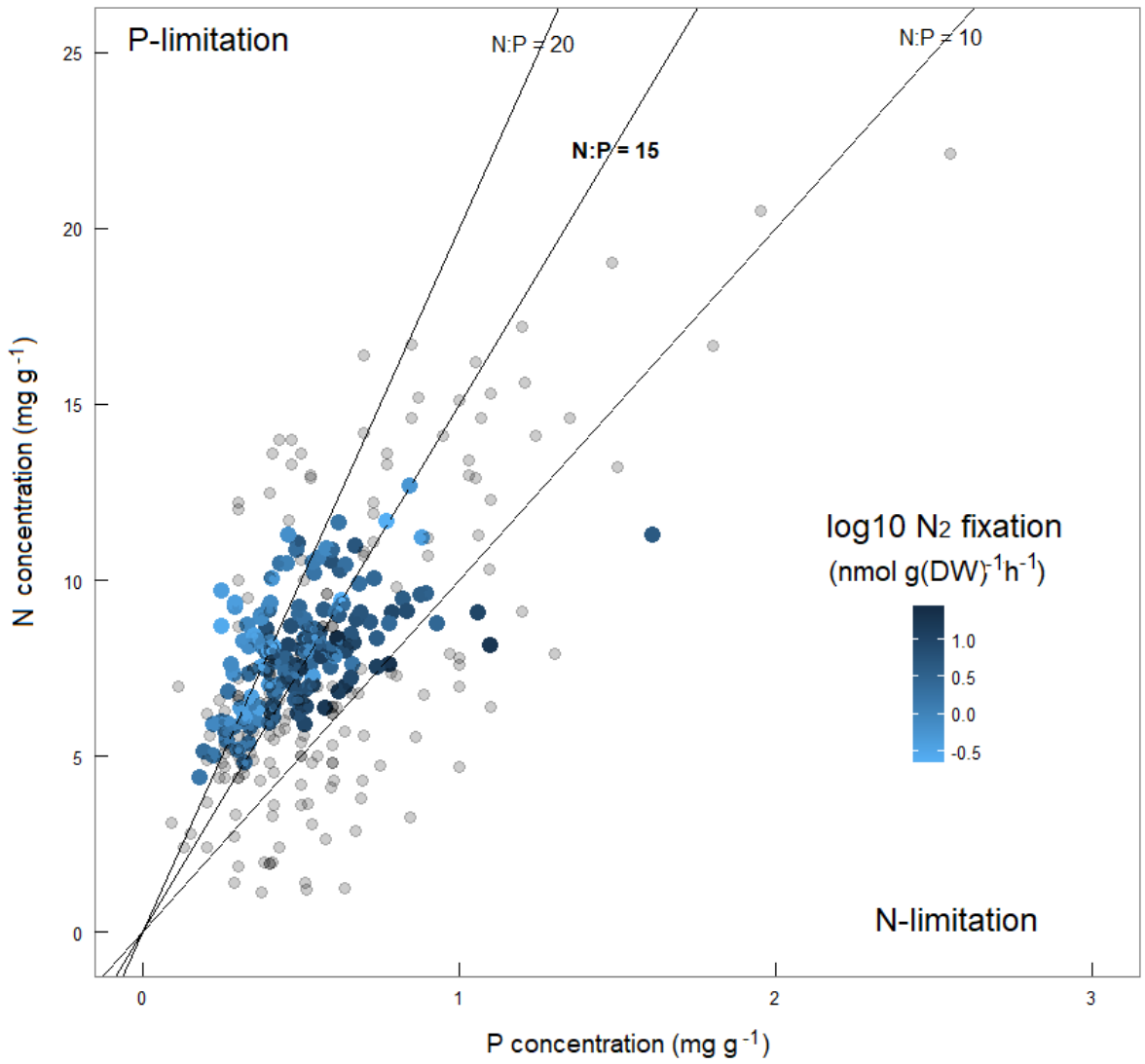

Fig. 6.7 The range of N and P concentration (mg g^{-1}) from this study (blue circles) superimposed on global distribution on N and P concentration in *Sphagnum* from ombrotrophic bogs (gray circles, for references see Fig.6.6). Blue gradient shows log_{10} scaled ARA N_2 fixation rates.

Table 6.1 Linear mixed effect model testing of relationships between N_2 fixation by ARA and a) N concentration, b) P concentration and c) N:P. Models structure is: N_2 fixation ~ X_{1-3} + SECTION + (1 + X_{1-3}|SITE), where X_1=N, X_2=P and X_3=N:P. Significant values are in **bold** ($p < 0.05$).

Continuous variable (Model name)	Fixed effects	Estimate	Std.Error	t-value	AICc
a) N_2 fixation (A0)	Intercept	0.5558	0.2930	1.897	283.5
	N	**-0.2608**	**0.115**	**-2.338**	
	Acutifolia	**-0.9354**	**0.1097**	**-8.526**	
b) N_2 fixation (B0)	Intercept	0.3344	0.2177	1.536	284.4
	P	**0.3013**	**0.1149**	**2.623**	
	Acutifolia	**-0.6526**	**0.1072**	**-6.090**	
c) N_2 fixation (C0)	Intercept	0.3808	0.1959	1.944	245.7
	N:P	**-0.4868**	**0.1433**	**-3.397**	
	Acutifolia	**-0.7898**	**0.1014**	**-7.792**	

Table 6.2 Best fit model showing the relationship between $^{15}N_2$ fixation and GPP and GM across the transect ($^{15}N_2$ fixation ~ GPP + GM + (1|SECTION/SITE). Significant values are in **bold** ($p < 0.05$).

Fixed effects	Estimate	Std.Error	t-value
Intercept	0.087	0.293	0.297
GPP	**0.216**	**0.065**	**3.340**
GM	**0.339**	**0.066**	**5.165**

Table 6.3 Results showing best fit model $^{15}N_2$ fixation ~ GPP + GM + (1|SITE) for a) Acutifolia and b) Cuspidata. For each model Akaike information criterion (AIC) and Bayesian information criterion (BIC) are presented. Significant values are in **bold** ($p < 0.05$).

a) Acutifolia, AIC = 136.6, BIC = 147.4, R^2 marginal = 0.18, R^2 conditional = 0.67, n = 65

Fixed effect	Estimate	Std.Error	t-value
Intercept	0.077	0.25	0.304
GPP	0.136	0.085	1.600
GM	**0.405**	**0.075**	**5.366**

b) Cuspidata, AIC = 118.6, BIC = 127.9, R^2 marginal = 0.20, R^2 conditional = 0.59, n = 48

Fixed effect	Estimate	Std.Error	t-value
Intercept	-0.008	0.239	-0.033
GPP	**0.443**	**0.142**	**3.127**
GM	0.225	0.154	1.459

Supplementary material

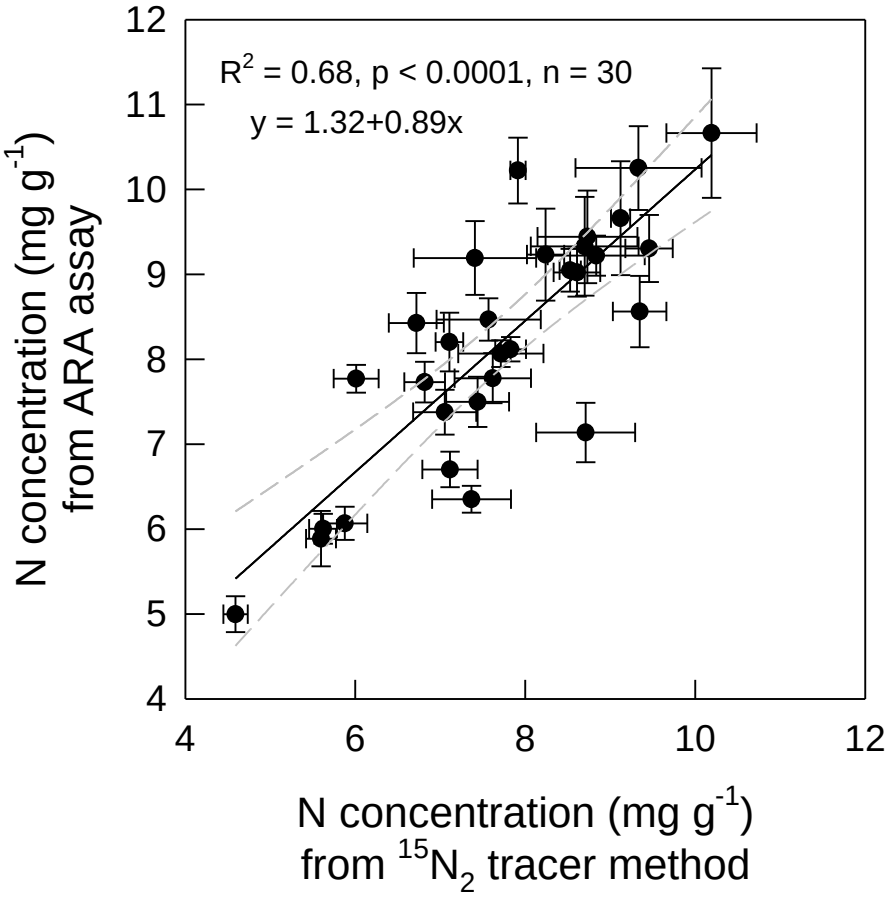

Fig.S6.1. The relationship between N concentration (mg g^{-1}) of *Sphagnum* samples obtained by acid digestion and by mass spectrometer. Each value is a mean of 5 subsamples used for ARA and 4 subsamples for $^{15}N_2$, with standard error bars. The dotted line is 95 % confidence interval.

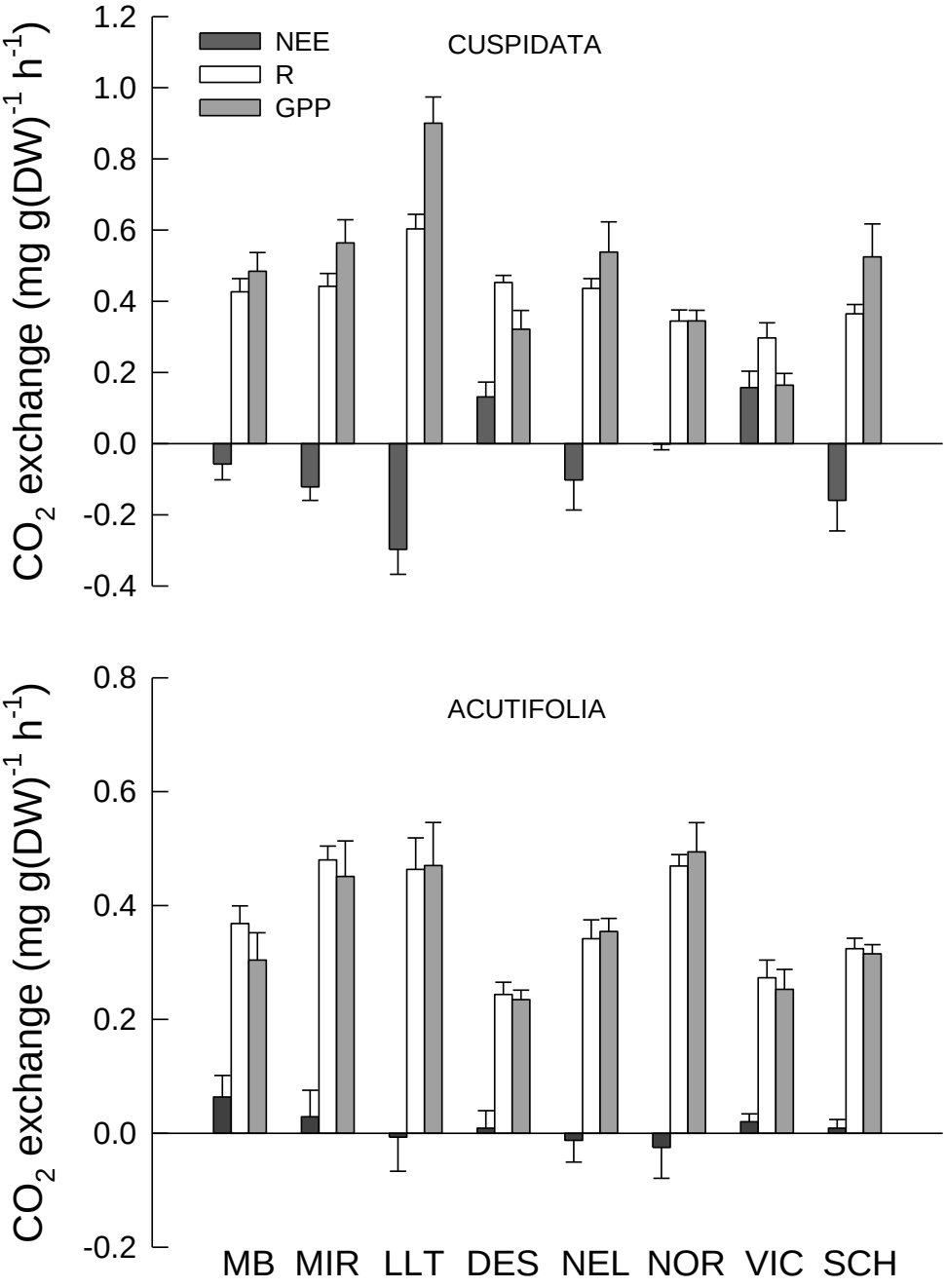

Fig.S6.2 The variability of NEE, R and GPP (mean ± SE) of *Sphagnum* sections (Cuspidata and Acutifolia) along the transect.

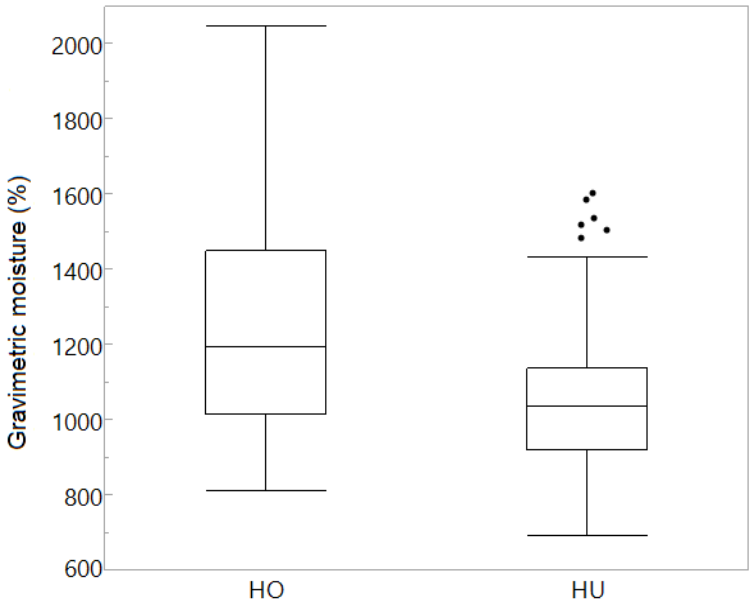

Fig.S6.3 Box and whisker plot showing GM (%) in hollows (HO; Cuspidata) and hummocks (HU; Acutifolia). Student t-test showed that HO > HU ($t(226.1) = -7.02$, $p < 0.0001$, $n = 257$). Horizontal line within the box represents median, while vertical lines represent 25th and 75th percentile, and dots over the vertical lines are outliers.

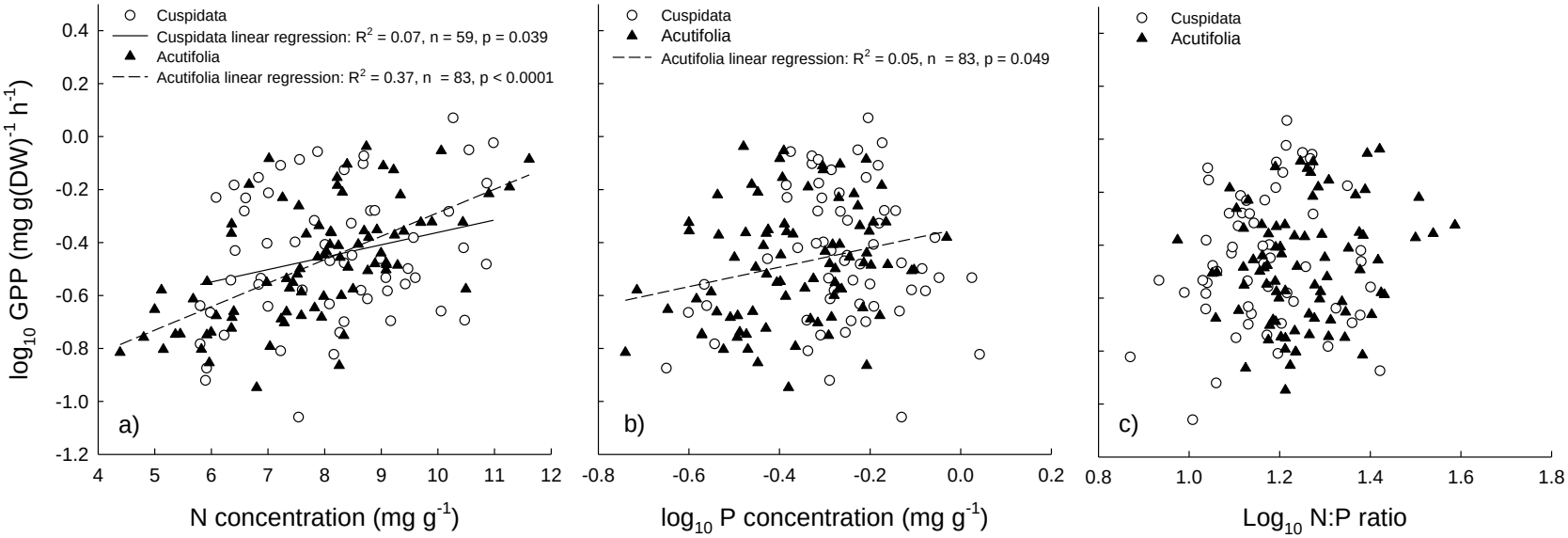

Fig.S6.4 The relationship between the GPP and a) N concentration, b) P concentration and c) N:P ratio in *Sphagnum* sections.

Table S6.1 Description of variables (mean ± standard deviation (SD), sample size (n)) used for data analyses.

Variable	Acutifolia			Cuspidata			Total		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
$^{15}N_2$ fixation	1.811	2.166	65	7.051	6.556	49	3.933	5.214	114
ARA	2.458	2.919	84	8.161	6.636	59	4.669	5.456	143
GPP	0.376	0.162	68	0.463	0.291	49	0.412	0.228	114
NEE	-0.002	0.109	68	-0.044	0.231	49	-0.019	0.171	114
R	0.374	0.114	68	0.415	0.133	49	0.391	0.123	114
GM	1010.2	169.8	68	1193.4	269.5	49	1086.9	234.4	114
N	7.855	1.552	84	8.161	1.511	59	7.981	1.538	143
P	0.466	0.194	84	0.567	0.178	59	0.508	0.193	143
N:P	18.41	5.484	84	15.488	4.521	59	17.205	5.292	143

CHAPTER 7

Conclusions and directions for future research

In addition to storing globally significant amounts of organic C (Yu *et al.*, 2010), northern peatlands store about 10% of terrestrial N, which has accumulated in peat since the last glaciation (Limpens *et al.*, 2006; Loisel *et al.*, 2014). In ombrotrophic peatlands, the primary N source is biological N_2 fixation. N_2 fixation is an important biogeochemical process in ombrotrophic bogs since N supply is required to support primary production. Thus, understanding the major factors driving N_2 fixation in bogs is essential to understand peatland responses to global change. In this thesis, I have identified environmental and biogeochemical factors (**in bold**, Fig.7.1) that drive the N_2 fixation process under natural climate variability and under increased nutrient deposition.

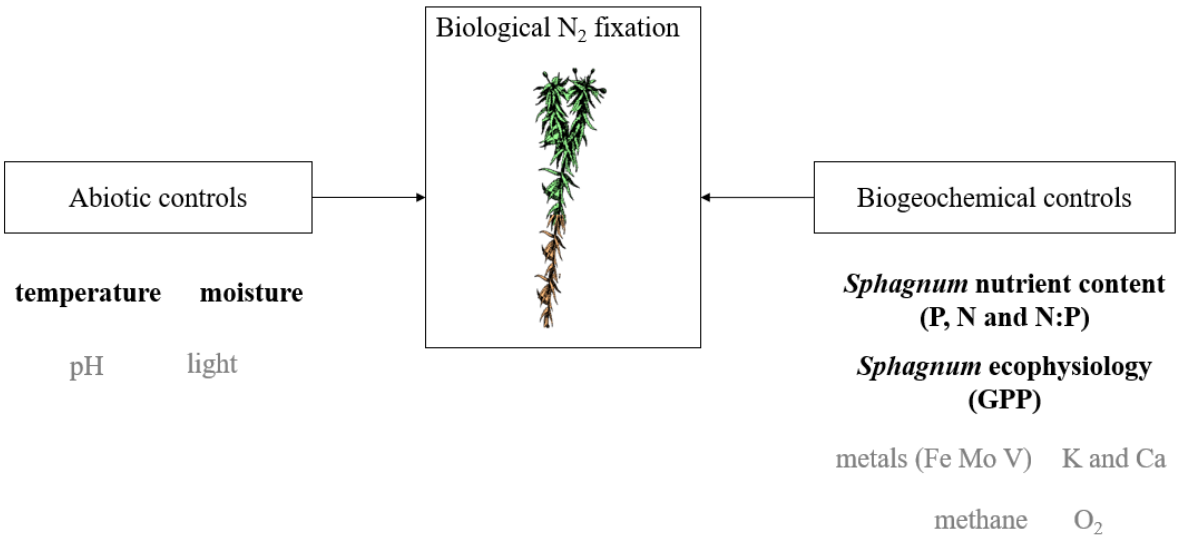

Fig. 7.1 Abiotic and biogeochemical factors that affect N_2 fixation in peatlands; **in bold** are the factors studied throughout this thesis.

7.1. Chapter synthesis and larger context

Chapter 3 is a comprehensive study of *in-situ* N_2 fixation rates in a representative cool-temperate ombrotrophic bog (Mer Bleue). The study investigates factors explaining the seasonal

and spatial variability of diazotrophic activity. The most important abiotic factors explaining seasonal variability of N_2 fixation were soil temperature and precipitation, while gravimetric water content in *Sphagnum* best explained spatial N_2 fixation variability. Chapter 3 demonstrates that easily measureable abiotic factors in peatlands can be used to predict a significant proportion of N_2 fixation variance. This study provides a framework for better estimation and prediction of N_2 fixation based on a limited set of *in-situ* measurements in bogs with hummock-hollow-pond features. In the future, using remote sensing and environment variables such as soil moisture, temperature and precipitation may help extrapolating N_2 fixation estimates in peatlands to larger geographical scales.

Chapter 3 is the first study that examines N_2 fixation rates at Mer Bleue and provides the first annual N_2 fixation estimate of 0.2 - 0.3 g fixed N m^{-2} y^{-1}. Combined with N deposition, which is about 0.8 - 1 g N m^{-2} y^{-1}, N_2 fixation rates can explain the annual N accumulation in live *Sphagnum* of 1.2 - 1.3 g N m^{-2} y^{-1}. At Mer Bleue, which has been exposed to moderate but long-term N deposition inputs (Turunen *et al*., 2004), about 20% of annual N inputs can be attributed to biological N_2 fixation. In contrast, in pristine peatlands with small N deposition inputs (< 0.1 g N m^{-2} y^{-1}, Vitt *et al.*, 2003), N_2 fixation is the primary source of N and can account for 50% or more (Hemond, 1983). Thus, increased atmospheric N deposition can alter the amount of N fixed *via* biological processes in ombrotrophic bogs (see Chapter 4).

Furthermore, Chapter 3 suggests that N acquired by *Sphagnum*-associated N_2 fixation remains in *Sphagnum* rather than being actively used by vascular plants. Instead, recent evidence of low $\delta^{15}N$ (Moore & Bubier 2009, in press) suggests that vascular plants depend more on re-mineralized N from peat and atmospheric N deposition than on N fixed by *Sphagnum*-associated diazotrophs. However, some of the N in peat may originate from free-living diazotrophs (Knorr

et al. 2015), which could help explain the large RERNA (~2.5 g N m^{-2} y^{-1}) deeper in the peat profile at Mer Bleue bog (Turunen *et al*., 2004 and Moore *et al.*, 2005). Studies focusing on N_2 fixation in peat could help understanding the diverse N sources and ultimately the N cycle in similar peatlands.

In Chapter 4, I demonstrated that long-term N additions in form of NH_4, NO_3 and NH_4NO_3 at Mer Bleue significantly decreased *Sphagnum*-associated N_2 fixation, while P addition increased N_2 fixation up to 5 times compared to controls. Incremental NH_4 additions resulted in a non-linear negative response of N_2 fixation, while NO_3 fertilization had a linear negative response. However, a negative effect on N_2 fixation was already observed at the lowest N additions of both species equivalent to 5 times the background N deposition. N_2 fixation rates in fertilized plots in a controlled laboratory setting with favorable conditions were as low as 0.01 g N m^{-2} y^{-1} . Compared to N_2 fixation estimates of 0.2-0.3 g N m^{-2} y^{-1} under ambient conditions at Mer Bleue (Chapter 3), such low rates have no ecological significance indicating that elevated N deposition can completely replace N_2 fixation as N input to ombrotrophic bogs. Chapter 4 also shows that both nutrient (N and P) additions significantly changed N and P concentrations in *Sphagnum*. N concentrations in *Sphagnum* significantly rose in response to all N species and amounts (1.6, 3.2 and 6.4 g N m^{-2} y^{-1}). N additions caused *Sphagnum*'s N:P ratio to rise to above 20 indicating strong P-limitation (Koerselman & Meuleman, 1996). In contrast, P additions significantly decreased N:P ratios in *Sphagnum* to 4 indicating strong N-limitation. Overall, I show that the N:P ratio rather than N and P individually represent the best explanatory variable for N_2 fixation. Recent studies found either inhibition (Kox *et al.*, 2016) or no effect (van den Elzen *et al.*, 2018) of N deposition on diazotrophic activity. P-limitation of the N_2 fixation process may explain these contrasting results. More similar case studies addressing these

questions could elucidate how *Sphagnum* as a host, and its nutrient status, limits the microbial N_2 fixation process especially in disturbed and polluted peatlands.

In Chapters 5 and 6, I tested the relationship between N_2 fixation and N:P ratio (established in Chapter 5), and GPP on a larger geographical scale across a transect of ombrotrophic bogs in eastern Canada. The transect encompasses bogs from the cool-temperate over the boreal to the subarctic region. The main aim of Chapter 5 was to constrain the natural variability of N, P concentrations and $\delta^{15}N$ in *Sphagnum* from hummocks and hollows along the transect. Both N and P concentrations declined from the south to the north, following a deposition gradient along the transect, while N:P ratio varied showing no clear pattern. $\delta^{15}N$ in *Sphagnum* linearly increased from -6 ‰ at the most southern site to around -1 ‰ at the most northern site and this trend was partially explained by a rising trend in water table and a decreasing trend in *Sphagnum* N concentrations. These results suggest that N_2 fixation becomes the primary source of N in less polluted and wetter bogs.

Chapter 6 contributes three major findings. First, I demonstrate that N_2 fixation estimates from ARA and $^{15}N_2$ tracer methods are linearly related under controlled environmental conditions across a wide geographical transect. The conversion ratio (R) between the two methods is 1.16, which is lower than the proposed theoretical value of 3-4 (Hardy *et al.*, 1968; Schwintzer & Tjepkema, 1994). The discrepancy indicates that the diazotrophic community involved in N_2 fixation is only partially captured by the ARA method. Limitations of ARA capturing diazotrophic activity of methane oxidizing communities have been previously reported (De Bont & Mulder, 1976; Larmola *et al.*, 2014). However, several other explanations may explain the lower R such as O_2 sensitivity of N_2 fixation in incubation chambers (Hardy *et al.*, 1973; Liengen, 1999) or the activation of alternative (V- and Fe-) nitrogenase enzymes in certain

microorganism due to micro-nutrient limitations (Smith *et al.*, 1987; Bellenger *et al.*, 2014). This thesis supports the claim that ARA should be used with caution when estimating N_2 fixation rates and calls for further investigations to better understand under which conditions these discrepancies in R occur.

Second, I show that a strong negative relationship exists between N_2 fixation and *Sphagnum* N:P ratios in bogs along the Eastern Canada transect. Compared to individual nutrient concentrations (N and P), N:P better explains variance in N_2 fixation rates. The relationship between N_2 fixation and N:P is stronger in *Sphagnum* section Cuspidata (found in hollows) than in Acutifolia (in hummocks). Similarly, larger N_2 fixation rates occur in hollows compared to hummocks.

Sphagnum N:P ratio in bogs has been widely used to establish the prevailing nutrient limitation: N limitation when N:P < 14, P limitation when N:P > 16 and NP co-limitation when 14<N:P<16 (Koerselman & Meuleman, 1996). Given that *Sphagnum* hosts diazotrophs, my results show that *Sphagnum* nutrient status may also determine microbial nutrient limitation and, thus, N_2 fixation activity. In ombrotrophic bogs, P inputs are limited and most bogs rely on P that has been acquired through weathering processes during their early formation (Walbridge & Navaratnam, 2006; Tipping *et al.*, 2014; Toberman *et al.*, 2015). Thus, P is strongly recycled within the peat column, but small P amounts are gradually lost over time (Wang *et al.*, 2015). Along with ongoing global change and =increased atmospheric N deposition, P-limitation may become more important than N-limitation in bogs, ultimately inhibiting the N_2 fixation process.

The third contribution of Chapter 6 is showing the link between *Sphagnum* GPP (photosynthesis) and the N_2 fixation process, which has never been studied before in peatlands. This study finds a positive relationship between $^{15}N_2$ fixation rates and GPP in *Sphagnum's*

section Cuspidata, suggesting that diazotrophy may be an important source of N for photosynthesis in hollows. This finding directly links the C and N cycle in ombrotrophic peatlands. In contrast, moisture was the only significant driver of N_2 fixation rates in hummock mosses (section Acutifolia) and no relationship with GPP was detected. Therefore, GPP in hummocks may rely on different sources of N than in hollows. Atmospheric N deposition and N re-mineralization from peat represent two possible N sources. This explanation is corroborated by results from Chapter 5 where $\delta^{15}N$ values in hummocks were found to be lower than in hollows, indicating that N sources other than N_2 fixation are more likely in hummocks. Furthermore, hummocks were generally more P-limited than hollows across the transect, suggesting different strategies of storing and utilizing nutrients (N and P) by *Sphagnum* and associated microbes between these two microforms. Chapter 6 shows that the C, N and P cycles are interconnected in ombrotrophic peatlands through diazotrophy, and these links should be further studied.

Finally, my thesis shows that several abiotic (moisture, soil temperature, and precipitation) and biogeochemical (N:P ratio in *Sphagnum* and GPP) factors affect the N_2 fixation process in ombrotrophic peatlands. However, a fraction of N_2 fixation variance remains unexplained after accounting for these factors. Future studies should particularly focus on factors such as O_2 and methane concentrations. The strong coupling between N_2 fixation and N:P ratio in *Sphagnum* in ombrotrophic peatlands represents an important finding especially since inputs of both nutrients have been undergoing large changes since the Industrial revolution.

7.2. Directions for future research

There are several knowledge gaps that need to be addressed to improve our understanding of biological N_2 fixation processes and their controls in peatlands. Here, I briefly discuss the most pertinent questions that arose from my thesis.

1) Identifying diazotrophs actively involved in the N_2 fixation process – While molecular studies have already identified a large diversity of diazotrophic communities by targeting a specific *nif* gene that encodes nitrogenase enzyme in peatlands (Zadorina *et al.*, 2009; Bragina *et al.*, 2012b), studies that focus on the functional expression of the *nif* gene in conjunction with biogeochemical rates would help determining which N_2 fixing microbes are active under specific environmental conditions. Furthermore, studies characterizing the functional relationship between *Sphagnum* and diazotrophs in peatlands are important to define if the type of interaction is either active (endosymbiotic), passive (non-symbiotic) or complex (involving several microbial groups; consortia). These studies may elucidate how nutrients (C, N and P) and energy are shared among mosses and microbes.
2) Improving methodology to measure N_2 fixation in peatlands – Due to its affordability and the ease of use, the ARA method (calibrated with $^{15}N_2$ tracer method) has been used widely to estimate N_2 fixation rates in peatlands. Recently, however, several reports suggested that ARA can underestimate N_2 fixation rates due to its inability to capture the activity of methane-oxidizing bacteria involved in diazotrophic activity (Larmola *et al*., 2014; Leppänen *et al.*, 2015; Warren *et al.*, 2017). Thus, the $^{15}N_2$ tracer method is likely to yield more accurate N_2 fixation measurements than the ARA method, especially in conditions where methane-oxidizing microbes may be metabolically active. However, the

$^{15}N_2$ tracer method is costly and still requires adaptations to make *in-situ* measurements possible. Thus, improving $^{15}N_2$ tracer methods and lowering its costs would be beneficial to better understand N_2 fixation processes and their controls in peatlands.

3) N_2 fixation not related to *Sphagnum* - Evidence from this thesis indicates that N acquired via biological N_2 fixation associated with *Sphagnum* is mainly incorporated in *Sphagnum*'s biomass, and that N_2 fixation at Mer Bleue is likely lowered by long-term moderate atmospheric N deposition. To achieve a better understanding of the fate of N acquired by N_2 fixation in ombrotrophic peatlands, studying an undisturbed peatland with potentially larger N_2 fixation rates would be essential. In nutrient-limited and undisturbed peatlands, the primary source of N is N_2 fixation due to low atmospheric N deposition. In ombrotrophic bogs, *Sphagnum* has adapted to retain and retranslocate N (Aldous, 2002a,b). Thus, it is unlikely that N acquired by associative N_2 fixation within *Sphagnum* is transferred towards vascular plants. Vascular plants therefore require other strategies to acquire N. One pathway would be through N mineralization of labile organic matter in peat. Alternatively, diazotrophy by free-living microbes and methane-oxidizers found deeper in the peat profile within the rooting zone of vascular plants may provide additional N in pristine environments (Knorr *et al.*, 2015). At Mer Bleue bog, the zone where free-living microbes and methane-oxidizers may be involved in additional N_2 fixation is located just above the water table. There, the largest methane-oxidation rates occur (Blodau *et al.*, 2007; Juutinen *et al.*, 2018). Exploring other potential N_2 fixation processes related to free-living diazotrophs deeper in the profile would therefore help constraining the ecosystem-level N budget.

4) Climate change impacts on N_2 fixation in peatlands – Increasing air temperatures and changing precipitation patterns will be most pronounced at high-latitudes (IPCC, 2014) where most northern peatlands are found. Given the importance of environmental controls (soil temperature, precipitation and moisture availability) on N_2 fixation in ombrotrophic peatlands, a better understanding of climate change impacts on environmental conditions and, thus, on N_2 fixation is necessary to predict changes to N cycles in peatlands. Ultimately, such changes may alter the storage of N and C in northern peatlands.

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