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Published in:
Science of the Total Environment

DOI:
[10.1016/j.scitotenv.2021.148676](https://doi.org/10.1016/j.scitotenv.2021.148676)

Publication date:
2021

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Alvarenga, D. O., & Rousk, K. (2021). Indirect effects of climate change inhibit N₂ fixation associated with the feathermoss *Hylocomium splendens* in subarctic tundra. *Science of the Total Environment*, 795, Article 148676. <https://doi.org/10.1016/j.scitotenv.2021.148676>



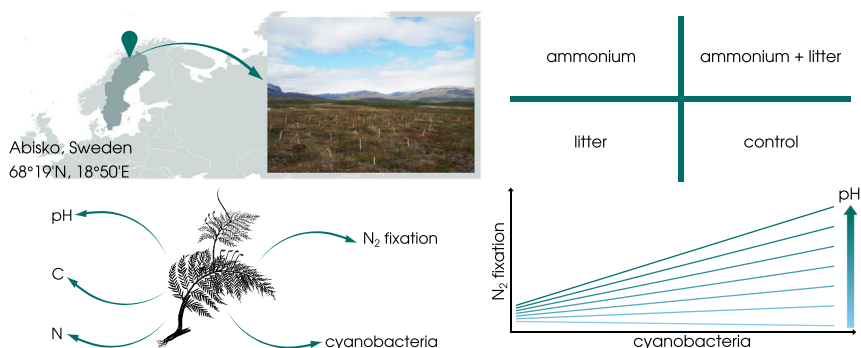
Short Communication

Indirect effects of climate change inhibit N₂ fixation associated with the feathermoss *Hylocomium splendens* in subarctic tundraDanillo O. Alvarenga^{a,b,*}, Kathrin Rousk^{a,b}^a Department of Biology, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen, Denmark^b Centre for Permafrost, University of Copenhagen, Øster Voldgade 10, DK-1350 Copenhagen, Denmark

HIGHLIGHTS

- Indirect effects of climate change also impact moss-cyanobacteria associations.
- Combined N and litter additions reduce cyanobacterial colonization and N₂ fixation.
- N additions acidify mosses, affecting cyanobacteria and N₂ fixation negatively.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 23 April 2021

Received in revised form 15 June 2021

Accepted 21 June 2021

Available online 1 July 2021

Editor: Elena PAOLETTI

Keywords:

Feathermoss

Cyanobacteria

Climate change

Nitrogen fixation

Symbiosis

Arctic tundra

ABSTRACT

Mosses can be responsible for up to 100% of net primary production in arctic and subarctic tundra, and their associations with diazotrophic cyanobacteria have an important role in increasing nitrogen (N) availability in these pristine ecosystems. Predictions about the consequences of climate change in subarctic environments point to increased N mineralization in soil and higher litter deposition due to warming. It is not clear yet how these indirect climate change effects impact moss-cyanobacteria associations and N₂ fixation. This work aimed to evaluate the effects of increased N and litter input on biological N₂ fixation rates associated with the feathermoss *Hylocomium splendens* from a tundra heath. *H. splendens* samples were collected near Abisko, northern Sweden, from a field experiment with annual additions of ammonium chloride and dried birch litter and the combination of both for three years. Samples were analyzed for N₂ fixation, cyanobacterial colonization, C and N content and pH. Despite the high N additions, no significant differences in moss N content were found. However, differences between treatments were observed in N₂ fixation rates, cyanobacterial colonization and pH, with the combined ammonium+litter treatment causing a significant reduction in the number of branch-colonizing cyanobacteria and N₂ fixation, and ammonium additions significantly lowering moss pH. A significant, positive relationship was found between N₂ fixation rates, moss colonization by cyanobacteria and pH levels, showing a clear drop in N₂ fixation rates at lower pH levels even if larger cyanobacterial populations were present. These results suggest that increased N availability and litter deposition resulting from climate change not only interferes with N₂ fixation directly, but also acidifies moss microhabitats and reduces the abundance of associated cyanobacteria, which could eventually impact the N cycle in the Subarctic.

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1. Introduction

While the nitrogen (N) fixing activities of most diazotrophic bacteria are restricted to anaerobic conditions, the cyanobacterial order Nostocales has evolved the exclusive capacity of differentiating vegetative cells into heterocytes, which allow them to fix atmospheric N under aerobic conditions (Kumar et al., 2010; Muro-Pastor and Maldener, 2019). As flexible and efficient diazotrophs, nostocalean cyanobacteria have acquired important roles in N biogeochemistry in oligotrophic environments such as arctic and subarctic tundra, where they are found either in biological soil crusts, lichens or associated with mosses (Stewart et al., 2011; Rousk et al., 2013a; Pushkareva et al., 2015; Rousk and Michelsen, 2017). Mosses can often be found as the dominant ground cover in arctic/subarctic tundra influencing ecosystem processes, promoting ecological stability and resilience and occasionally even taking up the sole responsibility for net primary production (Gornall et al., 2007; Turetsky et al., 2012). Since N is a limiting element for plants especially in high-latitude, low temperature environments (Du et al., 2020), moss associations with N₂-fixing cyanobacteria are important for the balance of both C and N in arctic and subarctic ecosystems (Solheim et al., 2004; Stewart et al., 2012; Rousk et al., 2017a).

However, N availability in these unpolluted, pristine ecosystems is likely to increase as a result of global change, including the rising atmospheric deposition of N oxides from fossil fuel combustion and runoff of reduced N from chemical fertilizers (Fowler et al., 2015; Zheng et al., 2020). Higher temperatures will also, albeit indirectly, lead to increased N availability in arctic and subarctic environments via increased N mineralization in soil and will enhance litter deposition from higher plant productivity (Borner et al., 2008; Chu and Grogan, 2010; Vowles and Björk, 2018). While small increases in N availability may stimulate N₂ fixation, larger and repeated additions of reactive N inhibit it instead (Rousk and Michelsen, 2016; Zheng et al., 2019). Nevertheless, the effects of global change impact moss-cyanobacteria associations in ways that are not fully understood yet. The impact of direct effects of climate change like increased temperatures on mosses and N₂ fixation have been evaluated in some works, which showed that, depending on moisture and season, warming could either increase or decrease moss-associated N₂ fixation rates (Sorensen et al., 2012; Lett and Michelsen, 2014; Rousk and Michelsen, 2017; Rousk et al., 2017b; Salemaa et al., 2019). On the other hand, it is still not clear yet how indirect effects affect moss-cyanobacteria associations and their N₂ fixation rates, in particular the persistence of cyanobacterial colonization of mosses when exposed to differences in environmental conditions caused by climate change.

Moss-associated N₂ fixation rates are higher in regions where N deposition is low (Leppänen et al., 2013). With increased N mineralization due to a warmer climate, higher N availability will likely inhibit N₂ fixation as previously shown (Rousk et al., 2013a). In addition, since both temperature and light intensity influences N₂ fixation activities of

moss-associated cyanobacteria (Gundale et al., 2012; Sorensen et al., 2012), increased litter input in the subarctic could reduce the light available to moss-associated cyanobacteria and therefore diminish their diazotrophic activities as well as their abundance. Litter could also hinder rain from reaching moss carpets and thereby reduce water availability, which is essential to sustain N₂ fixation, as well as release leachates that may either promote or inhibit N₂ fixation, depending on nutrient content (Rousk et al., 2014a, 2017b; Rousk and Michelsen, 2017). Different studies observed litter input stimulating (Sorensen and Michelsen, 2011; Rousk and Michelsen, 2017) or reducing (Jean et al., 2020) feathermoss-associated N₂ fixation rates.

This work aimed to evaluate indirect effects of climate change as increased N mineralization and litter deposition on moss-associated N₂ fixation rates from a tundra heath in northern Sweden. Our hypotheses were that a) ammonium additions would lead to a decrease in moss-associated N₂ fixation rates; b) blocking the light available for photosynthesis by litter would further reduce colonization by moss-associated nostocalean cyanobacteria and, consequently, N₂ fixation rates; and c) simultaneous ammonium and litter additions would lead to a combined, even larger decrease of cyanobacterial colonization and N₂ fixation activity. This was evaluated in samples of the feathermoss *Hylocomium splendens* from a field experiment near Abisko, northern Sweden, that were collected after four annual additions of ammonium and birch litter performed during three years.

2. Material and methods

2.1. Experimental design and sampling

To simulate indirect effects of climate change on subarctic tundra functions, a field experiment was established in an area near Abisko, in Swedish Lapland (68°19'N, 18°50'E), dominated by graminoids, forbs and shrubs (Fig. 1). Treatments were set up in June 2017 and consisted of 1 × 1 m plots that received yearly additions of either 5 L of ammonium solution (NH₄Cl 5 g N·m⁻²); dried birch (*Betula pubescens*) litter (90 g·m⁻², 37.5 C/N ratio) with 5 L of water; combined ammonium solution and birch litter; or 5 L of water for controls. All solutions were poured with a watering can on top of the vegetation, and on top of the added litter layer in the birch litter treatments. After the additions, another 5 L of water were poured onto each plot to avoid any build up on the surface of the vegetation and to further ensure that all solutions reached the soil instead. All additions used local water from a stream near the site. These additions are repeated every June, just at the beginning of the growing season. The ammonium additions (5 g N·m⁻²·yr⁻¹, or 50 kg N·ha⁻¹·yr⁻¹) are higher than the maximum background N deposition rates in the area (ca. 2 kg N·ha⁻¹) (Goth et al., 2019), but correspond to an amount of inorganic N expected to be released in a moderate projection of less than 3 °C warming in arctic tundra in 20 years (Mack et al., 2004).

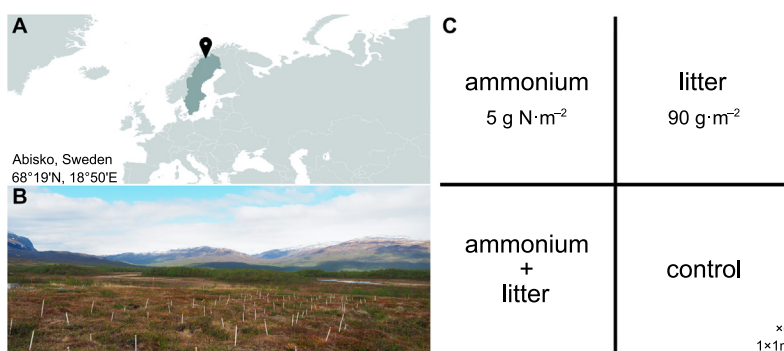


Fig. 1. Geographic location (A), general overview of the field (B) and treatments (C) of the experimental plots set up in Abisko, northern Sweden. Six replicates per treatment were used in the experiment.

Six replicates of each treatment were adopted with plots organized in randomized blocks. Samples of the feathermoss *Hylocomium splendens* Schimper were harvested from each of the replicate field plots in June 2020 one day after the annual addition and used in subsequent analyses. Hence, the plots received the treatments four times before sample collection. For each treatment, whole moss shoots (including the green, alive parts as well as the brown, senescent parts, ca. 2–5 cm long) were taken from at least ten different spots in each plot and composited to a single sample per field treatment ($n = 6$). Samples were collected by hand, placed in transparent plastic bags and kept under natural light and ambient temperature (5–13 °C on average in June). The samples were transported in the dark as cool as possible to Copenhagen, Denmark within 4 days of sampling. The field treatments caused no changes in the composition and productivity of plant communities in the experimental plots in comparison to controls, and thus did not introduce additional litter or shading into the experiment. More details about the field characteristics and plant composition can be found in Hicks et al. (2021).

2.2. Acetylene reduction assays

Acetylene reduction assays (ARA) were used to assess the N_2 fixation rates of the moss samples. For this, ten moss shoots from each field replicate were initially soaked in double-distilled water for 10 min. After rehydration, the shoots were transferred to 20 mL vials, which were sealed with rubber caps. A volume corresponding to 10% of the head space of each vial (i.e. 2 mL) was replaced with 2 mL of acetylene gas. The vials were then incubated for 24 h in growth chamber with 14 h of light at $125 \pm 5 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and 10 h of darkness at 10 ± 1 °C, close to the daily average temperature for June in Abisko. Acetylene was added to vials without samples to calculate residual ethylene in the acetylene gas used in the assay. The amount of acetylene reduced to ethylene in the vials, which was used as a proxy for the N_2 fixation rates in the samples, was quantified with an Agilent 8890 GC System gas chromatograph using the J&W CarboBOND column (Agilent, Santa Clara, USA). The volume of ethylene produced in the assay was calculated by comparisons with a 300 ppm ethylene standard curve.

2.3. Branch colonization assessment

The effects of each treatment on cyanobacterial colonization percentages of the sampled mosses were estimated by direct

observation of the same samples used in the ARA under fluorescence microscopy. Five moss shoots from each vial were placed on glass slides and ten random branches in each shoot were checked for red-glowing filaments that could be morphologically identified in the diazotrophic cyanobacterial order Nostocales (Komárek et al., 2014) under the green excitation filter of the Olympus BX61 fluorescence microscope (Olympus, Tokyo, Japan). The presence of any number of nostocalean filaments on a branch was considered a successful colonization of that branch.

2.4. Determination of moss pH and total C and N content

The five moss shoots that were used in the cyanobacterial colonization assessments were transferred back with the five moss shoots that remained in the 20 mL vials used in the ARA, to which 10 mL of double-distilled water were added. After 30 min of constant shaking at a moderate speed at room temperature, the pH in the liquids was measured with a pH meter. Next, the moss shoots were dried at 70 °C for 24 h and cut with sterile scissors until they became fine powders. Total C and N content in the samples was determined by the combustion method in 3–5 mg aliquots analyzed with the Euro EA elemental analyzer (EuroVector, Pavia, Italy) using orchard leaves (Leco, St. Joseph, USA) as standard.

2.5. Statistical analyses

To test whether ammonium, litter or the combination of these treatments affected N_2 fixation rates, cyanobacterial colonization, moss pH and elemental content (C, N) in the mosses, the data were analyzed by one-way ANOVAs followed by the Tukey's honest significant distance post-hoc test. As N_2 fixation, cyanobacterial colonization and pH were statistically confirmed to be affected by the treatments, they were further analyzed with two-way ANOVAs, which evaluated how these factors were affected by the interaction between treatments and factors. Linear regressions and Pearson correlation analyses were also performed to test for relationships between those three factors, and a multiple linear regression analyzed the interaction between cyanobacterial colonization and pH with N_2 fixation as the response variable. The analyses were performed using R 4.0.2 (<https://www.R-project.org/>) with the packages multcomp 1.4–13 (Hothorn et al., 2008), ggiraph (<https://github.com/davidgohe/ggiraph/>), ggiraphExtra (<https://github.com/cardiomoon/ggiraphExtra>) and ggplot2 3.3.2 (Wickham, 2016).

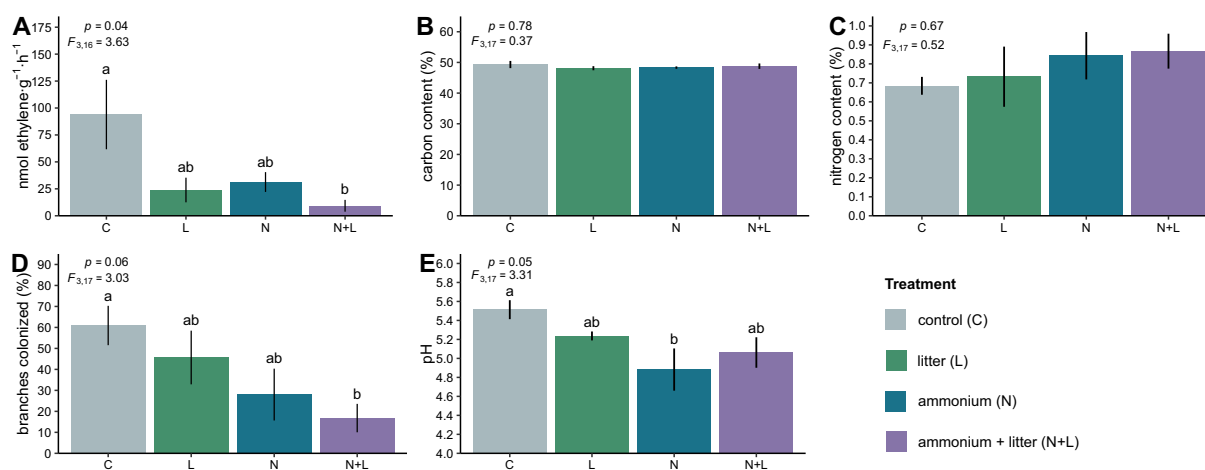


Fig. 2. Averages and standard errors for acetylene reduction (A), C and N content (B–C), branch colonization by N_2 -fixing cyanobacteria (D) and pH (E) of *H. splendens* samples from plots subjected to annual ammonium and/or birch litter additions in subarctic tundra, close to Abisko, northern Sweden. $n = 6$. Different lower case letters indicate significant differences between treatments according to Tukey's HSD test.

3. Results

Moss samples from control treatments had the highest acetylene reduction average, with 94 ± 32 ($\pm$ SE) $\text{nmol ethylene} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$, while the other treatments presented much lower average rates, ranging from 9 ± 5 to 31 ± 9 $\text{nmol ethylene} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ (Fig. 2A). Significant differences were observed in N_2 fixation rates between controls and the combined ammonium and birch litter additions (one-way ANOVA, $p = 0.04$, $F_{3,16} = 3.63$), with the former presenting the highest values, the latter the lowest values, and the treatments that received individual birch litter and ammonium additions in between. On the other hand, despite the high N additions no significant differences were found in C and N content among mosses subjected to the treatments, although slightly higher N content averages were indeed observed in the treatments that received ammonium additions, which ranged from 0.84 ± 0.12 to $0.86 \pm 0.09\%$, compared to $0.68 \pm 0.05\%$ in controls (Fig. 2B and C).

Differences between treatments were also observed in the percentages of *H. splendens* branches colonized by N_2 -fixing cyanobacteria (one-way ANOVA, $p = 0.06$, $F_{3,17} = 3.03$) and the pH resulting from different treatments (one-way ANOVA, $p = 0.05$, $F_{3,17} = 3.31$). The combined ammonium and birch litter treatment caused a significant reduction in the number of cyanobacteria colonizing the feathermoss branches (Fig. 2D), with percentages as low as 6% in samples from the combined ammonium and birch litter treatment against up to 92% branch colonization in controls. Ammonium additions significantly lowered the pH of the samples from an average of 5.5 ± 0.1 in the controls to 4.9 ± 0.2 and 5.0 ± 0.2 in the ammonium and the joint ammonium and litter treatments, respectively (Fig. 2E).

Cyanobacterial colonization of moss branches was significantly affected by the interaction between treatment and pH (two-way ANOVA, $p = 0.03$, $F_{3,13} = 4.05$), while the interaction between treatment and pH also affected acetylene reduction rates (two-way ANOVA, $p = 0.02$, $F_{3,12} = 5.22$). Linear regression analyses of the different factors showed a significant, positive relationship between percentages of moss branch colonization by cyanobacteria and pH ($p = 0.02$, $r^2 = 0.21$), branch colonization and acetylene reduction ($p = 0.03$, $r^2 = 0.20$) and pH and acetylene reduction ($p = 0.01$, $r^2 = 0.27$), of which 20–27% could be explained by the models according to the coefficients of determination obtained (Fig. 3). These relationships were also observed with Pearson correlation tests, which pointed to medium association strengths corresponding, respectively, to 50%, 48% and 55% positive correlation percentages between these relationships. Further, a multiple linear regression analyzing the interaction of cyanobacterial colonization and pH with acetylene reduction as the response variable indicated a statistically significant, positive relationship between these three factors ($p = 0.03$, $r^2 = 0.30$) (Fig. 4).

4. Discussion

In this work, we found a strong, positive link between N_2 fixation, cyanobacterial abundance and pH in *H. splendens*-associated communities from subarctic tundra responding to increased N availability and plant litter input. Since samples were collected 1 day after the last addition, this was the result of both immediate outcomes, observed one day after the last ammonium and birch litter additions, and longer term, chronic effects of ammonium and birch litter additions accumulated over the course of three years. Our results suggest that ammonium additions in subarctic tundra reduces cyanobacterial abundance and N_2 fixation rates while acidifying moss microhabitats, an effect that is slightly buffered by birch litter, but at the cost of a further decrease in cyanobacterial colonization (Fig. 2). In other words, the more acidic, litter-filled and/or N-rich moss habitats get, the fewer nostocalean cyanobacteria are likely to be found colonizing mosses and fixing N_2 (Fig. 3).

The rather high concentrations of ammonium added to the plots in this work did not lead to higher N content in the mosses, suggesting

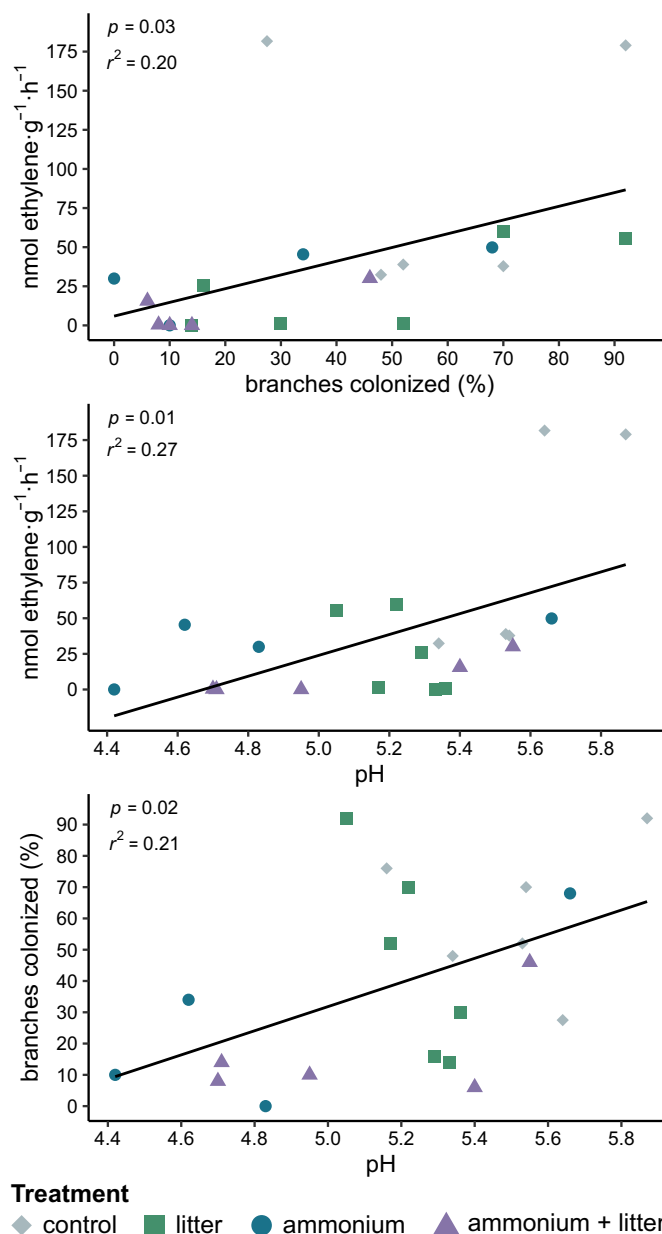


Fig. 3. Linear regression analyses of the relationships between cyanobacterial branch colonization, acetylene reduction rates and pH of *H. splendens* samples from northern Sweden in the different field treatments. $n = 6$.

that the ammonium additions were successfully washed through the moss carpet onto the soil. Here, it was possibly absorbed either by roots, microbial communities or other inhabitants of soil, or even lost through leaching. In the treatments receiving the combined ammonium and birch litter additions, where the ammonium solution was added on top of the litter, birch litter did not prevent the ammonium from reaching the soil, but it did increase the time necessary for the solution to percolate. Consequently, N may have stayed longer on the surface allowing more time for the ammonium solutions to be absorbed by the mosses and their associated bacteria, which may have also contributed to the observed additional reduction in N_2 fixation rates in comparison with the other treatments, especially compared to the ammonium-only treatments. This suggests that N mineralization in the litter may also affect moss-associated N_2 fixation. It is also possible that leachates containing dissolved organic N from litter, which have been previously shown to inhibit N_2 fixation (Salemaa et al., 2019), acted together with

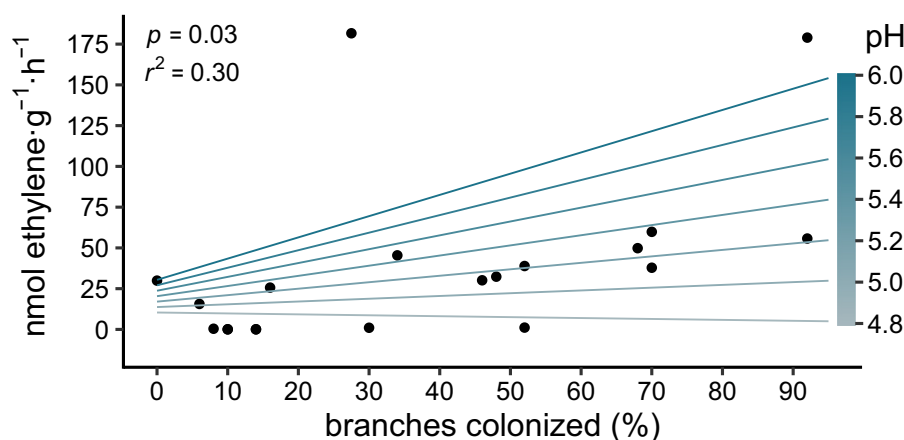


Fig. 4. Multiple linear regression analysis of relationship between *H. splendens* branches colonized by cyanobacteria, acetylene reduction and pH receiving annual ammonium and/or birch litter additions in a field experiment near Abisko, northern Sweden. $n = 6$.

ammonium additions and changes in light and moisture to produce the observed result.

Although mosses have been previously shown to uptake N from soil, their lack of vascular systems mean that soil is a limited source of N for these plants in comparison with atmospheric deposition (Ayres et al., 2006; Rousk et al., 2013b). Hence, the amount of N taken up from soil was likely negligible and did not affect moss N content, even though the ammonium additions led to slightly higher N availability in the soils (Hicks et al., 2021). However, the ammonium additions did change the pH of the moss, which affected associated cyanobacteria. Even though N did not accumulate in moss tissues, cyanobacterial activity was nevertheless still affected since the ammonium additions did lead to lower N₂ fixation rates and cyanobacterial colonization when combined with birch litter, partially confirming our first hypothesis. Lower N₂ fixation rates in nostoclean cyanobacteria are a known consequence of higher ammonium availability, triggering a negative feedback for the differentiation of vegetative cells into heterocytes (Flores et al., 2019). This can also be observed in moss-associated communities under both field and laboratory conditions, which can, however, recover their previous rates once N becomes a limiting factor again (Rousk et al., 2014b; Rousk and Michelsen, 2016).

Lower pH levels can also hinder cyanobacterial N₂ fixation due to the decreasing efficiency of nitrogenases under acidic conditions (Shi et al., 2012). Negative effects of low pH on cyanobacterial N₂ fixation rates have also been observed in subantarctic moss-associated communities (Smith, 1984). Several works have observed decreasing growth rates in *Nostoc* spp. cultures in response to increasingly acidic conditions (Allison et al., 1937; Kar and Singh, 1978; Giraldez-Ruiz et al., 1997; Katoh et al., 2003; Garby et al., 2017; Kannaujiya et al., 2020), which consequently further reduce N₂ fixation rates. Even acid-tolerant nostoclean cyanobacteria were shown to go through a reduction in biomass under lower pH levels (Gopalaswamy et al., 2007). Additionally, low pH was shown to inhibit the formation of hormogonia, which have a crucial role in the host colonization process, in plant-symbiotic *Nostoc* strains (Rasmussen et al., 1994).

Thus, it is difficult to ascertain whether ammonium had a direct effect on N₂ fixation by increasing N availability or an indirect effect by acidifying moss habitats. The observed reduction in pH probably reflects a longer term effect of N additions over the course of 3 years, while the direct inhibition of N₂ fixation caused by the N additions is mostly a transient, short term effect. Therefore, cyanobacterial N₂ fixation rates in arctic tundra could possibly be reduced in a future climate due to the combination of both the direct (inhibition of heterocyte differentiation and decreased nitrogenase efficiency) and indirect (decreased cyanobacterial abundance) effects of increased N availability. These effects can be exacerbated by increases in birch litter deposition, as the

combination of ammonium and birch litter additions may cause a significant reduction in N₂ fixation rates and cyanobacterial colonization, producing additive effects.

Decreased N₂-fixing cyanobacterial abundances suggested that, in a partial confirmation of our second hypothesis, they either leave the moss phyllosphere or their growth is limited as long-term effects of ammonium and birch litter additions, possibly due to changes in light availability, pH and/or N availability. This is the first time that a decline in cyanobacterial colonization in a moss as a result of increased N availability is shown. Decreases in the abundance of N₂-fixing cyanobacteria, however, do not necessarily elicit similar effects on the other members of moss microbiomes. Other N₂-fixing bacteria are also associated with mosses in northern environments (Holland-Moritz et al., 2021) and could potentially occupy the niche left by cyanobacteria. Nevertheless, with the exception of a few genera, most heterotrophic bacteria cannot fix N₂ under aerobic conditions (Zhang et al., 2020), thus making it difficult for most of the other N₂-fixing bacteria to occupy this niche. With the lack of N limitations making N₂-fixation by moss-associated communities redundant, an increased intra-phylum competition could possibly lead non-diazotrophic cyanobacteria to occupy this niche in a community shift that supplants N₂-fixing cyanobacteria in their favor provided that they still receive enough light.

Ammonium additions have more pronounced effects on subarctic soils than birch litter additions, causing a microbial community shift (Hicks et al., 2021). Nevertheless, litter from different plant species may influence N₂ fixation by moss-associated cyanobacteria in different ways according to their biochemical composition. Birch litter was previously shown to stimulate moss-associated cyanobacterial N₂ fixation rates and willow litter to inhibit them, effects that were attributed to a potentially higher P content in birch litter and to a higher N content in willow leachates (Sorensen and Michelsen, 2011; Rousk and Michelsen, 2017). On the other hand, paper birch litter was shown to inhibit N₂ fixation rates associated with *H. splendens*, possibly resulting from decreasing light availability and allelopathic phenols (Jean et al., 2020). In the present work, birch litter only caused significant inhibition of N₂ fixation when in combination with ammonium, possibly due to their joint effects on light availability and pH. Different methods, field conditions, chemical compositions and/or bacterial communities could be behind some of these contrasting results. In agreement with our third hypothesis, the interactions between N availability and litter seem to counteract the possible positive effects of phosphate-rich birch litter on the abundance of moss-associated cyanobacteria, despite causing a less dramatic impact on N₂ fixation.

In the last decades, the biological responses to ocean acidification have been the focus of an increasing number of works (Riebesell and Gattuso, 2015; Hong et al., 2017), but the acidification of terrestrial

environments, and in the subarctic tundra in particular, have received less attention. Similarly, most studies on the effects of climate change on cyanobacteria have focused on aquatic environments, which suggested a stimulation of their growth with warmer temperatures (Carey et al., 2012; Huisman et al., 2019), while much less is known about the effects of climate change on terrestrial and symbiotic cyanobacteria at this moment. Since there are substantial differences between the ecology of aquatic and terrestrial ecosystems and between free-living and symbiotic lifestyles, it is important to cover distinct models to have a broader understanding of the different biological impacts of climate change.

5. Conclusions

In addition to the direct impact of increasing temperatures shown in previous works, indirect effects of climate change in the subarctic will also cause short- and long-term, negative effects on moss-cyanobacteria associations. Increased N mineralization will likely result both in the redundancy of N₂ fixation by cyanobacteria and in the acidification of moss microhabitats. On the other hand, while birch litter can stimulate cyanobacterial N₂ fixation, it will in combination with lower pH levels result in an environment that is more hostile to cyanobacteria. In the long term, these effects could ultimately cause a decrease in the abundance of moss-associated cyanobacteria, which would eventually impact the N cycle in the subarctic via lower N₂ fixation rates.

CRediT authorship contribution statement

Danillo O. Alvarenga: Investigation, Formal analysis, Visualization, Software, Data curation, Writing – original draft. **Kathrin Rousk:** Conceptualization, Methodology, Investigation, Validation, Resources, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

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

Acknowledgements

We thank Prof. Johannes Rousk for setting up the field experiment and kindly providing the moss samples used in this work. We are grateful to Gosha Sylvester for helping with analyses of total carbon and nitrogen. We would also like to thank the anonymous reviewers for providing valuable criticism and suggestions that helped to improve and clarify this manuscript.

Funding

This work was supported by the Independent Research Fund Denmark (IRFD), Sapere Aude Grant (id: 7027-00011B).

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