Moss-associated nitrogen fixation across scales: from shoot to landscape

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Abstract

Biological nitrogen fixation (BNF) performed by moss-associated colonizing cyanobacteria is among the main sources of new nitrogen (N) inputs in pristine subarctic ecosystems. Moisture is the primary driver of BNF in cyanobacteria associated with mosses. Yet, knowledge of BNF and its potential drivers on smaller spatial scales is lacking. To address these knowledge gaps, we measured BNF along vertical layers (segments) representing different depths and age along moss shoots of two dominant species, *Pleurozium schreberi* and *Hylocomium splendens* over 6 weeks. Mosses were collected along a steep precipitation gradient in subarctic Northern Sweden, and factors known to affect BNF like contents of iron (Fe), phosphorus (P), nitrogen (N) and molybdenum (Mo), as well as pH were analysed. BNF capacity varied significantly and profoundly along the moss shoots and the precipitation gradient, and was highest in *H. splendens* and in mosses collected at the high end of the precipitation gradient. Contents of Fe, Mo and N generally increased with segment depth while P concentrations decreased and pH was stable across segments. While differences in BNF along the precipitation gradient were clear and largely explained by the importance of moisture for moss-associated BNF, nutrient content also seemed to affect BNF in mosses, although less pronounced. *P. schreberi* showed highest BNF in the middle segment, while BNF in *H. splendens* was highest in the lowest segments. Differences in cyanobacterial communities and different nutrient requirements may explain species specific BNF responses. This should be considered when aiming to upscale BNF to ecosystem level.

Introduction

Biological nitrogen fixation (BNF) is one of the main pathways for new N to enter pristine terrestrial ecosystems (Chapin, Matson, and Vitousek 2011). Mosses are widespread in the subarctic and other high latitude, nutrient limited terrestrial ecosystems, where they can form dense carpets with groundcover up to more than 70% (DeLuca et al. 2002; Gundale et al. 2012); Rousk, Sorensen, and Michelsen 2017). Feather mosses like *Pleurozium schreberi* and *Hylocomium splendens*, both common in these environments, are colonized by cyanobacteria capable of performing BNF (Rousk et al. 2013). BNF by cyanobacteria associated with these mosses is an important contributor of 'new' N to these systems (DeLuca et al. 2002) and can account for 1–3 kg N ha^{-1} $year^{-1}$ (Rousk and Michelsen 2017), which equals about 50% of total N input in these ecosystems (Goth et al. 2019). Yet, BNF by moss-associated cyanobacteria is greatly affected by abiotic factors and is promoted by moisture (Rousk, Pedersen, et al. 2017; Rousk, Jones, and DeLuca 2014; Rousk, Sorensen, and Michelsen 2018) as well as temperature (Rousk, Degboe, et al. 2017; Rousk, Sorensen, and Michelsen 2018) and light up to a certain point (Gundale et al. 2012) and is inhibited by increased deposition of N (Gundale, DeLuca, and Nordin 2011; Rousk et al. 2013). Other factors like molybdenum (Mo) and phosphorus (P) availability (Rousk, Degboe, et al. 2017) and pH (Smith 1984) can regulate BNF. Further, in tropical forests, iron (Fe) has been shown to affect BNF capacity (Winbourne, Brewer, and Houlton 2017) due to its importance for the nitrogenase enzyme that reduces N_2. Hence, BNF may be significantly affected by biogeochemical factors and climate change, and additions to our understanding of the N-cycle in high latitude ecosystems is key in

order to improve predictions for how these will respond to future changes in precipitation, temperature and nutrient availability.

These factors have previously been assessed at the level of entire moss carpets or moss-shoots, but few studies report systematically on the effects at a smaller scale, at different depths, and with that, age of moss shoots, although moisture, temperature and nutrient content are likely to differ along moss shoots. For instance, BNF has been found to be higher in the green, upper 4 cm (younger) than in the brownish (older) parts of moss shoots from *H. splendens* and *P. schreberi* likely due to effects of light (Leppänen et at. 2012). Similarly, Smith (1984) found the highest BNF in the moss *Brachythecium subplicatum* in the top of the moss shoot (1–3 cm from apex), corresponding to the highest density of colonizing cyanobacteria. Yet, another study (Gavazov et al. 2010) cut the top 3 cm of *H. splendens* into 1 cm segments and found a pattern of highest BNF in the middle segment, and lower BNF in the top and bottom segments. Thus, the highest activity seems to be found in the top 3 cm of moss shoots, but we lack an explanation for this pattern as well as knowledge if this pattern fits across varying species and locations.

Given that moisture is one of the most important drivers of moss-associated BNF (Rousk et al. 2018), it may also explain the vertical distribution of BNF along the moss shoots. Dry mosses are dormant and support no BNF activity as the colonizing cyanobacteria are only active when moist, and highest BNF rates are found in sufficiently moist mosses (Rousk et al. 2015; Rousk, Jones, and DeLuca 2014). Mosses cannot control water loss, as they have no vascular tissue, making them entirely dependent on moisture from the environment. The upper parts of moss carpets tend to dry out first, thus lowering evaporative water losses and preserving moisture in the lower parts which could maintain BNF activity longer. Fluctuations of drying and rewetting have a negative effect on BNF in feather mosses (Rousk, Jones, and DeLuca 2014) and promote high nutrient leaching and release from moss carpets (Read 1991; Wilson and Coxson 1999). We expect these variations along the moss shoots to affect BNF capacity, yet exact knowledge of where, on a vertical scale, BNF takes place in moss carpets, at what rates and which factors that control BNF is still scarce and knowledge of species-specific differences is limited. BNF may differ significantly within a few cm along a moss shoot, and assessing BNF at shoot level or in entire moss carpets may not correctly reflect BNF, if most of the process is happening in distinct layers and not evenly throughout the whole shoot or carpet.

The aims of this study were to assess (1) how moss-associated BNF varies along a steep precipitation gradient (from 300 to 1050 mm mean annual precipitation); (2) where along the moss shoots of the two common feather mosses, *P. schreberi* and *H. splendens* BNF by cyanobacteria takes place under contrasting precipitation regimes, and (3) to investigate which factors (Fe, P, N and Mo contents as well as pH) explain the vertical distribution of BNF along moss shoots from dry to wet habitats. We hypothesized that (H1) moss-associated BNF is highest at the site that receives the highest precipitation, (H2) highest BNF takes place in the middle segments of the moss shoots given that the balance between moisture and other factors is optimal here, and (H3) that Fe, Mo, P is positively linked to BNF, while N and low pH is negatively related to BNF.

Materials And Methods

Sampling sites, sample collection and sample handling

Moss samples were collected in October 2016 from three locations along a steep precipitation gradient stretching roughly 40 km in subarctic Northern Sweden, near Abisko. The mean annual air temperature in the Abisko area is 0.2°C with the warmest and coldest months July and February, average temperatures 11.9 and − 10.0°C, respectively (30-year mean 1986–2015, Abisko Scientific Research Station 2016). Collection sites were chosen by selecting sites with a similar vegetation type, a birch forest with a moss-dominated understory. Moss carpets (18x18cm) were taken by hand and placed in 8L plastic boxes. Six replicate samples were taken from each of the three sites with at least 5m distance between them. The site with the lowest mean annual precipitation (MAP) is close to Abisko Research Station, at 422 meters above sea level (masl) and 300 mm MAP (68°34'N, 18°82'E). The medium precipitation site, Låktatjåkka is at 487 masl and receives almost three times the precipitation of the Abisko site, 850 mm MAP and is ca. 30 km west of Abisko (68°42'N,18°32'E). At this site, only samples from *P. schreberi* could be obtained. The site with the highest MAP, Katterjåkk, at 473 masl receives 1100 mm MAP and is ca. 36 km west of Abisko (68°42'N, 18°18'E).

After transportation to Copenhagen, the samples were kept in growth chambers with 6 hours light (300 nmol m^{-2} s^{-1}) each day at a constant temperature of 0°C, reflecting the conditions at the time of sampling in the field. Samples were watered regularly to avoid desiccation and only with double distilled water (DD-H_2O) to avoid nutrient contamination. The field collection time was late fall just before onset of winter, outside the vascular plant growing season of the area (Lett and Michelsen 2014), and storage conditions were chosen to maintain the samples in their seasonal state until the start of the experiment.

Nitrogen fixation

As a measure of BNF, we used the acetylene reduction assay (ARA) (Hardy et al. 1968; Rousk et al. 2017). To provide material for ARA, separate moss shoots from each site were selected and cut into segments. The segments were identified first on *H. splendens* as it has a distinct yearly growth segments that are easily identifiable (Fig. 1B, C). The average length, 1cm, of these segments was used to decide the length at which to cut *P. schreberi* segments because this species does not have the same distinctive yearly growth (Fig. 1A). This choice of segment length for *P. schreberi* provided segments with changes in colour, from green to brown, which corresponded well with the changes in colour seen along the *H. splendens* segments (Fig. 1A, B). Segments (all with an average length of 1cm) were bundled together, top segments with top segments, middle segments with middle segments etc. (Fig. 1D). This selection provided six replicates (averaging > 30 segments each) of each of the three segment groups from each species from all sites, Abisko (dry), Låktatjåkka (medium), only *P. schreberi*, and Katterjåkk (wet) totalling 90 samples (n = 6).

Moss segments were put into 50mL transparent plastic tubes and kept in growth chambers set to 18 hours light (300 µmol m^{-2} s^{-1}) and a constant temperature of 15°C. Regular watering of the moss, using

DD-H_2O, immediately before each incubation was enough to keep the samples moist and to prevent desiccation. The chosen temperature, light and moisture conditions have been shown to conditions favourable for BNF in feather mosses (Rousk, Pedersen, et al. 2017).

For the ARA, airtight rubber septa sealed the tubes and 5ml of air was replaced with 5 ml acetylene gas using a syringe. This corresponded to 10% acetylene gas in the tubes. The samples were incubated in the growth chamber for ca. 24 hours. After the incubations, 6ml of air was sampled from each tube and injected into pre-vacated vials (Labco, Ceredigion, UK). The rubber septa were removed from the tubes and the tubes were placed in the growth chamber where they were kept until the next incubation. A total of six incubations were completed over 42 days to get a quantitative assessment of BNF potential. Incubation frequency was once every week except between incubations four and five with two weeks apart. The analysis of the ARA gas samples was done using a Shimadzu GC-14B Gas Chromatograph equipped with a Porapak N column, a FID detector and with injector, column and detector temperature at 250, 60 and 120°C, using He as carrier gas.

After the last ARA incubation, each group of segments was weighed and dried at 60°C. After drying, mosses were weighed again, cut into fine pieces and analysed for contents of Fe, P, Mo, C and N (below).

Moss nutrient and pH analyses

To analyse moss Fe, P and Mo content, an amount of 90–110 mg dry moss from each sample was placed in Teflon tubes. Sample material was limited making it necessary to use a modified acid digestion method. This meant using 3 ml of 16% HNO_3 diluted to 6 ml 8% HNO_3 after digestion, using the Plant Material program on a MARS^{tm} 6 microwave digestor, run twice to achieve complete digestion. Analyses of Fe, P and Mo contents were done using a Perkin Elmer PinAAcle 900T Atomic Absorption Spectrometer as in Goth et al. (2019). P and Mo analyses were done using graphite furnace vaporization and Fe was analysed using flame atomic absorption spectrophotometry. Verification of the digestion method and element analysis was obtained by analysis of standard material, and with blank subtraction and use of Perkin Elmer instrument calibration standards.

Analysis of total C and N in the moss segments required an amount of 4-5.5 mg ground, dry moss from each sample to be weighed and packed into tin capsules. The analysis was conducted using using an Eurovector CN analyser coupled to an Isoprime isotope ratio mass spectrometer.

For pH measurements, fresh moss was cut into small pieces and placed in 50 ml transparent plastic tubes with 15 ml DDH_2O. The tubes were gently shaken, no more than 30 seconds, to avoid damaging the moss cells and thus measuring pH on a mix of both surface and intracellular pH. Measurements were performed immediately after the shaking. Three replicates (> 20 segments each) were used for the pH measurements totalling 45 samples (n = 3). All samples were measured using a standard pH-meter (PHM240 pH/ION Meter, MeterLab, Radiometer Copenhagen).

Statistical analyses

Differences in ARA and measured factors, Mo, Fe, P and N contents as well as pH between species and segments were tested with two-way ANOVA for Abisko (dry) and Katterjåkk (wet), where both moss species could be collected. One-way ANOVA was used to test for differences between segments for Låktatjåkka (medium) which only provided one species, *P. schreberi*. Linear regression was used to test how ARA is related to the measured factors as well as relevant factor ratios (N, P, Fe, Mo). The relation between precipitation and ARA as well as precipitation and the measured factors was tested using linear regression. Normal distribution of data and homogenous variance in data were tested with diagnostics plots and transformation of data was performed when necessary to achieve normality and homogeneity. All data analyses were done in R (R Core Team. 2017.).

Results

Nitrogen fixation

Nitrogen fixation as measured with ARA varied strongly between sites along the precipitation gradient ($F = 264.3$ $DF = 442$ $P < 0.0001$) and between species across sites ($F = 48.8$ $DF = 443$ $P < 0.0001$) but between segments only for *P. schreberi* across sites ($F = 7.7$ $DF = 262$ $P = 0.0005$) and not for *H. splendens* ($F = 2.9$ $DF = 177$ $P = 0.056$) (Fig. 2; Table 1). The highest ethylene production was found at the wettest site (Katterjåkk) after 23 days in *H. splendens* in the lowest segment (the bottom) with a production of 61.1 ± 14.7 nmol g^{-1} h^{-1}, and the lowest in the driest site (Abisko) after 42 days in *P. schreberi* in the top segment with a production of 0.14 ± 0.03 nmol $g^{-1}h^{-1}$. There was a 10-fold increase in overall ethylene production between the driest site (Abisko) (0.35 ± 0.02 nmol $g^{-1}h^{-1}$) and the medium wet site (Låktatjåkka) (3.2 ± 0.4 nmol g^{-1} h^{-1}) and another 5-fold increase for both mosses at the wettest site (16.5 ± 1.6 nmol $g^{-1}h^{-1}$). *H. splendens* had the higher ethylene production rates than *P. schreberi*, but this was only clear in the samples from the wettest site, where the difference was more than 10-fold between species with an ethylene production of 1.5 ± 0.1 and 31.5 ± 2.1 nmol g^{-1} $h^{-1}$for *P. schreberi* and *H. splendens*, respectively. There was a consistent pattern of highest ethylene production in the middle segments of *P. schreberi* across all locations and incubation times. For *H. splendens*, ethylene production was highest in the lowest segments, albeit not significantly so (Fig. 3).

Table 1
Moss sample C:N-ratios and pH content.

Parameter	Species	Segment	Abisko (dry)	Låktatjåkka (medium)	Katterjåkk (wet)
C:N	*H. splendens*	1	65.74 (2.44)	NA	54.33 (0.80)
		2	70.14 (2.99)	NA	50.09 (0.75)
		3	69.04 (0.93)	NA	47.68 (2.53)
	P. schreberi	1	64.45 (2.85)	86.98 (1.77)	67.14 (2.90)
		2	64.04 (4.16)	94.60 (3.38)	61.14 (1.59)
		3	51.78 (2.56)	77.43 (5.28)	50.34 (2.65)
pH	*H. splendens*	1	4.53 (0.08)	NA	5.02 (0.13)
		2	4.44 (0.05)	NA	5.29 (0.09)
		3	4.59 (0.02)	NA	5.16 (0.30)
	P. schreberi	1	4.73 (0.10)	4.29 (0.04)	4.73 (0.10)
		2	4.66 (0.04)	4.31 (0.06)	4.57 (0.08)
		3	4.76 (0.05)	4.39 (0.08)	4.63 (0.05)

Numbers are means ± se.

Moss nutrients and pH

Contents of Fe and Mo varied along the precipitation gradient (but less pronounced than the ethylene production), and contents of Fe, P and Mo also varied within sites between species as well as across the moss segments within species (Fig. 4; Table 1). For instance, contents of Fe ranged from 0.56 ± 0.12 mg g^{-1} DW in the bottom segment in *H. splendens* from the wettest site, to a low of 0.12 ± 0.2 mg g^{-1} DW in the top segment in *P. schreberi* from the medium wet site. Overall, the content of Fe varied between sites, species and segments (Fig. 4; Table 1). There was a clear pattern of increasing Fe content with increasing segment depth for *P. schreberi* across all locations, but no clear pattern for *H. splendens* (Fig. 4, Table 1).

Mo content ranged between 0.37 ± 0.07 µg g^{-1} DW in the middle segments of *H. splendens* from the wettest site and 0.06 ± 0.01 µg g^{-1} DW in the top segment of *H. splendens* from the driest site. Overall, the content of Mo showed a pattern similar to Fe, with strong differences between sites, within sites between species and between segments within species (Fig. 4, Table 1), although with a less pronounced trend towards increasing content with increasing segment depth.

Moss P content was higher, 2.46 ± 0.07 mg g^{-1} DW, in the top segment of *P. schreberi* from Katterjåkk the wettest site than P in the bottom segment of *H. splendens* from Abisko the driest site, 0.47 ± 0.09 mg g^{-1}

DW. The content of P varied little between sites but greatly between segments and species within sites (Fig. 4; Table.1) and there was a pattern of decreasing P with increasing depth for all sites and both species.

Nitrogen content halved from a high of 9.65 ± 0.51 mg g^{-1} DW in the bottom segment of *H. splendens* from the wettest site, to a low of 4.88 ± 0.17 mg g^{-1} DW in the middle segment of *P. schreberi* from the medium wet site. Overall, moss N content was highest in the wettest site and in *H. splendens* from this location but the differences between sites were relatively small (Fig. 4; Table.1).

H. splendens from the wettest site had higher concentrations of Fe, Mo and N while *P. schreberi* from this site had the highest content of P.

pH varied across the precipitation gradient, but generally varied less between segments than between species within sites (Table 2) and pH was lower in *H. splendens* than in *P. schreberi* at Abisko (dry) but higher in *H. splendens* than in *P. schreberi* at Katterjåkk (wet) (Table 2).

Table 2
ANOVA. P-values and F-test values

Parameter	Fixed effect	Abisko (dry)		Låktatjåkka (medium)		Katterjåkk (wet)	
		P	**F**	**P**	**F**	**P**	**F**
ARA	Segment	< 0.001	13.9	< 0.001	9.0	**< 0.001**	12.5
	Species	0.019	5.6	NA	NA	**< 0.001**	1009
	Seg x Spec	0.003	6.0	NA	NA	**0.012**	4.5
Fe	Segment	< 0.001	53.4	0.001	11.2	**0.003**	7.2
	Species	0.087	3.1	NA	NA	**< 0.001**	53.6
	Seg x Spec	0.139	2.1	NA	NA	**0.004**	6.7
Mo	Segment	0.110	2.4	0.238	1.6	**0.022**	4.4
	Species	< 0.001	22.4	NA	NA	**< 0.001**	50.4
	Seg x Spec	0.897	0.1	NA	NA	**0.028**	4.0
P	Segment	< 0.001	9.3	< 0.001	12.0	**< 0.001**	12.1
	Species	< 0.001	24.8	NA	NA	**< 0.001**	51.8
	Seg x Spec	0.666	0.4	NA	NA	0.717	0.3
N	Segment	0.030	3.9	0.018	5.4	**< 0.001**	16.3
	Species	0.003	10.8	NA	NA	**< 0.001**	37.8
	Seg x Spec	0.016	4.8	NA	NA	**0.010**	2.5
C:N	Segment	0.052	3.3	0.015	5.6	**< 0.001**	15.8
	Species	< 0.001	14.1	NA	NA	**< 0.001**	24.1
	Seg x Spec	0.014	4.9	NA	NA	0.101	2.5
pH	Segment	0.169	2.1	0.541	0.7	0.929	0.1
	Species	0.002	14.8	NA	NA	**0.001**	17.7
	Seg x Spec	0.893	0.1	NA	NA	0.383	1.0

Correlations between BNF and nutrient content as well as precipitation along the moss shoots

BNF and measured elements showed varying and weak correlations across sites and species except for a few specific instances (Fig. 5). For *P. schreberi*, only the content of Mo at Låktatjåkka (medium) had a significant positive effect on BNF (R^2 = 0.395, P = 0.005). For *H. splendens* there were several significant

correlations between BNF and measured elements. Nitrogen (R^2 = 0.232, P = 0.043) content correlated positively with BNF at Katterjåkk (wet), while P content had a negative correlation at both Abisko (dry) (R^2 = 0.232, P = 0.043) and Katterjåkk (wet) (R^2 = 0.323 P = 0.014).

Men annual precipitation at the three locations Abisko (300 mm), Låktatjåkka (850 mm) and Katterjåkk (1050 mm) could not be correlated with BNF for *H. splendens* as it was only present in two locations. For *P. schreberi* the correlation between MAP and BNF was not linear but instead seemed saturated at the site with the medium MAP (Fig. 6).

Discussion

During this study, three important factors affecting BNF were kept constant: Temperature, light, and moss-moisture throughout the BFN measurements. This was done in order to attribute differences in BNF between moss samples to variance in other factors known to affect BNF as measured here, namely Fe, Mo, P and N content and pH. Constant and equal temperature, light and moisture for all segments is not expected under natural field conditions. Instead, the top segment is expected to receive more light than segments further down the moss shoot, but is also expected to be subject to larger fluctuations in temperature and moisture. The moisture and temperature conditions for BNF were optimal and equal for all segments, and hence, our measurements represent potential BNF along the entire moss shoot.

Nitrogen fixation along the precipitation gradient (H1)

We hypothesized (H1) that BNF would be highest in mosses collected at the endpoint of the precipitation gradient, the site that receives the most rain. We could partly confirm this first hypothesis as we found the highest BNF rates at the wet endpoint of the gradient for *H. splendens*, where activity was up to 100-fold higher here than at the driest site. For *P. schreberi*, however, activity was highest at the medium wet site (Låktatjåkka), but still higher at the wettest site than at the driest site. Further, BNF was also consistently higher for *H. splendens* compared to *P. schreberi* at the wettest site. These findings confirm previous results highlighting the importance of moisture for BNF in mosses (Rousk et al. 2014, 2017) and moss species-specific differences in associated BNF. For instance, large variation in BNF and associated dominant cyanobacteria has been found for the same moss species investigated here (Warshan et al. 2016) and cyanobacterial communities seem to be host specific (Ininbergs et al. 2011) and may have different requirements for sustaining BNF.

On the other hand, nutrient content (Mo, Fe) was higher in *H. splendens* collected from the wettest site, which could also promote BNF as shown elsewhere, including for arctic tundra (Rousk et al. 2017; Winbourne et al. 2017; Dynarski and Houlton 2018). Yet, higher precipitation could have led to higher nutrient availability e.g. through higher deposition or lateral or vertical transport in the moss carpet and thus, higher nutrient uptake by the mosses as a results of higher moisture conditions that would promote moss growth.

Nitrogen fixation along moss shoots (H2)

We hypothesized that the highest BNF takes place in the middle segments of the moss shoots (H2). We based this hypothesis on the assumption that the middle segments would have had the optimal conditions in regards to moisture and light under field conditions and thus the best conditions for BNF. However, our second hypothesis could only be partly confirmed since we found this for only one of the investigated moss species. Biological nitrogen fixation was highest in the middle segments of *P. schreberi* for all locations but no clear pattern could be found for *H. splendens*, even though activity tended to increase with moss shoot depth. These differences could be due to different morphologies between the investigated moss species. While *P. schreberi* has a well-defined stem and side branches, *H. splendens* is much more finely branched with several subbranches that may increase water holding capacity along the entire shoot in contrast to *P. schreberi* whose top segment likely dries out quickly which could hamper cyanobacterial colonization and BNF. On the other hand, the difference could be caused by the experimental setup and how the segments were cut. *H. splendens* has a distinct yearly growth in segments, and the length of these segments was used to determine segment length for *P. schreberi*. Perhaps smaller or larger segments are required from *P. schreberi* to capture the yearly growth and thus identify physiological changes along the moss shoots. We provided all segments in the study with equal moisture, light and temperature, and cyanobacteria associated with *H. splendens* could have adapted to these conditions whereas cyanobacteria associated with *P. schreberi* could not as they harbour different cyanobacteria communities with potentially different requirements (Warshan et al. 2016). Under natural conditions, these three factors would vary along the moss shoots, making it more likely to find this optimal compromise somewhere along the moss shoots.

We did not find the highest BNF in the greenest upper parts of *H. splendens* or *P. schreberi*, corresponding to segment 1, in contrast to reports in Leppänen et al. (2013). But instead, we found higher activity in the brownish middle and lower parts of the moss shoot, corresponding to segments 2 and 3. However, the previous study included what corresponds to all 3 segment units used in our project, into what they more crudely defined as the green upper part of the moss (upper 4 cm) making comparisons difficult. We found a different pattern in BNF associated with *H. splendens* than what was reported by Gavazov et al. 2010 who found that the middle segments had the highest BNF rates with moss sampled from the same region in Northern Sweden, which would correspond to our findings for *P. schreberi*. Yet, *H. splendens* in the previous study was collected from a fen habitat that has very different nutrient dynamics and hydrology compared to a birch forest where our samples originated from, likely also affecting moss shoot morphology. Smith (1984) found highest BNF rates in the upper 3 segments of *Brachythecium subplicatum* but the differences were much smaller than what we found here. Nevertheless, even though very few studies exist that assessed BNF along distinct segments along a moss shoot, it seems like the distribution of BNF along a moss shoot is driven by the moss host and less so by the environment as we collected different moss species from the same habitat and yet, found different patterns along the moss shoots. This could be due to moss species-specific differences in nutrient resorption (Liu et al. 2020), water holding capacity (Elumeeva et al. 2011) or other morphological (above) or chemical differences (below) between moss species.

It is worth noting, however, that the mosses from some locations had individuals with only 3 segments, while others provided individuals with 7 segments. Thus, distance between segments and soil varies between locations and species which might affect the impact of factors like temperature and moisture, due to varying influence of soil properties on segments, as well as cyanobacterial colonization

Nutrient content along moss shoots and correlation with BNF (H3)

Finally, we wanted to investigate if the nutrients, Fe, P, N and Mo, as well as pH, could explain variation in BNF along the shoots. We expected nutrient contents, C:N ratio and pH to vary between locations and between segments, and that Fe, P and Mo were positively linked to BNF while N and low pH were negatively related to BNF (H3). Indeed, almost all measured factors, except P, varied between sites with the highest Fe and Mo contents and highest pH in *H. splendens* from the wettest site, which also showed the highest BNF in this species. Phosphorus varied little between sites but instead varied greatly between segments and species within sites. All pH measurements showed slightly acidic conditions, which are not the perfect conditions for cyanobacteria to perform high BNF (Smith 1984; Liengen and Olsen 1997; Alvarenga and Rousk, 2021). Yet BNF measured in this study is still similar or even high compared to previous studies (Gavazov et al. 2010; Leppänen et al. 2013, Rousk et al. 2017), but comparisons with several previous studies are complicated as they express BNF on area and not mass basis.

The differences we found in nutrient content were significant, but small compared to the wide differences in BNF capacity. However, we did find the highest BNF rates at the wettest site with the highest Fe and Mo contents, and we found the highest BNF rates in *H. splendens*, the species with the highest contents of these elements. Within this site and species we also found the highest BNF rate in the segments with the highest content of Mo and Fe. This supports (H3) that Mo, Fe content could control BNF rates. Yet, the patterns were not consistent across sites, segments and mosses, and P even seems to have a negative effect on BNF.

The lack of a strong relation between Mo and BNF may be surprising as it has been shown that Mo availability can limit BNF in mosses from sites close to our sampling sites (Rousk et al. 2017). However, Mo content of the mosses in our study was below the proposed threshold of 250 ng Mo g^{-1} (Darnajoux et al. 2019) below which the nitrogenase enzyme shifts from the Mo-based to the vanadium(V)-based isoform. Indication of the dominance of the V-based nitrogenase has indeed been found in *H. splendens* (Rousk et al. 2017) and a lack of long-term promotion by Mo and P additions of BNF in the sites close by (Rousk & Rousk 2020). Hence, nutrient content cannot completely explain BNF along moss shoots, and moisture seems to remain the main factor driving BNF in mosses.

Conclusion

Nitrogen fixation measured by ARA in two common feather mosses collected along a steep precipitation gradient showed clear species-specific differences. There was a strong relationship between higher BNF

and sites with higher precipitation for *H. splendens*, but for *P. schreberi* there was no clear relation between high precipitation sites and BNF. Nitrogen fixation seems to be species-specific, and while *P. schreberi* had a clear and consistent pattern in BNF along the segments with highest activity in the middle segments across the precipitation gradient, *H. splendens* had overall much higher BNF but showed a temporal shift along the segments towards a higher production in the bottom segments. BNF seems weakly driven by measured variables (Fe, P, Mo, N and pH) and is likely overridden by moisture effects. Hence, we found clear differences in BNF along moss shoots of the common mosses *Pleurozium schreberi* and *Hylocomium splendens*, and while Fe, P and Mo contents and C:N ratio varied significantly between segments, the differences were not as pronounced as BNF. Further, BNF in mosses can vary significantly at small (moss depth or age) and large scales (precipitation gradients). Large variations in nutrients within just a few centimetre along the moss shoots as well as variation in precipitation can drive orders of magnitude differences in BNF in mosses. Similarly, large differences in BNF could be detected between moss species, which should be considered in ecosystem N budgets aiming to upscale moss-associated BNF.

Declarations

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Conflicts of interest not applicable

Ethics approval not applicable

Consent to participate not applicable

Consent for publication not applicable

Availability of data and materials the datasets used and/or analysed during the study are available from the corresponding author on reasonable request

Code availability the coding used during the study are available from the corresponding author on reasonable request

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Figures

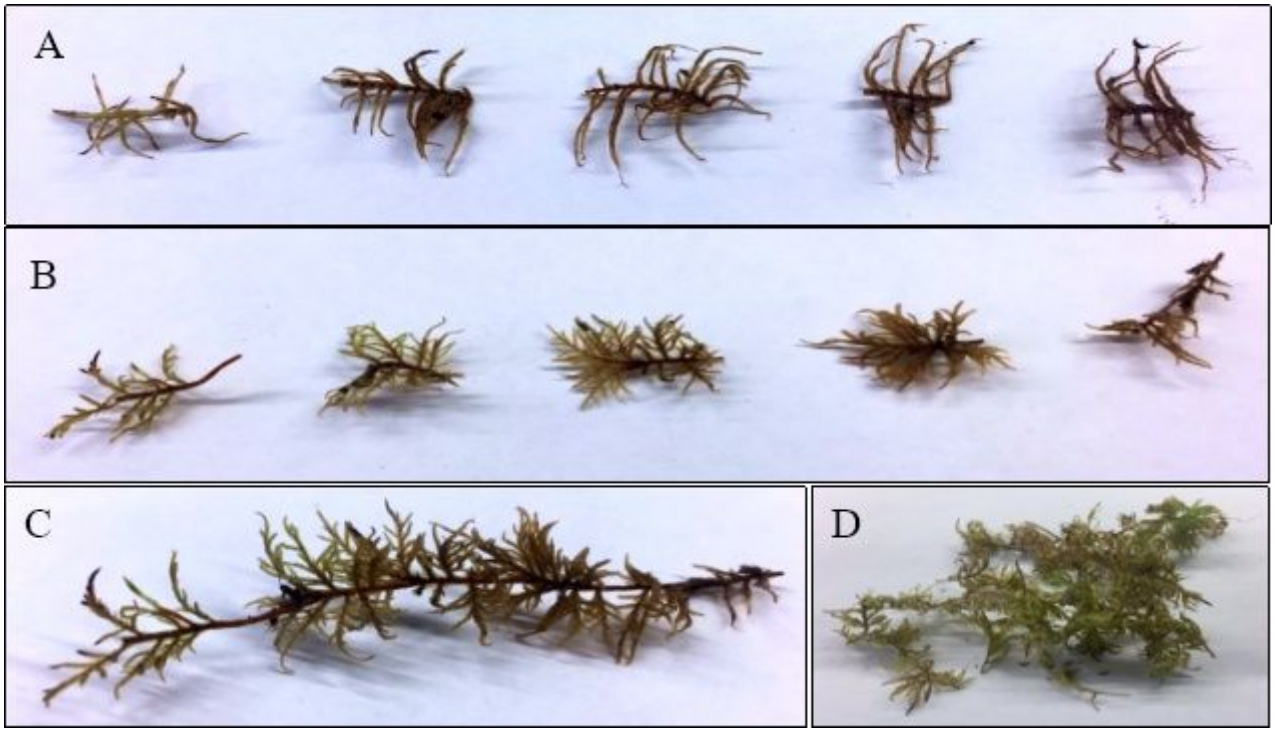

Figure 1

Moss shoots separated according to length. A) *Pleurozium schreberi,* segments from top (left) to bottom (right) and for B) *Hylocomium splendens* where each segment represents one years' growth from top (left) to bottom (right). C) Intact shoot of *H. splendens* with visible yearly growth segments and black lines indicating annual production. D) *H. splendens* top segments bundled together to form one sample.

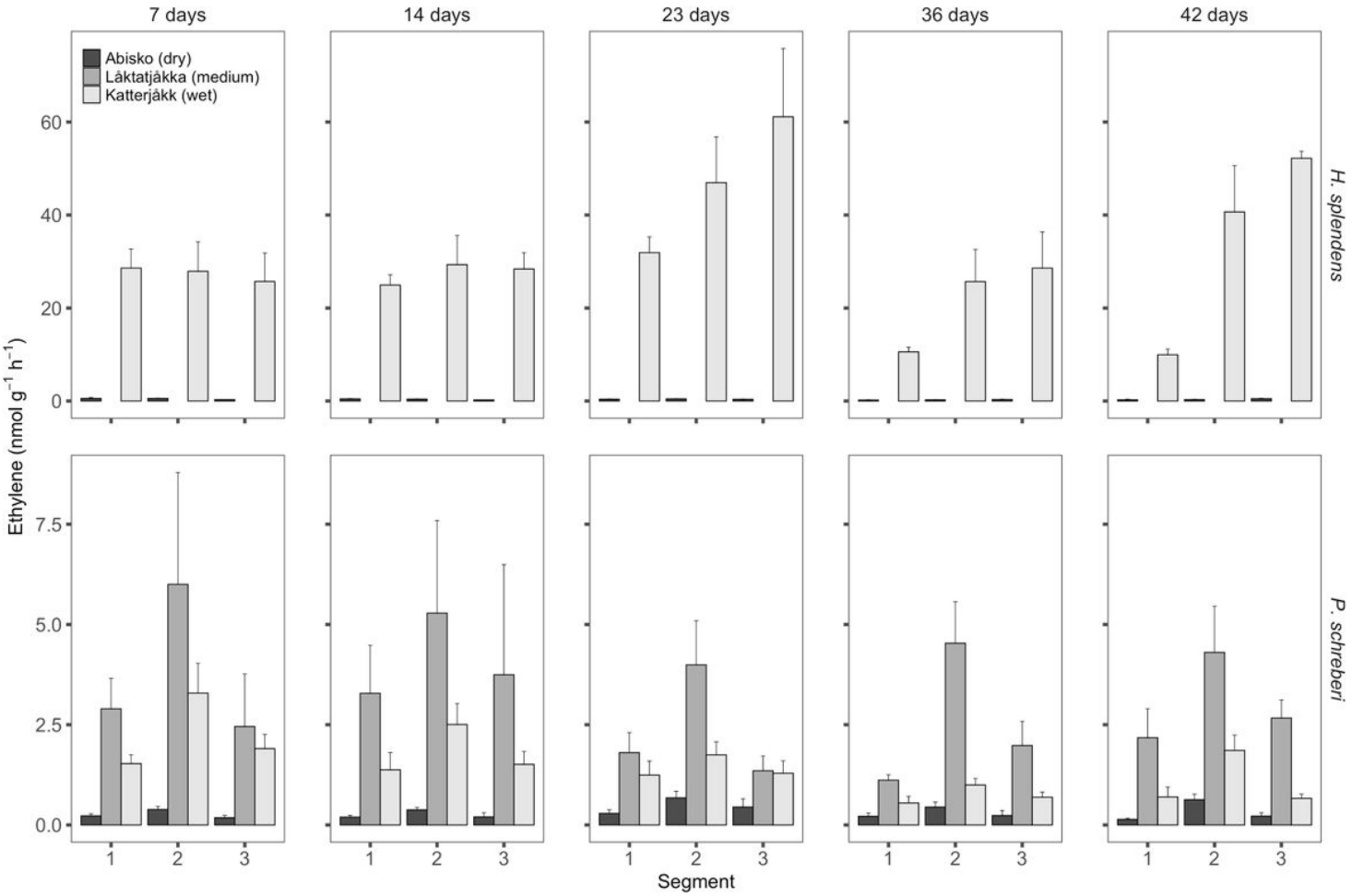

Figure 2

Ethylene production (nmol g^{-1} h^{-1})±SE (n=6) measured over 42 days in three different moss segments, top (1), middle (2) and bottom (3) from two different species of feather moss, *H. splendens* and *P. schreberi*, collected at three different locations with varying mean annual precipitation representing dry (Abisko), medium (Låktatjåkka) and wet (Katterjåkk) locations in The Subarctic.

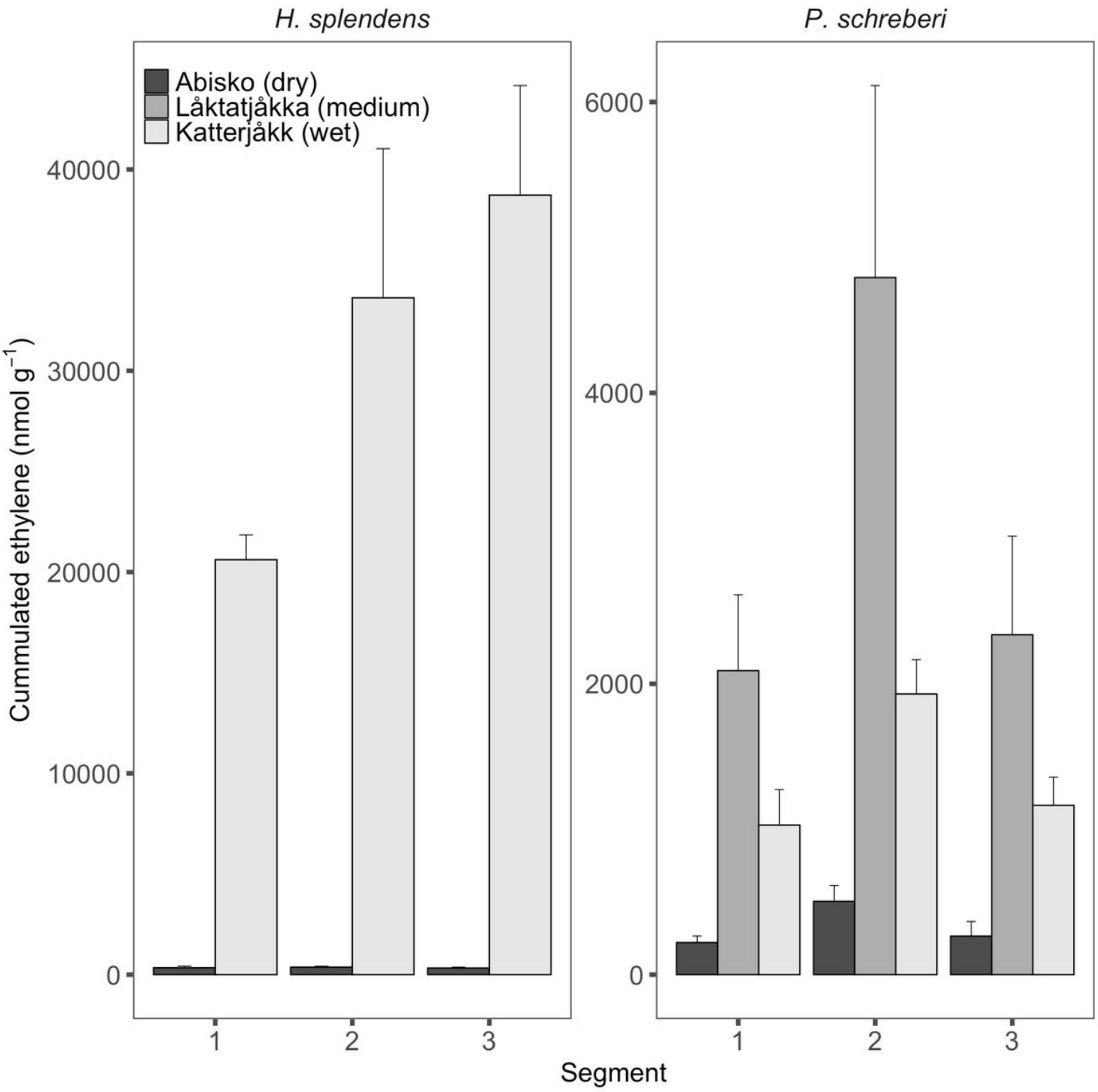

Figure 3

Ethylene production (nmol g^{-1})±SE (n=6) cummulated over 42 days in three different moss segments, top (1), middle (2) and bottom (3) from two different species of feather moss, *H. splendens* and *P. schreberi*, collected at three different locations with varying mean annual precipitation representing dry (Abisko), medium (Låktatjåkka) and wet (Katterjåkk) locations in The Subarctic.

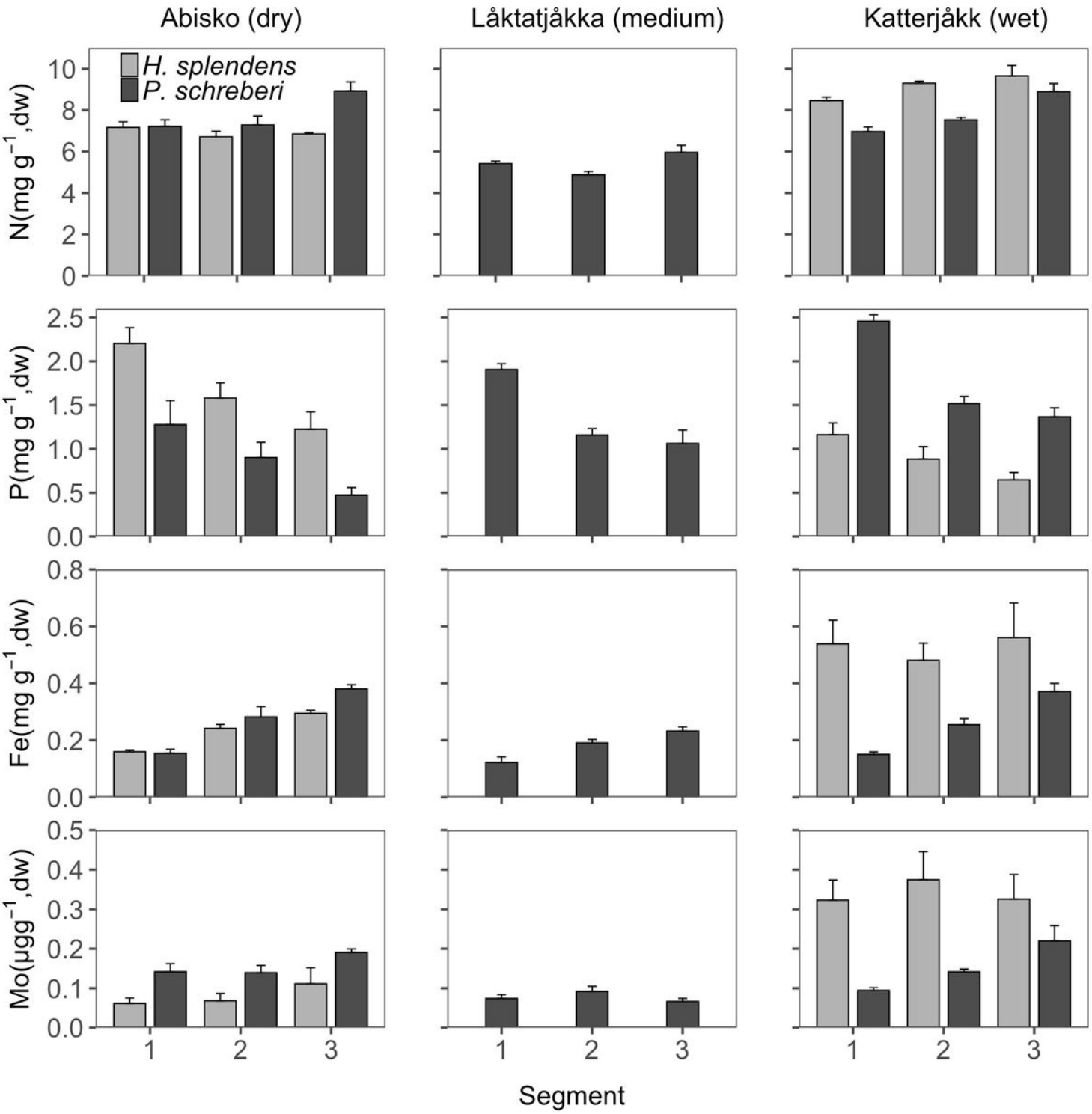

Figure 4

Contents of nitrogen (mg g^{-1} ,dw), phosphorus (mg g^{-1} ,dw), iron (mg g^{-1} ,dw) and molybdenum (µg g^{-1} ,dw)±SE (n=6) in three different moss segments, top (1), middle (2) and bottom (3) from two different species of feather moss, *H. splendens* and *P. schreberi*, collected at three different locations with varying mean annual precipitation representing dry (Abisko), medium (Låktatjåkka) and wet (Katterjåkk) locations in The Subarctic.

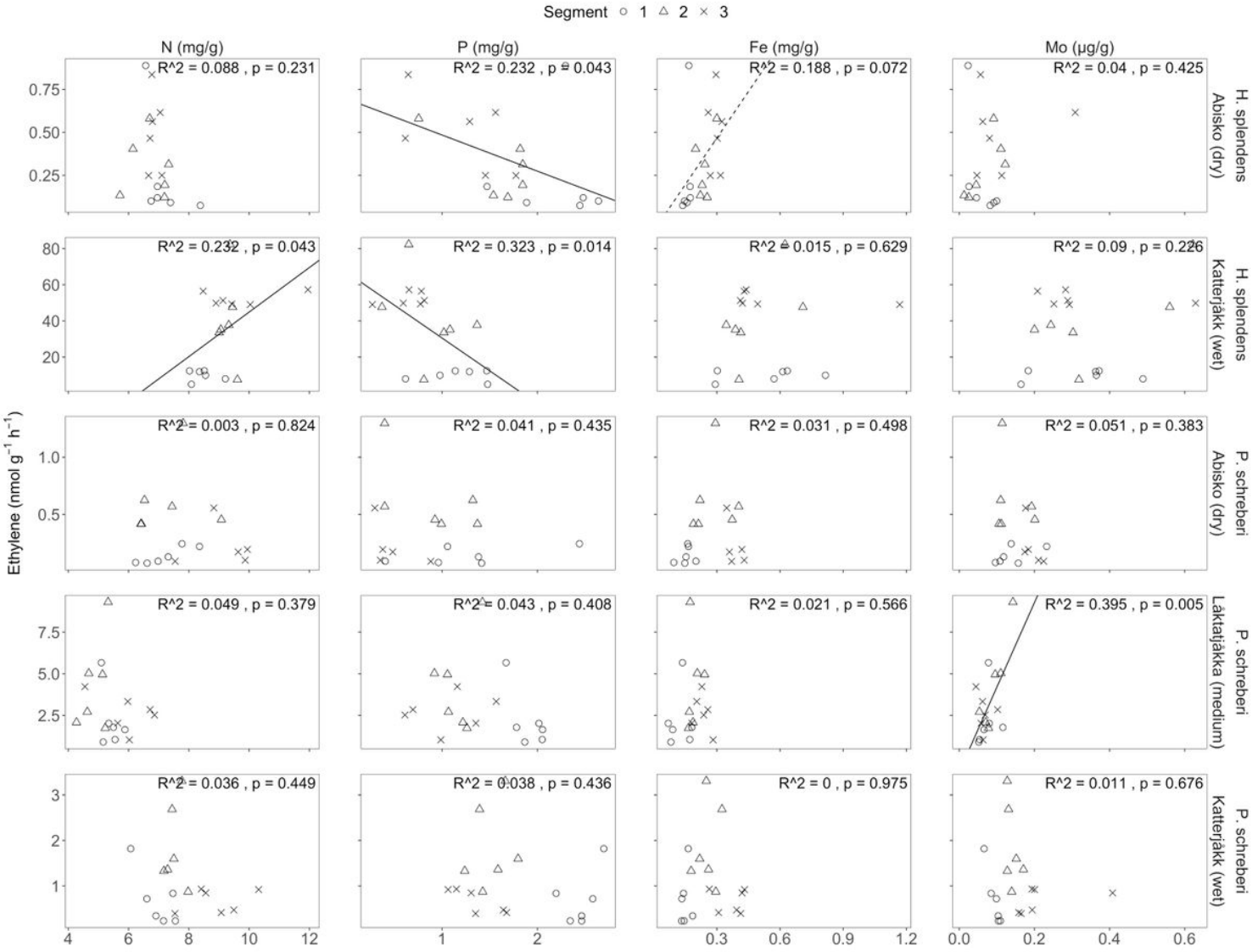

Figure 5

Relationship between ethylene production (nmol g^{-1} h^{-1}) and contents of nitrogen (mg g^{-1} ,dw), phosphorus (mg g^{-1} ,dw), iron (mg g^{-1} ,dw) and molybdenum (µg g^{-1} ,dw) (n=6) in three different moss segments, top (1), middle (2) and bottom (3) from two different species of feather moss, *H. splendens* and *P. schreberi*, collected at three different locations with varying mean annual precipitation representing dry (Abisko), medium (Låktatjåkka) and wet (Katterjåkk) locations in The Subarctic. Regressions lines and P values are given.

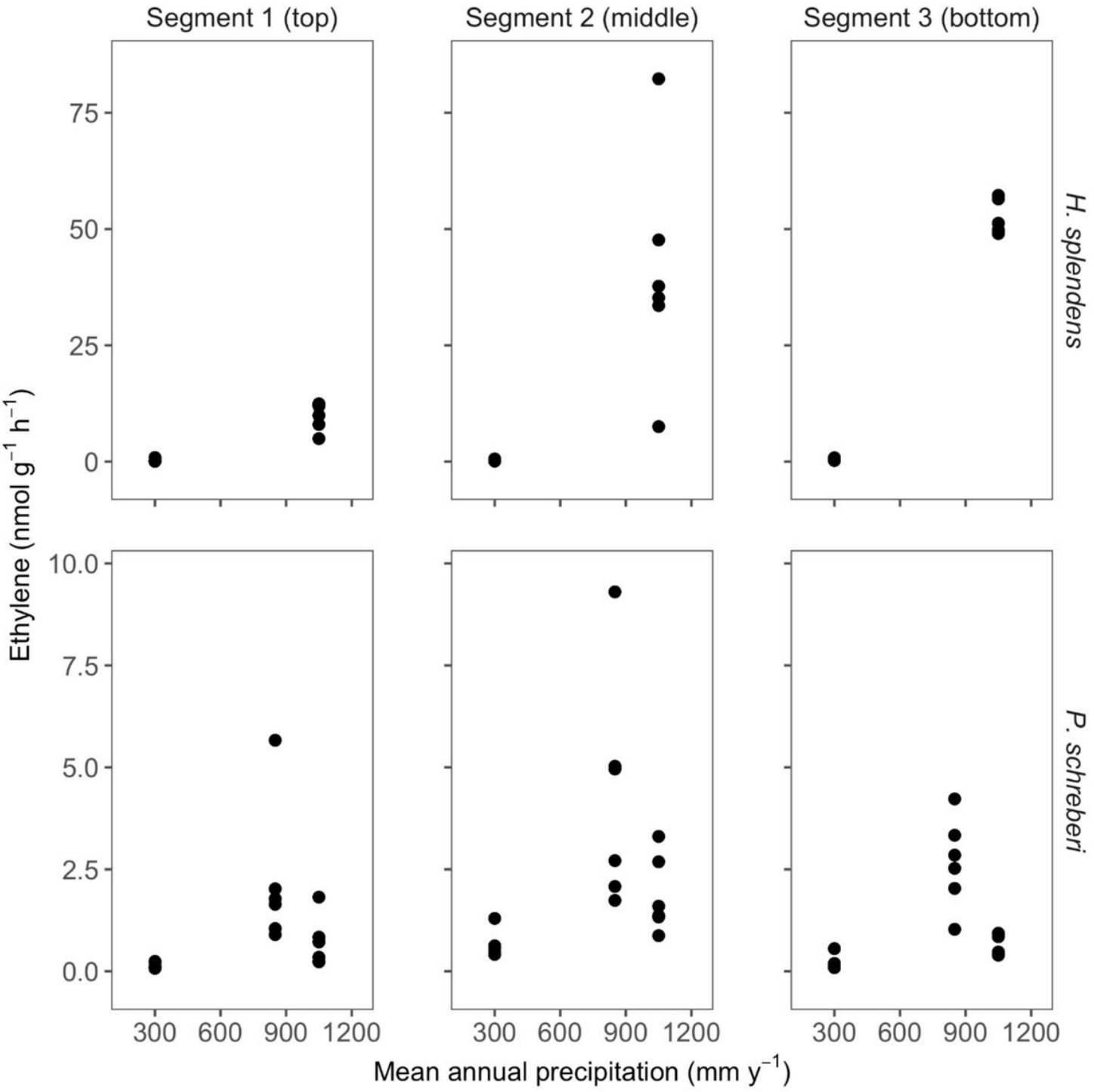

Figure 6

Relationship between ethylene production (nmol g^{-1} h^{-1}) (n=6) and mean annual precipitation in three different moss segments, top (1), middle (2) and bottom (3) from two different species of feather moss, *H. splendens* and *P. schreberi*, collected at three different locations with varying mean annual precipitation representing dry (Abisko), medium (Låktatjåkka) and wet (Katterjåkk) locations in The Subarctic.