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Extreme freeze-thaw cycles do not affect moss-associated nitrogen fixation across a temperature gradient, but affect nutrient loss from mosses

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\textbf{ARTICLE INFO}

\textbf{ABSTRACT}

Moss-associated nitrogen (N\textsubscript{2}) fixation performed by epiphytic, N\textsubscript{2}-fixing bacteria (diazotrophs) contributes significantly to ecosystem N input in pristine habitats. While we have some understanding of the effects of climate warming on moss-associated N\textsubscript{2} fixation, we lack data on effects of freeze-thaw cycles (FTCs) on diazotroph activity, although increased frequency of FTCs is predicted. We collected the widespread moss Pleurozium schreberi along a climate gradient (temperate, boreal, arctic) and exposed moss and associated diazotrophs to severe (20 °C difference, cycling between +10 and −10 °C) and mild (6 °C difference, ±3 °C) diurnal FTCs. We measured N\textsubscript{2} fixation in mosses over 8 weeks and assessed their nutrient loss (fixed N\textsubscript{2}, total dissolved N, ammonium, phosphate) during the FTCs. We expected lower nitrogenase activity in mosses exposed to more severe FTCs and different sensitivities of N\textsubscript{2} fixation towards FTCs along the climate gradient. However, no differences were found in N\textsubscript{2} fixation between mild and severe FTCs, but N\textsubscript{2} fixation in mosses from the temperate heath was less susceptible to FTCs than those from colder sites, suggesting adapted temperate diazotroph communities. Mosses lost little N, most at constant, positive temperatures, while more phosphate was lost from mosses exposed to FTCs, depending on the positioning along the climate gradient, mirroring nutrient demand and limitation. Our results show that moss-associated N\textsubscript{2} fixation is less susceptible towards FTCs than expected but nutrient loss from moss carpets can increase following FTCs, with consequences for nutrient pools and fluxes.

1. Introduction

Nitrogen (N) is an essential nutrient that often limits plant productivity in pristine ecosystems such as arctic tundra and boreal forests due to low N input via atmospheric deposition (<2 kg N ha\textsuperscript{−1} yr\textsuperscript{−1}; Penuelas et al., 2013) and slow N turnover (Hobbie, 1996). An important source for “new” N in these ecosystems is biological N\textsubscript{2} fixation performed by N\textsubscript{2} fixing bacteria (diazotrophs) that are free-living or associated with vascular plants, lichens and mosses (Ininbergs et al., 2011; Pushkareva et al., 2015; Rousk et al., 2015; Goth et al., 2019). Here, N\textsubscript{2}-fixing cyanobacteria associated with dominant moss species contribute between 1 and 3 kg N ha\textsuperscript{−1} yr\textsuperscript{−1} and is the major input of N to these ecosystems (DeLuca et al., 2002; Rousk and Michelsen, 2017). However, this key ecosystem process is strongly affected by abiotic factors such as nutrient availability, moisture conditions and temperature. For instance, moss-associated N\textsubscript{2} fixation is sensitive to increased N loads in boreal forests (Ackermann et al., 2012; Rousk et al., 2013a,b), and can be limited by molybdenum and phosphorus in the Arctic (Rousk et al., 2017a). Further, while it is well known that no moss-associated N\textsubscript{2} fixation occurs under dry conditions (Rousk et al., 2014, 2018), the effects of a change in temperature on moss-associated N\textsubscript{2} fixation remains ambiguous (Salazar et al., 2020) and is strongly linked to moisture conditions in the moss. For instance, increased temperatures in long-term field warming experiments in the Arctic reduced N\textsubscript{2} fixation in mosses as a result of drying of the moss (Sorensen and Michelsen, 2011; Sorensen et al., 2012), while increased temperatures (up to 30 °C) together with increased moss-moisture content promoted moss-associated N\textsubscript{2} fixation in laboratory incubations (Rousk et al., 2017b). Overall, increased N availability decreases moss-associated N\textsubscript{2} fixation, while P and micronutrients promote it but the responses of N\textsubscript{2} fixation towards nutrient additions are modulated by temperature and precipitation, i.e. location of the samples (Zheng et al., 2019). Further, drought severely inhibits- while increased precipitation promotes N\textsubscript{2} fixation (Zheng et al., 2020). Hence, moss-associated N\textsubscript{2} fixation seems...
especially sensitive to a change in moisture conditions.

In addition to changes in temperature and precipitation that could affect moss-associated N\textsubscript{2} fixation due to climate change, more extreme weather events such as freeze-thaw cycles (FTCs) are expected in the future (IPCC 2019). Increased frequency of FTCs is predicted to be most pronounced in high latitudes (Mellander et al., 2007; Henry 2008) but can also impact temperate regions significantly as a result of reduced snow cover due to climate warming (Grafman et al., 2001). Both drying-rewetting events and FTCs are extreme conditions for any microbial activity, given the low water availability during drying and freezing. Such events can disrupt microbial biomass (Soulides and Allison, 1961; Skogland et al., 1988; Stark and Firestone, 1995) and thereby, increase N availability in soils (Ivarson and Sowden, 1966; Deluca et al., 1992). However, the effects on microbial activity in soils depend on the severity, duration and frequency of the freezing event (Song et al., 2017). For instance, no effects on microorganisms (bacteria, amino acid uptake) in soils has been found when temperatures remained above −10\textdegree C (Lipson and Monson, 1998), but repeated diurnal FTCs between −4\textdegree C and +2\textdegree C reduced microbial biomass C in arctic soils (Larsen et al., 2002). While adequate data on FTCs effects on microbial activity in soil exists, we lack any knowledge on how FTCs affect moss-associated N\textsubscript{2} fixation, even though the effects are likely to be significant given the strong dependence of N\textsubscript{2} fixation on moisture availability, and the position of the mosses above the soil where FTCs are more frequent compared to the soil.

Free-living cyanobacteria have protective mechanisms against desiccation and salt stress (Sakamoto et al. 2009), tolerate very low temperature (Sand-Jensen and Jespersen, 2012) and can maintain N\textsubscript{2} fixation at temperatures at least down to −7\textdegree C (Davey and Marchant, 1983; Belnap, 2001) and can survive prolonged periods at −20\textdegree C without loss of activity (Nakatsu and Ino, 1987). Hence, cyanobacteria could be protected within the moss carpet and could maintain N\textsubscript{2} fixation activity during all phases of a freezing-thawing event with activity depending on the actual temperatures during the FTC, which further influence relative humidity and N\textsubscript{2} fixation activity. Nonetheless, severe FTCs (below −7\textdegree C) may inhibit N\textsubscript{2} fixation in mosses, directly as a result of low water availability and low temperatures, and indirectly as a result of increased N availability due to lysed cells. However, the patchy data on freeze-thaw effects on moss-associated N\textsubscript{2} fixation leaves this unknown.

Besides direct effects of FTCs on N\textsubscript{2} fixation activity via strong fluctuations in moisture availability and low temperatures, increased N availability as a consequence of microbial cell lysis could inhibit N\textsubscript{2} fixation in mosses (Ackermann et al., 2012; Roux et al., 2013a,b). In addition, mosses are non-vascular plants and release a substantial amount of nutrients (Liu et al., 2020), especially upon rewetting (Wilson and Coxon, 1999; Starsev and Liﬀers, 2006) that could affect the diazotrophs colonizers as well as ecosystem nutrient budgets.

To fill knowledge gaps on the effects of FTCs on N\textsubscript{2} fixation in mosses, we exposed Pleurozium schreberi, which dominates the understory in boreal forests and is common in arctic tundra and temperate heath vegetation, to freeze-thaw cycles with contrasting severity, a mild 6\textdegree C fluctuation (cycling between +3\textdegree C and −3\textdegree C) and a more severe 20\textdegree C fluctuation (cycling between +10\textdegree C and −10\textdegree C) over 8 weeks with daily FTCs. Mosses were collected from three different climates with daily FTCs. Mosses were collected in Northern Sweden near the Abisko Scientific Research Station (68°21′N, 18°49′E) from a subarctic birch forest in June 2016. For this area, the mean annual temperature is 0.5\textdegree C and the mean annual precipitation is 304 mm (mean from 1986 to 2015, http://polar. se/en/abisko-naturvetenskapliga-station/). The site consists of an open birch forest dominated by Betula pubescens, with an understory of the low shrubs Empetrum hermaphroditum, Vaccinium vitis-idaea, V. myrtillus and V. uliginosum. Covering 25–60% of the ground, Hylcomium splendens and Pleurozium schreberi are the dominant moss species at the site (Permin et al. unpublished). Close to this site, 25 FTCs (defined as more than 3 h above or below 0\textdegree C) have been recorded at the soil surface (0–3 cm) in the spring after snow melt (Konestabo et al., 2007).

2. Materials and methods

2.1. Site description and sampling

Pleurozium schreberi is a common moss in forest ecosystems, with a distribution from Greenland to Africa, from sea level to 5000 m elevation (Kuc, 1997). We collected P. schreberi in spring/early summer at three different sites in Scandinavia, forming a temperature gradient over a distance of 2000 km (see below). At each site, six moss mesocosms (21 × 21 × 10 cm) were collected with at least 1 m distance from each other and transported in plastic boxes to the laboratory in Copenhagen. Here, the mesocosms were placed in a climate chamber at 10\textdegree C/6\textdegree C and a 18 h/6 h day-night regime (see below) for equilibration until initiation of the experiment.

2.1.1. Subarctic site

Mosses were collected in Northern Sweden near the Abisko Scientific Research Station (68°21′N, 18°49′E) from a subarctic birch forest in June 2016. For this area, the mean annual temperature is 0.5\textdegree C and the mean annual precipitation is 304 mm (mean from 1986 to 2015, http://polar. se/en/abisko-naturvetenskapliga-station/). The site consists of an open birch forest dominated by Betula pubescens, with an understory of the low shrubs Empetrum hermaphroditum, Vaccinium vitis-idaea, V. myrtillus and V. uliginosum. Covering 25–60% of the ground, Hylcomium splendens and Pleurozium schreberi are the dominant moss species at the site (Permin et al. unpublished). Close to this site, 25 FTCs (defined as more than 3 h above or below 0\textdegree C) have been recorded at the soil surface (0–3 cm) in the spring after snow melt (Konestabo et al., 2007).

2.1.2. Boreal site

Mosses were collected from a boreal forest in Central Sweden, 15 km W of Uppsala (59°53′N, 17°21′E), at Fíby Urskog (87 ha; e.g. Kyschenko et al., 2017), May 2016. The moss samples were taken in a mixed Pinus sylvestris and Picea abies forest stand with an understory of V. and V. vitis-idaea. The mean annual temperature for the area is 5.7\textdegree C with a monthly precipitation of 474 mm (mean from 1986 to 2016, Swedish meteorological and hydrological institute, www.smhi.se). No FTCs are recorded as described above with at least 3 h above or below 0\textdegree C have been recorded at 5 cm depth in the spring in recent years (K. Clemensen, unpublished).

2.1.3. Temperate site

Mosses were collected in June 2016 at the former Climatice research site near Jægerspris, Denmark (55°53′N, 11°58′E). For more information on the site see Selsted et al., (2012). The site is a dry heath/grassland consisting of 30–40 cm tall vegetation dominated by a grass (Deschampsia flexuosa) and an evergreen dwarf shrub (Calluna vulgaris). Furthermore, a low cover of other herb and grass species is found, with an open moss cover beneath the canopy of vascular plants (Mikkelsen et al., 2008). For this area the mean annual air temperature is 8\textdegree C (mean from 1986 to 2016) and the mean annual precipitation for the same period is 613 mm (Danish Meteorological Institute, www.dmi.dk). Ca. 5 FTCs per year occur at the temperate site (K. S. Larsen, T. S. Mikkelsen, unpublished).

2.2. Experimental setup

Mosses from all sites were soaked in double distilled water (ddH\textsubscript{2}O) to ensure water saturation and to rinse off any excess nutrients. Single moss shoots were selected, the dead parts cut off and each moss sample
(consisting of several shoots) of approximately 4 cm length was weighed (3.0 g ± 0.8 fresh weight) and placed into transparent 50-ml centrifuge tubes. We placed six replicates per site in each of six climate chambers with daily fluctuations between + and −3 °C or between + and −10 °C (denoted with ± hereafter), as well as in chambers with constant temperatures, +3 °C, −3 °C, +10 °C or −10 °C. All chambers had a day/night length of 12 h with a photosynthetic active radiation (PAR) of 250 μmol m−2 s−1 during the light hours. In the cycled chambers, the temperature was set to positive during the day (12 h) and negative during the night (12 h).

2.3. Acetylene reduction assay

Nitrogen fixation was measured with the acetylene reduction assay (ARA) as in Rouk et al. (2017a). For this, the 50-ml centrifuge tubes were sealed with a rubber septum (Suba seal, Sigma-Aldrich, Søborg, Denmark), and 5 ml of air was replaced with 5 ml of acetylene, corresponding to 10% acetylene in the tube. The samples were incubated with acetylene once a week over an 8-week period for 24 h in order to measure activity during a full FTC. ARA was measured twice in the first week to be able to capture the direct stress effect of the FTCs on N2 fixation activity. After 24 h incubation, 6 ml of headspace was sampled from each tube and injected into a 6-ml pre-vacated Exetainer vial (Labco, Ceredigion, UK) for ethylene analysis on a gas chromatograph from each tube and injected into a 6-ml pre-vacated Exetainer vial. After 24 h incubation, 6 ml of headspace was sampled from each tube and injected into a 6-ml pre-vacated Exetainer vial (Labco, Ceredigion, UK) for ethylene analysis on a gas chromatograph (GC) 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. Control samples, moss samples incubated without acetylene, did not show any ethylene production. Ethylene in the acetylene gas was subtracted from the raw ethylene values produced by the samples. Moss samples were watered with 0.1 ml ddH2O twice a week (corresponding to 0.19 mm precipitation each time), except those samples incubated at constant negative temperature (−3 °C and −10 °C).

2.4. Nitrogen fixation with the 15N-N2 assimilation assay and nutrient leachate from moss

Another set of moss samples, prepared in the same way as described above, was labeled with 15N–N2 to assess the freezing-thawing mediated loss of fixed N2 from the moss carpet. Samples were watered with 2 ml ddH2O three times a week for the first three weeks, thereafter 3 ml once a week for the following five weeks, and the leachate was collected for analyses. The additional water was to obtain sufficient leachate for analyses. Leachates could only be obtained from samples incubated at +3 °C, +10 °C, ±3 °C and ±10 °C, thus, we only measured 15N–N2 assimilation for these four treatments. For the 15N-labeling, the 50-ml tubes were sealed with a rubber septum after one week, and 7.5 ml of air was replaced with 99% 15N–N2 (corresponding to 15% 15N2 in the tube). The samples were incubated for 24 h in their respective climate chambers. Leachates from the moss samples were collected each week. In order to have enough liquid for analyses, all leachates collected from the same sample were pooled to one sample. In addition, two replicates were pooled to get a sufficient amount of liquid. From each sample, 4 ml was analyzed for total dissolved N (TDN), ammonium (NH4+) and phosphate (PO43−) with a flow injection analyzer (FIAnalyser 5000, FOSS Tectar, Höganas, Sweden). The remaining liquid (~30 ml) was freeze-dried and analyzed for 15N using an isotope ratio mass spectrometer (Isoprime, Cheadle Hulme, UK) coupled to a CN elemental analyzer (Eurovector, Pavia, Italy).

2.5. Total N

After eight weeks, the experiment was terminated and mosses were dried at 70 °C for 24 h, weighed and ground. Of the ground moss, 4–5 mg were packed into tin capsules to determine total N concentration and 15N/14N on an Isoprime isotope ratio mass spectrometer (Isoprime) coupled to a CN elemental analyzer (Eurovector).

2.6. Statistical analysis

To test for differences in acetylene reduction, moss total N, and 15N–N, TDN, NH4+ and PO43− in leachates between the FTCs and between the sites, two-way ANOVAs followed by Tukey’s post hoc tests were performed. Nitrogen fixation rates across time, and 15N in moss tissue vs. 15N in leachate were assessed with regression analyses. Data was log-transformed to reach normality and homogeneity of variances prior to the analyses. All statistical analyses were run in R 3.6.3 (R Core Team, 2018).

3. Results

3.1. Nitrogen fixation in response to FTCs

Nitrogen fixation as assessed with ARA differed between time points (F = 20.6; p < 0.0001), sites (F = 15.7; p < 0.0001) and temperatures (F = 15.23; p < 0.0001), and the effects of temperature was dependent on the site (F = 5.7; p < 0.0001; Fig. 1; Supplementary Fig. S1). In mosses from the Subarctic, cumulative nitrogenase activity was almost double the rates at +3 °C compared to ±3 °C, −3 °C and −10 °C, while activity in the +10 °C and ±10 °C were similar and higher than in the ±3 °C, −3 °C and −10 °C treatments (F = 2.1; p = 0.03; Fig. 1a). Cumulative N2 fixation in the mosses from the boreal forest showed a similar pattern, albeit not significantly so (Fig. 1b), and nitrogenase activity in the mosses from the temperate heath did not show any significant response to the different treatments (Fig. 1c). The more severe FTCs (±10 °C) did not inhibit N2 fixation activity more than the less severe FTCs (±3 °C) in any of the investigated mosses. Rather the opposite was true for the mosses collected in the Subarctic – the less severe FTCs seemed to be more inhibiting for nitrogenase activity than the more severe ones (Fig. 1a).

Nitrogen fixation rates in mosses collected in subarctic and boreal forests responded more to the FTCs than N2 fixation in the temperate mosses. Nitrogen fixation rates at +10 °C and ±10 °C were similar and more than twice as high as activity at −10 °C in the subarctic and boreal mosses, while there were not differences in N2 fixation rates between the extreme FTCs in the temperate mosses. For the less extreme FTCs, N2 fixation rates at −3 °C and ±3 °C were similar, and lower than in the constant −3 °C treatments in the subarctic and boreal mosses, and again, no differences could be found between the treatments in the temperate mosses (Supplementary Fig. S1).

Overall N2 fixation activity (across all treatments and time points) was higher in mosses collected in the boreal forest (0.72 ± 0.04 nmol g dw−1 h−1) and in the Subarctic (0.41 ± 0.04 nmol g dw−1 h−1) compared to activity in the temperate heath (0.23 ± 0.03 nmol g dw−1 h−1; F = 13.07; p < 0.0001; Supplementary Fig. S1).

3.2. Moss nutrient content and release in response to FTCs

Total N was lowest in the subarctic mosses (F = 57.5; p < 0.0001; Fig. 2), but was not different between the treatments in any of the mosses collected along the climate gradient. Similarly, excess 15N in moss tissue at the end of the experiment was not different between the temperature treatments, but was higher in mosses collected from the boreal forest compared to mosses from the other two sites (F = 32.02; p < 0.0001; Fig. 3).

Fixed N was lost from all mosses, but the cumulated leached 15N (Fig. 4) always made up less than 1% of the total 15N retained in the moss (Fig. 3), and with subtle differences between the treatments (Fig. 4). For instance, most fixed N2 was leached from the boreal mosses (F = 26.8; p < 0.0001), least from the subarctic mosses without any treatment differences, and the temperate mosses fell in between. Yet, here, more N was leached from mosses in the ±10 °C FTCs compared to...
Fig. 1. Cumulative acetylene reduction (nmol g dw$^{-1}$) over 8 weeks in mosses collected from three different ecosystems (a subarctic birch forest, b boreal forest, c temperate heath) exposed to diurnal freeze-thaw cycles (±3°C, ±10°C) or kept at constant +3°C and −10°C. Given are means ± SE (n = 6). Different lower case letters indicate significant differences between the temperature treatments.

Fig. 2. Total N (TN, %) of moss tissue collected in three different ecosystems (a subarctic tundra, b boreal forest, c temperate heath) exposed to mild (±3°C) or severe (±10°C) freeze-thaw cycles, or kept at constant temperatures (−3°C, +3°C, −10°C, +10°C). Given are means ± SE (n = 6).
Fig. 3. Excess $^{15}$N in moss (μg g dw$^{-1}$) at the end of the experiment from three different ecosystems (a subarctic birch forest, b boreal forest, and c temperate heath) exposed to differently severe freeze-thaw cycles, ±3 °C and ±10 °C (grey bars), as well as in mosses kept at constant +3 °C and +10 °C (black bars). Given are means ± SE (n = 6).

Fig. 4. Loss of fixed N2 (excess $^{15}$N in leachate, μg N g dw$^{-1}$) from mosses collected in three different ecosystems exposed to differently severe freeze-thaw cycles, ±3 °C and ±10 °C (grey bars), as well as in mosses kept at constant +3 °C and +10 °C (black bars). Given are means ± SE (n = 3).
mosses kept at constant $+10 \degree{} C$ ($F = 6.5; p = 0.02$; Fig. 4c). Further, $^{15}N$ in moss tissue and $^{15}N$ in leachate was positively correlated in mosses from the boreal and temperate sites in the $+3 \degree{} C$ and $\pm10 \degree{} C$ treatments ($R^2 = 0.75; p = 0.001$).

The pattern of TDN and NH$_4^+$ loss was different. Loss of TDN was higher from mosses collected in the boreal forest than from the subarctic site ($F = 3.5; p = 0.05$) and more TDN was lost from the subarctic and temperate mosses kept at $+3 \degree{} C$ and $+10 \degree{} C$ compared to mosses in the FTCs from the same sites ($F = 3.3; p = 0.04$; Fig. 5). Cumulated TDN leaching loss (Fig. 5) made up only 0.3% or less of the total amount of N in the mosses (Fig. 2). All mosses lost similar amounts of NH$_4^+$, irrespective of origin, and the loss was higher in the constant $+10 \degree{} C$ treatments in the temperate moss compared to the $\pm10 \degree{} C$ FTCs ($F = 4.8; p = 0.009$; Fig. 6). A similar pattern was found in the boreal and subarctic mosses, albeit not significantly so (Fig. 6). Phosphate was lost from all mosses, but more from the subarctic mosses compared to mosses from the boreal forest and temperate heath ($F = 19.7; p < 0.0001$; Fig. 7). More P was lost from the subarctic mosses in both the $\pm3$ and $\pm10 \degree{} C$ FTCs compared to the constant $+3$ and $+10 \degree{} C$ treatment, and similarly, in the boreal mosses, more P was lost from the $\pm10 \degree{} C$ than from the other treatments. This pattern was not observed in the moss from the temperate heath. On the contrary, more P was lost from mosses in the $+3 \degree{} C$ and $+10 \degree{} C$ treatments compared to the $\pm3 \degree{} C$ and $\pm10 \degree{} C$ FTCs ($F = 6.7; p = 0.0003$; Fig. 7).

4. Discussion

We exposed mosses collected along a climate gradient to differently severe diurnal freeze-thaw cycles with freezing at nighttime and thaw at daytime and assessed associated N$_2$ fixation over an eight-week period. This allows us to address our hypotheses on severity of the FTCs, ecosystem differences and temporal patterns. Furthermore, we measured nutrients lost from the mosses during recurring FTCs that permits us to draw conclusions on the effects of FTCs on ecosystem nutrient cycling.

4.1. Nitrogen fixation response to the FTCs

We could not corroborate our expectation that severe FTCs inhibit moss-associated N$_2$ fixation (H1). While mosses kept at constant positive temperatures had generally higher N$_2$ fixation activity than mosses kept at constant negative temperatures, mosses from the Subarctic exposed to more severe fluctuations ($\pm10 \degree{} C$) showed higher activity than mosses exposed to fluctuations between $+3$ and $-3 \degree{} C$. Mosses collected from the boreal forest and temperate heath did not show any significant inhibition of nitrogenase activity in response to the temperature fluctuations, even though the boreal mosses followed the pattern of the subarctic mosses.

A temperature difference within 24 h of $20 \degree{} C$ as in the $\pm10 \degree{} C$ FTC is extreme. The lack of a negative effect on nitrogenase activity is surprising. However, $10 \degree{} C$ is above the mean annual temperature at our subarctic and boreal sites, and could promote cyanobacterial activity within the moss carpet. Cyanobacteria may be active and grow during the day, when temperatures are $\pm$3 and $-3 \degree{} C$. Once the freezing starts, some cyanobacteria die and cells lyse that could provide nutrients for the surviving cyanobacteria during thaw (Christensen and Tiedje, 1990). This is likely to happen more in the extreme treatment than in the less extreme treatment ($\pm3$ vs. $\pm10 \degree{} C$). Alternatively, cyanobacteria may survive these fluctuations in temperature and moisture, but cease to fix N$_2$ in the dark, which could explain similar activity in the $+10$ and $\pm10 \degree{} C$ FTCs. Nonetheless, cyanobacterial activity is sensitive to low moisture levels (Rousk et al., 2014), and N$_2$ fixation may be reduced in winter due to dehydration from freezing. In our study, moisture level during thaw may have been sufficient to sustain cyanobacterial activity. In addition, even though ice crystals cause plant tissues to dry out, mosses are efficient at absorbing water upon thawing, which can be

![Fig. 5. Loss of total dissolved N (μg N g dw$^{-1}$) from mosses collected in three different ecosystems exposed to differently severe freeze-thaw cycles, ±3 °C and ±10 °C (grey bars), as well as in mosses kept at constant +3 °C and +10 °C (black bars). Given are means ± SE (n = 3).](image-url)
Fig. 6. Loss of ammonium ($\mu$g NH$_4$–N g dw$^{-1}$) from mosses collected in three different ecosystems exposed to differently severe freeze-thaw cycles, ±3 °C and ±10 °C (grey bars), as well as in mosses kept at constant +3 °C and +10 °C (black bars). Given are means ± SE (n = 3).

Fig. 7. Loss of phosphate ($\mu$g PO$_4$–P g dw$^{-1}$) from mosses collected in three different ecosystems exposed to differently severe freeze-thaw cycles, ±3 °C and ±10 °C (grey bars), as well as in mosses kept at constant +3 °C and +10 °C (black bars). Given are means ± SE (n = 3).
quickly taken up via the moss leaves (Moffett et al., 2009) and could prevent complete desiccation of the cyanobacterial colonizers.

Bacteria can grow in subzero temperatures, down to –17 °C (Carpenter et al., 2000) and even at –20 °C (Tuorto et al., 2014), and cyanobacteria can withstand desiccation stress via solute production (Sakamoto et al., 2009; Sand-Jensen and Jespersen, 2012) and can sustain N2 fixation at least down to –5 °C (Belnap, 2001). Indeed, soil microbial biomass and community structure in boreal soils was not affected by a temperature difference of >-20 °C, cycled between –17 and 4 °C (Koponen et al., 2006). Nonetheless, repeated cycles (four) reduced respiration while denitrifying and nitrifying microbes remained active (Koponen et al., 2006). In contrast, in another study, N2O emissions decreased with repeated FTCs (Prieme and Christensen, 2001). However, this study was performed on organic farmed soils, which may respond very differently compared to soils from natural ecosystems. In a more comparable ecosystem to ours, a subarctic heath, Larsen et al. (2002) found ecosystem respiration during thaw in freeze-thaw cycled mesocosms to be between constantly frozen (–4 °C) and thawed (+2 °C) mesocosms, and activity decreased over time (18 diurnal cycles). Here, microbial biomass C decreased but activity was kept high in the FTCs, similar to our results.

Moss-associated nitrogenase activity was low in our experiment. This could be due to the fact that Pleurozium schreberi generally hosts fewer active diazotrophs compared to other common mosses such as Hylocomium splendens (Warshan et al., 2016). Yet, high nitrogenase activity has been found in P. schreberi in northern boreal forests (Rousk et al., 2013a, b), indicating that abiotic conditions in the specific sampling sites also affect diazotroph activity (see below).

The lack of a difference in nitrogenase activity between samples kept at +10 °C and +3 °C is surprising. However, the Q10 for N2 fixation in temperate and subarctic P. schreberi ranges between 2 and 8 (Rousk et al., 2017b), and is dependent on the moisture content, with higher Q10 values in more moist moss. Mosses in our experiment may have been below the moisture level (100% moss moisture) that leads to increased sensitivity of the nitrogenase enzyme to a temperature change. Further, biological process rates scale with temperature, and will start high and decrease over time. Hence, longer incubations underestimate process rates at higher temperatures compared to lower incubation temperatures at the same incubation time (Rousk et al., 2017b). Given that we incubated all samples for the same duration (24 h) – to be able to capture a complete FTC - N2 fixation rates in the samples kept at +10 °C are likely underestimates as measured nitrogenase activity is linked to incubation time during ARAs (Rousk et al., 2017b).

### 4.2. Ecosystem and temporal patterns

In contrast to our second hypothesis (H2), N2 fixation in mosses from the Subarctic was not differently susceptible to freeze-thaw cycles than mosses from the other sites. If anything, N2 fixation in mosses from the temperate heath showed the least response to the FTCs. The lack of a temperature effect on N2 fixation in the temperate moss could suggest that bacteria and mosses from this ecosystem are exposed to more frequent FTCs than mosses from the arctic and boreal biomes, and may therefore be adapted to FTCs. In the arctic and boreal biomes, snow cover is common in the winter, insulating the moss carpet, while a considerable snow cover is rare in the winter in Denmark. Snow cover affects thermal regime of the soil, and it has been shown that soil freezing and freezing intensity is negatively related to snow depth (Fitzhugh et al., 2001). Hence, snow cover is a strong determinant of freeze-thaw effects on microbial activity, not only in the soil but also within the moss carpet. A temperate heath may experience more FTCs during winter and spring, and could explain the lack of a response to our treatments. However, mosses from our temperate heath experience fewer FTCs compared to mosses from the subarctic site, but more than mosses from our boreal site. Hence, snow cover and associated exposure to FTCs can only partly explain our results, and further studies should address ecosystem specific responses to FTCs. Nonetheless, decreased snow cover and increased rain events in a future climate will enhance FTCs (Putkonen and Roe, 2003; Hentschel et al., 2009), especially in high latitudes that will lead to significant effects on microbial activity and nutrient availability (Yi et al., 2015). Unfortunately, we could not manipulate snow cover in our experimental setting, and different measurement depths for the FTC frequency across the ecosystems limits direct comparisons of the experienced FTCs. Nonetheless, we can still compare the response of N2 fixation in mosses from the different ecosystems under controlled conditions as we can test if the origin of the samples modulates their responses to the FTCs. I.e. assessing FTCs effects on moss-associated N2 fixation, and to ascertain how different, historical exposure to FTCs, as well as different mean temperatures and snow cover, modulates the responses to FTCs can only be done when all samples are exposed to the same conditions -irrespective of their origin.

The response of N2 fixation towards FTCs over time differed between the mosses; while there was a slight decrease in the ±10 °C compared to the +10 °C in the boreal mosses towards the end of the experiment, N2 fixation in the subarctic mosses exposed to ±10 °C FTCs was comparable to the activity in mosses kept at constant +10 °C and was higher than activity in the –10 °C at the end of the experiment (F = 3.8; p = 0.008) (Supplementary Fig. S1). On the other hand, nitrogenase activity in mosses from the temperate heath was lower in the ±10 °C FTCs compared to activity at +10 °C after eight weeks with diurnal FTCs (F = 3.3; p = 0.02). This only partly corroborates our third hypothesis (H3) but is in line with previous findings on soil microbial responses to repeated FTCs, where either inhibition (e.g. Larsen et al., 2002) or promotion (Song et al., 2017) was observed. Differences in effect sizes could be attributed to the frequency of FTCs and severity, and in our case, to sample origin. Moss and cyanobacterial activity in the Subarctic is constrained to the snow-free season lasting about 5 months per year that could have primed them for a quick resuscitation as soon as temperatures climb above freezing. In a comparative study of soil samples collected from temperate (susceptible) vs. high altitude (cold adapted) sites, 60 FTCs reduced respiration rates more in the temperate compared to the cold adapted soils, but reorganization of the microbial communities in the temperate soils led to induced tolerance to subsequent FTCs and maintained physiology (Stres et al., 2010). Hence, the net effect of FTCs on microbial activity between susceptible and cold-adapted communities could be non-distinguishable as a result of acclimation or adaptation of the susceptible communities. Indeed, N2 fixation in free-living cyanobacteria can become tolerant towards desiccation upon repeated cycles of drying-rewetting (Scherer and Zhong, 1991). In contrast, the majority of diazotrophs might have been killed in the first freeze-thaw cycle, due to lack of adaptation (Koponene et al., 2006; Sawicka et al., 2010), and the survivors may have utilized the now freed-up nutrients and commenced activity.

Overall N2 fixation rates were higher in mosses from the boreal and subarctic birch forest than mosses collected in the temperate heath. Given that we used the same moss species, P. schreberi, across ecosystems, our findings indicate that colonization by diazotrophs is not moss-species-specific, but rather depends on the abiotic conditions, in particular N deposition (Rousk et al., 2013a,b), humidity (Rousk et al., 2018) and light regime (Gundale et al., 2012) of the investigated ecosystem. This is an ongoing debate, and our findings support the environmental selection over species selection.

### 4.3. Consequences for the ecosystem nutrient budgets – nutrients lost during FTCs

We found that mosses lost little N under the present growth conditions, with less than 1% of fixed 15N lost in the time frame of the experiment, confirming that mosses retain N efficiently under N limiting conditions (Liu et al., 2020), and indicate that N demand of mosses at the temperate site was not saturated.

With climate change, more frequent FTCs are predicted in high
latitude ecosystems. Although N₂ fixation does not seem to be strongly and negatively affected by mild or severe FTCs and N loss was generally low, nutrient loss from the moss carpet during FTCs are likely to affect ecosystem nutrient pools. While more fixed \(^{15}\)N-N₂ was lost from the temperate moss during \(\pm 10\ ^\circ\)C cycling, less ammonium and total dissolved N was lost in the same treatment compared to \(\pm 10\ ^\circ\)C. Negligible and low amounts of fixed \(^{15}\)N-N₂ and total dissolved N were lost from the Subarctic mosses compared to the other mosses. We cannot rule out that initial differences in \(^{15}\)N-N₂ fixation between the moss samples could have led to differences in \(^{15}\)N lost from the samples. However, nitrogenase activity was higher in mosses from the Subarctic compared to mosses from the temperate heath, yet, mosses from the Subarctic lost less \(^{15}\)N, rendering this possibility unlikely. The discrepancy between the mosses rather indicates high N resorption efficiency in subarctic mosses that are adapted to low N availability. This is also reflected in the lower tissue N content in mosses from the Subarctic, that also lose less N than P, thereby contributing to the N limitation in ecosystems that have already slow N turnover rates due to low temperatures and low litter quality (Chapin 1983; Hobbie et al., 2002). High N resorption efficiency has been found in N-limited boreal mosses, which can resorb more than 60% of acquired N (Liu et al., 2020).

Phosphate on the other hand, was more easily lost from \(\pm 10\ ^\circ\)C FTCs in the subarctic and boreal mosses compared to the mosses kept at constant \(\pm 10\ ^\circ\)C, and no large effects were found in the 3 \(\pm\) FTCs. Hence, only the pattern of phosphate loss followed our expectations and we can only partly confirm our fourth hypothesis (H4). Mosses are very efficient at resorbing nutrients (Liu et al., 2020), especially mineral N, after drying-rewetting events (Startsev & Liefers et al., 2006). Mosses in our experiment could have resorbed leached ammonium during thaw in the \(\pm 10\ ^\circ\)C FTCs, reducing the loss from the moss. The loss of fixed N₂ showed a different pattern, with tendencies for larger losses in the severe FTC, indicating preferential resorption of certain N forms by the moss. The enhanced loss of ammonium in the \(\mp 10\ ^\circ\)C treatment indicates excess N within the moss tissue as a result of elevated N₂ fixation activity fulfilling the moss’ N demand (Rousk and Michelsen, 2016). Total N of the mosses was not affected by the extreme temperature fluctuations, but given the higher N content of mosses from the boreal forest and temperate heath compared to the subarctic mosses, these mosses have the potential to lose more N upon disturbances.

While the subarctic and boreal mosses somewhat follow our expectations with regard to P losses (larger losses in FTCs compared to constant temperatures), mosses from the temperate heath showed the opposite pattern, with higher P loss from the constant \(\pm 3\ ^\circ\)C and \(\pm 10\ ^\circ\)C compared to the FTCs. Mosses from the temperate region may be exposed more frequently to FTCs than mosses from subarctic tundra and boreal forests given the lack of an insulating snow cover during winter (as described above). Thus, a legacy of acclimation towards FTCs may have prevented P leach from the temperate mosses. Phosphate may be more limiting for mosses from the temperate ecosystem that receive more N from atmospheric deposition (9.8 kg N ha \(^{-1}\) year \(^{-1}\) at Riso, 20 km SE of the sampling site; Ellermann et al., 2019), compared to mosses from subarctic tundra (1.3 kg N ha \(^{-1}\) year \(^{-1}\) in Abisko, a few hundred meters away from the sampling site; Goth et al., 2019) and boreal forests (>2 kg N ha \(^{-1}\) year \(^{-1}\); Ackermann et al., 2012), that are likely more limited by N availability. Hence, depending on nutrient limitation, mosses release nutrients that are in excess of their demand and retain those most critical for growth.

N₂ fixation by moss- associated cyanobacteria seems to be hardly affected by FTCs despite large fluctuations in temperatures and moisture availability – independent of the ecosystem assessed. However, nutrient loss from the moss carpets is more dynamic and depends on exposure temperature. Nevertheless, given that e.g. moss-associated N₂ fixation can be limited by P availability in the Arctic (Rousk et al., 2017a), with increasing frequency of FTCs in the arctic and boreal biomes, more P may be lost from the moss carpet with consequences for ecosystem nutrient cycling and N₂ fixation.

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.

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Appendix A. Supplementary data

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Author contribution

AM, AP, KR and PP conceived and designed the experiment. PP performed the experiments and laboratory analyses. AM, AP, KR, PP analyzed the data. KR wrote the first draft of the manuscript with all authors contributing to the final draft.

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